Revealing a shortened version of SecA ($SecA^N$) that conceivably functions as a protein-conducting channel in the bacterial cytoplasmic membrane

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During biogenesis, nascent polypeptides of many proteins have to be translocated across biological membranes by relying on specific protein-conducting channels. It remains a great challenge to unequivocally identify the specific membrane-integrated channel proteins that translocate particular client proteins in living cells. In Gram-negative bacteria, proteins destined to the periplasmic compartment or outer membrane are all synthesized in the cytoplasm and have to be translocated across the inner (i.e., the cytoplasmic) membrane. The currently prevailing perception is that all these transmembrane translocations occur by using the same SecY channel on the inner membrane. Nevertheless, this perception, formed largely based on genetic and *in vitro* studies, has not yet been proved by direct analysis in living cells. Here, mainly via a systematic *in vivo* protein photo-crosslinking analyses mediated by a genetically incorporated unnatural amino acid, we revealed that, in contrary to the long-held view, nascent polypeptides of β-barrel OMPs are not translocated across the inner membrane via the SecY channel, but through a shortened version of SecA, designated as $SecA^N$, which exists as a membrane-integrated homo-oligomers. Furthermore, we demonstrated that $SecA^N$ is most likely part of the supercomplex that we revealed earlier as one which is responsible for the biogenesis of β-barrel OMPs in living cells and spans the cytoplasm, the inner membrane, the periplasm and the outer membrane.

Introduction

Many proteins have to be translocated across biological membranes during their biogenesis in both eukaryotic and prokaryotic cells (*1-4*). It has been revealed that such transmembrane translocations are commonly accomplished via the work of membrane-integrated protein-conducting channels (*2, 4-7*). In Gram-negative bacteria, nascent polypeptides of both outer membrane proteins and periplasmic proteins, all being synthesized by the cytoplasmic ribosomes , are believed to be mainly translocated across the inner (i.e., cytoplasmic) membrane through the SecY protein-conducting channel present in the SecYEG translocon either in a co- or post-translational manner (*2, 6, 7*). In the outer membrane of Gram negative bacteria (as well as that of eukaryotic mitochondria and chloroplasts), the major type of proteins are the β-barrel outer membrane proteins (β-barrel OMPs), which primarily comprise β-sheets that adopt a unique, highly stable cylindrical, barrel-like topology, and function in a variety of biological processes (*8, 9*). The nascent β-barrel OMPs, after crossing the inner membrane, will be further facilitated by protein factors located in the periplasmic compartment and on the outer membrane before reaching their final destination and to fold/assemble into their functional forms (*10-12*). Among these protein factors, SurA has been identified as the primary chaperone for escorting nascent β-barrel OMPs through the periplasmic compartment and for delivering them to the outer membrane (*13-17*). Additionally, the β-barrel assembly machine (BAM) complex, in which the BamA protein is a major component, has been found to be essential for the biogenesis of β-barrel OMPs (*18-20*). BamA has been revealed to be a protein that is integrated into the outer membrane via its C-terminal part but extends into the periplasmic compartment via its N-terminal POTRA domains (*21, 22*).

According to the currently prevailing perception, translocations of all nascent β-barrel OMPs and periplasmic proteins across the inner membrane in Gram-negative bacteria are largely accomplished by employing the same SecYEG translocon, whose membrane-integrated SecY protein is presumed to act as a protein-conducting channel (*2, 6, 7*). In addition, the cytoplasm-located motor protein SecA is considered to provide the driving force for the SecYEG translocon (*23*). As a highly conserved protein, SecY has its homologue in eukaryotic cells as the Sec61 protein that is integrated into the membrane of the endoplasmic reticulum (*2-5, 24-26*). The *secY* gene was initially identified as one whose suppressor mutations restored the export of β-barrel OMPs or periplasmic proteins that have a defective signal peptide (*27, 28*), as well as one whose defect resulted in a slow processing for precursors of both β-barrel OMPs and periplasmic proteins (*29, 30*). The SecY protein, by association with the SecE protein, was later found to act together with the SecA protein in supporting the translocation of the precursor form of an outer membrane protein across a reconstituted inner membrane vesicle (*31*). The protein-conducting channel is believed to be formed by the SecY protein largely based on its crystal structure (*32, 33*) as well as *in vitro* chemical crosslinking studies (*34, 35*).

The *secA* gene was initially identified as one whose defective mutation resulted in the accumulation of precursors of both β-barrel OMPs and periplasmic proteins (*36, 37*). The SecA protein was later found to be essential for translocation of both β-barrel OMPs and periplasmic proteins (*38, 39*). Subsequently, SecA was characterized to present both as a peripheral inner membrane protein (*40*) and as a cytoplasmic protein (*41*). Other studies showed that a portion of SecA is integrated in the inner membrane (*42-45*) and even exposed to the periplasmic compartment (*46-48*). The meaning of such various subcellular locations of SecA awaits further clarification. Furthermore, the SecA protein was proposed to undergo ATP-driven cycles of membrane insertion and de-insertion (*49, 50*). Additionally, it has also been reported that SecA alone, without SecYEG, is able to promote translocation of β-barrel OMPs across inner membrane vesicles or artificial lipid bilayers under *in vitro* conditions (*51-53*).

Our present understanding on how newly-synthesized proteins are exported across the inner membrane mainly stems from the genetic or *in vitro* studies. The genetic studies have resulted in the isolation of numerous export-defective mutants (*14, 15, 18, 19, 24, 26-30, 36, 37, 54*). The *in vitro* studies have exploited the cell-free synthesis/transport systems to characterize whether a protein factor plays a role for translocating unfolded substrate proteins across membrane vesicles (*16, 31, 52, 53*). Nevertheless, these commonly-taken approaches, though powerful, were unable to elaborate the exact function of each protein factor, or to pinpoint which specific client protein is actually translocated by which particular channel in living cells. For example, these approaches were unable to unequivocally clarify which membrane protein (e.g., SecY or others) on the inner membrane functions as the actual protein-conducting channel for a particular client protein, being an outer membrane protein, a periplasmic protein. In regard to the biogenesis of β-barrel 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 instance, if the prevailing perception is correct, then it is difficult to imagine how a single SecY channel translocates the nascent polypeptides of such dramatically different clients as β-barrel OMPs, periplasmic proteins. Besides, what are the structural and functional differences for the various subcellular forms of SecA?

In this study, we decided to take a more direct approach in an attempt to gain insights on the exact functions of such protein factors as SecY and SecA for protein export in Gram-negative bacterial cells. In particular, we performed a series of *in vivo* protein photo-crosslinking studies as mediated by genetically incorporated unnatural amino acid (*55*), an approach that we have been using over the past years (*17, 56-58*). Here, we first observed that although nascent polypeptides of periplasmic proteins directly interact with SecY, those of β-barrel OMPs does not. Further, we observed that precursors of β-barrel OMPs, but not of periplasmic proteins, could still be effectively processed when SecY becomes defective. Subsequently, we unveiled a shortened version of SecA that we designate as $SecA^{N}$, which directly interacts with not only nascent β-barrel OMPs but also the periplasmic region of BamA. In addition,

we demonstrated that the $SecA^N$ most likely exists as a homo-oligomer that is integrated in the inner membrane. These observations strongly implicate that our newly revealed $SecA^N$ protein, rather than the commonly believed SecYEG, functions as the translocon for translocating the nascent β-barrel OMPs in living bacterial cells.

Results

Nascent β-barrel OMPs do not, although nascent periplasmic proteins do, directly interact with SecY in living cells.

We first tried to clarify whether or not nascent polypeptides of both periplasmic proteins and β-barrel OMPs are translocated across the inner membrane through the SecYEG translocon (in which SecY is considered as the protein-conducting channel) in living cells, as commonly documented in the literatures (*2, 5, 7, 11*). For this purpose, we individually introduced the unnatural amino acid pBpa (*p*-benzoyl-L-phenylalanine) (*55-58*) at 21 somehow randomly selected residue positions across the polypeptide of nascent OmpF (an OMP) or 10 positions in nascent SurA (a periplasmic protein) before analyzing whether any of them interacts with SecY as revealed by photo-crosslinking analysis in living cells. Prior to the UV-irradiation, each pBpa variant of OmpF or SurA was first respectively expressed in the *ompF*-deleted or *surA*-deleted LY928 bacterial strain. The genome of LY928 strain was modified to encode the orthogonal amino-acyl tRNA synthetase and the orthogonal tRNA that are needed for pBpa incorporation, as we reported earlier (*17*).

With the pBpa variants of OmpF, our blotting analyses, however, failed to detect any photo-crosslinked product between nascent OmpF and the SecY channel (OmpF-SecY), which would appear as a band of ~ 89 kD, the combined molecular mass of OmpF (~ 40 kD) and SecY (~ 49 kD) (**Fig. 1A**; displayed are results of 12 of the 21 variants of OmpF). In contrast to this, for the pBpa variants of SurA, we successfully detected the photo-crosslinked product between nascent SurA and the SecY channel (SurA-SecY) of ~ 94 kD, the combined mass of SurA (~ 45 kD) and SecY, when pBpa was introduced at position 8 or 12 (in the signal peptide) of SurA (lanes 2 and 4, **Fig. 1B**). It is noteworthy to mention that we failed to detect any photo-crosslinked SurA-SecY product with pBpa variants in which the pBpa residue was placed at the mature part of SurA (lanes 8-11, **Fig. 1B**). The meaning of this is worth further investigations. Collectively, these observations indicate that, contrary to what has been commonly believed, only nascent polypeptides of periplasmic proteins, but not of β-barrel OMPs, directly interact with the SecY channel in living cells.

