

*Biological sciences-biochemistry*

**A shortened version of SecA (SecA<sup>N</sup>) functions as the protein-conducting channel for nascent  $\beta$ -barrel outer membrane proteins**

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During their biogenesis in cells, many proteins have to be translocated across biological membranes by protein-conducting channels. In Gram-negative bacteria, proteins destined to inner membrane, periplasm or outer membrane are synthesized in the cytoplasm and most of them are believed to be translocated across the inner membrane by the SecYEG translocon. Nevertheless, this perception, largely formed on the basis of genetic and *in vitro* studies, has hardly been proved by *in vivo* studies. Here, our *in vivo* photocrosslinking analysis mediated by genetically incorporated unnatural amino acid pBpA proved a direct interaction between the nascent periplasmic proteins and SecY, but strikingly, not between the nascent  $\beta$ -barrel outer membrane proteins (OMPs) and SecY. We then demonstrated that precursors of OMPs but not of periplasmic proteins are effectively processed in a SecY-defective strain. Subsequently, we discovered that a shortened form of SecA that lacks its C-terminal region, designated as SecA<sup>N</sup>, apparently functions as the translocon for nascent OMPs. In support of this, we discovered that SecA<sup>N</sup> directly interacts with BamA protein of the  $\beta$ -barrel assembly machine, and that SecA<sup>N</sup> is able to form a ternary complex with BamA and nascent OmpF in living cells. These observations strongly implicate that, in contrast to the common perception, the SecYEG only functions as the translocon for the nascent periplasmic proteins, not for the OMPs.

## Introduction

Many proteins have to be translocated across biological membranes during their biogenesis in living cells (1-6). Such translocation processes are carried out by membrane-integrated protein-conducting channels (7-11). In Gram-negative bacteria, both outer membrane proteins and periplasmic proteins are synthesized in the cytoplasm and translocated across the inner membrane through the co- or post-translational pathway (10, 12). The SurA protein has been identified as the primary chaperone for escorting nascent  $\beta$ -barrel outer membrane proteins (henceforth abbreviated as OMPs) across the periplasm (13-16). The BamA protein, as the major subunit of the essential  $\beta$ -barrel assembly machine (Bam) complex, is integrated into the outer membrane via its C-terminal  $\beta$ -barrel structure and extends into the periplasm via its N-terminal POTRA domains (17-19).

According to the currently prevailing perception, protein translocation across the inner membrane in Gram-negative bacteria is accomplished by the *sec* transcon, whose membrane integrated SecYEG complex acts as a protein-conducting channel while the peripheral SecA ATPase functions as the molecular motor (7, 8, 11, 20). The homologue of SecYEG in eukaryotic cells is designated as Sec61 and is integrated into the membrane of the endoplasmic reticulum (4, 7, 21-23). Initially, the *secY* gene was identified as one whose suppressor mutations restored the export of OMPs or periplasmic proteins that have a defective signal peptide (24, 25), as well as one whose defect resulted in a slow processing for precursors of both periplasmic proteins and OMPs (26, 27). The SecY and SecE proteins were found to act together with the SecA protein in supporting translocation of the precursor form of an outer membrane protein (28). The protein-conducting channel is believed to be largely formed by the SecY protein in light of its crystal structure (29, 30) as well as *in vitro* chemical crosslinking analysis (31, 32).

The *secA* gene was initially identified as one whose defect resulted in accumulation of precursors of both OMPs and periplasmic proteins (33, 34). The SecA protein was later found to be essential for translocation of both OMPs and periplasmic proteins (35, 36). SecA was subsequently characterized as a cytoplasmic as well as a peripheral and integrated membrane protein (37-40), and was demonstrated to be exposed to the periplasm (41-43). Nevertheless, the subcellular localization of SecA remains an issue of debate. Although the SecA protein was proposed to undergo ATP driven cycles of membrane insertion and deinsertion (44, 45), it was reported that a significant fraction of the SecA protein is permanently embedded in the inner membrane (39). In contrast to the conventional view, 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 (46-51).

