SUPPLEMENTARY DATA

Figure S1. Characterizing the affinity purification strategy for TF crosslinked RNCs
(A) IPTG induced expression of plasmid encoded TF matches endogenously expressed levels. Total lysates from wild-type and Δ*tig::kan* + pTig-TEV-Avi cells grown to mid-log phase in various IPTG concentrations were resolved by 12% SDS-PAGE under reducing conditions. TF steady-state levels were analyzed by immunoblotting using TF antiserum. The membrane was subsequently stripped and developed using S1 antiserum as the loading control.
(B) Untagged and TEV-Avi-tagged TF complement the simultaneous knockout of Δ*tig* Δ*dnaK*. Δ*tig* Δ*dnaK* cells transformed with pNde, pTig or pTig-TEV-Avi were serially diluted and spotted on LB plates containing 100 µg/ml of ampicillin and 70 µM IPTG. Cells were grown for 17 to 43 hr at indicated temperatures.
(C) Ribosome binding is required for TF-nascent polypeptide interaction. Δ*tig::kan* cells transformed with either pTig-TEV-Avi or pTigAAA-TEV-Avi (encoding a TF mutant deficient in ribosome binding) were grown to mid-log phase and harvested by centrifugation. Lysis, crosslinking, affinity purification and TEV elution of TF-RNCs were achieved as described in Figure 2. TEV eluates were split in half and analyzed by either non-reducing (non-red.) or reducing (red.) SDS-PAGE. Non-reducing gels were subjected to silver staining, while reducing gels were immunoblotted using TF or L23 antiserum.

Figure S2. Crosslinking analysis of purified TF-RNCs after filtering cells
Samples were treated as described in Figure 4A, except cells were not pre-treated with chloramphenicol before harvest, but rapidly filtered and flash frozen.

Figure S3. Quantifying expression levels for *ompF* derivatives
MG1655 Δ*ompF::cm* Δ*tig::tig-TEV-Avi* cells transformed with pRC10 containing indicated inserts were induced with IPTG unless otherwise noted. Cells were grown to an OD_{600} of 0.5 and harvested by centrifugation. Total RNA was purified and converted to cDNA using M-MuLV reverse transcriptase and random hexamers. Real time PCR was performed using the DyNAmo HS SYBR Green qPCR Kit. *ssrA* levels were measured for internal normalization.

Figure S4. Cells lacking TF show impaired outer membrane integrity
Wild-type and Δ*tig::kan* cells were grown to an OD_{600} of 0.4, and diluted 1:4 in LB media (A) only or containing indicated concentrations of (B) SDS/EDTA or (C) vancomycin. Cell growth was measured by determining OD_{600} at indicated time points.

Figure S5. Epitope tagged TF complements the chemical sensitivities of Δ*tig* cells
(A) Stationary cultures of wild-type cells transformed with pNde as well as Δ*tig::kan* cells transformed with pNde, pTig or pTig-TEV-Avi were spotted as 1:10 serial dilutions on LB plates containing 50 µg/ml of ampicillin, 70 µM IPTG and specified levels of drugs.
(B) Strains described in (A) were grown to an OD_{600} of 0.4 in LB media containing 100 µg/ml of ampicillin and 70 µM IPTG. Cultures were diluted 1:4 in LB media containing 50 µg/ml of ampicillin and 70 µM IPTG (i) only or supplemented with indicated concentrations of (ii) SDS/EDTA or (iii) vancomycin. Cell growth was determined by measuring optical density at 600 nm at indicated time points.

Figure S6. Comparing σ^E activities of WT and Δ*tig* cells

σ^E activity was measured at various optical densities in both wild-type and Δ*tig::kan* cells by monitoring β-galactosidase expression from a reporter integrated into the chromosome consisting of the σ^E-dependent promoter *rpoHP3* fused to *lacZ*.

Figure S7. Cotranslational translocation occurs more often for Sec-dependent substrates in Δ*tig* cells

(A) Wild-type and Δ*tig::kan* cells grown in maltose minimal media were pulse-labeled with ^{35}S-methionine for 30 sec. Labeling was stopped by adding 5% TCA. Cell extracts were immunoprecipitated using antisera specific for LamB, OmpF, OmpA and MBP. Resulting pull-downs were resolved by 12% SDS-PAGE. Running positions of precursor (p) and mature (m) forms are marked for each protein.