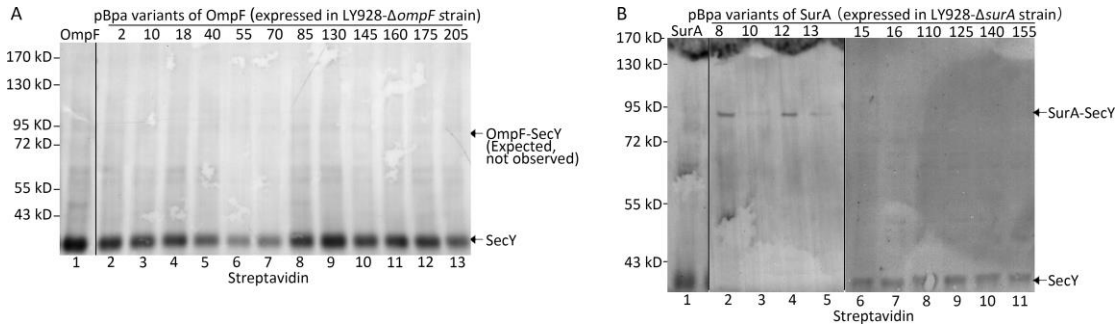


Figure 1. Nascent β-barrel OMPs (as represented by OmpF) do not, although nascent periplasmic proteins (as represented by SurA) do, directly interact with SecY in living cells.

(**A, B**) Blotting results for detecting photo-crosslinked 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* strain, probed with the streptavidin-alkaline phosphatase conjugate (streptavidin-AP conjugate), against the Avi tag that was linked to the SecY protein. Here, the residue positions are numbered by including the signal peptide. Protein samples were resolved by SDS-PAGE before being blotted. Samples of cells expressing the wild type OmpF or SurA (with no pBpa incorporation) were analyzed here as negative controls for the photo-crosslinking (lanes 1 in **A** and **B**). Indicated on the right of each gel are the positions of SecY, photo-crosslinked 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 β-barrel OMPs can be far more effectively processed than that of periplasmic proteins when SecY is defective.

To further clarify the role of the SecY channel in translocating β-barrel OMPs and periplasmic proteins, we tried to examine how the processing of their precursors would be affected when SecY becomes defective in the cells. For this purpose, we made use of the cold sensitive mutant strain, SecY39, in which protein export was previously reported to be only partially retarded at the permissive temperature of 37°C but dramatically retarded at the non-permissive temperature of 20°C due to a defect of the SecY protein (*59*). Our immunoblotting analyses, as shown in **Fig. 2A**, revealed that the processing of precursors of β-barrel OMPs, was far less defective when compared with that of periplasmic proteins in the SecY39 cells at such non-permissive condition.

This is first reflected by a significantly lower level of accumulation of precursors for β-barrel OMPs (pre-OmpA and pre-OmpF) than for periplasmic proteins (pre-MBP and pre-SurA) after the culturing temperature of the SecY39 strain was shifted from the permissive 37°C to the non-permissive 20°C and inoculated for 1 hour (lane 2, **Fig 2A**). Specifically, only a minor portion of the β-barrel OMPs, but at least half of the periplasmic proteins existed as precursor forms (lane 2, **Fig. 2A**). It is also reflected by a significantly more efficient processing of the accumulated precursors for β-barrel

OMPs (converting to their folded forms) than for periplasmic proteins (converting to their mature forms) during the 1 hour chasing period after protein synthesis in the cells was inhibited by the added chloramphenicol (*60*), as shown by data displayed in lanes 2-4 in **Fig 2A**. Specifically, most of the precursors for the β-barrel OMPs were converted to their folded forms, but little precursors for the periplasmic proteins were converted to their mature forms after the 1 hour chasing (lane 4, **Fig. 2A**). For comparison, when the SecY39 strain was cultured at the permissive temperature of 37°C, although the precursors of periplasmic proteins were accumulated (lane 2, **Fig. 2B**) to similar degree as shifted and incubated at 20°C for 1 hour (lane 2, **Fig. 2A**), their processing now becomes far more efficient, with little remain as precursors after the 1 hour chasing (lane 4, **Fig. 2B**). By contrast, both the accumulation (lane 2, **Fig. 2B**) and the processing of precursors for β-barrel OMPs after the 1 hour chasing (lane 4, **Fig. 2B**) observed at 37°C are largely comparable with those observed at 20°C (**Fig. 2A**).

Taken together, these observations indicate that the processing of precursors for β-barrel OMPs relies on the SecY channel in a far less degree than that for periplasmic proteins. These results are highly consistent with our *in vivo* protein photo-crosslinking results presented above in **Fig. 1**, indicating that nascent polypeptides of β-barrel OMPs do not, although periplasmic proteins do, directly interact with SecY in living cells. Furthermore, our results are 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 (*59*).

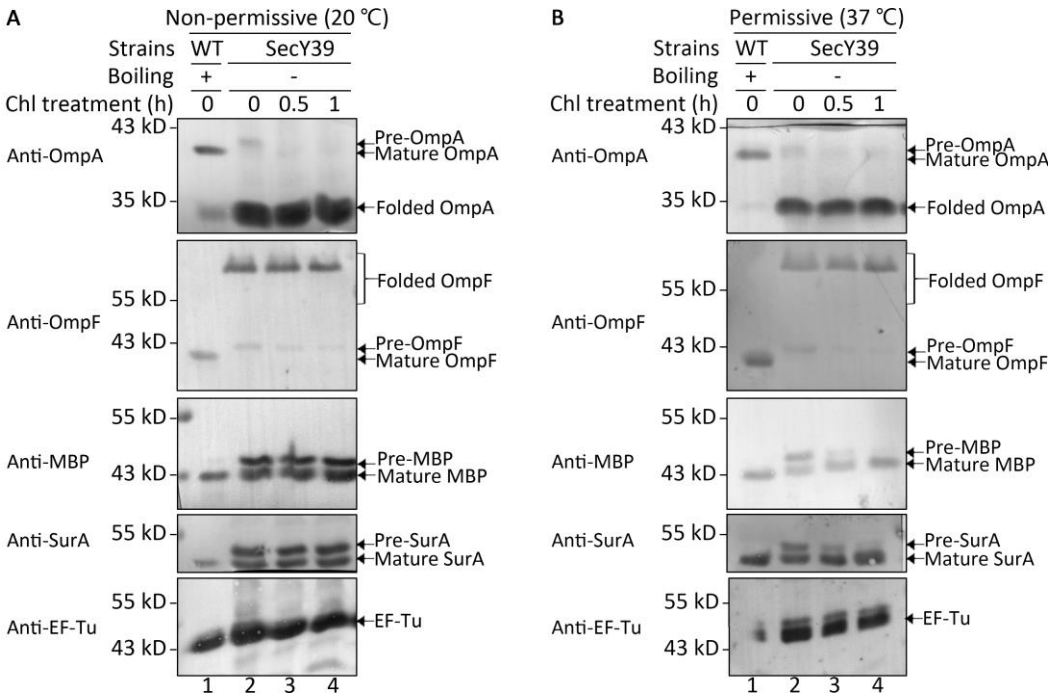


Figure 2. Precursors of β-barrel OMPs can be far more effectively processed than that of periplasmic proteins when SecY is defective.

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

Nascent polypeptides of β-barrel OMPs but not of periplasmic proteins interact with a shortened version of SecA (SecA^{N}).

Our results described above indicate that the SecY channel may translocate nascent periplasmic proteins, but unlikely the nascent β-barrel OMPs, across the inner membrane in living cells. Given the previous reports showing that a portion of the cellular SecA is embedded in the inner membrane (*42-45*), we then asked whether or not there is any possibility of this membrane-embedded form of SecA acting as the translocon for nascent β-barrel OMPs in living cells. For this to be true, such SecA proteins would have to directly interact with nascent β-barrel OMPs.

To this end, we first analyzed whether or not any of the aforementioned 21 pBpa variants of OmpF could form photo-crosslinked product with SecA in living cells. The immunoblotting result, probed with anti-SecA antibodies (with that of 5 representative OmpF variants being displayed in **Fig 3A)**, however, did not reveal any potential photo-crosslinked product between nascent OmpF and the SecA protein (OmpF-SecA), which would be ~ 142 kD, as the combined molecular mass of OmpF (~ 40 kD) and SecA (~ 102 kD). Nevertheless, the results revealed two unexpected photo-crosslinked product bands, one appeared to be ~ 80 kD and visible in most samples of pBpa variants of OmpF (indicated by the filled arrowheads in **Fig 3A**), the other appeared to be ~65 kD and visible in all cell samples (indicated by the non-filled arrowheads in **Fig 3A)**. Of note, these two bands were undetectable in samples of cells that were not exposed to UV light (**Fig. 3A**); the ~65 kD band was apparently unrelated to pBpa-mediated photo-crosslinking because it was also detected in the wild type OmpF control sample (lane 1, **Fig. 3A**). At this point, the most plausible explanations for these two unexpected photo-crosslinked products are such that the ~80 kD band represented a photo-crosslinked product between the pBpa variants of nascent OmpF (~40 kD) and a shortened version of SecA (with an apparent molecular mass of ~45 kD), while the ~65 kD band in turn represented a photo-crosslinked dimer of the shortened version of SecA (both explanations to be

further substantiated by multiple other results described below).

