

## FULL METHODS

### Bacterial strains and plasmids

All strains and plasmids used in this study are listed and described in Supplementary Tables 1 and 2. PCR reactions were performed with Phusion polymerase (New England Biolabs) or KOD polymerase (Novagen). Site-directed mutagenesis was performed using either the Quikchange (Stratagene) or Phusion (New England Biolabs) mutagenesis kits. All constructs were verified by sequencing. *E. coli* DH5 $\alpha$  strain was used for all cloning procedures.

For all experiments except cloning, we used *E. coli* strains lacking both Rmf and OmpT, because Rmf is known to induce dimerization and inactivation of ribosomes at stationary phase<sup>26</sup>, and the OmpT protease has been reported to proteolyze SecY<sup>27</sup>. The  $\Delta$ *rmf*  $\Delta$ *ompT* strain was constructed from individual deletion stains by P1 transduction<sup>28</sup>. The  $\Delta$ *rmf*  $\Delta$ *ompT* *secY*-CBP strain, in which the chromosomal *secY* gene is tagged with calmodulin-binding peptide (CBP) at its C-terminus, was constructed in the following way. We first synthesized a “CBP-RBS-Zeo” DNA cassette, containing a CBP-tag, a stop codon, a ribosome binding site, and a Zeocin resistance gene in this order. This cassette was amplified by PCR and electroporated into  $\Delta$ *rmf*  $\Delta$ *ompT* cells expressing Lamda Red recombinase from the pKD46 plasmid<sup>29</sup>. The resulting cells were selected on Zeocin-containing agar plates (Invivogen). Incorporation of the cassette into the chromosome was verified by PCR, DNA sequencing, and immunoblotting using CBP antibodies (Genscript). The  $\Delta$ *rmf*  $\Delta$ *ompT* *secY*-CBP strain was used, except for Figs. 2b and 2d, where the  $\Delta$ *rmf*  $\Delta$ *ompT* strain was employed. For all osmotic swelling/bursting experiments, we used a strain containing an additional deletion of the *glpF* gene because GlpF has been shown to efficiently permeate polyalcohol sugars, including xylitol<sup>30</sup>. The  $\Delta$ *rmf*  $\Delta$ *ompT* *secY*-CBP  $\Delta$ *glpF* was generated by P1 transduction (see Supplementary Table 1). The resulting strain did not show any xylitol permeability in osmotic swelling/bursting experiments.

The pTet-SecYEG plasmid, which expresses SecYEG under a tetracycline-inducible promoter, was made by PCR amplification of the SecE-SecY-SecG coding sequence from pBAD22-SecYEG plasmid<sup>9</sup> and its subsequent insertion into the pTet vector. The pACYC-SecYEG plasmid, which constitutively expresses SecYEG, was constructed by inserting the same SecYEG coding sequence into pACYC184 after fusing with a 200-bp DNA fragment containing the *E. coli rplN* promoter at its 5'-end. The plasmid pBAD-NC100 for expression of a SecM-stalled nascent chain was generated as follows: We first constructed the pBAD myc-SecM vector by sequentially inserting a DNA fragment coding for a myc-epitope between the PstI and SacI sites of pBAD His/C (Invitrogen), and a fragment coding for the 17-residue SecM stalling sequence (FSTPVWISQAQGIRAGP) between the PstI and EcoRI sites. Then we synthesized a DNA segment by PCR, which encodes the DsbA signal peptide fused to a sequence from an unrelated protein, and inserted it between the NcoI and PstI sites of the pBAD myc-SecM vector. Other plasmids for different nascent chain lengths and sequences were made by modifying the pBAD-NC100 plasmid. More information is available in Supplementary Table 2.