(B) 6-cm gel slices were excised from the first dimension (as in Figure S7A), digested with *Staphylococcus aureus* V8 protease, and resolved by 15% SDS-PAGE. Streaks emanating from precursor (p) or mature (m) MBP (indicated by arrows) respectively correspond to those containing or lacking their signal sequence.

EXTENDED EXPERIMENTAL PROCEDURES

General method for ribosome profiling in bacterial cells

Bacterial ribosome profiling for *Escherichia coli* has been successfully implemented for various laboratory strains including BW25113, MC4100 and MG1655.

Cell growth and harvest by centrifugation

A saturated culture was diluted to an OD_{600} of 0.1 in 1 l of LB media. Cells were grown at 37°C with vigorous shaking until reaching an OD_{600} between 0.4 and 0.5. Intact cells were pre-treated with chloramphenicol to a final concentration of 100 µg/ml for 2 min at standard growth conditions. The culture was poured over ice cubes supplemented with 100 µg/ml of chloramphenicol. Cells were spun down at 4500x*g* for 10 min at 4°C. The cell pellet was washed with 10 ml of pre-chilled resuspension buffer (10 mM $MgCl_2$, 100 mM NH_4Cl, 20 mM Tris pH 8.0, 1 mM chloramphenicol) and spun down at 3000x*g* for 5 min at 4°C. The cell pellet was next resuspended in 7.5 ml of pre-chilled lysis buffer (10 mM $MgCl_2$, 100 mM NH_4Cl, 20 mM Tris pH 8.0, 0.1% NP-40 *or* sodium deoxycholate, 0.4% Triton X-100, 100 U/ml of RNase-free DNase I (Roche), 0.5 U/µl of Superase•In (Ambion), 1 mM chloramphenicol). Resuspended cells were incubated on ice for 5 min and dripped over liquid nitrogen. Frozen cell pellets were cryogenically pulverized (Retsch MM301, 50 ml grinding jar, 25 mm grinding ball) at 15 Hz for 3 min in five sets with canisters re-chilled in liquid nitrogen between each cycle. The pulverized cells were transferred to a 50 ml Falcon and stored at -80°C.

Cell growth and harvest by filtration

A saturated culture was diluted to an OD_{600} of 0.1 in 200 ml of LB media. Cells were grown at 37°C with vigorous shaking until reaching an OD_{600} between 0.4 and 0.5. Cells were filtered using a 0.22 µm nitrocellulose membrane (GE, catalog no. E02WP09025) in a 90 mm glass filtration system (Kontes), scraped using a pre-warmed scoopula and immediately submerged in liquid nitrogen. The frozen cells were dislodged using a pre-chilled spatula. 0.65 ml of lysis buffer dripped over liquid nitrogen (10 mM $MgCl_2$, 100 mM NH_4Cl, 20 mM Tris pH 8.0, 0.1% NP-40 *or* sodium deoxycholate, 0.4% Triton X-100, 100 U/ml of RNase-free DNase I (Roche), 0.5 U/µl of Superase•In (Ambion), 1 mM chloramphenicol) was combined with the frozen cells for pulverization (Retsch MM301, 10 ml grinding jar, 12 mm grinding ball) at 15 Hz for 3 min in five sets with canisters re-chilled in liquid nitrogen between each cycle.

Lysate preparation

Pulverized cells were thawed in a 30°C water bath for 1 to 3 min (or until the lysate completely thawed) and incubated for 10 min in an ice-water bath. The lysate was spun down at 20,000x*g* for 10 min at 4°C. The clarified supernatant was collected and used immediately or frozen in liquid nitrogen and stored at -80°C. The concentration of the clarified lysate was determined by Nanodrop (Thermo Scientific) using a 1:100 dilution in 10 mM Tris pH 7.0.

Ribosome footprinting

25 Abs_{260} units of the clarified lysate (supplemented with 5 mM $CaCl_2$) were digested with 1500 enzyme units of micrococcal nuclease (Roche, catalog no. 10107921001) for 1 hr at 25°C and shaken at 1400 rpm in a thermomixer (Eppendorf). Digestions were quenched with EGTA to a final concentration of 6 mM. 1 enzyme unit for MNase is defined as an increase of 0.005 Abs_{260} units per min measured in a SpectraMax plate reader using 10 µg/ml of salmon sperm DNA as substrate supplemented with 5 mM $CaCl_2$ and 10 mM Tris pH 8.0 in a reaction volume of 0.1 ml.

