

# Bacterial protein translocation requires only one copy of the SecY complex in vivo

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The transport of proteins across the plasma membrane in bacteria requires a channel formed from the SecY complex, which cooperates with either a translating ribosome in cotranslational translocation or the SecA ATPase in post-translational translocation. Whether translocation requires oligomers of the SecY complex is an important but controversial issue: it determines channel size, how the permeation of small molecules is prevented, and how the channel interacts with the ribosome and SecA. Here, we probe in vivo the oligomeric state of SecY

by cross-linking, using defined co- and post-translational translocation intermediates in intact *Escherichia coli* cells. We show that nontranslocating SecY associated transiently through different interaction surfaces with other SecY molecules inside the membrane. These interactions were significantly reduced when a translocating polypeptide inserted into the SecY channel co- or post-translationally. Mutations that abolish the interaction between SecY molecules still supported viability of *E. coli*. These results show that a single SecY molecule is sufficient for protein translocation.

## Introduction

In bacteria, most secretory and membrane proteins are transported across or are integrated into the plasma membrane by a process that is similar to protein translocation across the ER membrane in eukaryotes (Driessen and Nouwen, 2008; Park and Rapoport, 2012). The translocation of these proteins is triggered by hydrophobic sequences, either cleavable signal sequences or noncleaved transmembrane (TM) sequences. The polypeptide substrates are translocated across the membrane through a hydrophilic channel formed by a conserved heterotrimeric membrane protein complex, the SecY complex in bacteria and archaea, and the Sec61 complex in eukaryotes. The complex consists of a large  $\alpha$ -subunit (SecY or Sec61p) that spans the membrane ten times, and two smaller, single-spanning  $\beta$ - and  $\gamma$ -subunits (called SecG and SecE in bacteria). The channel associates with different partners that provide the driving force for translocation. In bacteria, the SecY channel can either associate with the ribosome to translocate proteins during their synthesis (cotranslational translocation), or it can cooperate with the cytosolic ATPase SecA to transport proteins after completion of their synthesis (post-translational translocation). The exact mechanism of protein translocation is still poorly

understood. In particular, it is unclear whether one or more SecY/Sec61 complexes are required for translocation, an issue that impacts on several crucial aspects of translocation.

The question of how many SecY/Sec61 copies form the translocation channel has been controversial. Based on crystal structures of SecY complexes from different bacterial and archaeal species, it was proposed that a single copy of the complex forms the translocation channel (Van den Berg et al., 2004; Tsukazaki et al., 2008; Zimmer et al., 2008; Egea and Stroud, 2010). The channel has an hourglass shape (Van den Berg et al., 2004), with an empty cytoplasmic cavity and an extracellular cavity that is filled with a short helix, the plug, which moves out of the way during protein translocation. The constriction of the hourglass is formed by a pore ring of amino acids, which likely surround the polypeptide chain during its passage through the channel (Van den Berg et al., 2004). Translocation through a single SecY copy is supported by disulfide cross-linking experiments with a SecA-dependent translocation substrate, which indicate that both the signal sequence and the following polypeptide segment can be cross-linked to the same SecY molecule (Osborne and Rapoport, 2007). In addition, electron

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Abbreviations used in this paper: BM, bismaleimide; CBP, calmodulin-binding peptide; CuPh<sub>3</sub>, copper phenanthroline; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NEM, N-ethylmaleimide; TM, transmembrane.

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microscopy structures show that a single copy of Sec61/SecY complex is bound to a ribosome, with the cytoplasmic funnel of the translocation channel located underneath the ribosome tunnel (Ménétre et al., 2007, 2008; Becker et al., 2009; Frauenfeld et al., 2011). Based on the crystal structures, it has been proposed that the channel itself is responsible for maintaining the membrane barrier for small molecules (Van den Berg et al., 2004). Indeed, recent experiments support the view that the resting channel is sealed by both the pore ring and the plug domain; during translocation, the pore ring forms a gasket-like seal around the polypeptide chain, which prevents the permeation of small molecules (Park and Rapoport, 2011).

Other experiments suggest that the channel is formed from oligomers of the SecY/Sec61 complex. For example, fluorescence-quenching experiments indicate a channel diameter of 40–60 Å in intact microsomal membranes (Hamman et al., 1997), which is inconsistent with the pore being formed from just one copy of the Sec61 complex. It was argued that these experiments are more reliable than those performed in detergent or with reconstituted SecY molecules (Hamman et al., 1997; Lin et al., 2011). Because of the large pore size, it was proposed that the membrane barrier for small molecules is provided by BiP binding to the luminal end of the channel in the resting state, and by the translating ribosome binding to the cytoplasmic side of the channel during translocation (Hamman et al., 1998). Oligomers of the SecY/Sec61 complex have also been detected in intact membranes by cross-linking (Kaufmann et al., 1999; Schaletzky and Rapoport, 2006), fluorescence energy transfer (Mori et al., 2003; Snapp et al., 2004), and freeze-fracture electron microscopy experiments (Scheuring et al., 2005). However, it is unclear how the SecY/Sec61 molecules are arranged in these oligomers. A two-dimensional structure of the *E. coli* SecY complex in a lipid bilayer and cross-linking data indicate that two SecY molecules can associate back-to-back, i.e., with the long TM segments of the SecE subunits facing each other (Kaufmann et al., 1999; Breyton et al., 2002; Deville et al., 2011). Recent EM data show a synthetic signal peptide bound to only one of the two back-to-back-associated SecY molecules (Hizlan et al., 2012). Other orientations of the SecY molecules in the oligomers have also been suggested (Das and Oliver, 2011; Dalal et al., 2012). The oligomers detected in membranes cannot be very stable, as detergent-solubilized SecY complexes tend to behave as monomers, except under gentle solubilization conditions (Bessonneau et al., 2002).

Even if a single SecY/Sec61 complex is sufficient to form the pore of the channel, this does not necessarily mean that protein translocation can occur with just one copy. For example, it is conceivable that additional Sec61/SecY molecules stabilize the ribosome-channel junction (Schaletzky and Rapoport, 2006; Gumbart et al., 2009), or that a second SecY molecule prevents the dissociation of SecA from the translocating SecY copy, thereby enhancing the processivity of SecA during translocation of a polypeptide chain (Osborne and Rapoport, 2007; Deville et al., 2011; Dalal et al., 2012). In fact, several results suggest that a dimer may be required for at least SecA-dependent translocation. Using proteoliposomes with reconstituted SecY proteins, it was shown that a defective SecY

molecule can be rescued for translocation by linking it covalently with a wild-type SecY copy (Osborne and Rapoport, 2007). Furthermore, liposomes containing a single SecY molecule had a reduced translocation activity compared with vesicles containing dimeric SecY (Deville et al., 2011), and lipid nanodiscs containing only one SecY molecule did not stimulate ATPase activity of SecA, in contrast to nanodiscs containing two SecY copies (Dalal et al., 2012). The coexpression of two different mutant SecYs, each displaying a translocation defect, also resulted in increased translocation activity (Dalal et al., 2012). On the other hand, a recent study using fluorescence-correlation spectroscopy suggested that a single SecY complex molecule is sufficient for SecA-mediated translocation in vitro (Kedrov et al., 2011). However, these experiments were performed at extremely low SecY concentrations, which favor the dissociation of oligomers, and they were complicated by the fact that SecY is reconstituted into proteoliposomes in both orientations. Most importantly, they did not monitor directly the mobility of active SecY channels, as the translocation substrate was unlabeled. A major problem with all these studies is that they are performed at rather artificial conditions.

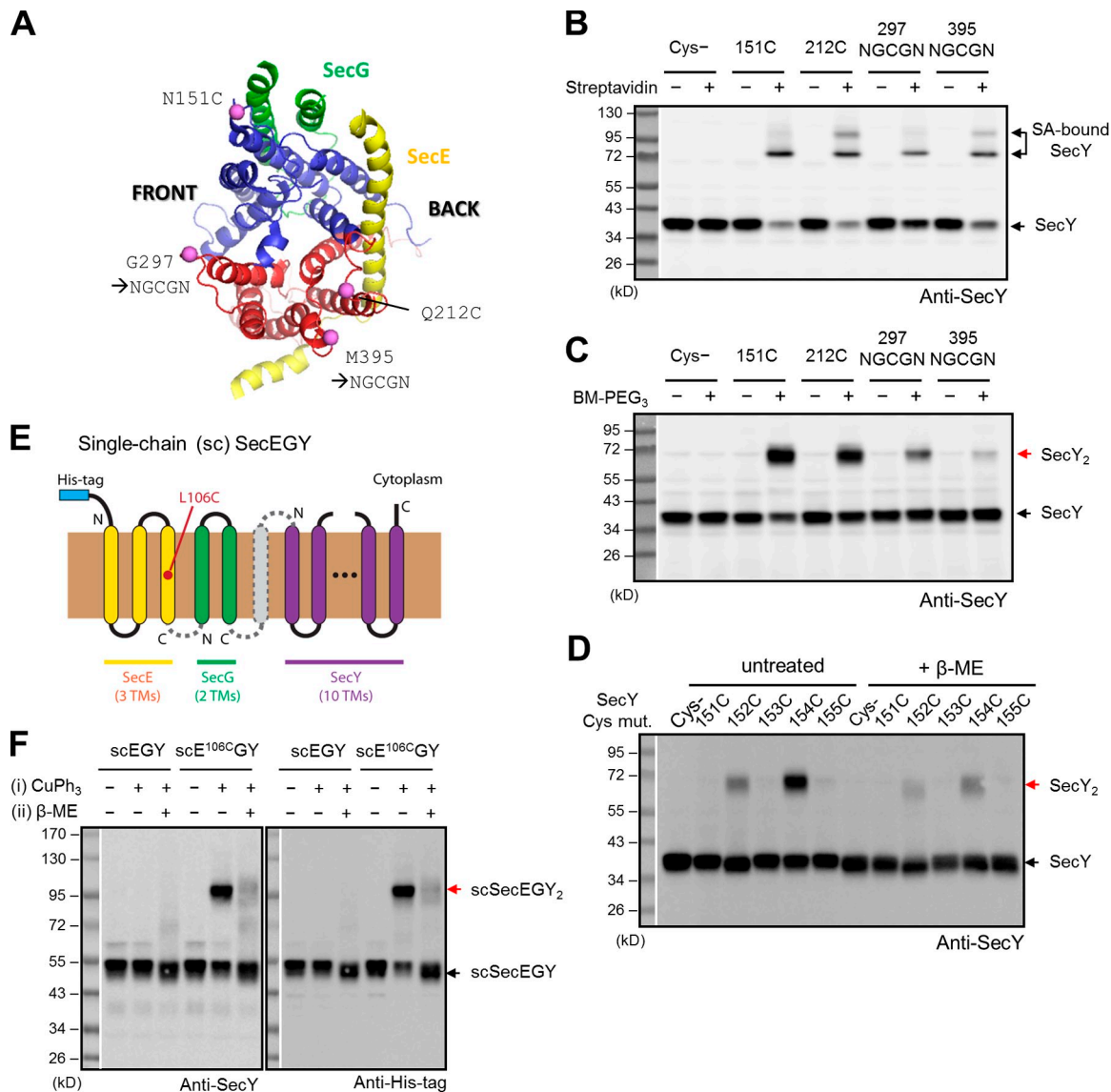
Here, we have tested whether SecY oligomers are required for protein translocation under physiological conditions. To this end, we have developed new methodology that allowed us to study defined translocation intermediates in intact *E. coli* cells. We use these methods to demonstrate that a single SecY molecule is sufficient for both co- and post-translational translocation.