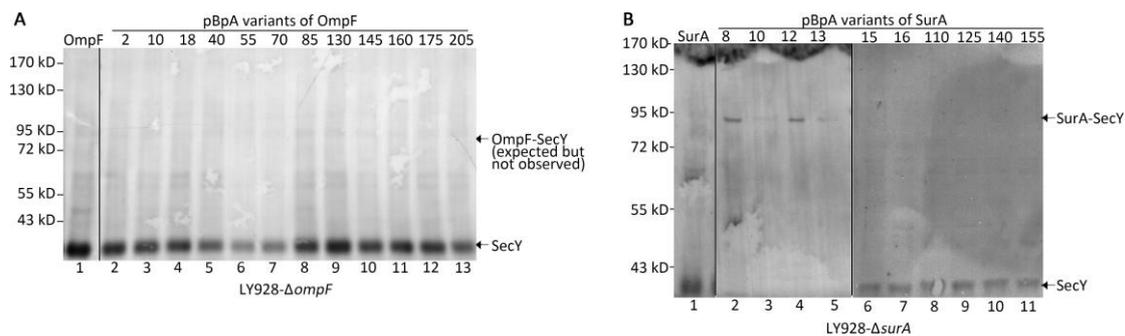
In regards to the biogenesis of OMPs and periplasmic proteins, as well as the function of SecYEG and SecA in living cells, many issues remain unresolved. For example, if the prevailing perception on SecYEG is correct, then how can a single SecYEG channel translocate the nascent polypeptides of such dramatically different clients as OMPs, periplasmic proteins and even inner membrane proteins? How can SecA exist in three different subcellular localizations and what does each form exactly do in living cells? In this study, mainly via unnatural amino acid mediated *in vivo* photocrosslinking (52), we first unexpectedly observed that although nascent polypeptides of periplasmic proteins directly interacted with SecY, those of OMPs did not. Further, we observed

that precursors of OMPs, but not of periplasmic proteins, could be effectively processed when SecY becomes defective. Subsequently, we demonstrated that a shortened version of SecA, designated as SecA<sup>N</sup>, directly interacts with the periplasmic part of BamA. These observations strongly implicate that SecA<sup>N</sup> 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 polypeptides of periplasmic proteins but not of OMPs directly interact with SecY

To clarify whether nascent polypeptides of both periplasmic proteins and OMPs are translocated across the inner membrane through the SecYEG translocon as commonly perceived, we first performed *in vivo* photocrosslinking analysis by individually introducing the unnatural amino acid pBpA (*p*-benzoyl-L-phenylalanine) (52) at 21 randomly selected residue positions across the polypeptide of OmpF (an OMP) or 10 positions of SurA (a periplasmic protein). The pBpA variants of OmpF or SurA were then each expressed in the *ompF*-deleted or *surA*-deleted LY928 bacterial strain, whose genome was modified to encode the amino-acyl tRNA synthetase and the tRNA needed for pBpA incorporation as we reported before (16). Strikingly, our blotting analysis against SecY failed to detect any photocrosslinked product between OmpF and SecY (**Fig. 1A**; displayed are results of 12 pBpA variants of OmpF), though successfully detected the photocrosslinked product between SurA and SecY when pBpA was introduced at signal peptide residue positions 8 and 12 in SurA (lanes 2 and 4, **Fig. 1B**). It should be pointed out that we did not detect any photocrosslinked SurA-SecY product when pBpA was introduced at the mature part of SurA protein (lanes 8-11, **Fig. 1B**). Collectively, these observations indicate that the nascent OMPs, unlike the periplasmic proteins, apparently do not directly interact with the SecYEG translocon in living cells.