The 30S subunit of *E. coli* ribosomes inhibits RNase I activity by an unknown mechanism (Datta and Burma, 1972). For this reason, we employed MNase, which also has the advantage of being able to be inhibited by addition of EGTA. We also observed that even for organisms where it is possible to use RNase I, ribosomal RNAs were typically less susceptible to digestion by MNase than by RNase I. This minimized contamination by ribosomal RNA.

10 to 55% (w/v) sucrose gradients (buffered in 10 mM $MgCl_2$, 100 mM NH_4Cl, 20 mM Tris pH 8.0, 1 mM chloramphenicol, 2 mM DTT) were prepared using a BioComp Gradient Master (BioComp Instruments) in polyclear centrifuge tubes (SETON, catalog no. 7030). Both digested and control samples were loaded on gradients and spun in an ultracentrifuge using an SW41 rotor at 35,000 rpm for 2.5 hr at 4°C. Gradients were fractioned using a BioComp Gradient Fractionator with the flow rate set to 0.2 mm per sec. Monosomes were manually collected by following the absorption trace at 254 nm using a BIO-RAD Econo UV monitor.

Library generation

Ribosome footprints were converted to a cDNA library as previously described (Ingolia, 2010).

Monosome fractions were denatured by adding SDS to a final concentration of 1% (w/v). 0.7 ml of denatured monosomes was extracted once with 0.7 ml of hot acid phenol, once with 0.7 ml of acid phenol and once with 0.6 ml of chloroform; precipitated; and resuspended in 20 µl of 10 mM Tris pH 7.0. 25 µg of RNA (quantified by Nanodrop) was mixed with 2x TBE-urea sample loading buffer (Invitrogen) and resolved on a 15% TBE-urea gel (Invitrogen) in 1x TBE (Ambion) at 200V for 65 min. A band between 28 to 42 bp was excised using 10 bp ladder (Invitrogen) as the standard, gel purified, precipitated and resuspended in 15 µl of 10 mM Tris pH 7.0. The 3' ends of RNA were dephosphorylated by T4 PNK (NEB) at 37°C for 1 hr; the enzyme was subsequently heat inactivated at 75°C for 10 min after which the RNA was precipitated and resuspended in 10 µl of 10 mM Tris pH 7.0. 5 pmol of RNA quantified by BioAnalyzer (small RNA kit, Agilent) was diluted to 5 µl in 10 mM Tris pH 7.0 and ligated at 37°C for 2.5 hr to 1 µl of 1 µg/µl Linker-1 (5'_App/CTGTAGGCACCATCAAT/3ddC_3', IDTDNA) supplemented with 8 µl of 50% sterile filtered PEG MW 8000, 2 µl of 10x T4 RNA ligase buffer (NEB), 2 µl of 100% DMSO (Sigma), 1 µl of 20 U/µl Superase•In (Ambion) and 1 µl of T4 ligase 2, truncated (NEB). The ligated products were precipitated and resolved on a 10% TBE-urea gel (Invitrogen) in 1x TBE at 200V for 50 min. A band between 45 and 60 bp was excised using 10 bp ladder as the standard, gel purified, precipitated and resuspended in 10 µl of 10 mM Tris pH 7.0. The gel extracted products were reverse transcribed with no more than five molar excess of Link-1 (5'_5phos/GATCGTCGGACTGTAGAACTCTGAACCTGTCGGTGGTCGCCGTATCATT/iSp18/CACTCA/iSp18/CAAGCAGAAGACGGCATACGAATTGATGGTGCCTACAG_3',

IDTDNA) using Superscript III (Invitrogen) in a 20 µl reaction volume at 50°C for 30 min. RNA products were hydrolyzed by the addition of NaOH (at a final concentration of 0.1 mM) and incubated at 95°C for 15 min. The reverse transcribed cDNA was resolved on a 10% TBE-urea gel in 1x TBE for 70 min at 200V. A band between 125 to 150 bp was excised using 10 bp ladder as the standard, gel purified and resuspended in 15 µl of 10 mM Tris pH 8.0. The cDNA was circularized with CircLigase^{TM} (EPICENTRE) in a 20 µl reaction volume at 60°C for 1 hr; the enzyme was heat inactivated at 80°C for 10 min after which the circDNA was stored at -30°C for further use.