## Results

### Interactions between resting SecY channels in the *E. coli* plasma membrane

We first investigated the oligomeric state of resting SecY channels by treating *E. coli* cells with the transcription inhibitor rifampicin, which leads to the depletion of translocating polypeptides. Single cysteines were introduced into periplasmic loops of SecY and a bismaleimide was added to intact cells to induce cross-links between two SecY molecules. The cysteines were placed in four of the five periplasmic loops of SecY on the periphery of the channel (Fig. 1 A). Cysteines at positions 297 and 395 were incorporated as part of a small, flexible insert to increase their accessibility to the cross-linking reagent. The mutants and a cysteine-lacking control SecY were expressed from a plasmid under the endogenous promoter and were functional, as they complemented a *secY* deletion (Fig. S1 A).

We first tested whether the introduced cysteines are accessible to a modification reagent (biotin-PEG<sub>2</sub>-maleimide) that has about the same size as the bismaleimide cross-linker. After treatment, modified proteins were detected after incubation with streptavidin by a mobility shift in SDS-PAGE. Streptavidin (SA)-bound SecY bands were visible for all cysteines (Fig. 1 B). Next, we incubated cells with bismaleimide-PEG<sub>3</sub> (BM-PEG<sub>3</sub>), a bi-functional cross-linker with an ~17 Å linker between the maleimide groups. The reaction was quenched and the samples were subjected to SDS-PAGE and blotting with SecY antibodies. Efficient cross-linking between two SecYs was seen with Cys151 and Cys212 (Fig. 1 C). Cross-linking with Cys297



**Figure 1. SecY complexes interact in vivo through different surfaces.** (A) View of *Thermotoga maritima* SecY complex from the periplasm. The N- and C-terminal halves of SecY are colored blue and red, respectively, SecG in green, and SecE in yellow. Balls in magenta indicate positions that were mutated in the *E. coli* protein to cysteines. Note that two cysteines are part of an inserted segment. (B) The accessibility of cysteine residues introduced into SecY was tested in intact *E. coli* by modification with biotin-PEG<sub>2</sub>-maleimide, followed by incubation with streptavidin (SA). The samples were analyzed by SDS-PAGE and immunoblotting with SecY antibodies (anti-SecY). The two bands correspond to one or two SecY molecules bound to tetrameric streptavidin. (C) The interaction of SecYs with the indicated cysteines was tested by addition of bismaleimide-PEG<sub>3</sub> (BM-PEG<sub>3</sub>) to resuspended intact *E. coli* cells, pretreated with rifampicin for 30 min. The samples were analyzed by SDS-PAGE and immunoblotting for SecY. SecY<sub>2</sub>, cross-linked SecY dimers. (D) The front-to-front interaction of SecYs with the indicated cysteines was tested by spontaneous disulfide bridge formation in vivo. Where indicated, the samples were treated with β-mercaptoethanol (β-ME). (E) Scheme of a SecY complex containing SecE, SecG, and SecY in a single polypeptide chain. The gray dotted segments are added linkers. Residue L106 at the back of SecE (see A) is indicated. (F) The back-to-back interaction of single-chain SecY complexes with a cysteine at position 106 of SecE was tested by disulfide bridge formation after addition of the oxidant CuPh<sub>3</sub> to cell lysates. Controls were performed with protein lacking a cysteine and with β-ME addition after cross-linking.

or 395 was significantly less efficient. Similar results were obtained when BM-PEG<sub>3</sub> was added directly to the bacterial culture, rather than to bacteria resuspended in buffer (Fig. S1 B). Cross-linking between Cys212 residues might have been expected, as this residue is close to the interface of a previously observed back-to-back SecY dimer (Fig. 1 A; Breyton et al., 2002). However, Cys151 is in the loop between TM segments 3 and 4, which lies on the opposite side of SecY (the front; Fig. 1 A). This front-to-front interaction may correspond to a dimer suggested by SecA–SecY cross-linking experiments

(Das and Oliver, 2011). A model of a front-to-front associated SecY dimer would indeed bring Cys151 of two SecYs close to one another (Fig. S1 C).

To provide further evidence for these two distinct interaction surfaces of SecY, we used disulfide bridge cross-linking. For the front-to-front interaction, we placed single cysteines at different positions in the loop between TM segments 3 and 4 and examined the formation of a disulfide-bridged dimer (Fig. 1 D). A cysteine placed at position 154 gave a prominent cross-link, even without addition of an oxidation reagent (Fig. 1 D). As expected,

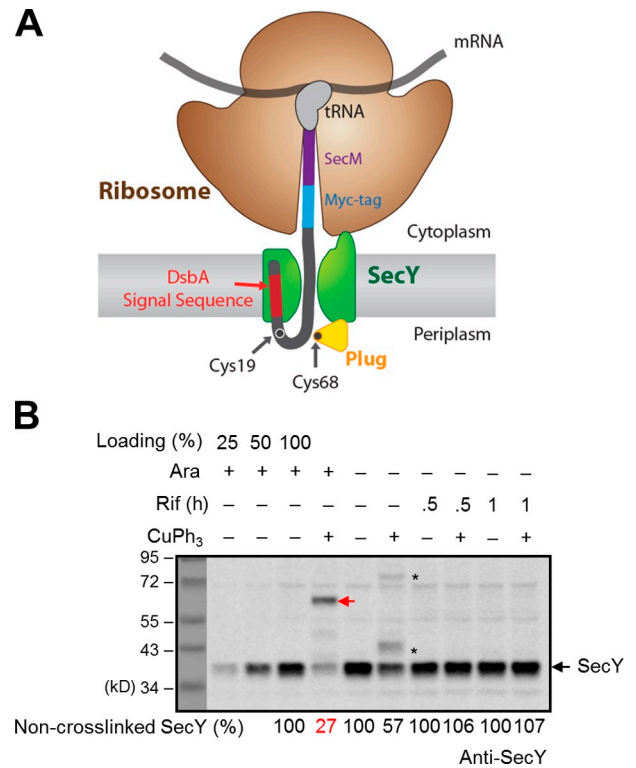
the intensity of the cross-linked product was greatly diminished upon addition of a reducing reagent ( $\beta$ -mercaptoethanol;  $\beta$ -ME). The spontaneous formation of a disulfide bridge suggests that two SecYs must come close to one another through their front surfaces.

To test for disulfide bridge formation between the back surfaces of SecY complexes, we used a SecE mutant with a cysteine at position 106 (Kaufmann et al., 1999). To exclude cross-linking between free SecE(106C) molecules that are not part of the SecY complex (Kaufmann et al., 1999), we generated a fusion protein containing SecE(106C), SecG, and SecY in one polypeptide chain (Fig. 1 E); the C terminus of SecE was linked to the N terminus of SecG with a short linker, and the C terminus of SecG was connected to the N terminus of SecY through an additional, unrelated TM segment. This fusion protein (scSecEGY) was stable and complemented a *secY*-null mutant (Fig. S1 A). When cells were lysed and treated with the oxidation reagent copper phenanthroline ( $\text{CuPh}_3$ ), a prominent disulfide-linked dimer of the fusion protein was observed after blotting with antibodies to either SecY or the N-terminal His-tag (Fig. 1 F). No dimer was seen when the cysteine was lacking or when  $\beta$ -ME was added after cross-linking. Thus, SecY complexes can interact with each other through their back surfaces. When one cysteine was introduced at the front (position 151), and another one at the back (position 212), the addition of the bismaleimide cross-linker led to a ladder of cross-linked SecY products (Fig. S1 D), indicating that the two interaction surfaces in the SecY complex can be used at the same time.