In light of these photo-crosslinking results, we next tried to elucidate whether or not the presumed shortened version of SecA indeed exists. For this purpose, we decided to analyze protein samples of the LY928 cells that neither expressing any pBpa variants of β-barrel OMPs nor being subjected to UV irradiation, as well as to employ a separating gel (of higher acrylamide concentration) that would resolve smaller proteins with a higher resolution (*61*). Remarkably, our immunoblotting analysis now clearly revealed one shortened version of SecA with an apparent size of ~ 45 kD that 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**). This indicates that this shorted version of SecA only contains the N-terminal region of the full length form of SecA, we thus designate it as $SecA^N$. In correlation to this, the ~ 80 kD photo-crosslinked product for the OmpF-G18pBpa variant was also detectable only with antibodies against the N-terminal region (lane 1), not with antibodies against the C-terminal region of SecA (lane 3), as shown in **Fig 3C**. It is noteworthy that we failed to detect photo-crosslinked products between any of the 10 pBpa variant of nascent SurA (as a representative of periplasmic protein) and either the full length SecA or $SecA^N$ (lanes 1, 3, 5, 7, 9, 11 and 13-16, **Fig. S1**). Interestingly, the ~ 65 kD photo-crosslinked product was once again detected in all the UV-irradiated samples (indicated by the arrowheads in **Fig. S1**), including the one in which no pBpa variants of SurA was expressed (lane 17, **Fig. S1**).

Taken together, these results strongly suggest the existence of a shortened version of SecA, $SecA^N$, which interacts with the nascent β-barrel OMPs, but not nascent periplasmic proteins, in living cells. Additionally, this $SecA^N$ is able to form a photo-crosslinked dimer (of ~ 65 kD) upon UV irradiation, thus likely exists as homo-oligomers in living cells. Such photo-crosslinked dimer of $SecA^N$ could be formed through an interacting pair of tyrosine residues at the interaction surfaces of the homo-oligomers, as observed in other proteins (*62*). In regards to the observation that the apparent size of the photo-crosslinked $SecA^N$ dimer is ~ 65 kD, instead of the expected ~ 90 kD (2 X 45 kD), it should be noted that the apparent electrophoretic mobilization rates of such photo-crosslinked products may differ significantly depending on the exact locations of the two residues that form the cross-linkage in the polypeptide chains, as observed before for other proteins (*63*).

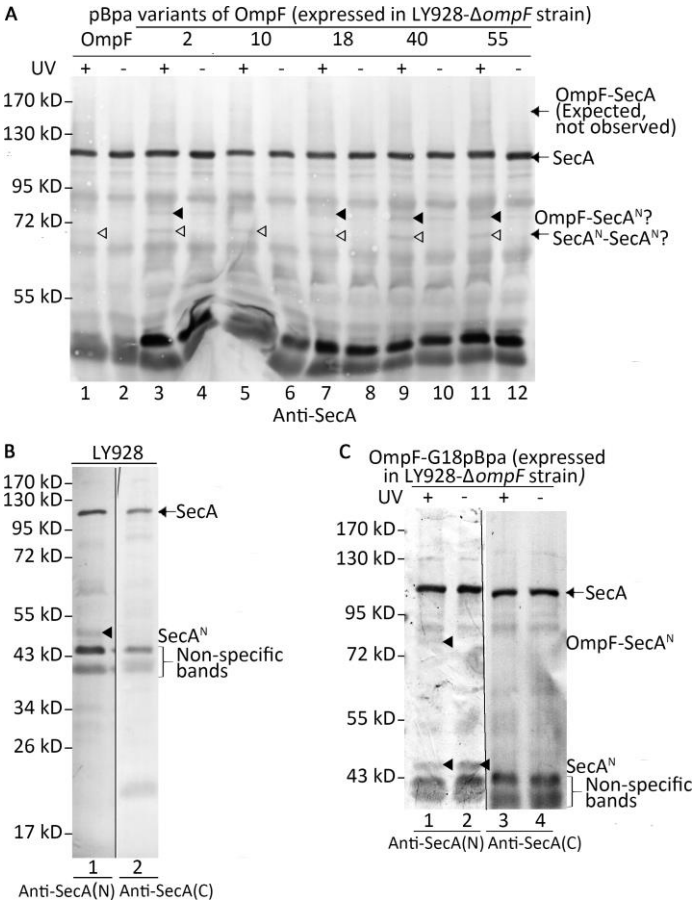


Figure 3. Nascent β-barrel OMPs but not nascent periplasmic proteins interact with a shortened version of SecA (SecA^N).

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

(**B**) Immunoblotting results for detecting the shortened version of SecA (SecA^N) after resolving on a high resolution gel, probed with antibodies against the N- (lane 1) or C-terminal (lane 2) region of SecA.

(**C**) Immunoblotting results for detecting the photo-crosslinked product between OmpF-G18pBpa and SecA^N, using antibodies against the N- (lane 1) or C-terminal (lane 3) region of SecA.

All protein samples were resolved by SDS-PAGE before subjecting to immunoblotting. The polymerized separating gel in (**A**) and (**C**) is 8%, while in (**B**) is 10%. Residue positions for OmpF (in panels **A** and **C**) 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 panel **A**). Indicated on the right of each gel are the positions of SecA, the shortened version of SecA (SecA^N), photo-crosslinked OmpF-SecA^N, and SecA^N-SecA^N; on the left are positions of molecular weight markers.

SecA^N is solely detected in the membrane fraction and apparently exists as homo-oligomers.

We subsequently tried to find out whether or not SecA^N is located in the membrane fraction, as to be required for it to function as a protein-conducting channel of nascent β-barrel OMPs. For this purpose, we first separated the membrane and soluble fractions of the cell lysates by differential centrifugation, according to a previously reported protocol (*42*). Our immunoblotting results, shown in **Fig 4**, clearly demonstrate that the SecA^N protein was detected almost wholly in the membrane fraction (lane 2), little in the soluble fraction (lane 5). Interestingly, similar to the membrane-integrated OmpF trimer (e.g., lane 1 vs lanes 2-4, **Fig. 2A**), we also observed that the SecA^N in the membrane fraction was detected as a smear on the gel if the membrane fraction was not boiled (lane 3, **Fig. 4**), with a significantly lower mobility than its monomeric form as detected in the boiled samples (lane 2, **Fig. 4**). This again indicates that SecA^N, like OmpF, is most likely a membrane-integrated protein that exists as homo-oligomers. This conclusion is further supported by our observation that crosslinked SecA^N dimers could also be effectively produced by directly subjecting the membrane fraction to UV exposure (lanes 1, **Fig. 4**) and that the SecA^N protein remained in the membrane fraction even after being treated with 8 M urea (lane 3, **Fig S2**) (*42*).

We next tried to purify the SecA^N protein from the membrane fraction of *E. coli* cells for further characterization, but without much success. We therefore decided to resolve the membrane fraction by SDS-PAGE, and to directly excise the gel slice around the ~ 45 kD position, where we identified SecA^N (as in lane 2, **Fig. 4**), for mass spectrometry analysis. As listed in **Table S1**, multiple matched SecA fragments were identified from this gel slice, confirming the existence of the SecA protein at this position of the gel. In addition, all the matched SecA fragments are derived from the N-terminal region, with the matching fragment closest to the C-terminus of the full length SecA to be ^{586}FYLSMEDALMR^{596} (**Table S1**). For comparison, we performed similar mass spectrometry analysis of the gel slice excised from the ~100 kD position, where the full length form of SecA was detected (as shown in lane 5, **Fig. 4**), after the soluble fraction was resolved by SDS-PAGE. Here, the results revealed a spectrum of matched peptide fragments spreading across the whole polypeptide chain of the full length SecA (**Table S1**).

In light of this, it is conceivable that SecA^N is generated from the full length SecA by making a cleavage at around Arginine 596. It follows that the molecular size of the SecA^N, assuming it to be ended at residue 596, would be ~65 kD (calculated by taking the average molecular weight of each amino acid reside as ~ 110 D), significantly larger than the apparent size of ~45 kD as estimated from its gel position (as shown in **Figs. 3B**, **3C** and **4**). Such discrepancy between the actual size calculated from its actual amino acid composition and the apparent size estimated from their gel position is not unusual for membrane proteins. For example, the membrane-integrated SecY protein always mobilizes to a position of ~ 30 kD on the

gel, as shown in **Fig. 1A** (also refer to lane 1, Fig. 2B in (*31*)), although its actual molecular size is 48.5 kD. It worth further investigation whether or not this cleavage of SecA is made by GlpG, a serine proteinase that is integrated in the inner membrane of bacterial cells (*64, 65*).

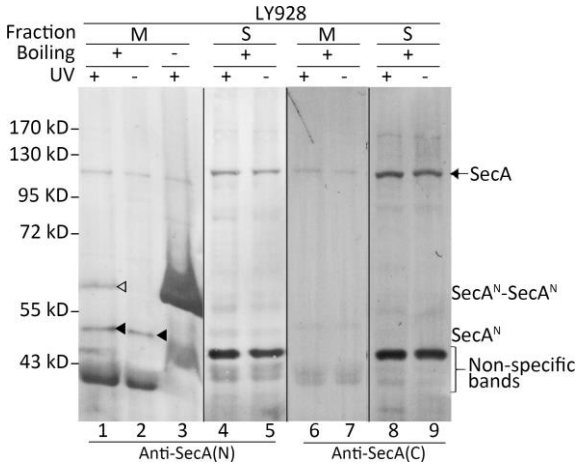


Figure 4. SecA^N is solely detected in the membrane fraction and apparently exists as homo-oligomers.