5 µl of circDNA was PCR amplified with 5'_AATGATACGGCGACCACCGA_3' and 5'_CAAGCAGAAGACGGCATACGA_3' using Phusion polymerase (NEB) for 7 to 10 cycles. Amplicons were resolved on an 8% polyacrylamide gel (Invitrogen) in 1x TBE at 180V for 45 min. A band between 125 to 150 bp was excised using 10 bp ladder as the standard, gel purified, precipitated and resuspended in 10 µl of 10 mM Tris pH 8.0. The PCR amplified DNA library was quantified by BioAnalyzer (high sensitivity DNA kit, Agilent) and sequenced by Illumina Genome Analyzer II. 10 to 13 pM solution was used for generating clusters with 5'_CGACAGGTTCAGAGTTCTACAGTCCGACGAT_3' as the sequencing primer.

Selective ribosome profiling in bacterial cells

Bacterial strains and plasmids

MC4100 Δ*tig::kan* and MG1655 Δ*tig::kan* were constructed by P1 transduction with strain specific P1-lysates.

All *tig* encoding plasmids used in this study were derived from pTrc99B (Amann et al., 1988), enabling *tig* expression under the control of an IPTG-inducible hybrid trp/lac promoter. pTrc99 was used to construct pTrc-Tig and pTrc-TigAAA. pNde, which is a derivative of pTrc99 that contains an NdeI restriction site, was used to construct pTig, pTig-Avi, pTig-TEV-Avi and pTigAAA-TEV-Avi. pNde derivatives required higher concentrations of IPTG to achieve endogenously expressed levels of TF when compared with pTrc99 based plasmids. Cloning of plasmids expressing C-terminally tagged TF was achieved by PCR, whereby the 3' end of the *tig* open reading frame was fused in frame with an AviTag (GSGLNDIFEAQKIEWHE) or a version cleavable by TEV protease (GSGENLYFQSGRSGLNDIFEAQKIEWHE).

MG1655 Δ*tig::tig-TEV-AviTag* Δ*ompF::cm* was constructed by P1 transduction using strain specific P1-lysates.

ompF derived plasmids were cloned between the EcoRI and KpnI restriction sites of pRC10 (Chaba et al., 2007), itself a derivative of pTrc99A (Amann et al., 1988). *ompF ss** had mutations at the following residues: N5D, V9E, A13D and V16E. *ompF Δ2-48* lacked codons 2 through 48, while *ompF Δ2-96* lacked codons 2 through 96. *ompF myo[1-50]* had 50 codons of human myoglobin inserted immediately following the signal sequence, but codon R54 was mutated by site directed mutagenesis from R(AGG) to R(CGG).

IPTG titration experiments

Cultures of MC4100 Δ*tig::kan* transformed with pTig-TEV-Avi were grown in LB media supplemented with 100 µg/ml of ampicillin and indicated concentrations of IPTG. MC4100 grown in LB was used as the wild-type control. Cells were treated with chloramphenicol to a final concentration of 100 µg/ml when they reached an OD_{600} of 0.45 and chilled on ice. Cells were next harvested by centrifugation, resuspended in SDS sample buffer and boiled for 5 min. Equal amounts were subjected to SDS-PAGE and western blotting using polyclonal antiserum specific for TF (lab collection).

Real time PCR

Cultures of MG1655 Δ*tig::tig-TEV-AviTag* Δ*ompF::cm* with plasmids containing *ompF* derivatives were grown in LB media supplemented with 100 µg/ml of ampicillin and 200 µM IPTG. 4 ml cultures were harvested by centrifugation and flash frozen. 0.3 ml of lysis solution (0.5% SDS, 30 mM NaAOc pH 5.5, 10 mM EDTA) was next added to the frozen cell pellet. Total RNA was extracted twice with acid phenol, once with chloroform, ethanol precipitated and resuspended in 10 mM Tris 7.0. 10 µg of total RNA was digested with 2 µl of DNase I (NEB) in 0.1 ml for 10 min at 37°C. 1 µl of 0.5 M EDTA was mixed in each digested sample and incubated for 10 min at 37°C. Total RNA was further purified using the RNA Cleanup Protocol from the QIAGEN RNeasy Mini Kit. 1 µg of purified total RNA was combined with 2 µl of 50 µM random hexamer solution (ABI), 1 µl of 20 U/µl Superase•In (Ambion), 1 µl of 10 mM dNTPs (NEB), 2 µl of 10x M-MuLV buffer (NEB) and 1 µl of 200 U/µl M-MuLV reverse transcriptase (NEB). This mixture was incubated at room temperature for 10 min, next at 42°C for 1 hr and finally at 90°C for 10 min. Real time PCR was performed using the DyNAmo HS SYBR Green qPCR Kit (Finnzymes) on a DNA Opticon Real Time Cycler (MJ Research). Primers were designed using SciTools (IDTDNA) for *ompF* and *ssrA*.