To test the stability of the SecY oligomers, we expressed simultaneously a SecY molecule with a cysteine at either position 151 or 212 from the endogenous promoter, and an additional SecY copy lacking a cysteine ( $\text{SecY}^{\text{Cys}^-}$ ) from the inducible arabinose promoter;  $\text{SecY}^{\text{Cys}^-}$  also contained a C-terminal calmodulin-binding peptide (CBP) tag ( $\text{SecY}^{\text{Cys}^-}$ -CBP) to distinguish it from the cysteine-containing SecY copy. If SecY forms stable oligomers, one would expect that the coexpression of the two SecY molecules over an extended time period (1 h) leads to defined assemblies that contain both cysteine-containing and -lacking SecY molecules, and the cross-linking yield should therefore decrease. Upon expression of  $\text{SecY}^{\text{Cys}^-}$ -CBP, the ratio of cysteine-free to cysteine-containing SecY molecules reached  $\sim 1.5:1$ , but the cross-linking efficiency of cysteine-containing SecY molecules was not reduced (Fig. S1, E and F). These data support the notion that the interactions between SecY molecules with both their front and back surfaces are relatively short-lived.

#### Translocation intermediates can be generated in vivo with just one SecY copy

Next, we tested whether the cotranslational insertion of a polypeptide into the translocation channel requires a SecY oligomer. To this end, we generated a translocation intermediate in vivo, using a previously established substrate (DsbA-SecM, formerly called NC100; Park and Rapoport, 2011). This is a polypeptide of 100 amino acids, containing the signal sequence of DsbA (Fig. 2 A), which targets the substrate to the cotranslational pathway (Schierle et al., 2003). A SecM-stalling sequence



**Figure 2. Saturation of SecY channels with a cotranslational translocation intermediate.** (A) Scheme of a cotranslational translocation intermediate generated with the substrate DsbA-SecM. The nascent chain contains the signal sequence of DsbA at the N terminus, a Myc-tag for detection, and a C-terminal SecM-stalling sequence. Its insertion into the SecY channel is monitored by disulfide bridge formation between cysteines in the nascent chain and SecY. (B) DsbA-SecM was expressed from the arabinose (Ara)-inducible promoter in cells producing SecY at approximately endogenous level using a GUG translational start codon (plasmid pACYC-SecYEG). After addition of the oxidant  $\text{CuPh}_3$  to the cell culture to induce disulfide bridge formation, the lysate was analyzed by SDS-PAGE and blotting with SecY antibodies. Where indicated, rifampicin (Rif) was added for different time periods. Red arrows and black asterisks indicate cross-links between SecY and substrate or endogenous proteins, respectively. For quantification of three independent experiments, see Fig. 3 C, right panel.

at the C terminus causes the ribosome to arrest translation, with the nascent chain associated as peptidyl-tRNA (Nakatogawa and Ito, 2001). DsbA-SecM was synthesized from the inducible arabinose promoter in cells expressing SecY from a plasmid under its native promoter. Expression of SecY at the endogenous level was achieved by changing the AUG translation initiation codon to the less efficient GUG codon. Insertion of the nascent chain into the SecY channel was monitored by disulfide bridge formation between a cysteine in DsbA-SecM (position 19, immediately following its signal sequence) and a cysteine in SecY (position 68). For these experiments we used a newly developed strain that lacks the chromosomal *secY* copy, whose presence leads to an underestimate of cross-linking efficiency to the nascent chain, as wild-type SecY lacks a cysteine at the appropriate position. Upon addition of the oxidant  $\text{CuPh}_3$  to intact cells,  $\sim 73\%$  of SecY was cross-linked to the nascent chain, as determined from the decrease of noncross-linked SecY (Fig. 2 B, lane 4; quantitation was confirmed by loading different amounts in lanes 1–3). As expected, no cross-linking between SecY and DsbA-SecM was observed when  $\text{CuPh}_3$  was omitted (lane 3) or



when the cells were treated with rifampicin to clear all translocation sites before addition of the oxidant (lanes 7–10). Also, this cross-linked product was not generated when DsbA-SecM expression remained uninduced, although minor cross-links to endogenous nascent chains were observed instead (lanes 5 and 6). The observed cross-linking yield of 73% is a lower estimate because some SecY channels may be occupied by endogenous substrates even after expression of DsbA-SecM, and disulfide formation is not 100% efficient. The fact that the cross-linking efficiency is substantially higher than 50% argues that only one molecule of SecY is required to accommodate the translocating DsbA-SecM chain.

To perform similar experiments with a post-translational substrate, we developed a new method to generate a translocation intermediate with a SecA-dependent substrate (OmpA-GFP). This polypeptide contains 79 amino acids, including the signal sequence of the post-translational substrate proOmpA, fused to a fast-folding GFP variant (Pédélec et al., 2006). With this substrate, one expects that the N terminus inserts into the SecY channel and that the folded GFP blocks further translocation through SecA and SecY (Fig. 3 A). To monitor insertion of the polypeptide into the channel, a cysteine was placed immediately after the signal sequence at position 21, and another cysteine into SecY (position 68). Efficient disulfide cross-linking between OmpA-GFP and SecY was observed upon addition of an oxidant to intact cells (Fig. 3 B, lane 2; the appearance of two cross-linked bands is due to incomplete unfolding of GFP in SDS sample buffer). No cross-linking was seen when either the substrate or SecY lacked the cysteine (lanes 6 and 8), when the cysteines were modified with *N*-ethylmaleimide (NEM) before addition of the oxidant (lane 3), or when the signal sequence was rendered nonfunctional by introducing two arginine residues (lane 10). As expected, the cross-linked product disappeared upon addition of the reducing agent  $\beta$ -ME (lane 4). These data demonstrate that a stable, post-translational translocation intermediate can be generated *in vivo*. With this substrate and SecY expressed at its endogenous level, ~67% of all SecY molecules could be cross-linked (Fig. 3 C, lane 4; controls as in Fig. 2 B). When the cysteine was placed at position 32 of OmpA-GFP, instead of position 21, the cross-linking efficiency was even higher (74%; see Fig. S2). Given that the cross-linking efficiency is substantially higher than 50%, only one molecule of SecY is required to accommodate the translocating OmpA-GFP chain.

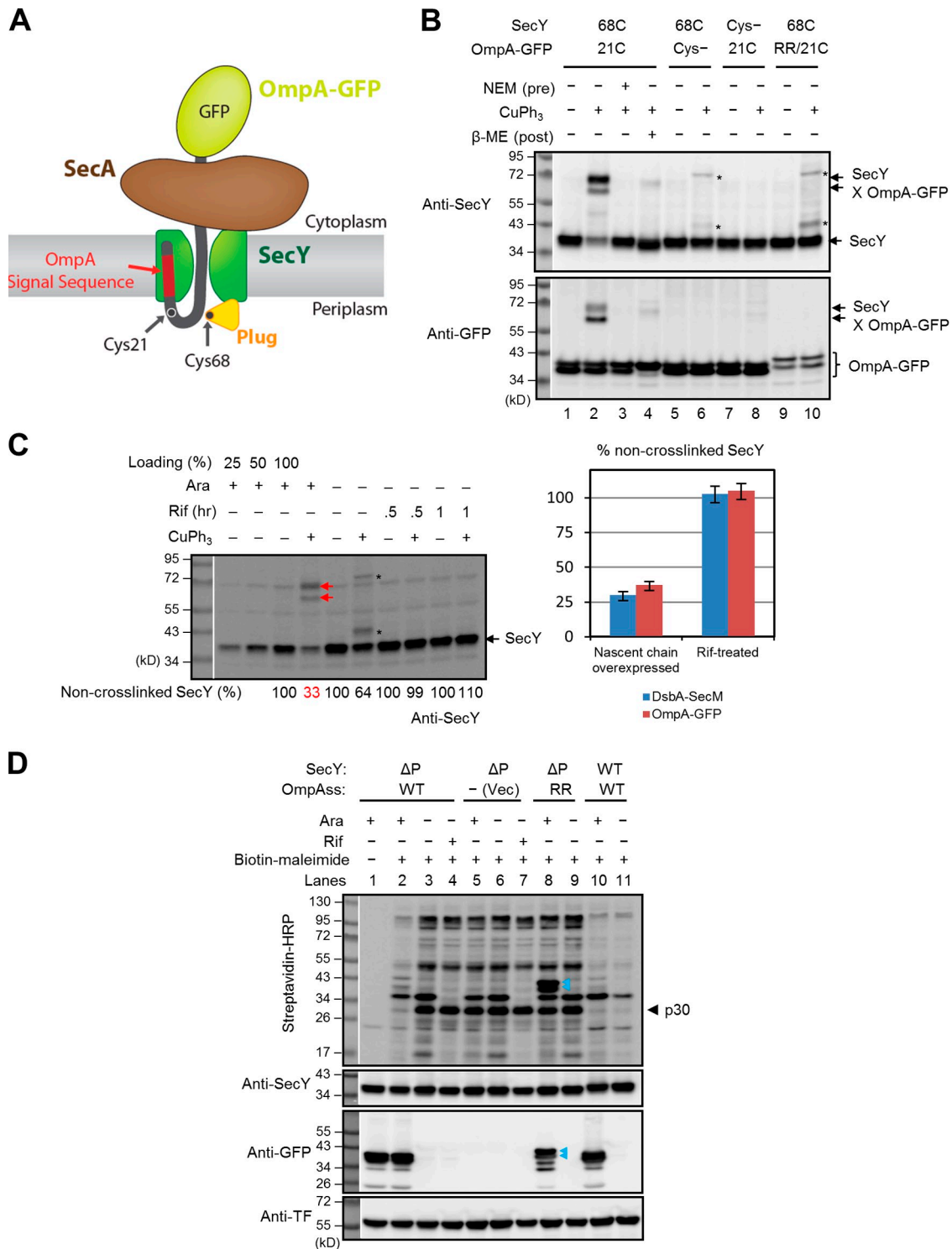
To further investigate whether the majority of translocation sites are occupied with OmpA-GFP, we tested whether the polypeptide substrate can block the permeation of small molecules through an open SecY channel. We have previously shown that SecY lacking its plug domain ( $\Delta$ P) is permeable to various small molecules, including the modification reagent biotin-maleimide and chloride ions, and that a cotranslationally inserted polypeptide chain can block the permeation of these molecules (Park and Rapoport, 2011). We now asked whether the post-translationally inserted polypeptide OmpA-GFP can do the same. When biotin-maleimide (biotin-PEG<sub>2</sub>-maleimide) was added to cells expressing SecY( $\Delta$ P), various cytosolic proteins, including a prominent protein of 30 kD (p30), were

