Discovery of a shortened version of SecA ($SecA^N$) that conceivably functions as a protein-conducting channel

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During biogenesis, nascent polypeptides of many proteins have to be translocated across biological membranes, relying on specific protein-conducting channels. It remains a great challenge to unequivocally identify the specific channel proteins for translocating particular client proteins in living cells. In Gram-negative bacteria, proteins destined to the inner membrane, periplasmic compartment or outer membrane are all initially synthesized in the cytoplasm and have to be translocated across the inner (i.e., the cytoplasmic) membrane. The prevailing perception is that all these translocations are accomplished by using the same SecYEG translocon. Nevertheless, this perception, formed largely based on genetic and *in vitro* studies, has yet been proved by direct *in vivo* analysis. Here, mainly via *in vivo* protein photocrosslinking analysis mediated by genetically incorporated unnatural amino acid pBpA, we revealed that, strikingly, the nascent β-barrel outer membrane proteins (OMPs) are translocated across the inner membrane by using $SecA^N$ (a shortened form of SecA), instead of SecYEG, as the translocon. This conclusion is made on the basis of our following observations. First, although direct interaction between the nascent periplasmic proteins and SecY, but not between nascent OMPs and SecY, was observed in living cells. Second, the processing of precursors of periplasmic proteins, but not OMPs, are severely reduced in a SecY-defective strain. Third, a shortened form of SecA, lacking its C-terminal region, thus designated as $SecA^N$, although does not interact with any nascent periplasmic proteins, directly interact with both nascent OMPs and the periplasmic region of the BamA protein, a key component for the assembly of OMPs. Fourth, $SecA^N$ is present wholly in the membrane fraction and as homo-oligomers. Fifth, an OmpF-$SecA^N$-BamA ternary complex (in addition to the OmpF-$SecA^N$ binary complex) could be clearly detected by our dual photocrosslinking analysis in living cells. An updated model for how proteins are translocated across the inner membrane of Gram-negative bacterial cells is proposed based on our new findings reported here.

Introduction

Many proteins have to be translocated across biological membranes during their biogenesis in living eukaryotic and prokaryotic cells (*1-4*). It has been demonstrated that such translocations are commonly carried out by membrane-integrated protein-conducting channels (*2, 4-7*). Nevertheless, it remains difficult to pinpoint whether a specific client protein is actually translocated by a particular channel in living cells. In Gram-negative bacteria (a prokaryote), both nascent outer membrane proteins and periplasmic proteins, initially synthesized in the cytoplasm, are believed to be translocated across the inner (i.e., cytoplasmic) membrane through such a protein-conducting channel either in a co- or post-translational manner (*2, 6, 7*). Downstream the inner membrane, protein factors in the periplasmic compartment and outer membrane will facilitate the nascent outer membrane proteins to reach their final destination as well as to fold/assembly into their functional forms (*8-10*). For example, the SurA protein has been identified as the primary chaperone for escorting nascent β-barrel outer membrane proteins (henceforth abbreviated as OMPs) across the periplasmic compartment (*11-15*). Additionally, the BamA protein, as the major subunit of the essential β-barrel assembly machine (Bam) complex, is integrated into the outer membrane via its C-terminal β-barrel structure and extends into the periplasmic compartment via its N-terminal POTRA domains (*16-18*).

According to the currently prevailing perception, translocations of all the nascent proteins across the inner membrane in Gram-negative bacteria are largely accomplished by the same *sec* translocon, for which the membrane-integrated SecYEG complex acts as a protein-conducting channel while the ATP-consuming molecular motor protein SecA provides the driving force (*2, 6, 7, 19*). The SecYEG is a highly conserved complex whose homologue in eukaryotic cells is named as the Sec61 complex that is integrated into the membrane of the endoplasmic reticulum (*2-5, 20-22*). The *secY* gene was initially identified as one whose suppressor mutations restored the export of OMPs or periplasmic proteins that have a defective signal peptide (*23, 24*), as well as one whose defect resulted in a slow processing for precursors of both OMPs and periplasmic proteins (*25, 26*). The SecY and SecE proteins were later found to act together with the SecA protein in supporting translocation of the precursor form of an outer membrane protein (*27*). The protein-conducting channel is believed to be largely formed by the SecY protein in light of its crystal structure (*28, 29*) as well as *in vitro* chemical crosslinking analysis (*30, 31*).

The *secA* gene was initially identified as one whose defect resulted in accumulation of precursors of both OMPs and periplasmic proteins (*32, 33*). The SecA protein was later found to be essential for translocation of both OMPs and periplasmic proteins (*34, 35*). SecA was subsequently characterized not only as a cytoplasmic but also as a peripheral- and integrated-membrane protein (*36-39*), and was demonstrated to be exposed to the periplasmic compartment (*40-42*). Paradoxically, on one hand, the SecA protein was proposed to undergo ATP-driven cycles of membrane insertion and

deinsertion (*43, 44*), on the other, a significant fraction of the SecA protein was reported to be permanently embedded in the inner membrane (*38*). In contrast to these conventional views, it has also been reported that SecA alone, without SecYEG, is able to promote translocation of OMPs across inner membrane vesicles or artificial lipid bilayers under *in vitro* conditions (*45-47*).

Our present understanding on how newly-synthesized proteins are exported across the inner membrane mainly stems from two approaches, the genetic studies that have resulted in the isolation of numerous export-defective mutants (*12, 13, 16, 17, 20, 22-26, 32, 33, 48*), and the *in vitro* biochemical analysis that exploits the cell-free synthesis/transport systems (*14, 27, 46, 47*). These commonly-taken approaches, though powerful, were unable to elaborate the exact function of each protein factor in living cells. It follows that these approaches are unable to clarify which membrane protein (SecY or others) on the inner membrane function as the actual translocon for a particular substrate protein (be an outer membrane protein, a periplasmic protein or an inner membrane protein). In regards to the biogenesis of OMPs and periplasmic proteins, as well as the function of SecYEG and SecA in living Gram-negative bacterial cells, many key issues remain to be resolved. For example, if the prevailing perception on the SecYEG translocon is correct, then how could a single SecYEG channel translocate the nascent polypeptides of such dramatically different clients as OMPs, periplasmic proteins and even inner membrane proteins? What are the structural and functional differences for the SecAs present in the different subcellular localizations?

In this study, we decided to take a more direct approach in an attempt to gain insights on the exact function of such protein factors as SecY and SecA for protein export in Gram-negative cells. In particular, we performed *in vivo* protein photocrosslinking studies between interacting proteins as mediated by genetically incorporated unnatural amino acid (*49*), an approach that we have extensively employed over the past years (*15, 50-52*). We first unexpectedly observed that although nascent periplasmic proteins directly interacted with SecY, those of OMPs did not. Further, we observed that precursors of OMPs, but not of periplasmic proteins, could still be effectively processed when SecY becomes defective. Subsequently, we demonstrated that a shortened version of SecA, which we designate as SecAN, directly interacts with both nascent OMPs, as well as the periplasmic region of BamA. In addition, we provided evidence to show that the $SecA^N$ exists in the inner membrane as a homo-oligomer. These observations strongly implicate that $SecA^N$ functions as the translocon for translocating the nascent OMPs, while SecYEG functions as the translocon for nascent periplasmic proteins in living bacterial cells.

Results

Nascent periplasmic proteins but not nascent OMPs directly interact with SecY in living cells

We first tried to clarify whether nascent polypeptides of both periplasmic proteins and OMPs are translocated across the inner membrane through the SecYEG translocon (with SecY being the protein-conducting channel) in living cells, as commonly documented in the literatures (*2, 5, 7, 9*). For this purpose, we first performed photocrosslinking analysis in living cells by individually introducing the unnatural amino acid pBpA (*p*-benzoyl-L-phenylalanine) (*49-52*) at twenty one somewhat randomly selected residue positions across the polypeptide of nascent OmpF (an OMP) or ten positions of nascent SurA (a periplasmic protein). Before UV-irradiation, each pBpA variant of OmpF or SurA was first respectively expressed in the *ompF*-deleted or *surA*-deleted LY928 bacterial strain, whose genome was prior modified to encode the amino-acyl tRNA synthetase and the tRNA that are needed for the pBpA incorporation as we reported before (*15*).

For the OmpF variants, our blotting analysis against SecY, however, failed to detect any photocrosslinked product, which would appear as a band at ~89 kD, the combined molecular mass of OmpF (~40 kD) and SecY (~49 kD) (**Fig. 1A**; displayed are results of twelve of the twenty one variants of OmpF). In contrast, for the SurA variants, we successfully detected the photocrosslinked product of ~94 kD, with SurA being ~45 kD and SecY ~49 kD, when pBpA was introduced at the signal peptide residue position 8 or 12 in SurA (lanes 2 and 4, **Fig. 1B**). It is noteworthy that we failed to detect any photocrosslinked SurA-SecY product when pBpA was placed at the mature part of SurA (lanes 8-11, **Fig. 1B**). Collectively, these observations indicate that, in contrast to the common perception, the nascent OMPs, unlike the nascent periplasmic proteins, apparently do not directly interact with the SecYEG translocon in living cells.