Immunoblotting results for detecting the forms of SecA in the membrane (M) and soluble (S) fractions of cell lysates of the LY928 bacterial strain, with or without UV irradiation, probed with antibodies against the N- (lanes 1-5) or C-terminal (lanes 6-9) region of SecA. The polymerized separating gel is 10%. Indicated on the right of the gel are the positions of SecA, SecA^N, photo-crosslinked SecA^N-SecA^N dimer, and on the left are positions of molecular weight markers.

Precursors of β-barrel OMPs and periplasmic proteins dramatically reduced their interaction with SecA^N and SecY, respectively, when they interact with a defective full length form of SecA.

In the *in vivo* protein photo-crosslinking experiments described above, we demonstrated that the nascent polypeptides of periplasmic proteins and β-barrel OMPs respectively interact with SecY (**Fig. 1B**) and SecA^N (**Fig. 3A** and **3C**). Nevertheless, we have not yet detected any direct interaction between these nascent polypeptides and the full length form of SecA in living cells (**Figs**. **3A**, **3C**, **S1**). These failures might be attributed to the transient nature of such interactions (thus unable to be captured by the *in vivo* protein photo-crosslinking technique that we applied here) or, less likely, to the lack of interaction between them in living cells. We therefore tried to clarify whether or not such interactions do occur in living cells. For this purpose, we tried to prolong such presumed transient interactions (thus to enable them to be captured by our *in vivo* photo-crosslinking) by replacing the wild type *secA* gene in the LY928 genome with the mutant *secA81* gene that encodes a temperature sensitive defective SecA protein, which is functional at 37°C but defective at 42°C (*66*). In particular, the mutant SecA81 protein possesses a Gly516Asp replacement (*66*). We demonstrated that SecA^N could still be effectively

produced from this *secA81* mutant gene under either permissive (lane 1) or non-permissive (lane 2) conditions, as shown by data displayed in **Fig. S3**.

With this LY928-SecA81 strain, we now detected the formation of photo-crosslinked products between nascent polypeptides of OmpF and the full length SecA, when pBpa was placed at residue position 10, 18 (in the signal peptide) or 55 (in the mature protein) in OmpF, as shown in **Fig. 5A** (lanes 3, 4 and 6), after the cells were shifted and cultured at the non-permissive temperature of 42°C for 1 hour. Similarly, photo-crosslinked products were detected when pBpa was placed at residue position 8 (in the signal peptide) in SurA (lane 2, **Fig 5B**). Of note, we detected two photo-crosslinked product bands between the nascent OmpF and full length SecA (**Fig. 5A**), which apparently indicate that each nascent polypeptide of OmpF interacts with two different sites of full length SecA. Whether this reflects the interaction between such nascent β-barrel OMPs and two forms of full length SecA (for example, the cytoplasmic and the membrane-associated/integrated forms) is worth further investigations. Furthermore, it should be pointed out that we did not detect any photo-crosslinked products when pBpa was placed in the mature part of SurA (lanes 8, 9, 10, 11, **Fig. 5B**).

It should be mentioned that under the non-permissive condition, precursors of neither β-barrel OMPs nor periplasmic proteins were effectively processed in LY928-SecA81 cells, as shown by data displayed in **Fig S4**, in consistent with what was reported before (*66*). Interestingly, we found that photo-crosslinked products between nascent OmpF and $SecA^N$ became hardly detectable (lanes 1-6, **Fig 5A**) while those between nascent SurA and SecY became significantly reduced (lanes 1-5, **Fig 5C**) in LY928-SecA81 cells cultured at such non-permissive temperature. These observations strongly suggest that nascent polypeptides of both periplasmic proteins and β-barrel OMPs directly but transiently 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 wild type bacterial cells. It follows that their prolonged upstream interactions involving the defective full length form of SecA decreased their downstream interactions involving the $SecA^N$ and SecY to occur in the mutant LY928-SecA81 cells under the non-permissive condition.

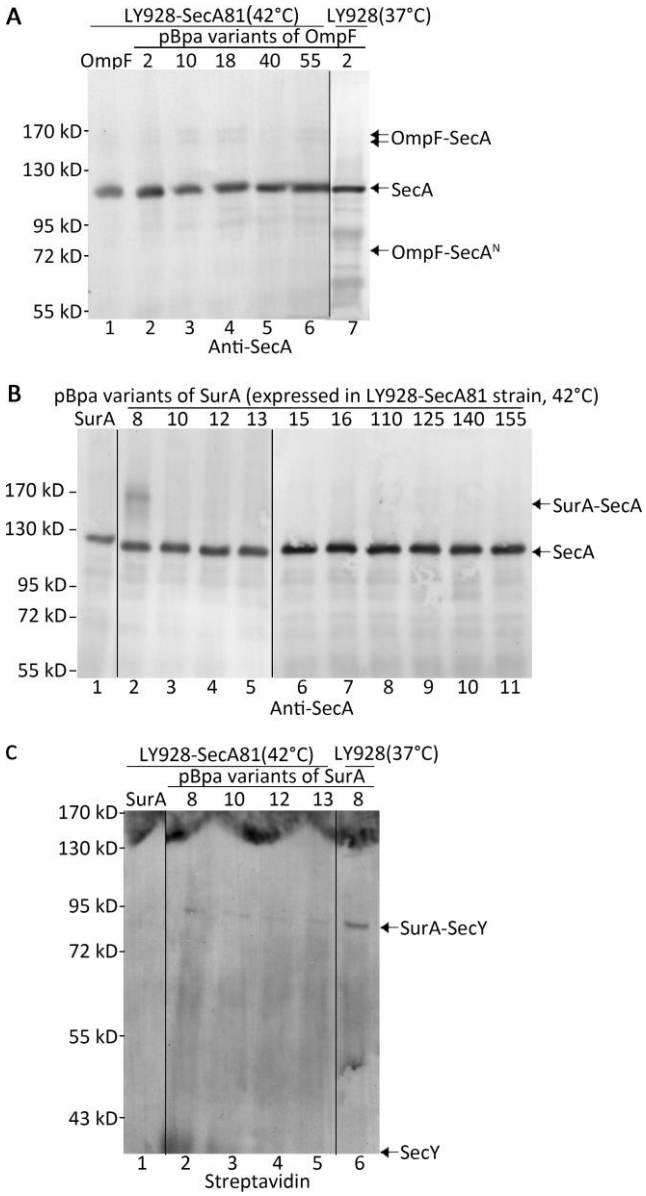


Figure 5. Precursors of β-barrel OMPs and periplasmic proteins dramatically reduced their interactions with SecA^N and SecY, respectively, when they interact with a defective full length form of SecA in the LY928-SecA81 mutant cells cultured under the non-permissive temperature of 42ºC.

(**A, B, C**) Immunoblotting results for detecting the *in vivo* protein photo-crosslinked products of the indicated pBpa variants of nascent polypeptides of OmpF (**A**) or SurA (**B**, **C**) expressed in the LY928-SecA81 mutant cells shifted and cultured at the non-permissive temperature of 42°C for 1 hour, probed with antibodies against SecA (**A, B**) or the streptavidin-AP conjugate against the Avi tag linked to SecY protein (**C**). Of note, lane 7 in panel **A** and lane 6 in panel **C** are respectively identical with lane 3 in **Fig 3A** and lane 2 in **Fig 1B**, and are shown here to solely indicate the expected positions of OmpF-SecA^N (**A**) and SurA-SecY (**C**). Here, the residue position is numbered by counting the signal peptide residues. The protein samples were resolved by SDS-PAGE before subjecting to the blotting analyses. Positions of SecA, OmpF-SecA, SurA-SecA or SurA-SecY are indicated on the right of the gels. Positions of the molecular weight markers are indicated on the left of the gels. Cells expressing the wild type OmpF or SurA (with no pBpa incorporation) were

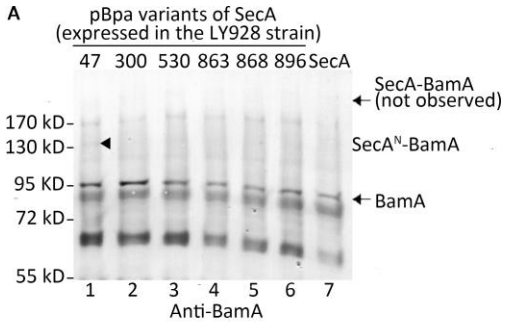
analyzed as negative controls for the photo-crosslinking (lanes 1 in **A, B** and **C**).