modified as demonstrated by blotting with HRP-conjugated streptavidin (Fig. 3 D, lane 3). The localization of the modified proteins in the cytosol was previously demonstrated by cell fractionation (Park and Rapoport, 2011). Most of the permeability is due to nontranslocating, resting channels, as the addition of rifampicin did not drastically change cytosolic protein modification (lane 4). When OmpA-GFP was expressed under the inducible arabinose promoter, the extent of modification was reduced to a level similar to that seen with cells expressing wild-type SecY (lane 2 vs. 10). Expression of a signal sequence mutant of OmpA-GFP did not have this effect (lane 8). Similar results were obtained when the permeation of chloride ions was tested after conversion of *E. coli* cells into spheroplasts (Fig. S3). Thus, most of the SecY channels can be occupied with the post-translational translocation substrate OmpA-GFP.

### The oligomeric state of the SecY channel engaged in translocation

We next tested whether the insertion of a polypeptide into the SecY channel changes the oligomeric state of SecY. The cotranslational (DsbA-SecM) or post-translational (OmpA-GFP) substrate was expressed under the arabinose promoter in cells expressing SecY with a single cysteine at either position 151 (at the front) or 212 (at the back). Cross-linking with the bi-functional reagent BM-PEG<sub>3</sub> showed that both the front-to-front and back-to-back interactions of SecY molecules were drastically reduced (Fig. 4, lane 4 vs. 2, lane 10 vs. 8, lane 16 vs. 14, and lane 22 vs. 20). Thus, it appears that SecY oligomers dissociate into monomers upon insertion of a translocating polypeptide chain. Because both front-to-front and back-to-back dimers dissociate, it seems likely that SecY molecules weakly associate with each other in the plane of the membrane, and that the equilibrium is shifted toward monomers upon insertion of a translocating polypeptide.

To investigate the possibility that a translocating SecY channel might associate with a nontranslocating SecY copy, we performed experiments under conditions where not all channels are occupied with a model substrate. We first tested whether a SecY channel containing the cotranslational substrate DsbA-SecM can be cross-linked back-to-back with a nontranslocating SecY channel. These experiments used the single-chain scSecEGY construct with a cysteine at position 106 of SecE for back-to-back cross-linking, and a cysteine at position 68 of SecY for cross-linking with the substrate. To achieve low occupancy of SecY with substrate, we reduced ribosome-associated nascent chains by expression of MazF, an RNase that cleaves mRNAs at ACA sequences (Zhang et al., 2003). The occupancy was indeed reduced (Fig. 5 A, lane 15). For the experiments, the cells were first treated with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to induce disulfide bridge formation between SecY and the substrate, and then cell extracts were treated with CuPh<sub>3</sub> to test for cross-linking between SecY molecules. Both cross-links between SecY and DsbA-SecM (lanes 9 and 10, 19 and 20) and between SecY and SecY (lanes 18 and 20) were observed, but no double cross-links containing two SecYs and substrate (lanes 10 and 20). Assuming that substrate binding does not



**Figure 3. Saturation of SecY channels with a post-translational translocation intermediate.** (A) Scheme of a post-translational translocation intermediate generated with SecA and the substrate OmpA-GFP. The translocating chain contains the signal sequence of OmpA at the N terminus and the “superfolder” GFP at the C terminus. Its insertion into the SecY channel is monitored by disulfide bridge formation between cysteines in the substrate and SecY. (B) The insertion of OmpA-GFP, containing a cysteine at position 21 (21C), into SecY containing a cysteine at position 68 (68C) was tested by disulfide bridge formation after the addition of the oxidant CuPh<sub>3</sub> to intact *E. coli* cells. Where indicated, the substrate or SecY lacked a cysteine or the cysteines were blocked with *N*-ethylmaleimide (NEM) before addition of CuPh<sub>3</sub>. As a control, a substrate was used with a defective signal sequence (RR-21C). Where indicated, disulfide bridges were reduced by β-mercaptoethanol (β-ME). All samples were analyzed by SDS-PAGE followed by blotting with SecY or GFP antibodies. (C) OmpA-GFP was expressed from the arabinose (Ara)-inducible promoter in cells producing SecY at approximately endogenous level using a GUG translational start codon (plasmid pACYC-SecYEG). After addition of CuPh<sub>3</sub>, the lysate was analyzed by SDS-PAGE and blotting with SecY antibodies. Where indicated, rifampicin (Rif) was added for different time periods. Red arrows and black asterisks indicate cross-links between SecY and substrate or endogenous proteins, respectively. The right panel shows quantification of the cross-linking efficiency between SecY and translocation substrates, based on the decrease of noncross-linked SecY in experiments such as shown in the left panel and Fig. 2 B. Three different experiments were analyzed (mean

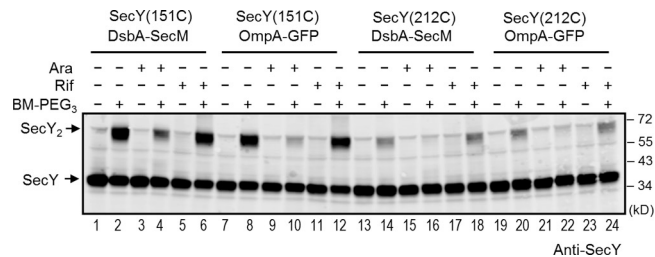
drastically change the distance between the cysteines in the interacting SecY molecules, it appears that substrate does not insert into back-to-back-associated SecY dimers.

Next, we performed similar experiments with the post-translational substrate OmpA-GFP. To increase the percentage of nonoccupied SecY channels, we reduced the expression of OmpA-GFP by changing the translation start codon from AUG to the less efficient GUG codon, and by adding rifampicin for 30 min to clear the SecY channels from most endogenous substrates (Fig. S4). As a result, the cross-linking efficiency of SecY to OmpA-GFP was drastically reduced (Fig. 5 B, lane 15). The cells were first treated with DTNB to induce SecY substrate cross-links, and then with CuPh<sub>3</sub> to test for SecY–SecY back-to-back cross-linking. While single cross-links were prominent (lanes 9, 10, 19, and 20; and lanes 18, 20), double-cross-links containing both two SecYs and substrate were weak (lanes 10 and 20). Thus, it appears that the majority of substrate molecules insert into monomeric SecY.

Next, we tested whether front-to-front-associated SecY molecules can insert a translocation substrate. Here, we exploited the fact that SecYs with a cysteine at position 154 spontaneously form a disulfide-bridged dimer. SecY also contained a cysteine at position 68 to test for the insertion of substrate containing a cysteine following the signal sequence. After inducing the expression of either DsbA-SecM or OmpA-GFP, the oxidant CuPh<sub>3</sub> was added to generate a disulfide bridge between SecY and substrate. With the cotranslational substrate, efficient cross-links between SecY and SecY (Fig. 5 C, lanes 13–16) and between SecY and DsbA-SecM (lanes 4, 8, 12, and 16) were observed, but no double cross-links (lanes 8 and 16). Thus, the cotranslational substrate does not insert into front-to-front-associated SecY dimers. With the post-translational substrate, weak double cross-links were seen (Fig. 5 D, lanes 8 and 16), indicating that the majority of molecules also inserts into monomeric channels.