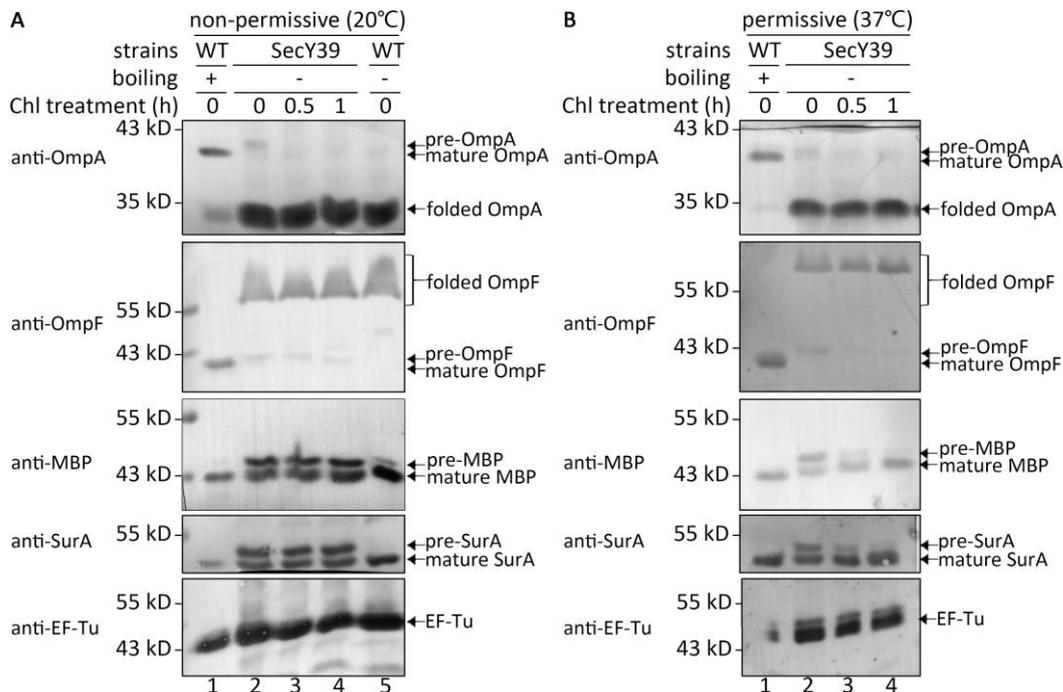
**Figure 1. Nascent polypeptides of periplasmic proteins but not of OMPs directly interact with SecY.**

(A, B) Blotting results for detecting the *in vivo* photocrosslinked products of the indicated pBpA variants of OmpF (A) or of SurA (B) expressed in the LY928-*ΔompF* or LY928-*ΔsurA* cells, probed with the Streptavidin-alkaline phosphatase (AP) conjugate against the AVI tag added on the SecY protein.

Residue positions are numbered by including the signal peptides. Protein samples were resolved by SDS-PAGE before subjecting to the blotting. Samples of cells expressing wild type OmpF or SurA (no pBpA incorporation) were analyzed as negative controls (lanes 1, A and B). Indicated on the right of each gel are the position of SecY, SecA, photocrosslinked SurA-SecY or OmpF-SecY (The SecY protein in lanes 2-5 in panel B mobilized at the bottom edge of the gel, thus hardly visible), and on the left are positions of molecular weight markers.

**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 OMPs and periplasmic proteins, we employed the cold sensitive mutant strain, SecY39, in which protein export was found to be partially retarded at the permissive temperature of 37°C and dramatically retarded at the non-permissive temperatures of 20°C (53). We observed a far less defect in the processing of precursor of OMPs in comparing with that of periplasmic proteins when this strain was cultured at the non-permissive temperature (Fig. 2A). This is reflected by a much lower level of accumulation of precursors for OMPs (OmpA and OmpF) than for periplasmic proteins (SurA and MBP), as shown in lane 2 of Fig 2A, as well as by a significantly more efficient processing of the accumulated precursors of OMPs than of periplasmic proteins in the 1 hour chasing period after adding chloramphenicol (that inhibits protein synthesis in cells (54)), as shown in lanes 2-4 of 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). Collectively, these observations indicate that the processing of the precursors for OMPs relies on the SecYEG translocon in a degree far less than that for periplasmic proteins. Our observation here is somehow consistent with what was reported before that the *secY39* mutation affected MBP export more strongly than it affected OmpA export (53).



**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 and OmpF) and periplasmic proteins (SurA and MBP) in the SecY39 cells cultured either at the non-permissive temperature (A), or permissive temperature (B); cells were treated with chloramphenicol (Chl) for the indicated length of time. 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 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 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.

### **The POTRA 2 domain of BamA directly interacts with a form of SecA that lacks its C-terminal region**

Our observations described above suggest that the SecYEG translocon does not directly translocate nascent OMPs across the inner membrane. We then addressed whether SecA functions to translocate nascent OMPs across the inner membrane. We initially failed to detect any direct interaction either between OmpF and SecA (one representative data is shown in lane 3, **Fig. 4A**) or between SurA and SecA when the pBpA variants were expressed in the LY928 strain, likely due to the transient nature of these interactions in living cells. We then observed such interactions when the *in vivo* photocrosslinking was performed under a SecA defective condition with the LY928-SecA81 strain that we particularly constructed for this study (55) (**Fig S1**) and meanwhile observed that precursors of neither OMPs nor periplasmic proteins were effectively processed within this cell (**Fig. S2**).