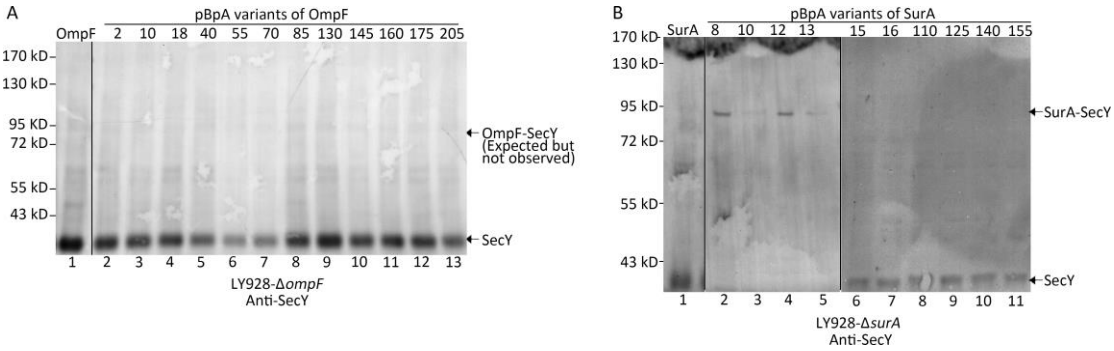

Figure 1. Nascent polypeptides of OMPs (OmpF), unlike that of periplasmic proteins (SurA), do not directly interact with SecY in living cells.

(**A, B**) Blotting results for detecting the photocrosslinked products of the indicated pBpA variants of OmpF (**A**) or of SurA (**B**) that were respectively expressed in the LY928-Δ*ompF* or LY928-Δ*surA* cells, probed with the streptavidin-alkaline phosphatase conjugate against the AVI tag linked to

the SecY protein. Here, the residue positions are numbered by including the signal peptide. Protein samples were resolved by SDS-PAGE before blotted against SecY. Samples of cells expressing the wild type OmpF or SurA (with no pBpA incorporation) were analyzed here as negative controls (lanes 1, **A** and **B**). Indicated on the right of each gel is the position of SecY, photocrosslinked OmpF-SecY (expected but not observed) or SurA-SecY, and on the left are positions of molecular weight markers. The SecY protein in lanes 2-5 in panel **B** mobilized to the bottom edge of the gel, thus hardly visible.

Precursors of OMPs but not of periplasmic proteins can be effectively processed when SecY is defective.

To further clarify the role of the SecYEG translocon in translocating nascent OMPs and periplasmic proteins, we employed the cold sensitive mutant strain, SecY39, in which protein export was previously reported to be partially retarded at the permissive temperature of 37°C and dramatically retarded at the non-permissive temperatures of 20°C due to a defect of the SecY protein (*53*). We found that processing of precursor of OMPs, OmpA and OmpF, was far less defective when compared with that of periplasmic proteins, maltose binding protein (MBP) and SurA, when the SecY39 strain was cultured at the non-permissive temperature of 20°C (**Fig. 2A**). This is reflected by a much lower level of accumulation of precursors for OMPs (pre-OmpA and pre-OmpF) than for periplasmic proteins (pre-MBP and pre-SurA) when the SecY39 strain was cultured at the non-permissive temperature without adding chloramphenicol, as shown in lane 2, **Fig 2A**. It is also reflected by a significantly more efficient processing of the accumulated precursors of OMPs (converting to the folded forms) than of periplasmic proteins (converting to the mature forms) during the 1 hour chasing period after protein synthesis in the cells was inhibited by the added chloramphenicol (*54*), as shown in lanes 2-4,**Fig 2A**. In comparison, when the SecY39 cell was cultured at the permissive temperature of 37°C, the differences in both precursor accumulation and processing are far less significant than those observed at the non-permissive temperature of 20°C (**Fig. 2B**). Taken together, these observations indicate that the processing of precursors for OMPs relies on the SecYEG translocon in a far less degree than that for periplasmic proteins. Our result is somehow consistent with what was reported before showing that the *secY39* mutation affected export of the periplasmic protein MBP in a higher degree than that of the outer membrane protein OmpA (*53*), and also supports our *in vivo* protein photocrosslinking results presented above in **Fig. 1**, that nascent periplasmic proteins but not OMPs directly interact with SecY in living cells.

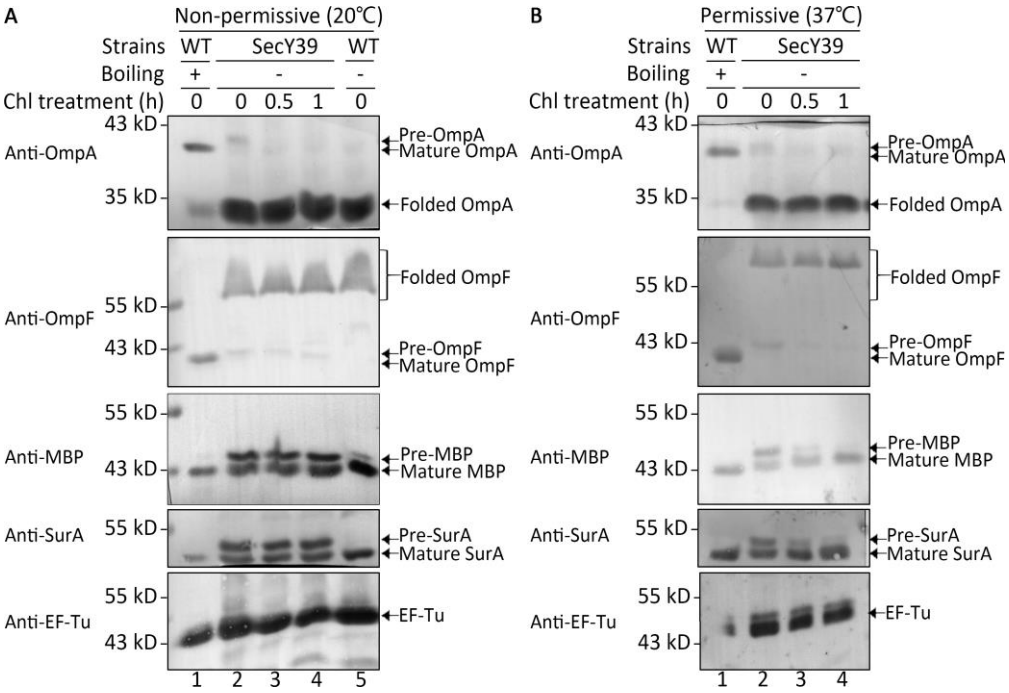

Figure 2. Precursors of OMPs but not of periplasmic proteins are effectively processed when SecY is defective.

Immunoblotting results for detecting the precursor, mature and folded forms of the indicated OMPs (OmpA or OmpF) and periplasmic proteins (SurA or MBP) in the cold-sensitive SecY39 cells cultured either at the non-permissive temperature of 20°C (**A**), or permissive temperature of 37°C (**B**); cells were treated with chloramphenicol (Chl) for the indicated length of time for the chasing experiments (lanes 2-4 in **A** and **B**). Protein samples of SecY39 cells were not boiled for the semi-native SDS-PAGE analysis (lanes 2-4 in **A** and **B**). Protein samples from wild type cells (WT) were boiled for indicating positions of the mature forms (that mobilizes with a slightly higher rate than the precursors) of OMPs and periplasmic proteins (lanes 1 in **A** and **B**), or not boiled for indicating positions of the folded forms of OMPs (lane 5 in **A**). The EF-Tu protein was analyzed here to indicate an equal loading of samples in lanes 2-5. Indicated on the right of each gel are positions of different forms of OMPs (precursor, mature and folded) and periplasmic proteins (precursor and mature), and on left are positions of the molecular weight markers.

Nascent OMPs but not periplasmic proteins interact with a shortened version of SecA ($SecA^N$).

Our observations described above apparently indicate that the SecYEG translocon does not directly translocate nascent OMPs across the inner membrane in living cells. Given that it has been previously demonstrated that a portion of the cellular SecA is embedded in the inner membrane (*36-39*), we then asked whether there is any possibility that this membrane-embedded form of SecA acts as the translocon for nascent OMPs in living cells. For this to be true, we presumed that such SecA proteins would not only have to directly interact with nascent OMPs, but may also interact with protein factors that participate in OMP biogenesis while located in the

periplasmic compartment. To this end we first analyzed whether the aforementioned twenty one pBpA variants of OmpF could form photocrosslinked product with SecA in living cells. The immunoblotting results for five representative OmpF variants, as displayed in **Fig 3A**, however, did not reveal any potential photocrosslinked OmpF-SecA product at ~142 kD, the expected combined molecular mass of OmpF (~40 kD) and SecA (~102 kD). Nevertheless, we observed two unexpected photocrosslinked product bands with a molecular mass of ~80 kD for most pBpA variants of OmpF (indicated by the stars in lanes 3, 7, 9, and 11, **Fig 3A**) or ~65 kD for all the cell samples (indicated by the arrowheads in lanes 1, 3, 5, 7, 9, and 11, **Fig 3A)**. It should be pointed out that these two bands were not detected in samples of cells unexposed to UV light (lanes 2, 4, 6, 8, 10 and 12, **Fig. 3A**), and that, strikingly, the ~65 kD band was detected even for the wild type OmpF that contains no pBpA (lane 1, **Fig. 3A**). At this point, the most likely explanation for these two photocrosslinked bands is such that the ~80 kD band represents a photocrosslinked product between the pBpA variants of OmpF (~40 kD) and a shortened form of SecA (~45 kD, instead of ~102 kD), while the ~65 kD band represents a crosslinked dimer of the shortened form of SecA (as substantiated by our data displayed in **Figures S1** and **5B**).