Cell growth and harvest for sequencing and lysis

Overnight cultures of MC4100 Δ*tig::kan* (transformed with indicated plasmids) were diluted to an OD_{600} of 0.02 in fresh LB media supplemented with 100 µg/ml of carbenicillin, 40 µg/ml of biotin and 10 µM IPTG for pTrc-Tig or 70 µM IPTG for pTig-Avi, pTig-TEV-Avi and pTig-AAA-TEV-Avi. Diluted cultures were grown at 37°C to an OD_{600} of 0.45 with vigorous shaking.

To harvest cells by centrifugation, 500 ml cultures were initially incubated with chloramphenicol to a final concentration of 100 µg/ml and shaken for 2 min at 37°C. To quickly chill the cells, cultures were poured over ice cubes of equivalent volume containing 100 µg/ml of chloramphenicol. Cells were then harvested by centrifugation at 4500x*g* for 10 min at 4°C, followed by one wash in ice cold buffer (50 mM HEPES pH 7.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM chloramphenicol). The pellet was resuspended in 3 ml of ice cold crosslinking/lysis buffer (50 mM HEPES pH 7.0, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM chloramphenicol, 1 mM PMSF, 0.4% Triton X-100, 0.1% NP-40, 100 U/ml of RNase-free DNase I) and flash frozen by dripping the cell suspension into liquid nitrogen. Frozen cell pellets were pulverized for cell lysis in a 50 ml stainless steel grinding jar (MM301/400, 25 mm grinding ball; Retsch) for 5 cycles, each at 15 Hz for 3 min.

To harvest cells by filtration, 200 ml cultures were filtered using a pre-warmed 90 mm glass filtration system (Kontes) with nitrocellulose membranes of 0.2 µm in pore size (MicroSep Cellulosic, GE). Harvesting by filtration was half as efficient in collecting cells

as harvesting by centrifugation, thus 1 l was cultured and filtered five independent times with each filter used once for 200 ml. Following the filtration of growth media, cells were quickly scraped off the membrane with a pre-warmed scoopula and immediately submerged in liquid nitrogen for flash freezing. Frozen cell pellets (from 200 ml of culture) were combined with 650 µl of frozen crosslinking/lysis buffer and subsequently pulverized in a 10 ml mixer mill jar using aforementioned milling conditions.

Crosslinking TF to ribosome-nascent chain complexes (RNCs)

Frozen cell powder was added in batches to the crosslinking/lysis buffer containing DSP (dithiobis succinimidyl propionate; Pierce) with constant stirring at room temperature, which roughly took 10 min in span. The amount of crosslinking/lysis buffer was equivalent to the volume of resuspended cells before pulverization, with the final concentration of DSP adjusted to 2.5 mM. Once the cell powder was completely dissolved, the thawed lysate was further incubated for 5 min. The crosslinking reaction was quenched with Tris pH 8.3 to a final concentration of 100 mM and further incubated for 5 min at room temperature with constant stirring. The quenched reaction was chilled on ice for 10 min before the lysate was clarified by centrifugation at 20,000x*g* for 10 min at 4°C.

Chemical crosslinking by EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide; Pierce) was done as described above for DSP crosslinking, except EDC was used at a final concentration of 20 mM at pH 5.8 and the reaction was quenched with 250 mM glycine, 100 mM Tris 8.0 and 4 mM $NaHCO_3$.

MNase digestion of crosslinked lysates

Clarified lysates were quantified by Nanodrop (Thermo Scientific). 25 Abs_{260} units (corresponding to 1 mg of total RNA) were taken for the undigested control. 450 Abs_{260} units (corresponding to 18 mg of total RNA) were subjected to nuclease digest for 1 hr at 25°C using 20250 enzyme units of MNase supplemented with 40 µl of Superase•In (Ambion). Reactions were quenched with EGTA pH 8.0 to a final concentration of 6 mM and chilled on ice.