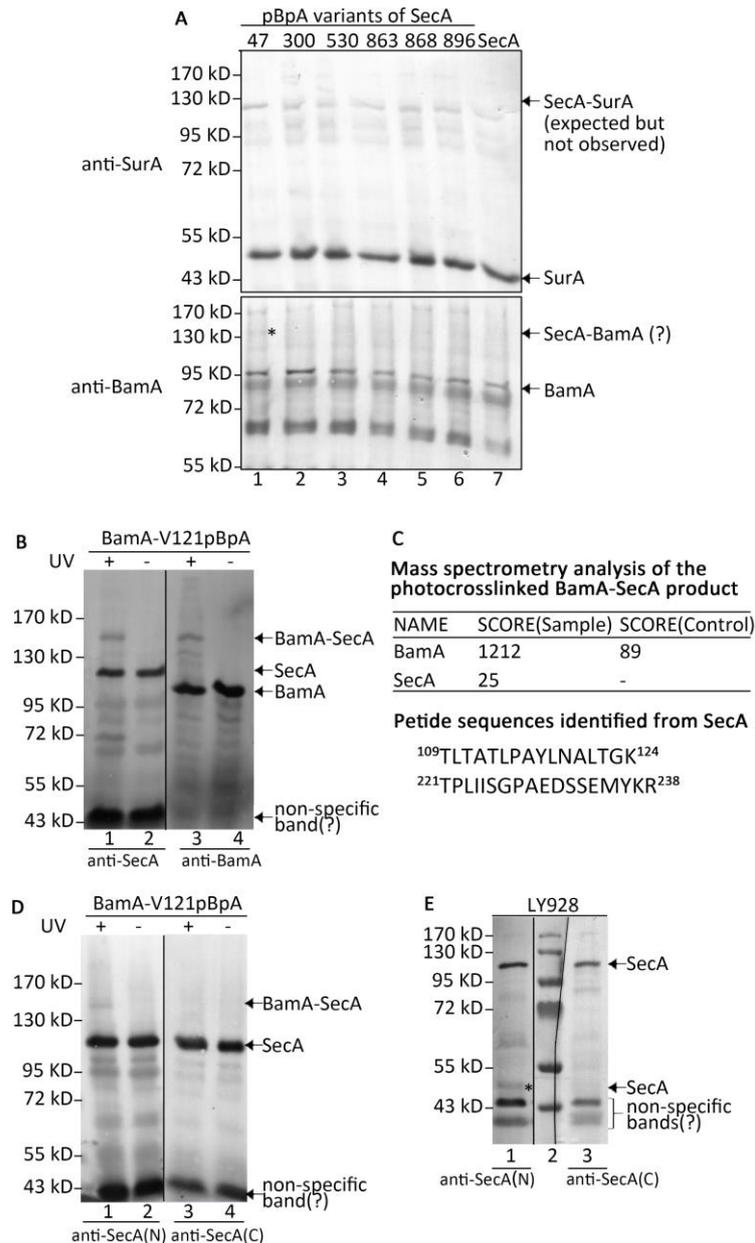
We next examined whether SecA interacts with protein factors that are known to participate in OMP biogenesis but localized outside the inner membrane, such as SurA and BamA (13-19). For this purpose, we first tried to find out whether pBpA individually introduced at six residue positions of SecA (at 47, 300, 530, 863, 868 or 896) that were reported to be exposed to the periplasm (43) was able to mediate photocrosslinking with SurA or BamA. Our immunoblotting analysis revealed no photocrosslinked SecA-SurA product for all these six variants, but a putative SecA-BamA product band of about 145 kD, being significantly lower than the expected combined molecular mass of BamA (95 kD) and SecA (102 kD) for one pBpA variant of SecA (lane 1, **Fig. 3A**).

To verify this putative interaction between SecA and BamA, we then performed a reciprocal photocrosslinking by introducing pBpA in the periplasm-located part of BamA. Remarkably, our immunoblotting analysis revealed the same BamA-SecA product, also of about 145 kD, when pBpA was introduced at the POTRA 2 domain of BamA (lane 1, **Fig. 3B**). It is noteworthy that this putative BamA-SecA band could also be clearly detected when probed with the streptavidin-AP conjugate against the AVI tag of BamA (lane 3, **Fig. 3B**).