We next tried to elucidate whether this ~80 kD photocrosslinked product was indeed generated from a certain shortened version of SecA. For this purpose, we first analyzed the protein samples of the LY928 cells that neither expressed any pBpA variants of OmpF nor were UV irradiated, and also by employing a separating gel (of higher acrylamide concentration) that would resolve smaller proteins (such as the shortened form of SecA) with a higher resolution (*55*). Remarkably, our immunoblotting analysis now clearly revealed one shortened form of SecA with a size of ~45 kD, that we designate as $SecA^N$ henceforth, because it could be detected only by antibodies against the N-terminal region (fragment 1-209) but not by antibodies against the C-terminal region (fragment 665-820) of SecA (lane 1 vs lane 2, **Fig 3B**). In correlation to this, for the OmpF-G18pBpA variant, we also detected the ~80 kD photocrosslinked product (as well as the ~45 kD form of SecA) only with antibodies against the N-terminal region (lane 1) but not against the C-terminal region of SecA (lane 3), as shown in **Fig 3C**. In contrast, similar analysis of the aforementioned 10 pBpA variants of SurA, as shown in **Fig S1,** did not reveal any photocrosslinked product between SurA and $SecA^N$ (with an expected combined molecular mass of ~85 kD). It is noteworthy that, similar to what was observed above for the OmpF variants (**Fig. 3A**), the ~65 kD photocrosslinked product (indicated by the arrowheads) was once again detected in all the UV-irradiated samples, including the one without expressing any pBpA variant of SurA (lanes 1, 3, 5, 7, 9, 11, 13-17, **Fig. S1**).

Taken together, these *in vivo* protein photocrosslinking results suggest that nascent OMPs but not periplasmic proteins interact with $SecA^N$, a shortened version of SecA that contains only the N-terminal region of the full length SecA. The unresolved ~65 kD photocrosslinked product, observed in all UV-irradiated cell samples irrespective of the presence of any pBpA variant of OmpF or SurA, most likely represents a

photocrosslinked dimer of $SecA^N$ that could be formed through an interacting pair of tyrosine residues upon UV irradiation as reported before (*56*). The apparent smaller size of this photocrosslinked product, being ~65 kD rather than the expected ~90 kD (45 kD + 45 kD) for a $SecA^N$ dimer, could be resulted from the fact that the crosslinking occurred through a tyrosine residue that is somehow located in the middle of the two $SecA^N$ polypeptide chains (such H-shaped crosslinked forms would mobilize at a rate significantly higher than a linear crosslinked form).

$SecA^N$ is located wholly in the membrane fraction and likely exists as homo-oligomers.

We subsequently tried to find out whether $SecA^N$ is located in the membrane fractions, as to be required for it to function as a protein-conducting channel, and whether it can be converted into photocrosslinked dimers by exposing such a membrane fraction to UV irradiation, as we speculated above. Our immunoblotting results, as shown in **Fig 3D**, demonstrate that, remarkably, the $SecA^N$ was almost wholly found in the membrane-containing precipitate fraction (lanes 1 and 2), but not in the cytoplasm-containing soluble fraction (lanes 4 and 5), and that the $SecA^N$ in the membrane fraction could be effectively converted into a crosslinked dimeric form of ~65 kD by UV exposure (lanes 1 vs 2)**.** With great interest, we also noticed that the $SecA^N$ in the membrane fraction could be detected in its monomeric form (~45 kD) only after the sample was boiled (lane 2, **Fig. 3D**), but detected as a smear with a significantly lower mobility if the membrane fraction was not boiled (lane 3, **Fig. 3D**). The most likely explanation for this phenomenon is that $SecA^N$ in the membrane exists as oligomers that are associated with the lipid components of the membrane, similar to the behavior of the OmpF (lane 1 vs lanes 2-5, **Fig. 2A**). In contrast, the full length form of SecA was detected largely in the soluble fraction (lanes 4, 5, 8 and 9), and only in a significantly lower level in the membrane fraction (lanes 1, 2, 3, 6, 7 and 10), and, again, could be detected with antibodies either against the N- or C-terminal region of full length SecA (**Fig 3D**). Taken together, these observations strongly indicate that $SecA^N$ exists as homo-oligomers that is integrated in the inner membrane.

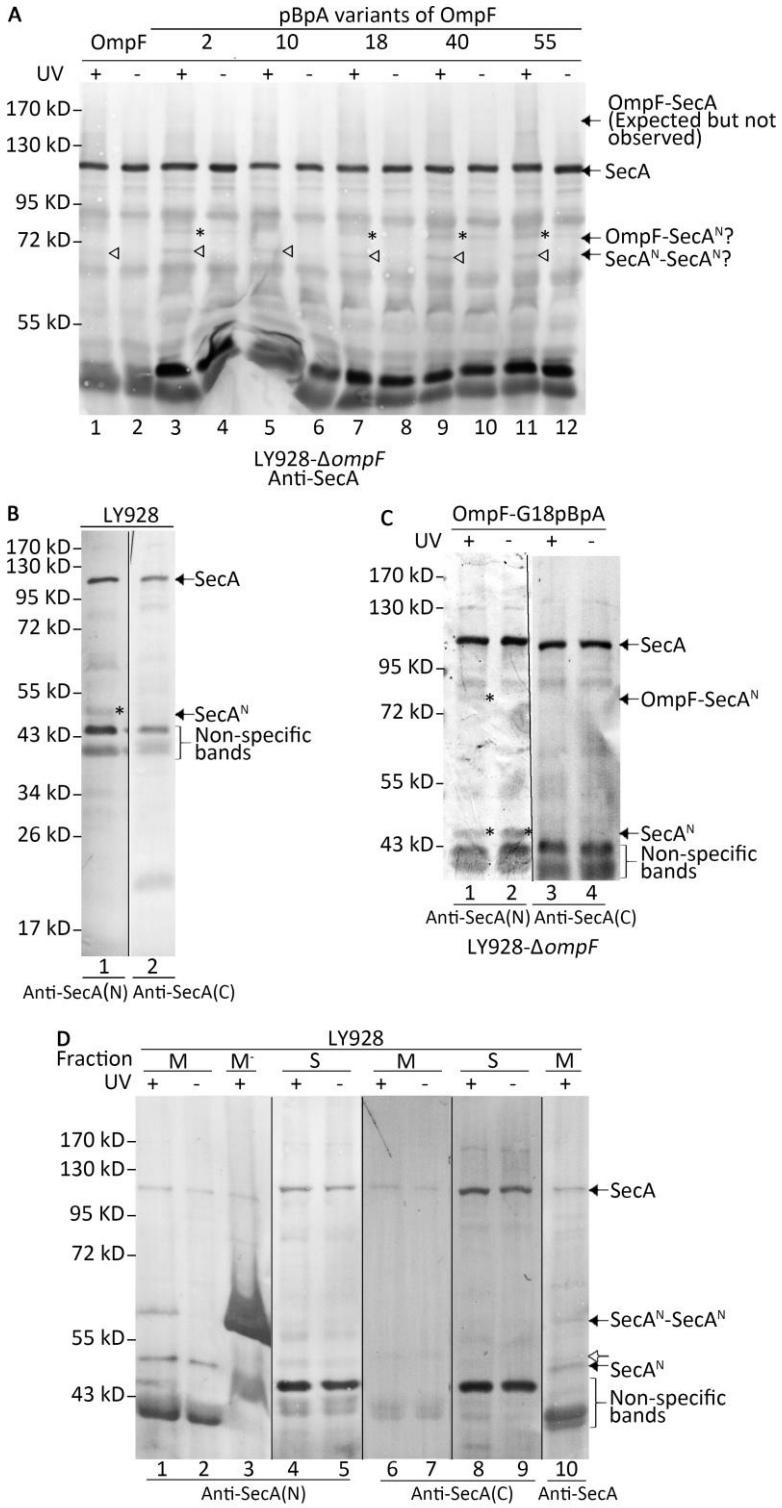

Figure 3. Nascent OMPs but not periplasmic proteins interact with a shortened version of SecA ($SecA^N$) that is located wholly in the membrane fraction and likely exists as homo-oligomers.

(**A**) Immunoblotting results for detecting the *in vivo* protein photocrosslinked products of the indicated pBpA variants of OmpF expressed in the LY928-Δ*ompF* cells, probed with antibodies against the full length SecA (i.e., a mixture of antibodies against the N-terminal fragment 1-209 and the C-terminal fragment 665-820 of SecA).

(**B**) Immunoblotting results for detecting the shortened form of SecA ($SecA^N$) after resolving on a

high resolution gel, using antibodies against the N- (lane 1) or C-terminal (lane 2) region of SecA.
(**C**) Immunoblotting results for detecting the photocrosslinked product between OmpF-G18pBpA and $SecA^N$, using antibodies against the N- (lane 1) or C-terminal (lane 3) region of SecA.
(**D**) Immunoblotting results for detecting the $SecA^N$ and full length SecA in the membrane (M) and soluble (S) fractions, as well as the photocrosslinked $SecA^N$ dimer after UV irradiation, using antibodies against the N- (lanes 1-4) or C-terminal (lanes 5-8) region of SecA. M^-: sample of membrane fraction not boiled.
Protein samples were resolved by SDS-PAGE before subjecting to immunoblotting. The polymerized separating gel in **A** and **C** is 8%, while in **B** and **D** is 10%. Residue positions are numbered by including the signal peptides. Samples of cells expressing wild type OmpF (with no pBpA incorporation) were analyzed as negative controls (lanes 1 and 2 in **A**). Indicated on the right of each gel are the positions of SecA, shortened version of SecA ($SecA^N$), photocrosslinked OmpF-$SecA^N$, $SecA^N$-$SecA^N$ and on the left are positions of molecular weight markers.

Precursors of both OMPs and periplasmic proteins interact with the full length form of SecA but not with $SecA^N$ and SecY, in the LY928-SecA81 mutant cells cultured under non-permissive conditions.