#### Dimerization-defective SecY mutants

To directly test the role of the back-to-back and front-to-front association of SecY molecules, we generated mutants in which these interactions are disrupted. We first screened for mutations with defective back-to-back association by placing different amino acids at position 106 of SecE, the point of closest contact of two SecY complexes. To this end, we expressed from a plasmid SecY complex containing mutant SecE and SecY with a cysteine at position 212; SecY dimer formation was determined after addition of BM-PEG<sub>3</sub>. Placing a bulky or negatively charged residue at position 106 of SecE reduced dimer formation only moderately, but introducing an arginine had a drastic effect, although the levels of SecE and SecY were only slightly reduced (Fig. 6 A). The residual dimer formation likely



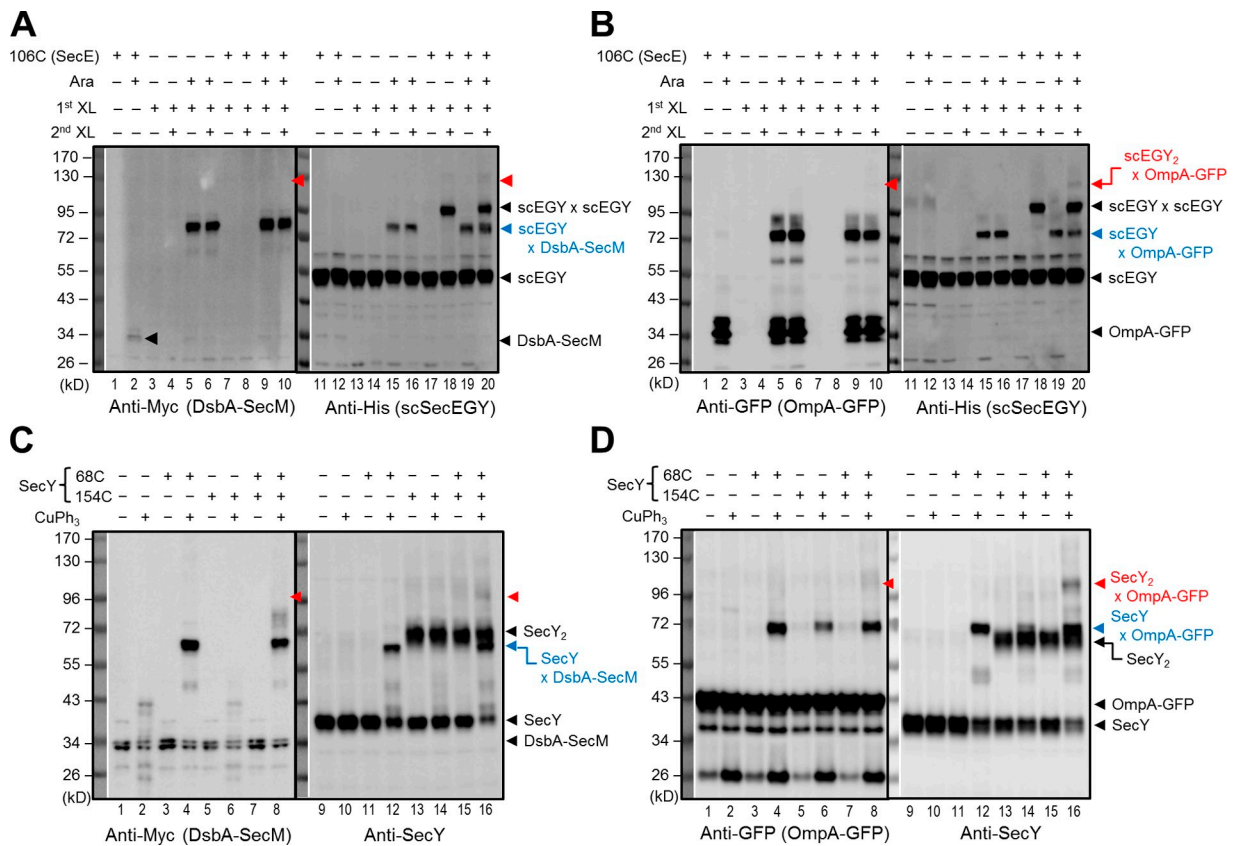
**Figure 4. Dissociation of SecY oligomers upon insertion of a translocating chain.** DsbA-SecM or OmpA-GFP was expressed from the arabinose (Ara)-inducible promoter in cells producing SecY at approximately endogenous level using a GUG translational start codon. SecY carried either a cysteine at the front (SecY-151C) or at the back (SecY-212C). Cross-linking of SecYs (SecY<sub>2</sub>) was tested after addition of bismaleimide-PEG<sub>3</sub> (BM-PEG<sub>3</sub>) to intact cells. Where indicated, cells were treated with rifampicin (Rif) before cross-linking.

originates from SecY complexes containing endogenous SecE. Indeed, when the same mutant was expressed in a strain lacking endogenous SecE, no dimer formation was observed (Fig. 6 B). Remarkably, this strain had no detectable growth defect (Fig. 6 C), suggesting that a back-to-back-associated SecY dimer is not required for protein translocation.

To disrupt the front-to-front association of SecY molecules, we introduced arginine residues into TM segment 3 of SecY, which is close to the predicted interaction surface. Based on the crystal structure (Tsukazaki et al., 2008; Zimmer et al., 2008), we placed two arginines at positions where their side chains would point away from the SecY complex. To avoid problems with membrane integration efficiency, we also replaced some of the less hydrophobic amino acids in TM3 with more hydrophobic ones. The resulting SecY mutant (TM3<sup>RR</sup>) was totally defective in front-to-front dimerization, as measured by cross-linking of SecY with a cysteine at position 151 after addition of BM-PEG<sub>3</sub> (Fig. 6 D). However, when present as the sole copy, the mutant SecY supported cell growth indistinguishably from the wild-type strain (Fig. 6 E). Thus, a front-to-front-associated SecY dimer is also not essential for protein translocation. A mutant of the SecY complex that carried mutations disrupting both front-to-front and back-to-back associations (SecY(TM3<sup>RR</sup>) and SecE(106R)) was also able to complement a strain lacking both SecE and SecY and grew with wild-type kinetics (Fig. S5). Thus, even the simultaneous disruption of both interactions has no deleterious effect on translocation.

One potential caveat to our conclusion is that a small amount of dimers may still be formed in the mutants and might be essential for the viability of cells. To exclude this possibility, we expressed wild-type and mutant SecY at different levels and monitored both dimer formation and cell viability. To express SecY at reduced levels, we used plasmids with different origins

and SD). (D) OmpA-GFP with either a wild-type (WT) or defective (RR) signal sequence was expressed under the arabinose (Ara) promoter in cells producing wild-type SecY or SecY lacking its plug domain ( $\Delta$ P). Controls were performed with an empty vector (vec) and without Ara induction. Biotin-maleimide was added to the cells, and the modification of proteins was probed by SDS-PAGE and blotting with HRP-conjugated streptavidin. The samples were also probed with SecY, GFP, and trigger factor (TF; loading control) antibodies. Where indicated, cells were pretreated with rifampicin (Rif) before addition of biotin-maleimide. p30, a prominently modified cytosolic protein. The blue arrowheads indicate biotinylation of translocation-incompetent OmpA-GFP carrying a defective signal sequence.



**Figure 5. Preferential insertion of substrate into monomeric SecY complex.** (A) A single-chain SecY complex with a cysteine at position 106 of SecE for back-to-back cross-linking was expressed under the endogenous promoter together with the cotranslational substrate DsbA-SecM under the arabinose (Ara) promoter. The level of DsbA-SecM was reduced by expression of the RNase MazF. Insertion of the nascent chain was monitored by disulfide bridge cross-linking between DsbA-SecM and SecY after addition of DTNB to intact cells (first XL). After homogenization, a cell extract was treated with the oxidant CuPh<sub>3</sub> to induce cross-links between SecY molecules (second XL). The samples were analyzed by SDS-PAGE and immunoblotting for Myc (substrate) and His (channel) tags. The red arrow indicates the position expected for double cross-links of SecY with SecY and DsbA-SecM. (B) As in A, but with the post-translational substrate OmpA-GFP. The expression level of substrate was reduced by the use of a GUG translational start codon, and SecY channels were cleared of endogenous substrates by addition of rifampicin for 30 min. (C) SecY with a cysteine at position 154 for spontaneous front-to-front disulfide bridge formation was expressed together with DsbA-SecM. Channel insertion of DsbA-SecM was monitored by disulfide bridge formation between substrate and SecY after addition of CuPh<sub>3</sub>. Controls were performed with SecY lacking cysteines for SecY-SecY (154C) or SecY-substrate (68C) cross-linking. The red arrow indicates the position expected for double cross-links of SecY with SecY and DsbA-SecM. (D) As in C, but with OmpA-GFP. The cross-linking between substrate and position 154 of SecY (lane 6) might be explained by assuming that the proOmpA signal sequence is mobile (more so than that of DsbA), so that it comes close to position 154 at the lateral gate.

of replication and different translation start codons for the *secY* gene. With these constructs, the level of SecY expression could be varied from 127% compared with the endogenous SecY level in wild-type cells all the way down to 5% (Fig. 7 A). As expected, lowering the SecY level decreased the cross-linking efficiency, both for the front-to-front and the back-to-back associations (Fig. 7 B). However, the decrease was less pronounced than expected from a bimolecular cross-linking reaction between randomly colliding SecY complexes, suggesting that the SecY-SecY interactions have a finite lifetime. When tested in a *secY*-null strain, the reduction of SecY levels to ~60% had no effect on growth (Fig. 7 C; left). A slight growth defect was seen at a level of 43% and a major defect at 23%. Importantly, the results were similar with a SecY mutant that is defective in front-to-front association (Fig. 7 C; middle). A slight difference in viability was seen at very low levels of SecY expression (~25% of wild-type; Fig. 7 C), which is explained by the reduced stability of the SecY mutant (see immunoblots in Fig. 6 D). Similar results were obtained with a SecY complex

compromised in back-to-back dimer formation (Fig. 7 C, right). These data indicate that disruption of dimer formation has a negligible effect on cell growth, even when SecY levels are close to the minimum required for cell viability. Thus, a single copy of SecY is sufficient to support protein translocation.

## Discussion

Our experiments provide strong evidence that a single SecY complex is sufficient for protein translocation in vivo. This implies that the pore of the translocation channel is formed from just one SecY molecule and that additional copies of SecY are not essential for moving a polypeptide co- or post-translationally through the channel.