We next isolated this putative photocrosslinked BamA-SecA product and subjected it to mass spectrometry analysis. Although the results revealed presence of both BamA and SecA, interestingly, the score for SecA is rather low and that the two identified peptide fragments of SecA were both derived from the N-terminal region of SecA (**Fig. 3C**), indicating that SecA in the photocrosslinked SecA-BamA product is possibly a shortened form. We then tried to clarify this by performing immunoblotting analysis with antibodies against either the N-terminal region (fragment 1-209) or the C-terminal region (fragment 665-820) of SecA. Results presented in **Fig. 3D** demonstrate that this putative SecA-BamA product could be detected only by antibodies against the N-terminal region (lanes 1 and 2 vs lanes 3 and 4) of SecA. In light of these observations, it is conceivable that the SecA in the photocrosslinked SecA-BamA product is

composed of its N-terminal region of about 50 kD. A careful look of our immunoblotting results probed with anti-SecA antibodies (lanes 1 and 2 in **Fig. 3B**; **Fig. 3D**) identified a dense but apparently non-specific band at the 50 kD position (given its far smaller size than the SecA protein).

In an effort to unveil this shortened version of SecA, we then resolved the protein samples using a separating gel that resolves smaller proteins with a higher resolution (56). With this modification, our immunoblotting analysis then did reveal this shortened version of SecA that could only be detected by antibodies against the N-terminal region (lane 1 vs lane 3, **Fig 3E**). However, the level of this form of SecA is apparently much lower than that of full length SecA (lane 1, **Fig 3E**). This is somehow consistent with previous observations that only about one sixth of the total amount of SecA is integrated into the inner membrane (37, 39). In retrospect, the reason for this shortened SecA form to be ignored before is most likely due to the co-existence of the nearby non-specific protein bands (as shown in **Fig. 3B** and **3D**). Collectively, these observations strongly indicate that a shortened form of SecA interacts with BamA in living cells. We henceforth designate this form of SecA as SecA<sup>N</sup>.

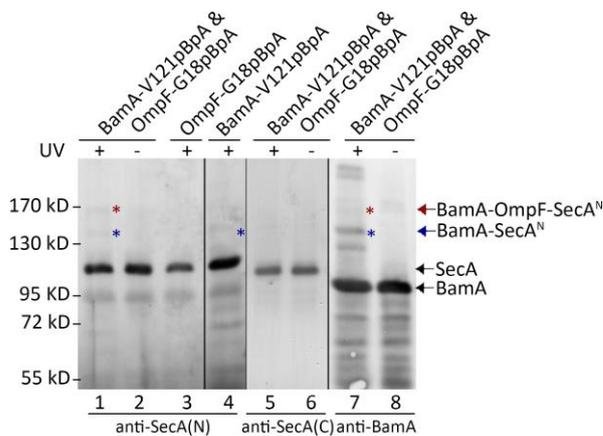


**Figure 3. The POTRA 2 domain of BamA directly interacts with a shortened form of SecA.**

(A) Immunoblotting results of *in vivo* photocrosslinked products of the indicated pBpA variants of SecA expressed in LY928 cells, probed with antibodies against SurA (top) or BamA (bottom). (B) Blotting results of *in vivo* photocrosslinked product of BamA-V121pBpA expressed in LY928 cells, using antibodies against SecA (lanes 1 and 2) or streptavidin-AP conjugate against the AVI tag of BamA (lanes 3 and 4). (C) Results of mass spectrometry analysis of the putative photocrosslinked BamA-SecA product generated from BamA-V121pBpA. Shown are the protein scores for BamA and SecA, as well as the two peptide sequences identified for SecA. (D) Immunoblotting results of the *in vivo* photocrosslinked product of BamA-V121pBpA using antibodies against the N- (lanes 1 and 2) or C-terminal (lanes 3 and 4) region of SecA. (E) Immunoblotting results for detecting the shortened version of SecA after resolving on a high resolution gel, also using antibodies against the N- (lane 1) or C-terminal (lane 3) region of SecA. Protein samples were resolved by SDS-PAGE before subjecting to immunoblotting. The polymerized separating gel in panel A, B, C and D is 5%, while in panel E is 10%. Indicated on the right of each gel are positions of the indicated forms of proteins and on the left are Positions of molecular weight markers. Cells expressing wild type SecA (no pBpA introduced) were analyzed as negative controls (lane 7, panel A).