In the *in vivo* protein photocrosslinking experiments described above, we observed that nascent OMPs directly interact with $SecA^N$ (**Fig. 3A** and **3C**) and that the nascent periplasmic proteins directly interact with SecY, (**Fig. 1B**) but not yet observed any direct interaction between the nascent OMPs or periplasmic proteins and the full length form of SecA. These failures might be explained as such that either they interact in a very transient manner (thus could not be captured by our *in vivo* protein photocrosslinking) or, less likely, they do not interact at all in living cells. To clarify this, we decided to try to prolong such transient interactions (thus allow them to be captured by our *in vivo* photocrosslinking) by replacing the wild type *secA* gene in the LY928 genome with the mutant *secA81* gene that encodes a temperature sensitive SecA protein being functional at 37°C but defective at 42°C (*57*). The mutant SecA81 protein possesses a Gly516Asp replacement, which presumably only affects the full length form of SecA but most likely not the $SecA^N$. Our data presented in **Fig S2** clearly indicate that the $SecA^N$ is effectively formed under both permissive (lane 1) and non-permissive (lane 2) conditions in this LY928-SecA81 mutant strain.

With this LY928-SecA81 strain, we could now indeed detect the photocrosslinked OmpF-SecA (~142 kD; lanes 3, 4 and 6, **Fig 4A**) or SurA-SecA (~147 kD; lane 2, **Fig 4B**) when the cells were cultured at the non-permissive temperature of 42°C (under this condition, precursors of neither OMPs nor periplasmic proteins were effectively processed, as shown by our data displayed in **Fig S3**, consistent with what was reported before (*57*). Remarkably, in the LY928-SecA81 cells cultured at the non-permissive temperature, we neither detected the photocrosslinked ~80 kD OmpF-$SecA^N$ (lanes 1-6, **Fig 4A**) nor the ~90 kD SurA–SecY product (lanes 1-5, **Fig 4C**). These observations could be best explained as follows. Nascent periplasmic proteins and nascent OMPs directly interact with the full length form of SecA (in the cytoplasm) before they respectively interact with SecY and $SecA^N$ (in the inner

membrane) in the wild type cells, but the prolonged interaction of the former (upstream) interaction prevented the latter (downstream) interaction to occur in the mutant LY928-SecA81 cells under the non-permissive condition. The two photocrosslinked OmpF-SecA bands may have resulted from the interaction between the introduced pBpA residue and two different sites of SecA.

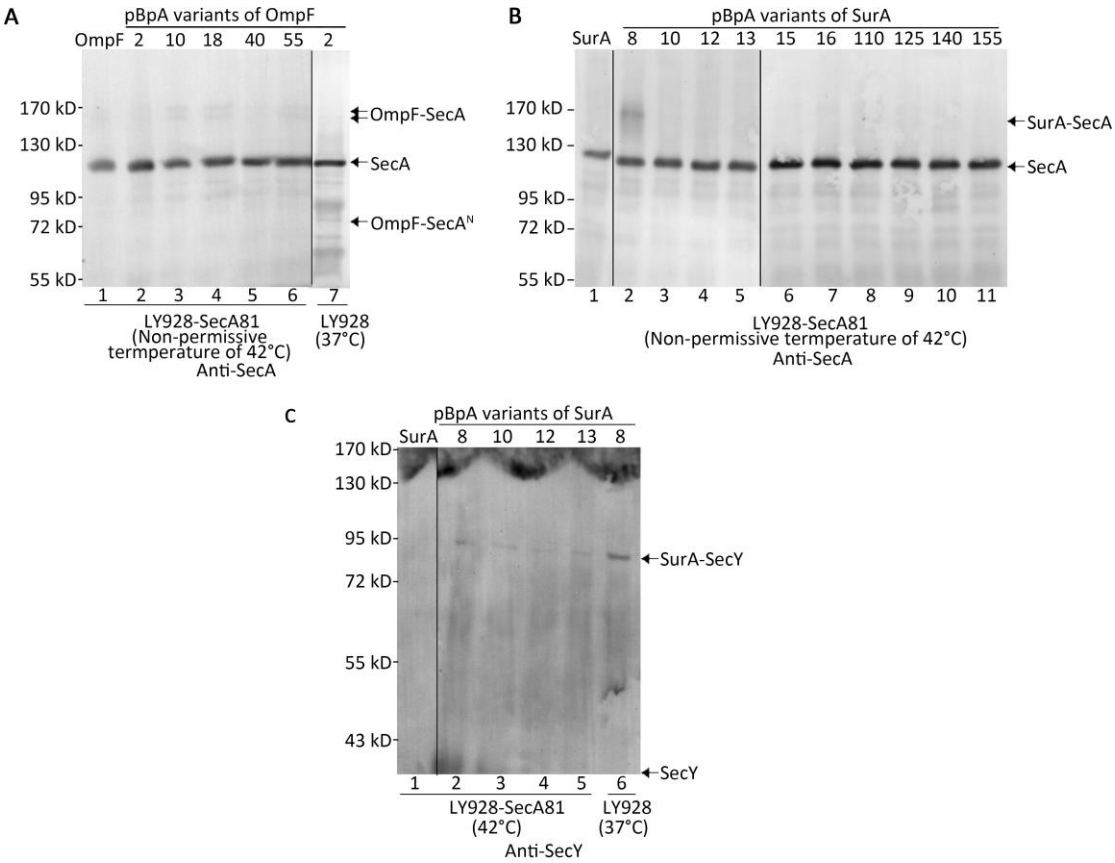

Figure 4. Precursors of both OMPs (OmpF) and periplasmic proteins (SurA) interact with the full length form of SecA, but not with SecA^N and SecY, respectively, in the LY928-SecA81 mutant cells cultured under the non-permissive temperature of 42°C.

(**A, B, C**) Immunoblotting results for detecting the *in vivo* protein photocrosslinked products of the indicated pBpA variants of OmpF (**A**) and SurA (**B**) expressed in the LY928-SecA81 mutant cells cultured at the non-permissive temperature of 42 °C, probed with antibodies against SecA (**A, B**) or the streptavidin-alkaline phosphatase conjugate against the AVI tag linked to the SecY protein (**C**). Lane 7 in panel **A** and lane 6 in panel **C** are identical with lane 3 in **Fig 3A** and lane 2 in **Fig 1B**, respectively, and used here to solely indicate the expected position of OmpF-SecA^N or SurA-SecY. The residue position numbering includes the signal peptide. The protein samples were resolved by SDS-PAGE before subjecting to immunoblotting analysis. Position of SecA, OmpF-SecA, SurA-SecA or SurA-SecY is indicated on the right of the gels. Positions of the molecular weight markers are indicated on the left of each gel. Cells expressing the wild type OmpF or SurA (with no pBpA incorporation) were also analyzed as negative controls (lanes 1 in **A, B** and **C**).

SecA^N directly interacts with the periplasmic POTRA 2 domain of BamA in living

cells

We next examined whether the $SecA^N$ interacts with protein factors that are known to participate in OMP biogenesis but located in the periplasmic compartment outside the inner membrane, such as SurA (*11-15*) and BamA (*16-18*). For this purpose, we first tried to find out whether pBpA individually introduced at six residue positions of SecA (at 47, 300, 530, 863, 868 or 896) that were previously reported to be exposed to the periplasmic compartment (*42*) was able to mediate photocrosslinking with SurA and/or BamA. In remarkable support with what we observed above (**Fig 3A** and **3C**), our immunoblotting analysis revealed a putative $SecA^N$-BamA product band, of ~140 kD, when pBpA was introduced at residue position 47 in the N-terminal region of full length SecA (lane 1, **Fig. 5A**), but with no potential photocrosslinked SecA-BamA product (of ~197 kD; with BamA being ~95 kD). In contrast, we failed to detect any photocrosslinked $SecA^N$-SurA product with the six pBpA variants of SecA in the LY928 cell (**Fig. S4**), suggesting that $SecA^N$ on the inner membrane may not directly interact with SurA present in the periplasmic compartment.

To verify this putative interaction between $SecA^N$ and BamA, we then performed a reciprocal photocrosslinking by individually introducing pBpA at nine residue positions in the periplasmic domains of BamA. Remarkably, our immunoblotting analysis revealed a BamA-$SecA^N$ product, also of ~140 kD, that could only be detected by antibodies against the N-terminal region (lane 1) but not against the C-terminal region (lane 3) of SecA, when pBpA was introduced at residue positions 121 or 129 in the POTRA 2 domain of BamA (**Fig. 5B**, only the results for BamA-V121pBpA is displayed).

We next isolated this putative photocrosslinked BamA-$SecA^N$ product (instead of the OmpF-$SecA^N$ product described in **Fig. 3A** and **3C** simply due to its higher level) and subjected it to mass spectrometry analysis. Although the results revealed the presence of both BamA and SecA, interestingly, the score for SecA was rather low and, in addition, the two identified peptide fragments for SecA (as shown in **Fig. 5C**) were both derived from the N-terminal region of SecA, once again indicating that $SecA^N$, instead of the full length SecA, is most like present in the photocrosslinked product. Taken together, these data clearly indicate that the $SecA^N$ is able to directly interact with the periplasmic region of the BamA protein.