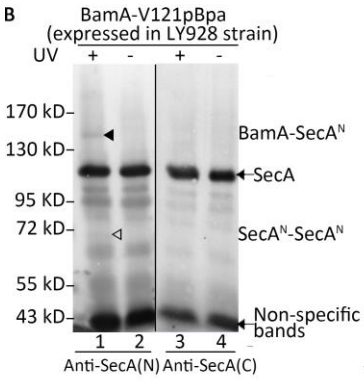
SecA^N directly interacts with the periplasmic POTRA 2 domain of BamA protein in living cells

In an attempt to provide further evidence to show that SecA^N may function as the transmembrane protein-conducting channel for nascent β-barrel OMPs, we next examined whether or not SecA^N interacts with protein factors that have been demonstrated to participate in OMP biogenesis but are located in the periplasmic compartment outside the inner membrane, such as SurA (*13-17*) and BamA (*16-18*). For this purpose, we first tried to find out whether pBpa individually introduced in SecA at 6 residue positions (i.e., 47, 300, 530, 863, 868 or 896) that were previously reported to be exposed to the periplasmic compartment (*48*) is able to mediate photo-crosslinking with SurA and/or BamA. Remarkably, our immunoblotting results, shown in **Fig. 6A**, revealed a putative photo-crosslinked product between SecA^N and BamA, of ~140 kD, when pBpa was placed at residue position 47 in SecA (lane 1, filled arrowhead), but no potential photo-crosslinked product between the full length form of SecA and BamA (which should be ~197 kD; with BamA being ~95 kD and SecA ~102 kD) was detected by anti-BamA antibodies. We however did not detect any photo-crosslinked product between SecA^N and SurA with these 6 pBpa variants of SecA in the LY928 cell (**Fig. S5**), suggesting that SecA^N on the inner membrane may not directly interact with SurA protein present in the periplasmic compartment.

We then tried to verify this putative interaction between SecA^N and BamA by performing the reciprocal photo-crosslinking such that pBpa was individually introduced at 9 residue positions in the periplasmic domains of BamA (*21, 22*). The immunoblotting results, displayed in **Fig. 6B**, clearly revealed the photo-crosslinked BamA-SecA^N product that could be detected only by antibodies against the N-terminal region (lane 1, filled arrowhead) but not by antibodies against the C-terminal region (lane 3) of SecA, when pBpa was introduced at residue position 121 or 129, both of which are located in the POTRA 2 domain of BamA (only results for BamA-V121pBpa is displayed in **Fig. 6B**). The putative photo-crosslinked SecA^N dimer was once again detected here (lane 1, unfilled arrowhead, **Fig. 6B**)

We subsequently isolated this putative photo-crosslinked BamA-SecA^N product by affinity chromatography and subjected it to mass spectrometry analysis. Although it revealed the presence of both BamA and SecA, interestingly, as shown in **Fig. 6C**, the score for SecA was rather low and the two matched peptide fragments of SecA were both derived from the N-terminal region of SecA. This once again supports the conclusion that SecA^N is present in the photo-crosslinked product. Taken together, these data clearly demonstrate that SecA^N is able to directly interact with the periplasmic region of the BamA protein.





C

Mass spectrometry analysis of the putative photocrosslinked BamA-SecA^N product

NAME	SCORE(Sample)	SCORE(Control)
BamA	1212	89
SecA	25	-

SecA-matched peptide sequences :

^{109}TLTATLPAYLNALTGK^{124}

^{221}TPLIISGPAEDSSEMYKR^{238}

Figure 6. SecA^N directly interacts with the periplasmic POTRA 2 domain of BamA in living cells. (**A**) Immunoblotting results for detecting the *in vivo* protein photo-crosslinked 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* photo-crosslinked 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 photo-crosslinked BamA-SecA^N product generated from BamA-V121pBpa. Shown are the protein scores for BamA and SecA, as well as the two matched peptide sequences from 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.

Our *in vivo* photo-crosslinking data described above indicate that both OmpF (**Fig. 3**) and BamA (**Fig. 6**) interact with SecA^N. In light of this, we then asked whether or not a ternary BamA-OmpF-SecA^N complex is formed in living cells. For this purpose, we tried to perform a dual *in vivo* protein photo-crosslinking analysis by co-expressing both OmpF-G18pBpa (that forms photo-crosslinked product with SecA^N, as shown in

Fig. 3) and BamA-V121pBpa (that also forms photo-crosslinked product with SecA^N, as shown in **Fig. 6**) variants in LY928 cells. Blotting results, shown in **Fig. 7**, revealed the presence of such a photo-crosslinked ternary BamA-OmpF-SecA^N complex, which could be probed either with antibodies against the N-terminal region of SecA (lane 1, red arrowhead), or with streptavidin-AP conjugate against the Avi tag linked to BamA (lane 7, red arrowhead), but not with antibodies against the C-terminal region of SecA (lane 5). As expected, a binary BamA-SecA^N complex was also clearly detected here (lanes 1, 4 and 7, **Fig. 7**, blue arrowheads). Collectively, these results once again strongly suggest that the SecA^N protein, by interacting with the BamA protein, translocates nascent β-barrel OMPs across the inner membrane.

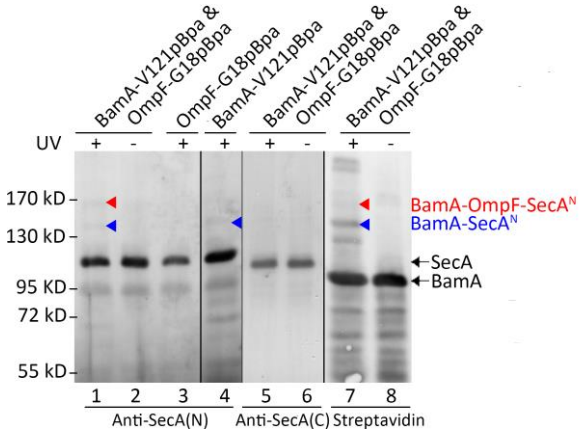


Figure 7. A ternary BamA-OmpF-SecA^N complex is formed in living cells.

Blotting results for detecting photo-crosslinked 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 using 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 putative transmembrane domain in which a GXXXG transmembrane domain interaction-motif is identified

We have demonstrated that SecA^N is most likely a membrane-integrated homo-oligomeric protein (**Fig. 4**). In light of this, we then tried to find out whether or not there are any potential transmembrane domains along the polypeptide chain of SecA^N; and if so, whether or not any GXXXG motif, that has been implicated in mediating transmembrane domain interactions (*67, 68*), can be identified in them. According to the results of mass spectrometry analysis on SecA^N (**Table S1**), we speculated above that the cleavage is made around residue Arg596. We then subjected the N-terminal 596 residues of SecA to TMpred software prediction (*69, 70*), as provided by the online server

(http://www.ch.embnet.org/software/TMPRED_form.html). As a result, two potential transmembrane domains, being ^{145}PLFEFLGLTVGINLPGMPAPA^{165} and ^{488}AAIVAQAGYPAAVTIATNMAG^{508} were revealed from this putative SecA^{N} sequence.

To our satisfaction, we revealed a single GXXXG (being ^{151}GLTVG^{155}) motif across the whole polypeptide chain of *E. coli* SecA, and what is more, this motif is present in one of these two potential transmembrane domains. It worth further investigation on whether or not this GXXXG motif play a key role for the SecA^{N} monomers to self-assemble into homo-oligomers in the inner membrane. Of note, we further revealed that such a GXXXG motif, as well as the predicted potential transmembrane domain containing it, is also found in the SecA proteins of other bacterial species, as listed in **Table 1**. It should be pointed out that the structure of SecA^{N}, presumably existing as a membrane-integrated homo-oligomer according to our experimental data presented here, is most likely significantly different from that of the SecA^{N} equivalent in the structure of the full length SecA as has been reported (*71*).

Table 1. The GXXXG motifs found in one SecA transmembrane domain predicted by the TMpred online server are highly conserved in different bacterial species.

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

[1]Listed here is the percentage of amino acid sequence identity for the SecA of the indicated bacterial species when compared with the amino acid sequence of *E. coli* SecA.

[2]TM: residue ranges and the total number of residues (in the brackets) of one of the predicted transmembrane domain in the SecA proteins.

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

Discussion

This study was conducted in an initial attempt to clarify whether or not nascent polypeptides of both β-barrel OMPs and periplasmic proteins are translocated across the inner membrane through the SecYEG translocon in living cells, as commonly documented (*2, 5, 7, 11, 29*). Our *in vivo* protein photo-crosslinking analysis mediated by a genetically incorporated unnatural amino acid, however, did not reveal any

direct interaction between nascent β-barrel OMPs and SecY (**Fig. 1A**), though proved a direct interaction between nascent periplasmic proteins and SecY (**Fig. 1B**). We then demonstrated that precursors of β-barrel OMPs, unlike that of periplasmic proteins, could be effectively processed even under a SecY defective condition (**Fig. 2A**). Afterwards, we unveiled that a shortened version of SecA that lacks its C-terminal region, thus being designated as $SecA^N$, which most likely functions as the translocon for nascent β-barrel OMPs to get across the inner membrane in living cells, as supported by the following experimental evidences. First, nascent β-barrel OMPs, but not periplasmic proteins, interact with $SecA^N$ in living cells (**Figs. 3A** and **3C**). Second, $SecA^N$, containing ~ 600 residues out of the 901 residues of SecA (**Fig. 3B**, **Table S1**), is solely present in the membrane fraction (**Fig. 4**) and most likely exists as homo-oligomers (**Figs. 3A**, **4**, **S1**). Third, precursors of β-barrel OMPs no longer interact with $SecA^N$ when they interact with a defective full length form of SecA in a temperature sensitive secA mutant strain (**Fig. 5**). Fourth, $SecA^N$ and BamA directly interact with each other most likely in the periplasm (**Figs. 6A**, **6B**). Fifth, a BamA-OmpF-$SecA^N$ ternary complex is formed in living cells (**Fig. 7**). Sixth, two transmembrane domains with one of which containing a GXXXG motif was predicted in $SecA^N$ (**Table 1**). Seventh, precursor processing of β-barrel OMPs relies largely on ATP but hardly on the transmembrane proton gradient, while that of periplasmic proteins relies on both as energy sources (**Fig. S6**).