Our cross-linking experiments show that SecY complexes can associate with one another in the plane of the membrane by back-to-back and front-to-front associations. A back-to-back interaction of SecY molecules has been observed previously (Kaufmann et al., 1999; Breyton et al., 2002; Dalal et al., 2012),



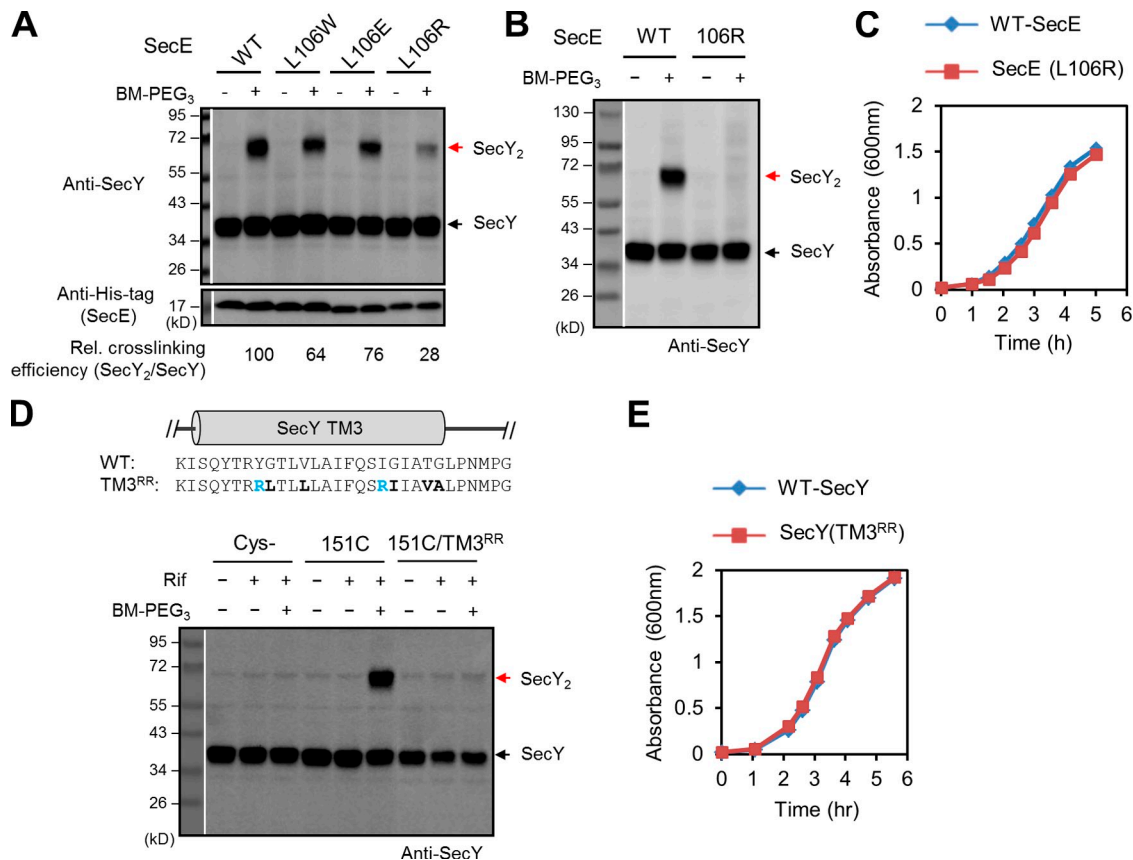


Figure 6. **Mutants disrupting the association of SecY complexes in the membrane.** (A) The back-to-back association of SecY complexes was tested using a cysteine at position 212 and the bi-functional cross-linker bismaleimide-PEG<sub>3</sub> (BM-PEG<sub>3</sub>). Mutations were introduced at position 106 of SecE to test their effect on SecY dimer (SecY<sub>2</sub>) formation. The samples were analyzed by SDS-PAGE and immunoblotting for SecY and His tag (SecE). The cross-linking efficiency was quantitated and is expressed relative to that with wild-type SecE. (B) As in A, but with the chromosomal SecE copy deleted. (C) The growth rate of the cells used in B was compared. The data shown are from a single representative experiment out of two repeats. (D) The front-to-front association of SecY complexes was tested using a cysteine at position 151 and the bi-functional cross-linker bismaleimide-PEG<sub>3</sub> (BM-PEG<sub>3</sub>). Mutations were introduced in TM3 of SecY, as indicated (TM3<sup>RR</sup>), to disrupt SecY dimer (SecY<sub>2</sub>) formation. Where indicated, cells were treated with rifampicin (Rif) before cross-linking. (E) The growth rate of the cells used in D was compared. The data shown are from a single representative experiment out of two repeats.

and a front-to-front association was suggested on the basis of SecA-SecY photo-cross-linking experiments (Das and Oliver, 2011). Our results now show that, at least in the resting state, SecY molecules can interact with both surfaces inside the membrane of intact *E. coli* cells. In fact, it is possible that surfaces other than the front and back of a SecY complex can also mediate dimerization. Both the front-to-front and back-to-back interactions of SecY complexes appear to be transient, as demonstrated by competition experiments with noncross-linkable SecY molecules. The interactions can be abolished by introducing positive charges into TM segments at the front or back of the SecY complex, likely because these charges lead to repulsion of SecY complexes in the hydrophobic environment of the membrane. Taken together, our experiments argue against a defined oligomeric state of the SecY complex in native membranes and suggest that SecY complexes interact relatively weakly inside the lipid bilayer, although the actual lifetime of the interactions remains to be determined. A weak interaction between SecY complexes is consistent with fluorescence-correlation spectroscopy experiments, which show little dimer formation when SecY is present at low concentrations in liposomes (Kedrov et al., 2011). It also explains why SecY complexes

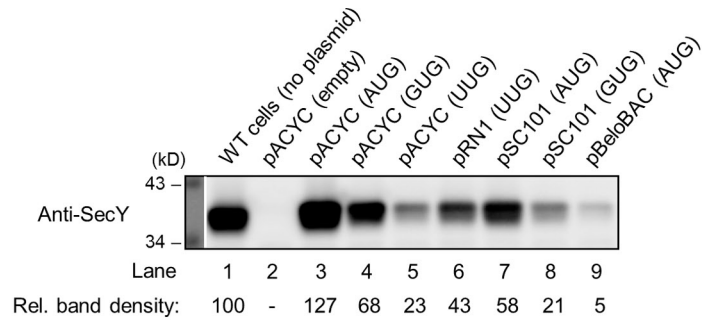
are monomeric upon solubilization in detergent (Bessonneau et al., 2002).

Using new methods to assemble translocation intermediates *in vivo*, we show that short translocating polypeptides insert into monomeric SecY. With an excess of substrate, almost all SecY complex molecules can be occupied with either a co- or post-translational translocation substrate. These experiments show that even if oligomers formed through interaction surfaces other than the front or back of the SecY complex, such oligomers would not be required for the insertion of a translocation substrate into the channel. Polypeptide insertion occurs into monomeric SecY channels even under conditions where there are plenty of free SecY molecules available. In fact, for a cotranslational substrate, both back-to-back and front-to-front dimers are entirely incompatible with translocation, as shown by the absence of dimer cross-links with SecY containing a translocating polypeptide. For a post-translational substrate, some molecules inserted into dimeric SecY, but most still preferred monomeric SecY. Taken together, these results indicate that the insertion of a polypeptide chain into the SecY channel requires only a single SecY copy. In principle, it is possible that later stages of translocation require additional SecY copies. However, the fact that

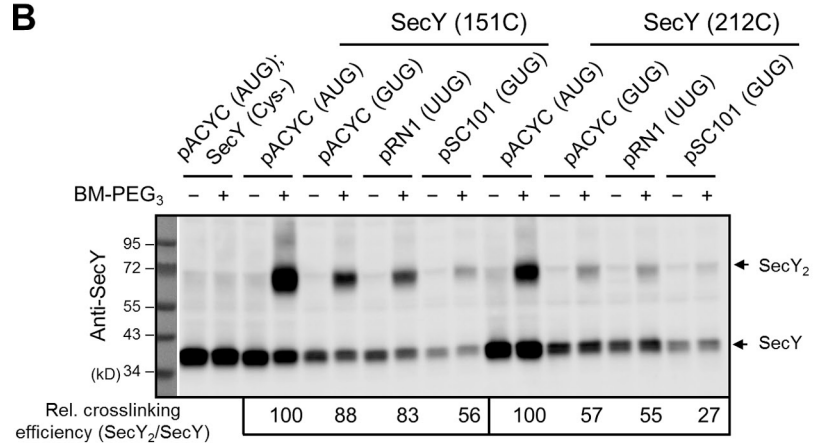
Figure 7. **Oligomerization-defective SecY mutants have a negligible growth defect.**

(A) SecY was expressed at different levels by using plasmids with different origins of replication (approximate copy numbers: 10–12 for pACYC, 20–40 for pRN1, 5 for pSC101, and 1–2 for pBeloBAC) and different translation start codons for the secY gene (shown in brackets). Samples from equal numbers of cells were analyzed by SDS-PAGE and immunoblotting with antibodies to the C terminus of SecY. In lanes 2–9, the endogenous SecY copy was tagged with CBP at the C terminus, which makes it nondetectable by SecY antibodies. The levels of SecY are given relative to that in wild-type cells (lane 1). (B) SecY with a cysteine at position 151 (the front) or 212 (the back) was expressed at different levels from various plasmids. Cross-linking between SecYs was induced by addition of bismaleimide-PEG<sub>3</sub> (BM-PEG<sub>3</sub>) and the samples were analyzed by SDS-PAGE and immunoblotting with SecY antibodies. (C) Wild-type (WT) SecY complex or mutant complexes defective in either front-to-front association (SecY (TM3<sup>RR</sup>)) or back-to-back association (SecE(106R)) were expressed at different levels from various plasmids in a strain lacking endogenous SecY. The growth of colonies at 37°C was analyzed at different time points after transformation. N.D., not determined.