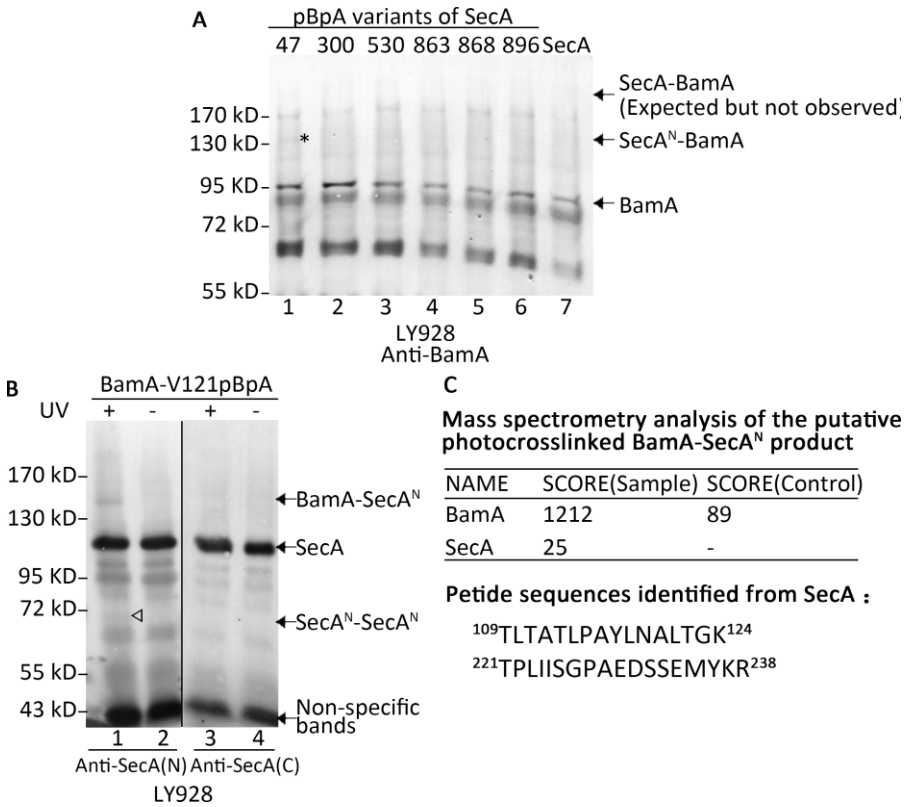

Figure 5. SecA^N directly interacts with the periplasmic POTRA 2 domain of BamA in living cells. (**A**) Immunoblotting results for detecting the *in vivo* protein photocrosslinked products of the indicated pBpA variants of SecA that were expressed in LY928 cells, probed with antibodies against BamA. (**B**) Blotting results for detecting the *in vivo* protein photocrosslinked product of BamA-V121pBpA that was expressed in LY928 cells, using antibodies against the N-terminal region (lanes 1 and 2) or C-terminal region (lanes 3 and 4) of SecA. (**C**) Results of mass spectrometry analysis of the putative photocrosslinked BamA-SecA^N product generated from BamA-V121pBpA. Shown are the protein scores for BamA and SecA, as well as the sequences of the two peptides identified for SecA. For **A** and **B**, protein samples were resolved by SDS-PAGE before subjecting to blotting. Indicated on the right of the gels are positions of BamA, SecA, SecA^N-BamA or BamA-SecA^N, on the left are positions of the molecular weight markers.

A ternary BamA-OmpF-SecA^N complex is formed in living cells.

In light of our aforementioned observations that either OmpF (**Fig. 3**) or BamA (**Fig. 5**) interacts with SecA^N, we then tried to detect a ternary BamA-OmpF-SecA^N complex in living cells. For this purpose, we simultaneously introduced pBpA into both BamA (at residue position 121) and OmpF (at residue position 18). We then performed this dual *in vivo* protein photocrosslinking within the LY928 cells that co-expressed BamA-V121pBpA and OmpF-G18pBpA. Blotting analysis using antibodies against the N- or C-terminal region of SecA, as well as streptavidin-AP conjugate against the AVI tag linke to BamA, remarkably, indeed revealed the presence of a photocrosslinked ternary BamA-OmpF-SecA^N complex in addition to a binary BamA-SecA^N complex (lanes 1 and 7, **Fig. 6**). Consistent with results presented in **Fig. 5B**, such a ternary complex could only be detected by antibodies against the N-terminal region (lane 1)

not by antibodies against the C-terminal region of SecA (lane 5, **Fig. 6**). Collectively, these observations once again strongly suggest that the $SecA^N$ protein, by interacting with the BamA protein, translocates nascent OMPs across the inner membrane.

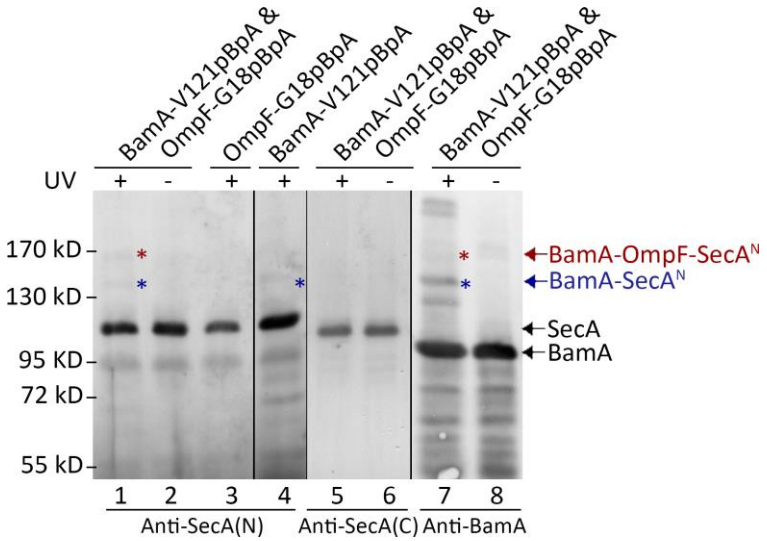

Figure 6. A ternary BamA-OmpF-$SecA^N$ complex is formed in living cells.

Blotting results for detecting photocrosslinked products formed in the LY928 cells expressing the indicated pBpA variants of BamA and/or OmpF, using antibodies against the N-terminal (lanes 1-4) or C-terminal region of SecA (lanes 5 and 6), or the streptavidin-AP conjugate against the AVI tag linked to BamA (lanes 7 and 8).

Protein samples were resolved by SDS-PAGE before subjecting to the blotting. Indicated on the right of the gel are positions of BamA, SecA, BamA-$SecA^N$ or BamA-OmpF-$SecA^N$, on the left are positions of the molecular weight markers.

$SecA^N$ contains a potential transmembrane region with the sole GXXXG motif in the full length SecA

Our results presented above strongly indicate that $SecA^N$, likely as a homo-oligomer, may form a protein-conducting channel in the inner membrane for nascent OMP proteins to pass through. We then tried to find out whether there is any potential transmembrane region in $SecA^N$. Before that, we need to speculate where on the polypeptide chain the cleavage is made in the full length SecA to generate $SecA^N$. If we assume the cleavage is made (in the cytoplasm) by a typical soluble serine protease that recognize such basic residues as lysine and arginine, then Arg416 could be the putative cleavage site, because it locates in a loop and that such a cleavage produces an N-terminal region of ~47 kD (*58*). If we assume that the cleavage is made by GlpG, the serine proteinase integrated in the inner membrane (*59, 60*), then ^{351}Ser-Asp^{352} is a putative cleavage site and produces an N-terminal region of ~40 kD. Here we made our prediction based on a cleavage at residue Arg416 and by using the TMpred software (*61, 62*) provided by the online server (http://www.ch.embnet.org/software/TMPRED_form.html). As displayed in **Fig 7A**, we identified one potential transmembrane domain (colored red) with the indicated orientation when subjected the N-terminal 416 residues (orange) of SecA to TMpred

software prediction. It should be pointed out that the structure of this presumably membrane-integrated SecA^N, when determined in the future, may differ significantly from the N-terminal region of SecA that we displayed in **Fig. 7A**, taken from the determined structure of the soluble full length SecA (*58*).

Then, to our satisfaction, we revealed that the single GXXXG (being GLTVG, as shown in Fig. **7B**) motif across the whole polypeptide chain of the SecA is located in this potential transmembrane region. Such a GXXXG motif has been identified as being commonly found in transmembrane helices of integral membrane proteins and also important for strong interactions between the transmembrane domains (*63*). The orientation of such a potential transmembrane domain matches well with our identification of residue T47 (colored blue in **Fig 7A**) in SecA^N as one that is exposed to the periplasmic compartment and directly interacts with the POTRA 2 domain of BamA (lane 1, **Fig. 5A**). We revealed that such a GXXXG motif is also found in all the single transmembrane region predicted by the TMpred software provided by the online server in SecA proteins of other bacterial species, as represented by those listed in **Table 1**, suggesting that such a membrane-embedded SecA^N is likely present in all these bacterial species.

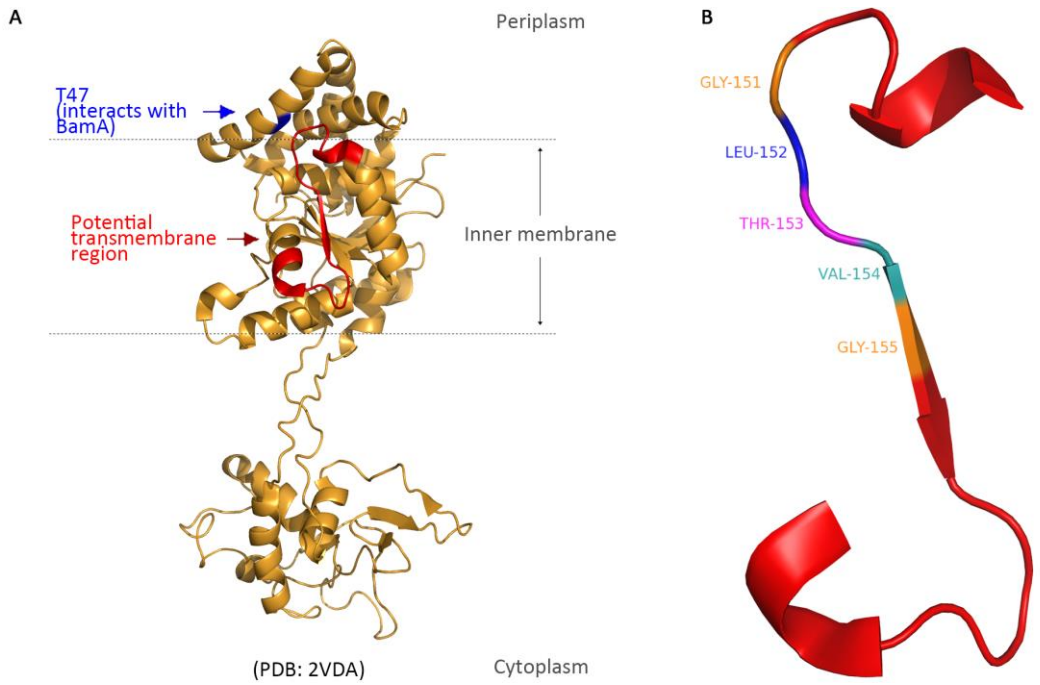

Figure 7. Structure of the N-terminal region (residue 1-418) derived from the determined structure of the full length SecA (A) and the sole GXXXG (GLTVG) motif identified in SecA (B). The potential transmembrane region is colored red and residue T47 that we identified as one interacting with the periplasmic POTRA 2 domain of BamA protein is colored blue in (**A**).