### **Cell culture and protein expression**

Unless otherwise indicated, *E. coli* cells were grown and induced as follows. Cells harboring the indicated plasmids were picked from freshly-transformed colonies and inoculated into LB medium supplemented with appropriate antibiotics (100 µg/mL for ampicillin and 50 µg/mL for chloramphenicol). Cultures were grown at 37°C to log phase ( $OD_{600nm} \sim 0.4-0.6$ ) before induction. To induce SecYEG from the pTet vector (Figs. 1 and 3a), 200 ng/mL anhydrotetracycline (aTet) was added for 30 min. To overexpress nascent chains, 0.2% arabinose was added for 1 hr except in Figs. 2b and 2d, where the induction was for 2 hrs. When both SecYEG (from pTet) and a nascent chain were co-expressed (Fig. 3b-d), 0.07% arabinose was added first to *E. coli* cultures at log phase, 200 ng/mL aTet after 15 min, and induction was

continued for additional 40 min. Where indicated, 100 µg/mL rifampicin was added to cultures for 1 hr at 37°C.

SecY was expressed from the endogenous *rplN* promoter on either the pACYC-SecYEG (Figs. 2c, 2e-g, 3e-f) or the pRSY (Fig. 2d) vectors. The latter also encodes rare-codon tRNAs and the SRP pathway components (Ffh, 4.5S RNA, and FtsY) from their own endogenous promoters. For the experiment in Fig. 2b, a fused vector (pBAD-NC100/SecYEG) was used, which codes for both NC100 and SecY, together with pR2HQ4, a vector overexpressing the SRP components and rare-codon tRNAs. Details about these plasmids and their constructions are given in Supplementary Table 2. When a nascent chain was intended to saturate the SecY channels, vectors contained a GUG start-codon for SecY instead of AUG (Figs. 2b-c, 2e-g, 3e-f). Saturation of GUG-SecY with the NC100 chains was not affected by plasmid combinations or by overexpression of the rare-codon tRNAs and the SRP components.

### ***In vivo* biotinylation and subcellular fractionation**

*E. coli* cells were grown at 37°C in LB medium to log phase and induced as described above. After an aliquot of the culture was taken, 0.4 mM biotin-PEG<sub>2</sub>-maleimide (BM; Pierce) was directly added to the culture for 30 min at room temperature. When a nascent chain was overexpressed, the incubation was shortened to 15 min. After quenching the reaction with 20 mM β-mercaptoethanol and further incubation for 15 min on ice, the cells were collected by brief centrifugation. For SDS-PAGE analysis, the same number of cells were lysed in SDS-sample buffer and loaded on a gel (0.1 OD<sub>600nm</sub> per lane). For subcellular fractionation, the cells were resuspended in ice-cold spheroplasting buffer (100 mM Tris-HCl pH 8.0, 18% sucrose). Conversion of cells into spheroplasts was carried out by addition of 2 mM EDTA and 0.1mg/mL hen egg lysozyme and for 10-min on ice. The spheroplasts were sedimented by centrifugation (9000 rpm, 10 min), and the supernatant containing periplasmic proteins was removed. The spheroplasts were resuspended in 100 mM Tris-HCl pH 8.0, 150 mM NaCl and lysed by sonication. The lysate was subjected to

centrifugation for 1 hr at 51,000 rpm in a TLA100.3 rotor (Beckman) to pellet the membranes. The supernatant (cytosolic proteins) and the membranes were analyzed by SDS-PAGE.

### **Osmotic swelling/bursting experiments**

Cells were placed on ice for a few minutes and harvested by centrifugation at 5,000 rpm for 7 min. After resuspension in 1/15 culture volume in 20 mM Tris-HCl pH 7.2 and 18% sucrose (0.619 *m*), the cells were converted to spheroplasts by addition of 2 mM EDTA and 0.1 mg/mL lysozyme at 4°C. When nascent chains were expressed, 4 mM MgSO<sub>4</sub> was added 3 min after initiation of spheroplasting to avoid potential adverse effects of EDTA on ribosome-nascent chain complexes. Efficient (>95%) spheroplasting was verified by phase contrast microscopy and immediate lysis upon dilution into water by measuring the turbidity. To determine permeability for various small molecules, the spheroplast suspension was rapidly mixed at room temperature and with a 19-fold volume of an iso-osmotic solution of xylitol (0.616 *m*) or KCl (0.342 *m*). The absorbance at 500 nm was recorded in a spectrophotometer. The time between the mixing and measurement was about 5 sec. To obtain more accurate iso-osmotic solutions, osmotic coefficients were taken into account (1.05 for sucrose, 0.90 for KCl, and 1.00 for xylitol). For measurement of chloride permeability, 10 μM valinomycin was included in the KCl solution. When the rate of turbidity decrease was determined, initial slopes were calculated by linear regression. These numbers were normalized with respect to the initial turbidity.