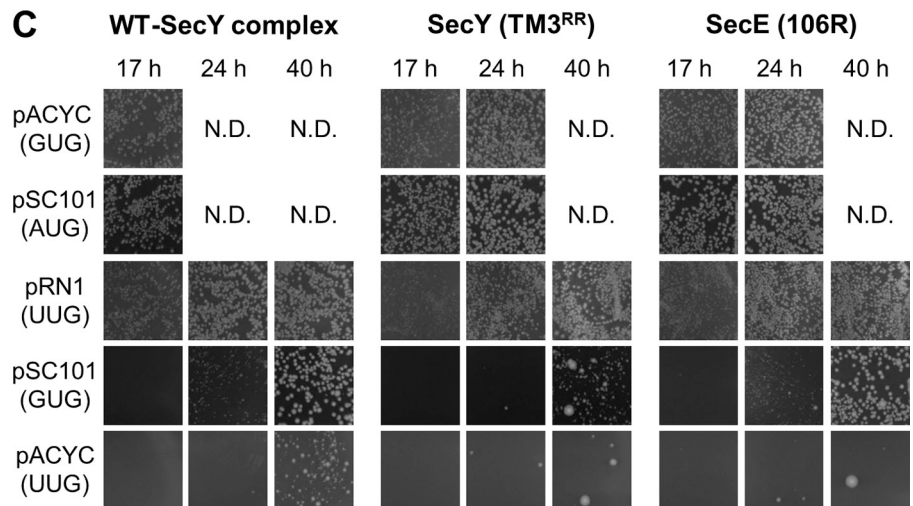
**A**



**B**



**C**



SecY mutants with disrupted back-to-back or front-to-front associations grew at the same rate as wild-type cells suggests that oligomers are not essential for the entire translocation process. This conclusion is consistent with recent fluorescence-correlation experiments (Kedrov et al., 2011).

Our results do not exclude the possibility that oligomerization of SecY complexes may facilitate protein translocation. For example, a nontranslocating SecY molecule might contribute to SecA binding to the channel. Such an assumption could explain the previous observations that a defective SecY mutant can be rescued for translocation by its covalent association with a wild-type SecY copy (Osborne and Rapoport, 2007); a weakened interaction of the translocating SecY copy with SecA might be compensated for by additional interactions of

a nontranslocating SecY copy with SecA. A similar interpretation could apply to the observation that two defective SecY mutants can form an active complex (Dalal et al., 2012), that SecA has a substrate-stimulated ATPase activity only when interacting with two SecY molecules in lipid nanodiscs (Dalal et al., 2012), and that cross-linked SecY dimers have an increased translocation activity in single-molecule experiments (Deville et al., 2011). In all these cases, a nontranslocating SecY copy might boost the activity of the translocating SecY copy. However, our data indicate that these nontranslocating SecY copies are not essential for translocation.

The fact that cotranslational translocation requires only one SecY copy is consistent with mechanisms derived from electron microscopy data: the ribosome is bound to a single

SecY channel in such a way that a nascent polypeptide can move directly from the ribosome tunnel into the SecY channel (Ménéret et al., 2007, 2008; Becker et al., 2009; Frauenfeld et al., 2011). Our new data, in conjunction with previous results (Park and Rapoport, 2011), also show that the pore residues of a single SecY molecule form a gasket-like seal that prevents the permeation of small molecules during co- and post-translational translocation. Given the structural conservation of the channel, it is likely that in eukaryotes the pore and membrane barrier are also formed from a single copy of the Sec61 complex.

For SecA-mediated translocation, the new results raise the interesting question of how an interaction of SecA with a single SecY molecule can push a polypeptide chain through the membrane. In one model, SecA would be processive, moving an entire polypeptide chain through the SecY channel without dissociating from it. The interaction of SecA with a single SecY molecule is in fact strong in the ATP-bound state, a state that could be visualized in a crystal structure of the complex (Zimmer et al., 2008). However, in the ADP-bound state, SecA has a weaker affinity for SecY (Zimmer et al., 2008), raising the possibility that SecA would dissociate if it did not have additional binding sites with the membrane. It had been assumed that these sites are provided by a nontranslocating SecY copy (Osborne and Rapoport, 2007), but it now appears that this mechanism is not essential.

A major aspect of the work presented here is the development of methodology to generate protein translocation intermediates in vivo. All previous experiments on the mechanism of protein translocation had been done in vitro or had monitored in vivo heterogeneous populations of nascent chains. Our ability to generate both co- and post-translational translocation intermediates together with the structural information that is available for the SecY complex, SecA, and other translocation components, now opens a new era in the field in which detailed mechanistic questions can be asked in a physiological context.

Finally, it should be pointed out that the question addressed in this paper, i.e., the oligomeric state of the SecY complex, is relevant to other membrane proteins for which oligomerization is considered. For example, as in the case of SecY, it has been generally difficult to determine the oligomeric state of G protein-coupled receptors or ion channels (Duffield et al., 2003; Hern et al., 2010; Grage et al., 2011; Kasai et al., 2011; Dixon et al., 2012), and in several cases, it has been controversial whether oligomers are functionally important. Much of the uncertainty comes from the fact that membrane proteins often interact only weakly through hydrophobic surfaces inside the membrane. Thus, the in vivo cross-linking methodology and the manipulation of interaction surfaces, developed by us for SecY, may help to test the role of oligomers in these cases as well.

## Materials and methods

### Bacterial strains and plasmids

Strains and plasmids used in this study are listed and described in Tables S1 and S2. Standard PCRs and site-directed mutagenesis were performed with KOD polymerase (EMD Millipore) and were verified by sequencing. *E. coli* strain DH5 $\alpha$  was used for all cloning procedures.

*E. coli* chromosomal *secY*- and *secE*-null strains were constructed by standard  $\lambda$ Red recombination techniques (Datsenko and Wanner, 2000). In brief, to make a *secY*-null strain (EP63), EP61 ( $\Delta$ *rmf*  $\Delta$ *ompT*; see Table S1) cells were transformed with  $\lambda$ Red recombinase-encoding pKD46 together with pTet2-SecYEG, which expresses the wild-type SecYEG complex under a tetracycline-inducible promoter. The pTet2-SecYEG plasmid also includes the *p15A* origin of replication and a kanamycin resistance gene. After induction of  $\lambda$ Red recombinase, the cells were electroporated with a PCR product containing a hygromycin B resistance gene, flanked by short sequences homologous to the chromosomal *secY* locus (the PCR was performed using the forward primer 5'-TCGAAGCTGGCGGG-TAAAATCGAGGAATAAGTAGCAGATGGGTAAAAAGCCTGAAGCTC-3', the reverse primer 5'-GAACCTTCATTTTACTCTCCGTAAGTCTCGGGC-GACCAATTATTCCTTTGCCCTCGGA-3'; and pAG32 as a template). Colonies were selected on LB agar plates containing 50  $\mu$ g/ml kanamycin, 100  $\mu$ g/ml hygromycin B, and 100  $\mu$ g/ml anhydrotetracycline (aTet). Removal of the chromosomal *secY* gene was verified by PCR. The resulting strain cannot form a colony on plates without aTet, but grows normally when transformed by a plasmid constitutively expressing wild-type SecY (e.g., pACYC-SecYEG). To generate strains deleted in chromosomal *secE* (Fig. 6, B and C), EP52 ( $\Delta$ *rmf*  $\Delta$ *ompT*::*kan secY-CBP::zeo*) cells were transformed with pKD46 and pACYC-SecYEG (containing either the wild-type or L106R mutant *secE* copy).  $\lambda$ Red recombinase-induced cells were electroporated with a PCR product containing a streptomycin resistance marker, flanked by short sequences homologous to the chromosomal *secE* locus (the PCR was performed using the forward primer 5'-CGTTTCGTTG-GTTTGCCTCGCGATCGCGGGGTAAAATGTTGTAGAAAACCTTGA-CAGGTTGGTTATGAGGGAAGCGGTGATCGC-3', the reverse primer 5'-CAAAACCGGAAAACGCTGAACGACGTACCAGCGCTTTTATGGA-GCTTCAGACATATGAATATCCTCCTTATTGCCGACTACCTTGGTG-3'; and pCDFDuet-1 as a template). Selection was performed on plates containing 40  $\mu$ g/ml chloramphenicol and 30  $\mu$ g/ml streptomycin. A *secY/secE*-null strain (EP66) was constructed in essentially the same way, starting with the *secY*-null strain. The deletion of chromosomal *secE* was verified by PCR.

### Growth of bacteria

Experiments were performed with the following strains: EP62 ( $\Delta$ *rmf*  $\Delta$ *ompT secY-CBP::zeo*) for Figs. 1 (B and C), 6 (A and D), 7 (A and B), and S1 B; EP52 ( $\Delta$ *rmf*  $\Delta$ *ompT::kan secY-CBP::zeo*) for Fig. 3 D; EP53 ( $\Delta$ *rmf*  $\Delta$ *ompT secY-CBP::zeo*  $\Delta$ *glpF::kan*) for Fig. S3; and EP63 ( $\Delta$ *rmf*  $\Delta$ *ompT*  $\Delta$ *secY::hph*) for Figs. 1 (D–F), 2 B, 3 (B and C), 4, 5, 6 E, 7 C, S1 (A, D–F), S2, and S4. To express SecY complexes, the cells were transformed with pACYC-SecYEG, which expresses all subunits of the SecY complex from a constitutive endogenous promoter (the *rplN* promoter). When overexpression of a translocation substrate was needed, pBAD-NC100 (for DsbA-SecM) or pBAD-OmpA-GFP was additionally introduced into the cells. When maximum occupancy of translocation sites by DsbA-SecM or OmpA-GFP was intended (Figs. 2 B, 3 [C and D], 4, S2, and S3), the GUG start codon was used for the SecY gene in pACYC-SecYEG. For the competition experiments in Fig. S1 (E and F), the cells were cotransformed with pACYC-SecYEG containing the indicated SecY cysteine mutants and pBAD-SecY<sup>CBP</sup>EG encoding cysteine-free SecY with a C-terminal calmodulin-binding peptide (CBP) tag. *E. coli* cells were picked from freshly transformed colonies, inoculated into LB medium supplemented with appropriate antibiotics (100  $\mu$ g/ml ampicillin for pBAD and pRN1 plasmids; 40  $\mu$ g/ml chloramphenicol for pACYC-SecYEG; and 15  $\mu$ g/ml chloramphenicol for pSC101 and pBeloBAC plasmids), and grown at 37°C to log phase (OD<sub>600nm</sub> = ~0.4–0.6) before induction and any other manipulations. To induce overexpression of DsbA-SecM and OmpA-GFP, 0.15% arabinose was added for 30 min at 37°C.