Table 1. Transmembrane regions predicted by the TMpred online server and the GXXXG motifs identified in them for SecA proteins of various bacterial species.

Bacterial species	Identity (%)[1]	TM[2]	Orientation	Score[3]	GXXXG motif
Escherichia coli	100	145-165 (21)	Outside-inside	735	GLTVG (151-155)
Lactobacillus delbrueckii	48	143-161 (19)	Outside-inside	443	GLTVG (149-153)
Stenotrophomonas maltophilia	62	141-161 (21)	Outside-inside	722	GLSVG (151-155)
Bacillus subtilis	50	141-159(19)	Outside-inside	172	GLTVG (149-153)
Pseudomonas aeruginosa	64	145-162 (18)	Outside-inside	310	GLSVG (151-155)
Mycobacterium tuberculosis	35	145-165 (17)	Inside-outside	555	GLTVG (158-162)
Streptococcus pyogenes	44	143-161 (19)	Outside-inside	581	GLSVG (149-153)

[1]Listed here is the identity between the SecA amino acid sequence of the indicated bacterial species and that of E. coli.

[2]TM: predicted transmembrane region

[3]Here, we included the TM regions predicted with a score<500.

Discussion

This study was conducted in an initial attempt to clarify whether both OMPs and periplasmic proteins are translocated across the inner membrane through the SecYEG translocon in living cells, as commonly documented (*2, 5, 7, 9, 25*). Our *in vivo* protein photocrosslinking analysis mediated by genetically incorporated unnatural amino acid residue, though proved a direct interaction between nascent periplasmic proteins and SecY, strikingly, did not reveal any direct interaction between nascent OMPs and SecY (**Fig. 1**). We then demonstrated that precursors of OMPs but not of periplasmic proteins could be effectively processed under a SecY defective condition (**Fig. 2A**). Afterwards, we revealed that a shortened form of SecA that lacks its C-terminal region, thus designated as $SecA^N$, most likely functions as the translocon for nascent OMPs in living cells, as supported by the following observations in living cells. First, nascent OMPs but not periplasmic proteins interact with $SecA^N$ in living cells (**Fig. 3A**, **3C** and **S1**). Second, $SecA^N$ is present solely in the membrane fraction and likely as homo-oligomers (**Fig. 3B** and **3D**). Third, precursors of both OMPs and periplasmic proteins interact with the full length form of SecA, but not $SecA^N$ and SecY, respectively, in the LY928-secA81 mutant cells cultured under the non-permissive temperature of 42°C (**Fig. 4**). Fourth, $SecA^N$ directly interacts with BamA (**Fig 5A**), and reciprocally, BamA directly interacts with $SecA^N$ (**Fig. 5B**). Fifth, a BamA-OmpF-$SecA^N$ ternary complex was detected (**Fig. 6**). Sixth, a transmembrane region that contains a GXXXG motif, being conserved in SecA from other bacterial species, was identified in $SecA^N$ (**Fig. 7**, **Table 1**). Seventh, processing of precursors of OMPs relies largely on ATP but hardly on the transmembrane proton gradient, while that of periplasmic proteins relies on both ATP and transmembrane protein gradient as energy sources (**Fig. S5**).

Our observations described here, in combination with revelations reported before (*11-18, 38, 45, 64*), strongly implicate that SecA^N, instead of SecYEG, is responsible for translocating nascent OMPs across the inner membrane. Here, as depicted in **Fig. 8**, we emphasize the following points. First, as the translocon of nascent OMPs, SecA^N, likely exists as a homo-oligomer (shown as a dimer in **Fig. 8** although its exact oligomeric size remains to be resolved), is integrated into the inner membrane by interacting with a SecYEG protomer, which in turn interacts with a second SecYEG protomer that likely functions as the translocon for nascent periplasmic proteins (*65, 66*). Second, the full length form of SecA, in the cytoplasm (in both fully soluble and membrane associated forms) delivers the signal peptide-containing nascent OMPs and nascent periplasmic proteins to the membrane-integrated SecA^N and SecYEG translocons. Third, SecA^N is likely part of a supercomplex that we proposed earlier for the biogenesis of OMPs in living cells (*15*) and contain SecA, SecYEG, SurA, PpiD and BamA, spanning the cytoplasm, the inner membrane, the periplasm and the outer membrane.

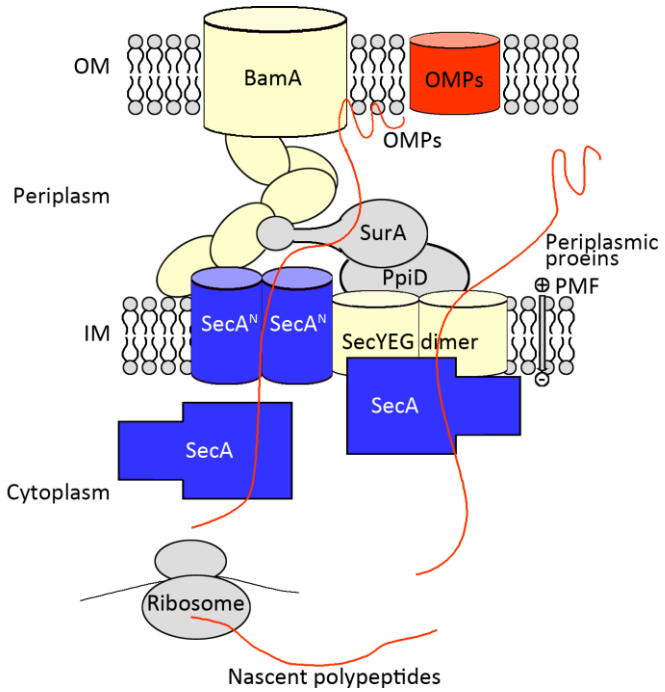

Figure 8. A model illustrating the respective translocation of nascent OMPs and nascent periplasmic proteins through SecA^N and SecYEG across the inner membrane in Gram-negative bacteria.

One has to ask why the SecA^N protein has been ignored in the past decades by people working on SecA. We speculate the following as possible reasons for this. First, it may have been considered as a degradation product due to its significantly smaller size than the full length form of SecA. Second, this form, almost wholly present in the membrane fraction (as shown in **Fig. 3D**), exists in the whole cell extract at a level far lower than that of the full length form of SecA, as shown by our data presented in **Fig. 3B**, as well as reported by others, who demonstrated that only about one sixth of the total amount of SecA is integrated into the inner membrane (*36, 38*). Third, since this

form is integrated into the inner membrane, it may have escaped the commonly performed immunoprecipitation analysis in the early days (*67*). Fourth, the co-existence of $SecA^N$ with some nearby non-specific protein bands added further confusion for its unequivocal identification (as shown in **Fig. 3**).

We searched the literatures in an attempt to seek for earlier hints on the presence of $SecA^N$ in bacterial cells. Interestingly, we did notice the presence of a SecA form with a size very close to $SecA^N$ that could either be immunoprecipitated by antibodies against the N-terminal region of SecA (as shown in lanes 7 and 8, Fig. 2, in (*68*)) or be detected in the inner membrane fraction of *E. coli* cells by antibodies against the full length SecA (Fig. 4 in (*42*)). Moreover, it was also reported that the N-terminal fragment of about 240 residues of the SecA protein from either *E coli* (*36*) or *B. Subtilis* (*69*) is integrated into the membrane. Many issues remain to be resolved for $SecA^N$. For example, how $SecA^N$, likely as homo-oligomers, forms the protein conducting channel in the inner membrane? Where and How $SecA^N$ is generated from the full length SecA? How $SecA^N$ interacts and works with full length SecA present in the cytoplasm?

In retrospect, the perception that SecYEG is directly responsible for translocating both OMPs and periplasmic proteins has been mainly derived from genetic and *in vitro* reconstitution studies (*25, 27*). Our study reported here represents an attempt to clarify this perception by performing experiments in living cells. Consistent with what was reported before, our data also indicate that SecYEG, though does not serve as the direct conducting channel, does play an important role for translocating nascent OMPs across the inner membrane in living cells (**Fig. 2**). This can be explained as follows. First, SecYEG is responsible for translocating such periplasm-located quality control factors as SurA for OMP biogenesis (*11-13, 15*). Second, SecYEG, as a highly hydrophobic membrane-integrated protein, most likely anchors $SecA^N$ (which possess only one putative transmembrane region) in the inner membrane. These points are supported by such reported observations as such that an overproduction of SecYEG resulted in an increased level of membrane-integrated SecA (*41*), and that the additional of SecYEG significantly increased the efficiency of SecA in functioning as protein-conducting channels in liposomes (*70*).

Unresolved issues regarding the relationship between SecYEG and $SecA^N$ include the following. Is $SecA^N$ indeed anchored in the inner membrane by SecYEG as we presumed here? Do $SecA^N$ and SecYEG have any functional coordination in translocating OMPs and periplasmic proteins (such that the two groups of proteins are produced in certain desired ratio)? How does the cytoplasmic full length SecA effectively partition nascent OMPs and nascent periplasmic proteins to $SecA^N$ and SecYEG, respectively? The molecular mechanism for $SecA^N$ to function as a protein-conducting channel, as well as the structural and functional interaction between $SecA^N$ and SecYEG undoubtedly merits further investigations.