Our new findings described here, in combination with previous revelations (*13-20, 44, 51, 72*), strongly implicate that $SecA^N$ and SecYEG are responsible for translocating nascent polypeptides of β-barrel OMPs and periplasmic proteins, respectively, across the inner membrane. Here, as schematically illustrated in **Fig. 8**, we emphasize the following points. First, as the translocon of nascent β-barrel OMPs, $SecA^N$, likely exists as a homo-oligomer (arbitrarily shown as a dimer in **Fig. 8,** although its exact oligomeric form remains to be elucidated). Second, the integration of $SecA^N$ into the inner membrane is presumed to be partially rely on its interaction with a SecYEG protomer, which in turn interacts with a second SecYEG protomer that likely functions as the translocon for nascent periplasmic proteins (*73, 74*). Third, the full length form of SecA in the cytoplasm (being membrane associated or not) is presumed to deliver the signal peptide-containing nascent β-barrel OMPs and nascent periplasmic proteins to the membrane-integrated translocons $SecA^N$ and SecYEG, respectively. Fourth, $SecA^N$ is proposed to be part of the supercomplex we revealed earlier as one that is responsible for the biogenesis of β-barrel OMPs in living cells (*17*) and spans the cytoplasm, the inner membrane, the periplasm and the outer membrane.

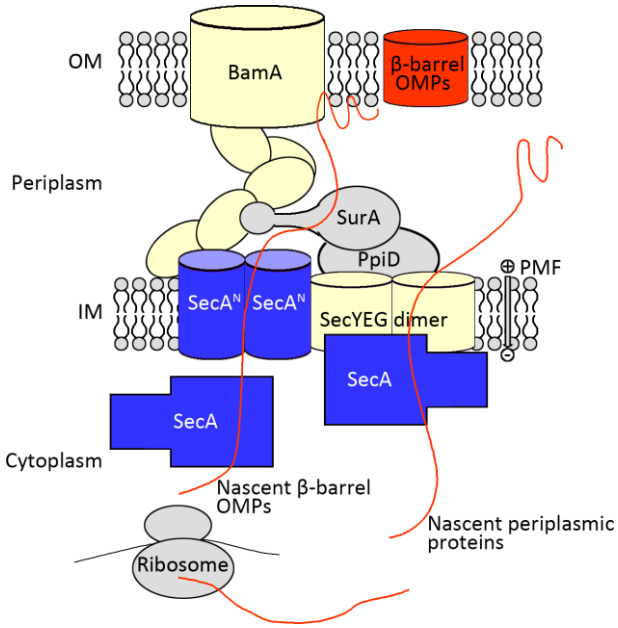


Figure 8. A schematic illustration on the translocation of nascent polypeptides of β-barrel OMPs and periplasmic proteins across the inner membrane in Gram-negative bacteria by using the SecA^N and SecYEG translocons, respectively.

One may ask why the SecA^N protein has not been unveiled over the past decades by people working on protein translocation across the inner membrane in bacteria. We speculate the following as possible reasons. First, SecA^N could have been considered as a degradation product due to its significantly smaller apparent size than the full length form of SecA. Second, SecA^N, almost wholly resides in the membrane fraction (as shown in **Fig. 4**) and presents in the whole cell extract at a level far lower than that of the full length form of SecA, as also shown by our data presented in **Fig. 3B**. Third, since SecA^N is integrated into the inner membrane (from which we found it uneasy to purify it), it may have thus escaped the commonly performed immunoprecipitation analysis in the early days (*41*). Fourth, in using the antibodies that are available to us (*75*), we noticed the existence of multiple non-specific protein bands at the position where SecA^N is found, which may have added further difficulty for it to be noticed (as shown in **Fig. 3A**).

Despite of these, we still searched the literatures in an attempt to seek for earlier hints on the presence of SecA^N in bacterial cells. Interestingly, we did notice that a SecA form whose monomeric size is very close to SecA^N was either be immunoprecipitated from the lysates of *E. coli* cells by antibodies against the N-terminal region of SecA (as shown in lanes 7 and 8, Fig. 2, in (*40*)) or be detected in the inner membrane fraction by antibodies against the full length SecA (Fig. 4 in (*48*)). Moreover, it was also reported that the N-terminal fragment of about 240 residues of the SecA protein from either *E coli* (*42*) or *B. Subtilis* (*76*) is integrated into the membrane. Many issues remain to be resolved on the structure and function of SecA^N. For example, how SecA^N, as homo-oligomers, forms the protein conducting

channel in the inner membrane? Where in the bacterial cell (in the cytoplasm or on the inner membrane) and How $SecA^N$ is generated from the full length form of SecA (by using the membrane-integrated GlpG serine protease or not)? How $SecA^N$ on the inner membrane interacts and works with the full length SecA present in the cytoplasm?

In light of our new findings reported here, it needs to be reappraised on the long-held perception that SecYEG is directly responsible for translocating nascent polypeptides of both β-barrel OMPs and periplasmic proteins in bacterial cells (*2, 5, 7, 11*). In retrospect, this perception has been mainly derived from genetic mutation and *in vitro* reconstitution studies (*29, 31*), as we described above. Consistent with what was reported before, our data also indicate that SecY, though not serving as the actual conducting channel, does play certain role for translocating nascent β-barrel OMPs across the inner membrane in living cells (**Fig. 2**). This might be explained as follows. First, SecYEG is responsible for translocating such periplasm-located quality control factors as SurA, which play important roles for OMP biogenesis, across the inner membrane (*13-15, 17*). Second, SecYEG, as a highly hydrophobic membrane-integrated protein, may partially anchor the homo-oligomeric $SecA^N$ in the inner membrane. This speculation is partially supported by such previously reported observations as that an overproduction of SecYEG would result in an increased level of membrane-integrated SecA (*47*), and that the addition of SecYEG would significantly increase the efficiency of SecA in functioning as protein-conducting channels in liposomes (*77*).

Unresolved issues regarding the relationship between SecYEG and $SecA^N$ include the following. Is $SecA^N$ indeed anchored in the inner membrane by SecYEG? Do $SecA^N$ and SecYEG have any functional coordination in translocating β-barrel OMPs and periplasmic proteins (such that, for example, the two groups of proteins are produced in certain desired ratio)? How does the cytoplasmic full length form of SecA effectively partition nascent β-barrel 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 translocons undoubtedly merits future investigations.

Methods

Bacteria strains and plasmid construction

All bacteria strains and plasmids used in this research are respectively listed in **Tables S2** and **S3**. The pYLC-OmpF, pYLC-BamA, pYLC-SurA or pYLC-SecA plasmid was constructed by isolating the encoding gene (including its promoter) via PCR, using the *E. coli* genomic DNA as template before the DNA fragment was inserted into the pYLC plasmid vector through restriction enzyme free cloning (*78*). The pYLC is a low copy plasmid that we derived from the pDOC plasmid (*17*). 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 in the *ompF* or *surA* genes 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 and was described before (*17*). 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 of the separated fractions

The separation of soluble and membrane fractions was performed according to (*42, 79*) 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 unlysed 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.

Mass spectrometry analysis

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 bands 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 (*17*). The protein bands around 45 kD after the membrane fraction was resolved by SDS-PAGE and Coomassie Blue staining, or those around 100 kD after the soluble fraction was resolved by SDS-PAGE and Coomassie Blue staining were similarly excised from the gel and applied to LC-MS/MS identification.

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, cultured at the permissive temperature of 37°C to the mid-log phase (as the control) or shifted the mid log phase cells to and incubated at the non-permissive temperature (20°C for SecY39, 42°C for SecA81) for 1 hour before 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.

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Supplemental methods

Treatment of SecY39 mutant cells with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP)

A single colony of SecY39 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 mid-log phase, treated with CCCP (10 μM) for 30 min, then incubated in glucose (0.2 %) for 20 min before further cultured in the presence of chloramphenicol (34 μg/mL) for 30 min or 1 hour. The treated cells were 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 (as shown in **Fig. S6**).

Supplemental figures

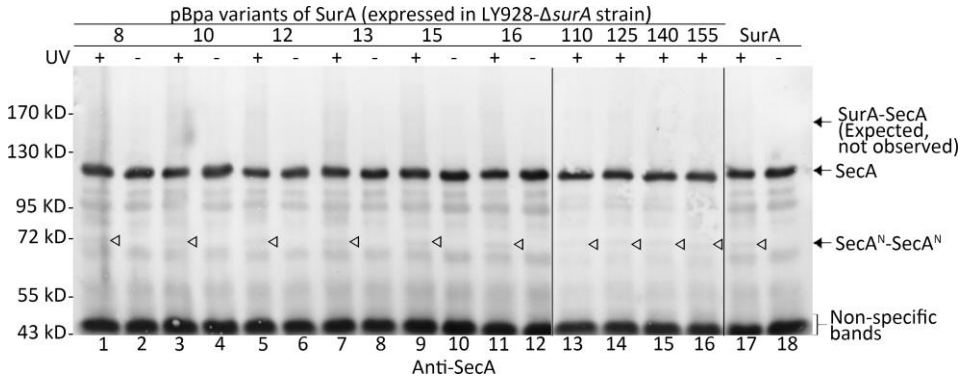

Figure S1. Nascent periplasmic protein SurA does not directly interact with SecA^N.
Immunoblotting results for detecting the *in vivo* protein photo-crosslinked products of the indicated pBpa variants of SurA expressed in the LY928-Δ*surA* cells, probed with antibodies against SecA. Samples of cells expressing wild type SurA (with no pBpa incorporation) were analyzed as the negative control (lanes 17 and 18). Indicated on the right of the gel are the positions of SecA, photo-crosslinked SecA^N dimer (SecA^N-SecA^N), photo-crosslinked SurA-SecA (expected but not observed), and on the left are positions of the molecular weight markers.