### A BamA-OmpF-SecA<sup>N</sup> ternary complex is formed in living cells

In light of our aforementioned observations that nascent OmpF interacts with SecA (**Fig. S1**) and BamA interacts with SecA (**Fig. 3**), we then addressed whether a ternary complex could be formed among BamA, nascent OmpF and SecA<sup>N</sup>. For this purpose, we simultaneously introduced pBpA into both BamA (at residue position of V121) and OmpF (at residue position of G18). We then performed this dual *in vivo* photocrosslinking within the LY928 cells co-expressing BamA-V121pBpA and OmpF-G18pBpA. Blotting analysis using antibodies against the N-terminal region of SecA or streptavidin-AP conjugate against the AVI tag of BamA, remarkably, indeed revealed the presence of a photocrosslinked ternary complex of BamA-OmpF-SecA<sup>N</sup> and a binary complex of BamA-SecA<sup>N</sup> (lanes 1 and 7, **Fig. 4**). Consistent with results presented in lane 3, **Fig. 3D**, such a ternary BamA-OmpF-SecA<sup>N</sup> could not be detected by antibodies against the C-terminal region of SecA (lane 5, **Fig. 4**). Collectively, these data strongly suggest that the SecA<sup>N</sup> protein, by interacting with the BamA protein, translocates nascent OMPs across the inner membrane.



**Figure 4. A BamA-OmpF-SecA<sup>N</sup> ternary complex is formed in living cells.**

Blotting results of the photocrosslinked products formed in the LY928 cells expressing the indicated pBpA variants of BamA and 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 of BamA (lanes 7 and 8).

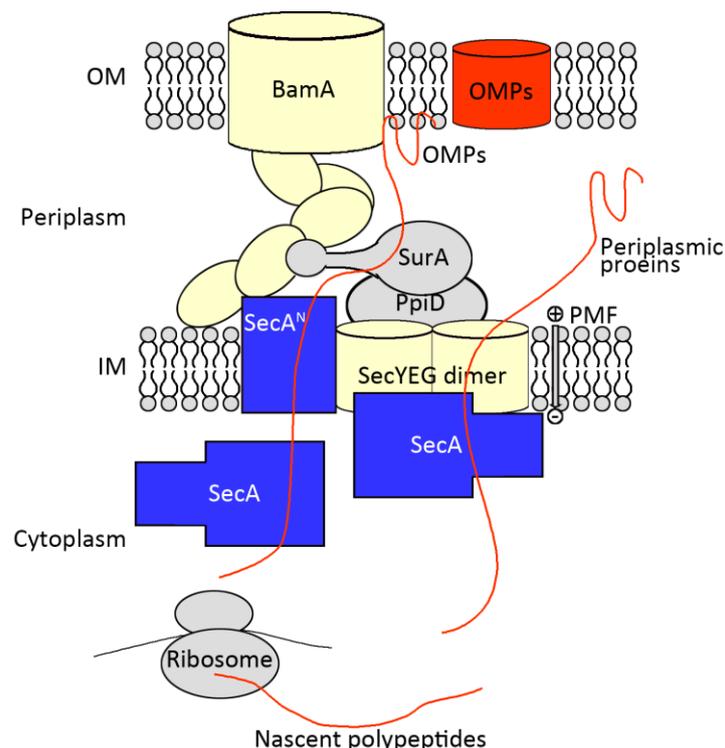
Protein samples were resolved by SDS-PAGE before subjecting to the blotting. Indicated on the right of the gel are positions of BamA, SecA, as well as the photocrosslinked BamA-OmpF-SecA<sup>N</sup> and BamA-SecA<sup>N</sup> complexes, on the left are positions of the molecular weight markers.

### Discussion

This study was performed 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 perceived (7, 8, 26). Our *in vivo* photocrosslinking analysis mediated by genetically incorporated unnatural amino acid, 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 in a SecY defective strain (**Fig. 2A**). Afterwards, we revealed that a shortened form of SecA that lacks its C-terminal region, SecA<sup>N</sup>, most likely functions as the translocon for nascent OMPs in living cells, as supported by the following observations. First, SecA<sup>N</sup> directly interacts with BamA in living cells (**Fig. 3A**). Second, reciprocally, BamA directly interacts with SecA<sup>N</sup> (**Fig. 3B**). Third, a BamA-OmpF-SecA<sup>N</sup> ternary complex was detected (**Fig. 4**). Fourth, processing of precursors of OMPs relies largely on ATP but hardly on the transmembrane proton gradient, while that of periplasmic proteins relies on both as energy sources (**Fig. S3**).