Methods

Bacteria strains and plasmid constructions

All bacteria strains and plasmids used in this research are respectively listed in **Tables S1** and **S2**. The pYLC-OmpF, pYLC-BamA, pYLC-SurA or pYLC-SecA plasmid was constructed by isolating the encoding gene (including its promoter) via PCR, using *E. coli* genomic DNA as the template before the DNA fragment was inserted into the pYLC vector through restriction enzyme free cloning (*71*). The pYLC is a low copy plasmid that we derived from the pDOC plasmid (*15*). The site-specific mutagenesis for introducing the TAG amber codon into the specific gene in the plasmids to generate pBpA variant proteins was performed using the phusion site-directed mutagenesis kit (New England Biolabs, Massachusetts, USA).

pBpA mediated *in vivo* protein photo-crosslinking

The pYLC-OmpF or pYLC-SurA plasmids carrying the TAG mutations were respectively expressed in LA928-Δ*ompF* and LA928-Δ*surA* cells for *in vivo* protein photo-crosslinking analysis. The LA928 strain was generated by us for pBpA incorporation purposes as we described before (*15*). The cells were cultured at 37°C in LB medium containing pBpA (200 µM), grown to the mid-log phase, before irradiated with UV light (365 nm) for 10 min in a Hoefer UVC-500 crosslinker. The UV irradiated cells were harvested by centrifugation, resuspended in 100 µL SDS-PAGE loading buffer before boiled for 5 min. Protein samples were separated by SDS-PAGE before subjected to blotting analyses.

Separation of soluble and membrane fractions and UV irradiation

The separation of soluble and membrane fractions was performed according to (*36*) with some modifications. Briefly, the LY928 cell was cultured to mid-log phase, collected by centrifugation, resuspended and washed with PBS buffer, lysed (in PBS buffer containing 0.1 mM PMSF) by sonication before centrifuged at 10,000 x g for 5 min to remove the cell debris and un-lysed cells. The supernatant was then centrifuged at 22,000 x g for 20 min to separate the soluble and membrane fractions. Both fractions were irradiated with UV light (365 nm) for 10 min in a Hoefer UVC-500 crosslinker, before subjected to SDS-PAGE and blotting analyses.

Purification and mass spectrometry analysis of *in vivo* photocrosslinked product

Photo-crosslinked product of BamA-V121pBpA was purified by affinity chromatography using streptavidin resin. The eluted sample was resolved by SDS-PAGE before the gel was subjected to either blotting analysis or Coomassie Blue staining. The protein band at around 145 kD was then excised from the gel and applied for liquid chromatography-tandem mass spectrometry (LC-MS/MS) identification as we described before (*15*).

Chloramphenicol treatment of the SecY39 and SecA81 cells

A single colony of either the SecY39 or SecA81 mutant strain was inoculated in 3 mL of LB medium containing 50 µg/mL Kanamycin, then cultured at the permissive temperature of 37°C to the mid-log phase (as the control), or first incubated at the non-permissive temperature (20°C for

SecY39, 42°C for SecA81) for 1 hour, then treated with chloramphenicol (34 μg/mL) for 30 min or 1 hour. Treated cells were then collected by centrifugation, resuspended in loading buffer before incubated at 37°C for 10 min for semi native SDS-PAGE analysis. Protein samples were separated by SDS-PAGE before subjected to blotting analysis.

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Conflict of Interest

We declare that we have no conflict of interest to this work.

Author Contributions

Feng Jin designed and performed the experiments, Zengyi Chang designed some experiments. Feng Jin and Zengyi Chang analyzed the data and prepared the manuscript.

References

1. Palade, G. (1975) Intracellular aspects of the process of protein synthesis, *Science (New York, N.Y.) 189*, 867.
2. Rapoport, T. A., Jungnickel, B., and Kutay, U. (1996) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes, *Annual review of biochemistry 65*, 271-303.
3. Blobel, G. (2000) Protein targeting (Nobel lecture), *Chembiochem : a European journal of chemical biology 1*, 86-102.
4. Chang, Z. (2016) Biogenesis of Secretory Proteins. In: Ralph A Bradshaw and Philip D Stahl (Editor-in-Chief), Encyclopedia of Cell Biology, Vol 1, Waltham, MA: Academic Press, 535-544.
5. Wickner, W., and Schekman, R. (2005) Protein Translocation Across Biological Membranes, *Science (New York, N.Y.) 310*, 1452-1456.
6. Mitra, K., Frank, J., and Driessen, A. (2006) Co- and post-translational translocation through

the protein-conducting channel: analogous mechanisms at work?, *Nature structural & molecular biology 13*, 957-964.

7. Driessen, A. J., and Nouwen, N. (2008) Protein translocation across the bacterial cytoplasmic membrane, *Annual review of biochemistry 77*, 643-667.
8. Rigel, N. W., and Silhavy, T. J. (2012) Making a beta-barrel: assembly of outer membrane proteins in Gram-negative bacteria, *Current opinion in microbiology 15*, 189-193.
9. Rollauer, S. E., Sooreshjani, M. A., Noinaj, N., and Buchanan, S. K. (2015) Outer membrane protein biogenesis in Gram-negative bacteria, *Philosophical transactions of the Royal Society of London. Series B, Biological sciences 370*.
10. Chang, Z. (2016) The function of the DegP (HtrA) protein: Protease versus chaperone, *IUBMB life 68*, 904-907.
11. Lazar, S. W., and Kolter, R. (1996) SurA assists the folding of Escherichia coli outer membrane proteins, *Journal of bacteriology 178*, 1770-1773.
12. Rouviere, P. E., and Gross, C. A. (1996) SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins, *Genes & development 10*, 3170-3182.
13. Sklar, J. G., Wu, T., Kahne, D., and Silhavy, T. J. (2007) Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in Escherichia coli, *Genes & development 21*, 2473-2484.
14. Hagan, C. L., Kim, S., and Kahne, D. (2010) Reconstitution of outer membrane protein assembly from purified components, *Science (New York, N.Y.) 328*, 890-892.
15. Wang, Y., Wang, R., Jin, F., Liu, Y., Yu, J., Fu, X., and Chang, Z. (2016) A Supercomplex Spanning the Inner and Outer Membranes Mediates the Biogenesis of beta-Barrel Outer Membrane Proteins in Bacteria, *The Journal of biological chemistry 291*, 16720-16729.
16. Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M., and Tommassen, J. (2003) Role of a Highly Conserved Bacterial Protein in Outer Membrane Protein Assembly, *Science (New York, N.Y.) 299*, 262-265.
17. Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J., and Kahne, D. (2005) Identification of a multicomponent complex required for outer membrane biogenesis in Escherichia coli, *Cell 121*, 235-245.
18. Hagan, C. L., Silhavy, T. J., and Kahne, D. (2011) beta-Barrel membrane protein assembly by the Bam complex, *Annual review of biochemistry 80*, 189-210.
19. Rapoport, T. A. (2007) Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes, *Nature 450*, 663-669.
20. Deshaies, R. J., and Schekman, R. (1987) A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum, *The Journal of cell biology 105*, 633-645.
21. Gorlich, D., Prehn, S., Hartmann, E., Kalies, K. U., and Rapoport, T. A. (1992) A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation, *Cell 71*, 489-503.
22. Stirling, C. J., Rothblatt, J., Hosobuchi, M., Deshaies, R., and Schekman, R. (1992) Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum, *Molecular biology of the cell 3*, 129-142.
23. Emr, S. D., Hanley-Way, S., and Silhavy, T. J. (1981) Suppressor mutations that restore export of a protein with a defective signal sequence, *Cell 23*, 79-88.