Isolating monosomes by sucrose density gradients

10 to 55% (w/v) sucrose density gradients were prepared as described for general ribosome profiling. Monosomes from nuclease treated samples were collected for the total control by sucrose density gradients, except DTT was omitted from the gradient buffer.

Affinity purification of TF crosslinked RNCs

Nuclease treated lysates were loaded on 25% sucrose cushions (50 mM Tris pH 7.5, 1 M NaCl, 10 mM $MgCl_2$, 1 mM chloramphenicol, 1 mM PMSF) and spun at 45,000 rpm for 4.5 hr at 4°C in a Ti45 rotor (Beckman). As soon as the supernatant was discarded, the pellet was quickly washed with wash buffer (50 mM Tris pH 7.0, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM chloramphenicol, 1 mM PMSF, 0.4 % Triton X-100, 0.1 % NP-40). The ribosomal pellet was resuspended in 1.25 ml of wash buffer and incubated overnight on ice.

For each sample, 220 µl of the matrix (50% slurry of Strep-Tactin sepharose; IBA, Göttingen, Germany) was equilibrated in wash buffer. The resuspended ribosome fraction was incubated with the matrix for 1 hr on an overhead roller at 4°C. The matrix was centrifuged at 500x*g* for 1 min. The supernatant was carefully aspirated by pipette, removing unbound ribosomes. The matrix was washed three times in wash buffer for 45 min at 4°C on an overhead roller, followed by centrifugation at 500x*g* and aspiration of the supernatant by pipette. The matrix was next washed once with cleavage buffer (50 mM Tris pH 7.0, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM chloramphenicol).

130 µl of cleavage buffer plus 5.8 µM TEV protease (lab collection) was added to the matrix for specific elution of crosslinked TF-RNCs and incubated for 30 min at room temperature on an overhead roller. The matrix was centrifuged at 500x*g* after which the supernatant was collected and saved. TEV cleavage was repeated using the conditions described above. 120 µl of cleavage buffer was added to the matrix to capture residual TF-RNCs trapped in the slurry. The collected eluates were pooled for subsequent isolation of mRNA footprints (see *Library generation* under General ribosome profiling section). Typical yields per sample ranged between 50 to 100 µg of RNA as measured by Nanodrop. The affinity matrix was washed once with wash buffer for 30 min and another time with cleavage buffer and saved for gel analyses.

Crosslinking analysis by polyacrylamide gel electrophoresis and immunoblotting

Samples were mixed with SDS sample buffer (625 mM Tris pH 6.8, 50% glycerol, 15% β-mercaptoethanol, 0.05% bromophenol blue) unless indicated differently and resolved on a 12% SDS polyacrylamide gel. For non-reducing SDS-PAGE, sample buffer lacking reducing agents was used. SDS gels were visualized by standard methods for silver staining.

For immunoblotting, samples were first subjected to SDS-PAGE, then electro-transferred to PVDF membranes (Roth, Germany) and probed with indicated antisera (lab collection). Alkaline phosphatase conjugated to secondary antibodies were used for ECF detection using an FLA-3000 PhosphoImager (Fuji Photo Film, Tokyo, Japan). Bands were quantified using Multi-Gauge (Fuji Photo Film, Tokyo, Japan).

Sequencing analysis

Sequences were imaged by Illumina Genome Analyzer II and analyzed by GAPipeline v.0.3.0; sequences were next aligned to the bacterial genome using Bowtie v.0.12.0 as previously described (Ingolia, 2010; Ingolia et al., 2009). NC_012759.fna (RefSeq) was used for MC4100. Matrix and phasing parameters were determined from ϕX DNA. Bacterial genomes were obtained at NCBI Reference Sequence Bank (http://www.ncbi.nlm.nih.gov/RefSeq).

Assigning footprints to the genome

Genome-aligned reads with more than two mismatches were excluded. Moreover, only uniquely aligned sequencing reads were included to facilitate the analysis; thus it was assumed that non-unique alignments (i.e. sequences that matched to multiple places in the genome exactly at the nucleotide level) came from non-coding sequences, such as rRNA and tRNA molecules.

Because footprint reads varied in length, scores were fractioned and distributed across internal residues, in contrast to footprints derived from eukaryotic systems in which the 5' position was scored once for each sequencing read (Guo et al., 2010; Ingolia et al., 2009). For footprints between 25 and 40 nt in length, 12 nt were trimmed from each end with the remaining residues given a score of 1/N in which N equals the number of positions leftover after discarding the 5' and 3' ends. By blurring the signal across the central residues, the certainty to which the ribosome could be positioned was lowered by one to five codons (Figures 1C through 1E).