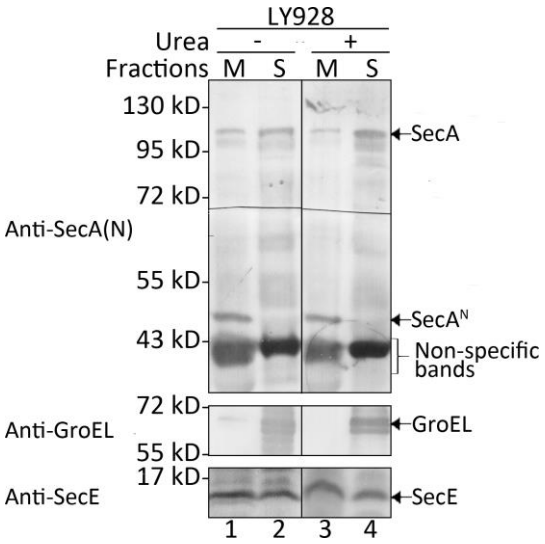

Figure S2. SecA^N in the membrane fraction could not be removed by treatment with 8 M urea. Here, the membrane (M) and soluble (S) fractions of the lysed LY928 cells were separated according to a previously reported protocol (*1*) and was obtained by centrifugation at a relatively low speed, at which usually only stable large membrane protein complexes will be ended in the pellet (*2*). GroEL and SecE were analyzed here as marker proteins of the cytoplasm and inner membrane, respectively. For comparison, we treated both the membrane and soluble fractions with 8 M urea. We hardly detected any SecA^N protein in the soluble fraction, but repeatedly detected part of the SecE proteins in the soluble fraction (lanes 2 and 4), apparently indicating that SecA^N, but not secE, exists as large complexes in the membrane. Additionally, a small portion of the full length SecA was repeatedly detected in the membrane fraction and could not be removed even by the treatment with 8 M urea, somehow consistent with what was reported before (*1, 3*). The Positions of SecA, SecA^N, GroEL and SecE are indicated on the right and positions of the molecular weight markers indicated on the left.

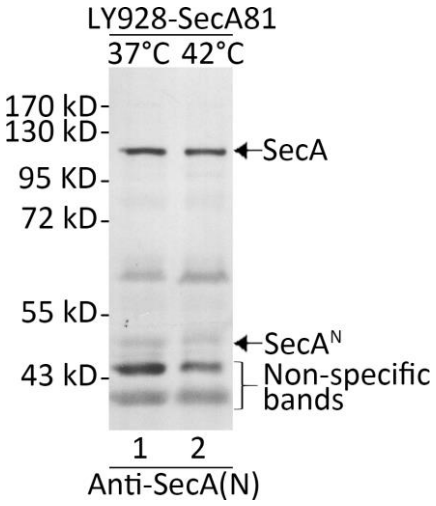

Figure S3. SecA^N is effectively generated under both permissive (37°C) and non-permissive (42°C) conditions in the LY918-SecA81 strain.

Immunoblotting results for detecting the full length SecA and SecA^N in LY928-SecA81 cells grown either under permissive (lane 1) or non-permissive (lane 2) temperature, probed with antibodies against the N-terminal region of SecA. Indicated on the right of the gel are the positions of SecA and SecA^N, and on the left are positions of molecular weight markers.

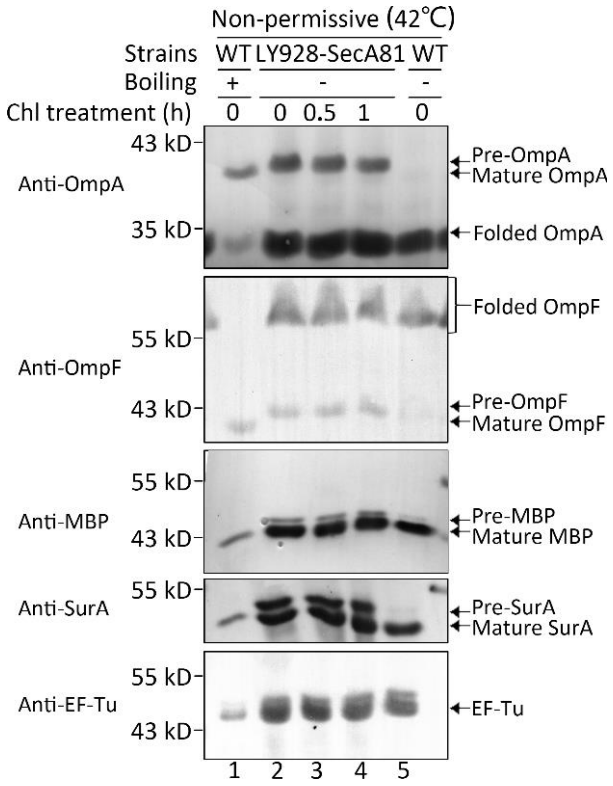

Figure S4. Precursors of neither OMPs nor periplasmic proteins are effectively processed under a SecA-defective condition.

Immunoblotting results (using the indicated antibodies) for detecting the precursor, the mature

and the folded forms of the indicated OMPs (OmpA and OmpF) and periplasmic proteins (SurA and MBP) produced in the LY928-SecA81 cells that were cultured at the non-permissive temperature of 42°C (when SecA becomes defective) and treated with chloramphenicol for the indicated length of time in the chasing experiments. The protein samples of the LY928-SecA81 cells were not boiled before loading for the semi-native SDS-PAGE analysis (lane 2-4). The protein samples from the wild type cells were boiled for indicating the positions of the unfolded mature forms (with a higher mobility than the precursors) of the OMPs and the periplasmic proteins (lane 1), or not boiled for indicating the positions of the folded forms of the OMPs (lane 5). The EF-Tu protein was analyzed here to indicate the equal loading of samples in lanes 2-5. Indicated on the right of each gel are positions of the different forms of the OMPs (precursor, mature and folded) and periplasmic proteins (precursor and mature). Positions of the molecular weight markers are indicated on the left of each gel.

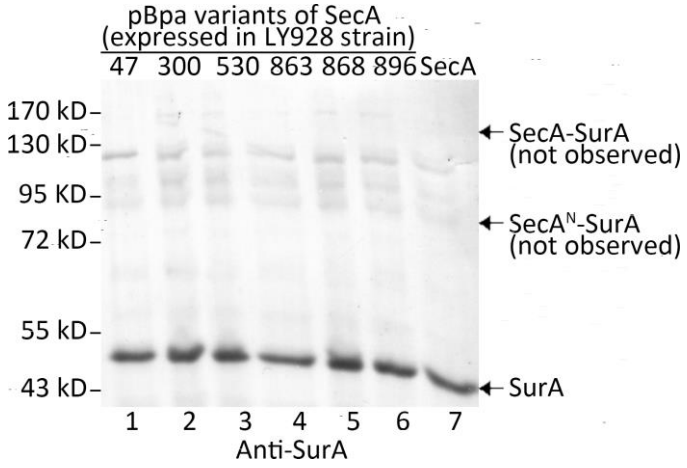

Figure S5. SecA^N (or SecA) does not directly interact with the periplasmic chaperone SurA in living cells

Immunoblotting results for detecting the *in vivo* protein photocrosslinked products of the indicated pBpA variants of SecA expressed in the LY928 cells, probed with antibodies against SurA. Sample of cells expressing wild type SecA (with no pBpA incorporation) were analyzed as the negative control (lane 7). Indicated on the right of the gel are positions of SurA, photo-crosslinked SecA^N-SurA and SecA-SurA (both were not observed), and on the left are positions of molecular weight markers.

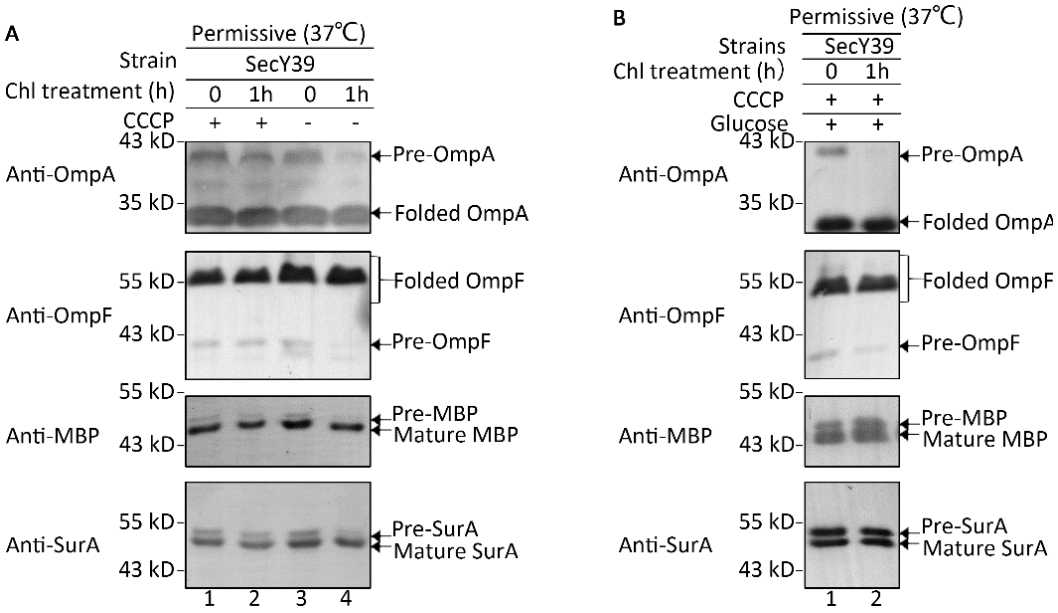

Figure S6. Processing of precursors for OMPs depends on ATP but that for periplasmic proteins depends on both ATP and the transmembrane proton gradient as energy sources.