Our observations described here, in combination with revelations reported before (13-19, 57), strongly implicate that SecA<sup>N</sup> is responsible for translocating nascent OMPs across the inner membrane. Here, as illustrated in **Fig. 6**, we emphasize the following points. First, as the translocon of OMPs, SecA<sup>N</sup> (whose oligomeric status 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 (58, 59). Second, the full length form of SecA in the cytoplasm delivers the signal peptide-containing nascent OMPs and nascent periplasmic proteins to SecA<sup>N</sup> and SecYEG translocons, respectively. Third, a supercomplex containing SecA, SecA<sup>N</sup>, SecYEG, SurA, PpiD (16, 60) and BamA, spanning the cytoplasm, the inner membrane, the periplasm and the outer membrane functions for the biogenesis of OMPs in living cells.



**Figure 6. A model illustrating the translocation of nascent OMPs, through SecA<sup>N</sup>, and nascent periplasmic proteins, through SecYEG, across the inner membrane in Gram-negative bacteria.**

We searched the literatures in an attempt to seek for earlier hints on the presence of SecA<sup>N</sup> in bacterial cells. Interestingly, we did notice the presence of a SecA form with a size very close to SecA<sup>N</sup> that could either be immunoprecipitated by antibodies against the N-terminal region of

SecA (as shown in lanes 7 and 8, Fig. 2, in (61)) or be detected in the inner membrane fraction of *E. coli* cells by antibodies against the full length SecA (Fig. 4 in (43)). Moreover, it was also reported that the N-terminal fragment of about 240 residues of the SecA protein from either *E. coli* (37) or *B. Subtilis* (62) is integrated into the membrane. Many issues remain to be resolved for SecA<sup>N</sup>. For example, how SecA<sup>N</sup> forms the protein conducting channel in the inner membrane? How SecA<sup>N</sup> is generated from the full length SecA? How SecA<sup>N</sup> interacts and works with full length SecA 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 (26, 28). Our study reported here represents an attempt to clarify this perception by performing experiments in living cells. As observed before, our data also indicate that SecYEG 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 periplasm-located quality control factors for OMP biogenesis such as SurA (13-16). Second, SecYEG most likely anchors SecA<sup>N</sup> in the inner membrane. This is indicated by such reported observations as that an overproduction of SecYEG resulted in an increased level of membrane-integrated SecA (42), and that the additional of SecYEG significantly increased the efficiency of SecA in functioning as protein-conducting channels in liposomes (50). Unresolved issues regarding the relationship between SecYEG and SecA<sup>N</sup> include the following. Is SecA<sup>N</sup> indeed anchored in the inner membrane by SecYEG as we presumed in our model? Do SecA<sup>N</sup> and SecYEG have any functional coordination in translocating OMPs and periplasmic proteins? How does the cytoplasmic SecA effectively partition nascent OMPs and nascent periplasmic proteins to SecA<sup>N</sup> and SecYEG, respectively? The molecular mechanism for SecA<sup>N</sup> to function as a protein-conducting channel, as well as the structural and functional interaction between SecA<sup>N</sup> and SecYEG undoubtedly merit 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 template before the DNA fragment was inserted into the pYLC vector through restriction enzyme free cloning (63). The pYLC is a low copy plasmid that we derived from the pDOC plasmid (16). 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* photo-crosslinking

The pYLC-OmpF or pYLC-SurA plasmids carrying the TAG mutations were respectively expressed

in LA928- $\Delta ompF$  and LA928- $\Delta surA$  cells for *in vivo* photo-crosslinking analysis. The LA928 strain was generated by us for pBpA incorporation purposes as we described before (16). The cells were cultured at 37°C in LB medium containing pBpA (200  $\mu$ m), grown to the mid-log phase, before irradiated with UV (365 nm) for 10 min in a Hoefer UVC-500 crosslinker. The UV irradiated cells were harvested by centrifugation, re-suspended in 100  $\mu$ L SDS-PAGE loading buffer before boiled for 5 min. Protein samples were separated by SDS-PAGE before subjected to 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 (16).

### **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  $\mu$ g/mL Kanamycin strain, then cultured at the permissive temperature of 37°C to the mid-log phase (for 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  $\mu$ g/mL) for 30 min or 1 hour. Treated cells were then collected by centrifugation, re-suspended 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, analyzed the data and prepared the manuscript. Zengyi Chang supervised this study and prepared the manuscript.

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