### ***In vivo* expression and crosslinking of nascent chains**

To disulfide-crosslink a nascent chain with SecY, 0.25 mM CuPh<sub>3</sub> was added directly to the induced culture. After gentle rocking at room temperature for 10-20 min, the cells were collected by centrifugation and resuspended in ice-cold TMP100 buffer (50 mM Tris-acetate pH 7.2, 25 mM Mg(OAc)<sub>2</sub>, 0.1 M KOAc) containing 20 mM N-

ethylmaleimide (NEM). The cells were lysed by sonication and the samples analyzed by non-reducing SDS-PAGE and immunoblotting. Where indicated, the samples were treated with 2%  $\beta$ -mercaptoethanol or 0.2 mg/mL RNase A before loading them onto the gel.

### **Testing for SRP-dependence of nascent chain insertion**

WAM121 cells (Supplementary Table 1) were transformed with pRARE/SecYEG and pTac-NC100 (Supplementary Table 2). Cells from a saturated culture, grown in LB medium supplemented with 0.1% arabinose, were washed three times with RM medium (1x M9 salt, 1 mM MgSO<sub>4</sub>, 2% casamino acids and 0.2% glucose) supplemented with ampicillin and chloramphenicol, and inoculated into fresh RM medium for 3.5 hrs at 37°C. Incubation was then continued either in the absence or presence of 0.1% arabinose. After an additional 1 hr, overexpression of the nascent chain was induced for 2 hrs by addition of 1mM isopropyl thiogalactopyranoside (IPTG). *In vivo* crosslinking experiments were performed as above.

### **Mal-PEG modification of RNCs**

pBAD-NC100 plasmids containing single cysteines at various positions were transformed into  $\Delta rnf \Delta ompT$  cells harboring the pRARE plasmid. After induction of nascent chain expression, the cells were harvested and lysed by sonication in TMP100 buffer. Mal-PEG ((methyl-PEG<sub>12</sub>)<sub>3</sub>-PEG<sub>4</sub>-maleimide; Pierce) was prepared as a 125 mM solution in dimethyl sulfoxide (DMSO) and added to the cell homogenate at 2 mM final concentration. After incubation for 1 hr at room temperature, the reaction was stopped by addition of 33 mM NEM. The samples were subjected to SDS-PAGE and analyzed by immunoblotting with myc antibodies.

### **Flow cytometry**

To monitor the membrane potential of cells, SecY was induced for the indicated time period. The cells were diluted to  $\sim 5 \times 10^6$  cell/mL into phosphate buffered saline containing 2  $\mu$ M bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DIBAC4(3); Invitrogen). After a few minutes, the cell suspension was injected to a flow cytometer (FACScalibre; BD) for analysis. The flow rate was set at "low". Signals from cells were readily distinguishable from background signals as a separate population in forward (FSC) vs. side scattering (SSC) plots. This population was gated to exclude background events from further analysis. The DIBAC4(3) signal was measured in the green fluorescence channel (FL1-H). 50,000 events were counted in each experiment.

### **SDS-PAGE, immunoblotting, and densitometry analysis**

SDS-PAGE was performed using Bis-Tris gels (Invitrogen) with MES-SDS running buffer. Images of immunoblots were taken with a CCD-based device (Fujifilm LAS-3000) and a standard ECL reagent. Image J software was used for densitometry analysis.

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