Immunoblotting results, using the indicated antibodies, for detecting precursor, mature and folded forms of the indicated OMPs (OmpA and OmpF) and periplasmic proteins (SurA and MBP) in secY39 cells cultured at the permissive temperature of 37°C , either treated with carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) before adding chloramphenicol (**A**) or treated with CCCP and glucose before adding chloramphenicol (**B**). Protein samples of secY39 cells (lanes 1-4 in **A**) were not boiled for the semi-native SDS-PAGE analysis. Indicated on the right of each gel are the positions of the different forms of the OMPs (precursor and folded) and periplasmic proteins (precursor and mature), on the left are positions of the molecular weight markers.

The data presented here was aimed to address whether or not there is a difference in energy sources for precursor processing of OMPs and periplasmic proteins. To this end, we again utilized the SecY39 strain in which the processing of precursors of both OMPs and periplasmic proteins was known to become slow and can thus be monitored more effectively (*4*). We first cultured the SecY39 cells at the permissive temperature of 37°C and in the presence of CCCP, which is known to disrupt the proton gradient and meanwhile decrease the ATP level in cells (*5*). Our immunoblotting analysis clearly demonstrated that processing of precursors of both OMPs and periplasmic proteins were blocked in our chasing experiment when protein synthesis in cells was inhibited by adding chloramphenicol (lane 2, panel **A**). We then repeated this experiment by adding glucose, which raises the ATP level through glycolysis but could not recover the transmembrane proton gradient that was disrupted by CCCP. Under this condition we observed an effective precursor processing for OMPs but not for periplasmic proteins (lane 2, panel **B**). Collectively, these observations strongly suggest that precursor processing of OMPs relies largely on ATP, hardly on the transmembrane proton gradient as an energy source, while that of periplasmic proteins relies on both ATP and the transmembrane proton gradient as energy sources.

Supplemental tables

Table S1. SecA-matched peptide sequences revealed by mass spectrometry analysis of the gel slices excised from the ~ 45 kD (where $SecA^N$ was detected) or ~100 kD (where SecA was detected) position after the membrane or soluble fraction was respectively resolved by SDS-PAGE (as shown in **Fig. 4**).

The ~45 kD position ($SecA^N$)	The ~ 100 kD position (full length SecA)
^{57}GEVLENLIPEAFAVVR^{72}	^{109}TLTATLPAYLNALTGK^{124}
^{83}HFDVQLLGGMVLNER^{97}	^{125}GVHVVTVNDYLAQR^{138}
^{104}TGEGKTLTATLPAYLNALTGK^{124}	^{221}TPLIISGPAEDSSEMYK^{237}
^{109}TLTATLPAYLNALTGK^{124}	^{221}TPLIISGPAEDSSEMYKR^{238}
^{125}GVHVVTVNDYLAQR^{138}	^{330}DGEVIIVDEHTGR^{342}
^{203}LHYALVDEVDSILIDEAR^{220}	^{449}GQPVLVGTISIEK^{461}
^{239}VNKIIPHLIR^{248}	^{462}SELVSNELTK^{471}
^{278}GLVLIEELLVK^{288}	^{483}FHANEAAIVAQAGYPAAVTIATNMAGR^{509}
^{330}DGEVIIVDEHTGR^{342}	^{545}HDAVLEAGGLHIIGTER^{561}
^{348}RWSDGLHQAVEAK^{360}	^{586}FYLSMEDALMR^{596}
^{349}WSDGLHQAVEAK^{360}	^{643}KQLLEYDDVANDQRR^{657}
^{449}GQPVLVGTISIEK^{461}	^{644}QLLEYDDVANDQRR^{657}
^{462}SELVSNELTK^{471}	^{664}NELLDVSDVSETINSIR^{680}
^{537}IKADWQVR^{544}	^{739}ILAQSIEVYQR^{749}
^{545}HDAVLEAGGLHIIGTER^{561}	^{833}MPEEVEELEQQR^{844}
^{586}FYLSMEDALMR^{596}	^{851}LAQMQQLSHQDDDSAAAAALAAQTGER^{877}

Table S2. *E. coli* strains used in this work

Strain	Relevant genotype (and reference)	Source
BW25113	Wild type cells (*6*)	Keio collection, Japan
SecY39	A temperature sensitive *secY* mutant (Arg 357 replaced by His) (*4*)	Kind gift from Prof. Koreaki Ito
LY928	Modified BW25113 strain; The genome was modified to encode the orthogonal amino-acyl tRNA synthetase and the tRNA, both needed for pBpa incorporation (*7*)	Our lab
LY928-SecA81	Modified LY928 strain; the *secA* gene was replaced by a temperature sensitive *secA* mutant (Gly 516 replaced by Asp) (*8*)	This work
LY928-Δ*ompF*	Modified LY928 strain; The *ompF* gene was deleted	This work
LY928-Δ*surA*	Modified LY928 strain; The *surA* gene was deleted	This work

Table S3. Plasmids used in this work.

Plasmid	Description	Source
pYLC	A low copy plasmid that we derived from the pDOC plasmid with a mutation in the replication origin (*7*); Expressing the ampicillin and kanamycin resistant genes	Our lab
pYLC-OmpF	Expressing the wild type OmpF protein (controlled by its natural promoter)	This work
pYLC-OmpF-M2pBpA pYLC-OmpF-I10pBpA pYLC-OmpF-G18pBpA pYLC-OmpF-V40pBpA pYLC-OmpF-G55pBpA pYLC-OmpF-E70pBpA pYLC-OmpF-Y85pBpA pYLC-OmpF-A130pBpA pYLC-OmpF-A145pBpA pYLC-OmpF-T160pBpA pYLC-OmpF-F175pBpA pYLC-OmpF-E205pBpA	Expressing the pBpA variants of OmpF with the indicated residue replacement	This work
pYLC-SurA	Expressing the wild type SurA protein (controlled by its natural promoter)	This work
pYLC-SurA-L8pBpA pYLC-SurA-G10pBpA pYLC-SurA-A12pBpA pYLC-SurA-M13pBpA pYLC-SurA-A15pBpA pYLC-SurA-N16pBpA pYLC-SurA-T110pBpA pYLC-SurA-Y125pBpA pYLC-SurA-E140pBpA pYLC-SurA-Q155pBpA	Expressing the pBpA variants of SurA protein with the indicated residue replacement	This work
pYLC-SecA-Avi	Expressing the wild type SecA protein containing a Avi tag at the C-terminus	This work
pYLC-SecA-T47pBpA-Avi pYLC-SecA-S300pBpA-Avi pYLC-SecA-T530pBpA-Avi pYLC-SecA-D863pBpA-Avi pYLC-SecA-A868pBpA-Avi pYLC-SecA-C896pBpA-Avi	Expressing the indicated pBpA variants of the SecA protein that contains an Avi tag at the C-terminus	This work
pYLC-Avi-BamA	Expressing the wild type BamA protein that contains an Avi tag at the N-terminus	This work

pYLC-Avi-BamA-V121pBpA pYLC-Avi-BamA-T129pBpA	Expressing the indicated pBpA variants of the BamA protein that contains an Avi tag at the N-terminus	This work
pBad-SecY-Avi	Expressing the wild type SecY protein that contains an Avi tag at the C-terminus; expressing the ampicillin resistant gene	Our lab
pBad-Avi-SecY	Expressing the wild type SecY protein that contains an Avi tag at the N-terminus; expressing the tetracycline resistant gene	This work

Table S4. Antibodies used in this work.

Antibody	**Animal immunized**	**Type**	**Dilution**	**Source**
Anti-OmpA	Rabbit	Polyclonal	1/10000	Our lab
Anti-OmpF	Mouse	Polyclonal	1/10000	Our lab
Anti-SurA	Rabbit	Polyclonal	1/10000	Our lab
Anti-SecA	Rabbit	Polyclonal	1/5000	Our lab
Anti-MBP	Mouse	Monoclonal	1/5000	TrangGen Bioteck, (Beijing, China)
AP-conjugated anti-mouse IgG	Goat	Polyclonal	1/10000 or 1/5000	Jackson ImmunoResearch (Pennsylvania, USA)
AP-conjugated anti-rabbit IgG	Goat	Polyclonal	1/10000	Jackson ImmunoResearch (Pennsylvania, USA)
Streptavidin AP-conjugated (probing the Avi tag)			1:8000	Roche (California, USA)

Supplemental References

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