24. Shultz, J., Silhavy, T. J., Berman, M. L., Fiil, N., and Emr, S. D. (1982) A previously unidentified gene in the spc operon of Escherichia coli K12 specifies a component of the protein export machinery, *Cell 31*, 227-235.
25. Ito, K., Wittekind, M., Nomura, M., Shiba, K., Yura, T., Miura, A., and Nashimoto, H. (1983) A temperature-sensitive mutant of E. coli exhibiting slow processing of exported proteins, *Cell 32*, 789-797.
26. Shiba, K., Ito, K., Yura, T., and Cerretti, D. P. (1984) A defined mutation in the protein export gene within the spc ribosomal protein operon of Escherichia coli: isolation and characterization of a new temperature-sensitive secY mutant, *The EMBO journal 3*, 631-635.
27. Brundage, L., Hendrick, J. P., Schiebel, E., Driessen, A. J. M., and Wickner, W. (1990) The purified E. coli integral membrane protein SecYE is sufficient for reconstitution of SecA-dependent precursor protein translocation, *Cell 62*, 649-657.
28. Berg, B. v. d., Clemons, W. M., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., and Rapoport, T. A. (2004) X-ray structure of a protein-conducting channel, *Nature 427*, 36-44.
29. Li, L., Park, E., Ling, J., Ingram, J., Ploegh, H., and Rapoport, T. A. (2016) Crystal structure of a substrate-engaged SecY protein-translocation channel, *Nature 531*, 395-399.
30. Joly, J. C., and Wickner, W. (1993) The SecA and SecY subunits of translocase are the nearest neighbors of a translocating preprotein, shielding it from phospholipids, *The EMBO journal 12*, 255-263.
31. Cannon, K. S., Or, E., Clemons, W. M., Jr., Shibata, Y., and Rapoport, T. A. (2005) Disulfide bridge formation between SecY and a translocating polypeptide localizes the translocation pore to the center of SecY, *The Journal of cell biology 169*, 219-225.
32. Oliver, D. B., and Beckwith, J. (1981) E. coli mutant pleiotropically defective in the export of secreted proteins, *Cell 25*, 765-772.
33. Oliver, D. B., and Beckwith, J. (1982) Identification of a new gene (secA) and gene product involved in the secretion of envelope proteins in Escherichia coli, *Journal of bacteriology 150*, 686-691.
34. Cabelli, R. J., Chen, L., Tai, P. C., and Oliver, D. B. (1988) SecA protein is required for secretory protein translocation into E. coli membrane vesicles, *Cell 55*, 683-692.
35. Cunningham, K., Lill, R., Crooke, E., Rice, M., Moore, K., Wickner, W., and Oliver, D. (1989) SecA protein, a peripheral protein of the Escherichia coli plasma membrane, is essential for the functional binding and translocation of proOmpA, *The EMBO journal 8*, 955-959.
36. Cabelli, R. J., Dolan, K. M., Qian, L. P., and Oliver, D. B. (1991) Characterization of membrane-associated and soluble states of SecA protein from wild-type and SecA51(TS) mutant strains of Escherichia coli, *The Journal of biological chemistry 266*, 24420-24427.
37. Ulbrandt, N. D., London, E., and Oliver, D. B. (1992) Deep penetration of a portion of Escherichia coli SecA protein into model membranes is promoted by anionic phospholipids and by partial unfolding, *The Journal of biological chemistry 267*, 15184-15192.
38. Chen, X., Xu, H., and Tai, P. C. (1996) A significant fraction of functional SecA is permanently embedded in the membrane. SecA cycling on and off the membrane is not essential during protein translocation, *The Journal of biological chemistry 271*, 29698-29706.
39. Eichler, J., and Wickner, W. (1997) Both an N-terminal 65-kDa domain and a C-terminal 30-kDa domain of SecA cycle into the membrane at SecYEG during translocation, *Proceedings of the National Academy of Sciences of the United States of America 94*, 5574-5581.

40. Young Jae, K., Rajapandi, T., and Oliver, D. (1994) SecA protein is exposed to the periplasmic surface of the E. coli inner membrane in its active state, *Cell 78*, 845-853.
41. van der Does, C., den Blaauwen, T., de Wit, J. G., Manting, E. H., Groot, N. A., Fekkes, P., and Driessen, A. J. (1996) SecA is an intrinsic subunit of the Escherichia coli preprotein translocase and exposes its carboxyl terminus to the periplasm, *Molecular microbiology 22*, 619-629.
42. Ramamurthy, V., and Oliver, D. (1997) Topology of the Integral Membrane Form of Escherichia coli SecA Protein Reveals Multiple Periplasmically Exposed Regions and Modulation by ATP Binding, *Journal of Biological Chemistry 272*, 23239-23246.
43. Economou, A., and Wickner, W. (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion, *Cell 78*, 835-843.
44. Economou, A., Pogliano, J. A., Beckwith, J., Oliver, D. B., and Wickner, W. (1995) SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF, *Cell 83*, 1171-1181.
45. Yang, Y. B., Lian, J., and Tai, P. C. (1997) Differential translocation of protein precursors across SecY-deficient membranes of Escherichia coli: SecY is not obligatorily required for translocation of certain secretory proteins in vitro, *Journal of bacteriology 179*, 7386-7393.
46. Hsieh, Y. H., Zhang, H., Lin, B. R., Cui, N., Na, B., Yang, H., Jiang, C., Sui, S. F., and Tai, P. C. (2011) SecA alone can promote protein translocation and ion channel activity: SecYEG increases efficiency and signal peptide specificity, *The Journal of biological chemistry 286*, 44702-44709.
47. Zhang, H., Hsieh, Y. H., Lin, B. R., Yu, L., Yang, H., Jiang, C., Sui, S. F., and Tai, P. C. (2013) Specificity of SecYEG for PhoA precursors and SecA homologs on SecA protein-conducting channels, *Biochemical and biophysical research communications 437*, 212-216.
48. Tormo, A., Almiron, M., and Kolter, R. (1990) surA, an Escherichia coli gene essential for survival in stationary phase, *Journal of bacteriology 172*, 4339-4347.
49. Chin, J. W., Martin, A. B., King, D. S., Wang, L., and Schultz, P. G. (2002) Addition of a photocrosslinking amino acid to the genetic code of Escherichiacoli, *Proceedings of the National Academy of Sciences of the United States of America 99*, 11020-11024.
50. Zhang, M., Lin, S., Song, X., Liu, J., Fu, Y., Ge, X., Fu, X., Chang, Z., and Chen, P. R. (2011) A genetically incorporated crosslinker reveals chaperone cooperation in acid resistance, *Nature chemical biology 7*, 671-677.
51. Fu, X., Shi, X., Yan, L., Zhang, H., and Chang, Z. (2013) In vivo substrate diversity and preference of small heat shock protein IbpB as revealed by using a genetically incorporated photo-cross-linker, *The Journal of biological chemistry 288*, 31646-31654.
52. Ge, X., Wang, R., Ma, J., Liu, Y., Ezemaduka, A. N., Chen, P. R., Fu, X., and Chang, Z. (2014) DegP primarily functions as a protease for the biogenesis of beta-barrel outer membrane proteins in the Gram-negative bacterium Escherichia coli, *The FEBS journal 281*, 1226-1240.
53. Baba, T., Jacq, A., Brickman, E., Beckwith, J., Taura, T., Ueguchi, C., Akiyama, Y., and Ito, K. (1990) Characterization of cold-sensitive secY mutants of Escherichia coli, *Journal of bacteriology 172*, 7005-7010.
54. Rosenbaum, M., Halvorson, H., Preston, W. S., and Levinthal, C. (1955) Developmental of phage T2 desoxyribosenucleic acid in the absence of net protein synthesis, *Journal of bacteriology 69*, 228-229.

55. Schagger, H. (2006) Tricine-SDS-PAGE, *Nature protocols 1*, 16-22.
56. Malencik, D. A., and Anderson, S. R. (1987) Dityrosine formation in calmodulin, *Biochemistry 26*, 695-704.
57. Nakatogawa, H., Mori, H., Matsumoto, G., and Ito, K. (2000) Characterization of a mutant form of SecA that alleviates a SecY defect at low temperature and shows a synthetic defect with SecY alteration at high temperature, *Journal of biochemistry 127*, 1071-1079.
58. Gelis, I., Bonvin, A. M., Keramisanou, D., Koukaki, M., Gouridis, G., Karamanou, S., Economou, A., and Kalodimos, C. G. (2007) Structural basis for signal-sequence recognition by the translocase motor SecA as determined by NMR, *Cell 131*, 756-769.
59. Maegawa, S., Ito, K., and Akiyama, Y. (2005) Proteolytic action of GlpG, a rhomboid protease in the Escherichia coli cytoplasmic membrane, *Biochemistry 44*, 13543-13552.
60. Akiyama, Y., and Maegawa, S. (2007) Sequence features of substrates required for cleavage by GlpG, an Escherichia coli rhomboid protease, *Molecular microbiology 64*, 1028-1037.
61. Hofmann, K. (1993) TMBASE-A database of membrane spanning protein segments, *Biol. Chem. Hoppe-Seyler 374*, 166.
62. Hu, H. J., Holley, J., He, J., Harrison, R. W., Yang, H., Tai, P. C., and Pan, Y. (2007) To be or not to be: predicting soluble SecAs as membrane proteins, *IEEE transactions on nanobioscience 6*, 168-179.
63. Teese, M. G., and Langosch, D. (2015) Role of GxxxG Motifs in Transmembrane Domain Interactions, *Biochemistry 54*, 5125-5135.
64. Yang, Y. B., Yu, N., and Tai, P. C. (1997) SecE-depleted membranes of Escherichia coli are active. SecE is not obligatorily required for the in vitro translocation of certain protein precursors, *The Journal of biological chemistry 272*, 13660-13665.
65. Bessonneau, P., Besson, V., Collinson, I., and Duong, F. (2002) The SecYEG preprotein translocation channel is a conformationally dynamic and dimeric structure, *The EMBO journal 21*, 995-1003.
66. Breyton, C., Haase, W., Rapoport, T. A., Kuhlbrandt, W., and Collinson, I. (2002) Three-dimensional structure of the bacterial protein-translocation complex SecYEG, *Nature 418*, 662-665.
67. Liebke, H. H. (1987) Multiple SecA protein isoforms in Escherichia coli, *Journal of bacteriology 169*, 1174-1181.
68. Oliver, D. B., and Beckwith, J. (1982) Regulation of a membrane component required for protein secretion in Escherichia coli, *Cell 30*, 311-319.
69. Takamatsu, H., Nakane, A., Oguro, A., Sadaie, Y., Nakamura, K., and Yamane, K. (1994) A Truncated Bacillus subtilis SecA Protein Consisting of the N-Terminal 234 Amino Acid Residues Forms a Complex with Escherichia coli SecA51(ts) Protein and Complements the Protein Translocation Defect of the secA51 Mutant, *The Journal of Biochemistry 116*, 1287-1294.
70. Hsieh, Y.-h., Zhang, H., Wang, H., Yang, H., Jiang, C., Sui, S.-f., and Tai, P. C. (2013) Reconstitution of functionally efficient SecA-dependent protein-conducting channels: Transformation of low-affinity SecA-liposome channels to high-affinity SecA-SecYEG-SecDF·YajC channels, *Biochemical and biophysical research communications 431*, 388-392.
71. Lee, D. J., Bingle, L. E., Heurlier, K., Pallen, M. J., Penn, C. W., Busby, S. J., and Hobman, J. L.

(2009) Gene doctoring: a method for recombineering in laboratory and pathogenic Escherichia coli strains, *BMC microbiology 9*, 252.