Quantifying ribosome density

To measure the overall expression rate for each gene (expressed as the density across an ORF in reads per kilobase million), the number of reads aligned to an ORF was normalized by its overall length and the total number of reads aligned to all ORFs. When comparing expression rates for each gene between replicates (Figure 1F), the sum of reads between them had to exceed 100 counts, ensuring that measurements were reliable and not dictated by sampling error (Ingolia et al., 2009). When comparing expression rates between different genes of the same operon (Figure 1G), each gene required at least 100 counts. The list of transcription units were found at http://ecocyc.org (Keseler et al., 2009) for MG1655 and applied to MC4100.

To measure ribosome density across each gene, the reads at each position were normalized by the total number of reads aligned to all ORFs only (i.e. in units of reads per kilobase). Ribosome densities were visualized by MochiViewv v.1.45 (Homann and Johnson, 2010), an open source genome browser. The genes identified in Figures 1D and 1E were found by browsing MochiView, indicating that other unidentified short ORFs are likely to exist.

Meta-gene analyses

The average ribosome density across nucleotides 280 to 400 was used as the mean ribosome density for each gene. To obtain the meta-gene profile illustrated in Figure 1B, the ribosome density profile for each well-expressed gene (i.e. those with an overall density greater than 0.08 reads per base) was scaled by its own mean ribosome density. This gave differently expressed genes similar expression rates (i.e. equal weighting). Each mean normalized ribosome density profile was next aligned by its start codon (or by its stop codon) and averaged across each position—that is if the transcript was long enough to be included for averaging. Thus the output was an average of numerous mean normalized ribosome density profiles.

Measuring enrichment efficiency

To generate meta-gene profiles illustrated in Figures 3A and 4B, meta-gene ribosome density profiles were separately computed for both footprints derived from affinity purified TF-RNCs and those derived from all footprints. But ORFs encoding less than 135 codons were excluded from this analysis, because their inclusion would inflate the overall range in enrichment efficiency near the N-terminus. In addition, 100 codons from the 5' end were trimmed and excluded when deriving meta-gene ribosome density profiles starting from the stop codon only. Ratios between the two meta-gene read density profiles were taken across each position (i.e. enriched over total), yielding the average mean normalized enrichment efficiency.

Individual enrichment efficiency profiles shown in Figure 3B were determined for each gene by taking the ratio across the coding sequence between the ribosome density profile computed for affinity purified footprints and the ribosome density profile computed for the total collection of footprints. However, these density profiles were scored differently than previously described, with the score at each position also containing the scores of ±20 nucleotides from it. This was done because many genes were marked by regions lacking continuous density.

To assess initial chaperone engagement for each gene as in Figures 3C and 4C, individual enrichment efficiency profiles were computed for very well-expressed genes, here defined as those having a read density of more than 1.5 reads per base. After measuring when each enrichment efficiency profile first crossed a threshold of 1.3 arbitrary units, the collection of these lengths (in codons) was represented as a histogram. Genes that did not meet this threshold were excluded from this analysis. Genes were also excluded if they already exceeded the threshold at the first codon, which accounted for less than 1% of ORFs.

Gene-by-gene correlations across enrichment efficiency profiles (from very well-expressed genes with a read density of more than 1.0 read per base) were determined between DSP replicates or DSP and EDC experiments as in Figure 4D.

To measure the overall enrichment efficiency as in Figure 6A, the sum of the enriched footprint density was divided by the sum of the total footprint density per gene, but excluding 100 codons from the 5' end. Genes were separated based on their GO (gene ontology) annotation (i.e. GO=0005737, GO=0019866, GO=0009279) and compared with the total set using the rank sum test from MatLab v.7.5.0. GO annotations were found at http://ecocyc.org (Keseler et al., 2009) for MG1655 and converted for MC4100.