To monitor growth of *E. coli* cells producing a reduced level of the SecY complex (Fig. 7 C), competent EP63 cells were transformed with 50 ng of each SecY-expressing plasmid. Cells were first recovered at 37°C for 1 h in LB medium without antibiotics and plated on LB agar medium supplemented with appropriate antibiotics.

### In vivo biotinylation and streptavidin gel-shifting assays

*E. coli* cultures were grown to log phase, and treated with 100  $\mu$ g/ml rifampicin for 30 min. After harvesting 4 ml of each culture, the cells were resuspended in 1.5 ml of buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA. After addition of 0.4 mM biotin-PEG<sub>2</sub>-maleimide (Thermo Fisher Scientific), the suspension was incubated for 1 h at 4°C. After quenching with 20 mM  $\beta$ -mercaptoethanol for 20 min at 4°C, the cells were washed three times with 1 mL of the buffer and then resuspended in 200  $\mu$ l



of buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM  $\beta$ -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1.5 kU rlysozyme (EMD Millipore), 16 U/ml Benzonase nuclease (EMD Millipore), and 0.5% Triton X-100. 25  $\mu$ g streptavidin (Genscript) was added to a 40- $\mu$ l aliquot of the reaction, and binding was allowed for 1 h at 4°C. The samples were then supplemented with 0.1% SDS, 12.5% glycerol, and bromophenol blue for SDS-PAGE.

#### Cysteine cross-linking experiments

Cells were grown to log phase and pelleted by brief centrifugation. Where indicated, *E. coli* cultures were supplemented with 100  $\mu$ g/ml rifampicin for 30 min at 37°C before harvest. For cross-linking of SecY molecules with a bi-functional cross-linker, the cells were resuspended at the original volume in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA. Cross-linking was initiated by addition of 0.4 mM bismaleimide-PEG<sub>3</sub> (Thermo Fisher Scientific) and continued for 1 h at 4°C under gentle rocking. The reaction was stopped by addition of 30 mM  $\beta$ -mercaptoethanol (incubation for 20 min at 4°C). The cells were collected and subjected to SDS-PAGE and immunoblotting. For Figs. 4, 6, 7 B, and S1 (B, E, and F), the cross-linking reactions were performed by directly adding 0.6 mM bismaleimide-PEG<sub>3</sub> to *E. coli* cultures in LB medium, without prior pelleting and resuspension. Relative SecY-SecY cross-linking efficiencies in Figs. 6 A and 7 B were calculated from the ratios of the band intensity of cross-linked and noncross-linked SecY (SecY<sub>2</sub>/SecY).

To cross-link single-chain SecEGY molecules through a disulfide bridge, the cells were resuspended in 1/10 culture volume of buffer containing 50 mM Tris-HCl, pH 7.5, and 300 mM NaCl, and homogenized by brief sonication. Cross-linking was performed by addition of 0.5 mM CuPh<sub>3</sub> for 30 min at 23°C. The reaction was stopped by addition of 25 mM NEM and the samples were subjected to nonreducing SDS-PAGE. For reduction of the disulfide bridge, 2%  $\beta$ -mercaptoethanol was added and the samples were incubated for 30 min at 50°C before SDS-PAGE.

For formation of a disulfide bridge between SecY and substrate, 0.25 mM CuPh<sub>3</sub> was directly added to a 1-ml aliquot of *E. coli* culture, followed by gentle rocking for 10 min at 23°C. The reaction was quenched by addition of 20 mM NEM for 20 min at 4°C. The cells were pelleted and resuspended in SDS sample buffer for nonreducing SDS-PAGE.

Double disulfide-cross-linking of a substrate molecule and two single-chain SecY molecules (Fig. 5, A and B) was performed as follows. For DsbA-SecM, EP63 cells harboring pBAD(MazF)-NC100 and pACYC-scSecEGY were grown to mid-log phase. 0.15% arabinose was first added to the culture for 1 h to overproduce the nascent chains, and then 200 ng/ml aTet was added for the next 20 min to induce MazF expression. For OmpA-GFP, EP63 cells harboring pBAD-OmpA-GFP (GUG) and pACYC-scSecEGY, 0.15% arabinose was first added for 20 min to induce OmpA-GFP expression, followed by the addition of 100  $\mu$ g/ml rifampicin for the next 30 min to block further synthesis of OmpA-GFP and endogenous proteins. Then, disulfide bridge formation between SecY and substrate was induced by addition of 1 mM DTNB in 25 mM Tris-HCl, pH 7.5, for 10 min at 23°C. The cells were transferred to 4°C, collected by centrifugation, and washed twice with buffer containing 50 mM Tris-OAc, pH 7.2, 20 mM Mg(OAc)<sub>2</sub>, and 100 mM NH<sub>4</sub>Cl before resuspension in 1/8 of culture volume. After breaking the cells by brief sonication, 0.5 mM CuPh<sub>3</sub> was added to the lysate to induce disulfide cross-linking between single-chain SecEGY molecules. After 15 min at 23°C, the reaction was stopped by addition of 20 mM NEM. The samples were analyzed by nonreducing SDS-PAGE.

#### Measuring permeation of small molecules through the SecY channel

*E. coli* cells harboring pACYC-SecYEG (WT or  $\Delta$ P with a GUG-start codon) and pBAD-OmpA-GFP were grown in LB medium supplemented with 50  $\mu$ g/ml chloramphenicol and 100  $\mu$ g/ml ampicillin to log phase. Expression of OmpA-GFP was induced by the addition of 0.15% arabinose for 30 min at 37°C. Where indicated, 100  $\mu$ g/ml rifampicin was added instead to the cultures for 30 min at 37°C. Permeation of biotin-PEG<sub>2</sub>-maleimide and of chloride ions were measured as described previously (Park and Rapoport, 2011). In brief, to test permeation of biotin-PEG<sub>2</sub>-maleimide, 0.4 mM biotin-PEG<sub>2</sub>-maleimide (Thermo Fisher Scientific) was directly added to an aliquot of the culture at 23°C for 30 min. After quenching by addition of 30 mM  $\beta$ -mercaptoethanol, the cells were collected and subjected to SDS-PAGE and blotting. To test permeation of chloride ions, cells were pelleted and resuspended in 20 mM Tris-HCl, pH 7.2, and 18% sucrose, and converted into spheroplasts by addition of EDTA and lysozyme. The spheroplast suspension was rapidly mixed in a cuvette with a 19-fold volume of an isotonic solution of KCl (0.342 mol/kg) containing 10  $\mu$ M valinomycin, and the absorbance at 500 nm was recorded in a spectrophotometer.

#### SDS-PAGE, image acquisition, and densitometry analysis

SDS-PAGE was performed using 4–12% Bis-Tris gels (Invitrogen and Bio-Rad Laboratories) with either MES-SDS or MOPS-SDS running buffer (Invitrogen). For each SDS-PAGE experiment, samples from equal numbers (OD<sub>600nm</sub>) of cells were loaded on lanes. Rabbit polyclonal antibodies against the C terminus of *E. coli* SecY were described previously (Cannon et al., 2005). Mouse monoclonal antibodies recognizing an internal segment of *E. coli* SecY were a gift from Ian Collinson (University of Bristol, Bristol, England, UK). Mouse monoclonal anti-GFP (clones 7.1 and 13.1), anti-Myc (9E10), and anti-His antibodies (A00186) were obtained from Roche, Sigma-Aldrich, and Genscript, respectively. Rabbit polyclonal anti-trigger factor (TF) antibodies (A01329) were obtained from Genscript. A standard ECL reagent was used to develop immunoblots. All images including immunoblots and agar plates were taken with the CCD-based device LAS-3000 (Fujifilm) using the acquisition software Image Reader LAS-3000 (Fujifilm). The levels of images were adjusted using Adobe Photoshop. ImageJ software (National Institutes of Health, Bethesda, MD) was used for densitometry analysis (using raw 16-bit images).

#### Online supplemental material

Fig. S1 shows the functionality of SecY mutants and the interaction between resting SecY complexes in vivo. Fig. S2 shows that the majority of SecY channels can be occupied with the OmpA-GFP substrate. Fig. S3 shows that insertion of OmpA-GFP reduces chloride permeation through the open SecY channel. Fig. S4 shows that the occupation of SecY with OmpA-GFP can be reduced by lowering the expression level. Fig. S5 demonstrates that a SecY mutant defective in both front-to-front and back-to-back dimerization is as functional as wild-type SecY. Tables S1 and S2 list the strains and plasmids used in this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201205140/DC1>.

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