In vitro and in vivo analyses

In vitro analysis of N-terminal enzymatic processing of nascent chains

S135 translation extracts were prepared from MC4100 Δ*tig* cells using a modified version of an established protocol (Müller and Blobel, 1984). Cultures were grown at 37°C in S135 medium (0.9% tryptone/peptone, 0.08% yeast extract, 0.56% NaCl, 1 ml/l of 1M NaOH, 0.08% glucose) to an OD_{600} of 1.2 and harvested by centrifugation. Cell pellets were resuspended in S135 buffer (10 mM triethanolamine (TEA) pH 7.5, 14 mM $Mg(OAc)_2$, 60 mM KOAc) containing 1 mM dithiothreitol (DTT) and 0.5 mM phenyl methane sulfonyl fluoride (PMSF) [1 ml of S135 buffer per 1 g of wet pellet]. Cells were lysed using a French Pressure Cell. Lysates were next cleared by centrifugation at 30,000x*g* for 30 min at 4°C. 60 µl of 1 M TEA pH 7.5, 0.6 µl of 1 M DTT, 1.6 µl of 1 M $Mg(OAc)_2$, 6 µl of 1 mM each of all amino acids except methionine, 6 µl of 1 mM methionine, 2 µl of 0.25 M ATP, 27 µl of 0.2 M phosphoenolpyruvate and 2.4 µl of 2 mg/ml pyruvate kinase were mixed per ml of supernatant (also known as S30 extract) and incubated for 1 to 2 hr at 37°C. Extracts were cooled on ice and dialyzed three times using a 12 to 14 kDa cut-off against S135 buffer containing 1 mM DTT. Following dialysis, 1 ml aliquots were ultracentrifuged in a TLA-100.2 rotor at 135,00x*g* for 13 minutes at 4°C. 750 µl of supernatant from each aliquot were combined and dialysed three times against S135 buffer lacking DTT but containing 1 mM tris(2-

carboxyethyl)phosphine (TCEP). The final S135 extract was aliquoted, snap-frozen in liquid nitrogen and stored at -80°C.

Each translation reaction (in a total volume of 25 µl) contained 2.5 µl of compensation buffer (392 mM TEA pH 7.5, 1.35 M KOAc, 99 mM $Mg(OAc)_2$, 8 mM spermidine); 6.25 µl of power mix (12.8% polyethylene glycol MW 6000, 48 mM phosphoenolpyruvate, 32 mM creatine phosphate, 1 mM TCEP, 0.16 mM each of all amino acids except methionine, 10 mM ATP, 2 mM each of GTP, CTP and UTP; buffered at pH 7.0); 40 µg/ml of creatine phosphate kinase; 300 ng of purified template DNA; 0.5 µl of purified T7 polymerase; 2 µl of S135 extract; 0.5 µl of ^{35}S-methionine at 10 µCi/l; 100 nM $CoCl_2$ and specified concentrations of PDF and MAP. Reactions were incubated in a thermomixer shaken at 900 rpm for 30 min at 37°C. Control reactions were supplemented with actinonin to a final concentration of 30 ng/ml. All reactions were terminated by adding 35 µl of ice cold 10% TCA. Samples were incubated on ice for 30 min and centrifuged at 20,000x*g* for 30 min at 4°C. Precipitants were resuspended in alkaline sample buffer, run on 11% Tris-tricine gels, stained by Coomassie Brilliant Blue and dried before the autoradiogram was developed.

In vivo analysis of tig overexpression on the processing of nascent polypeptide chains

MC4100 Δ*acrA::kan* cells were transformed with pTrc99, pTrc-Tig or pTrc-TigAAA. Serial dilutions of overnight cultures were spotted on LB plates containing 100 µg/ml of ampicillin and indicated concentrations of IPTG and/or actinonin and incubated for 16 hours at 37°C.

In vivo complementation analysis

MC4100 Δ*tig::kan* Δ*dnaK52* cells (Vorderwülbecke et al., 2004) were transformed with pNde, pTig or pTig-TEV-Avi. Cells were grown on selective LB agar plates containing 70 µM IPTG for 21 hours at 30°C to facilitate the episomal expression of *tig* or *tig*-TEV-Avi.

Colonies were struck to singles twice on LB plates containing 100 µg/ml of ampicillin and 70 µM IPTG before they were inoculated and grown overnight at 30°C in LB media containing 100 µg/ml of ampicillin and 70 µM IPTG. 1:10 serial dilutions were spotted on LB plates containing 100 µg/ml of ampicillin and 70 µM IPTG, and incubated for 17 hours at 34°C, 21 hours at 30°C or 43 hours at 25°C.

SDS/EDTA and vancomycin sensitivity assay

As shown in Figures 6C and 6D, MG1655 cells harboring pTrc99 (empty vector) and the corresponding MG1655 Δ*tig::kan* mutant cells containing pTrc99 or pTrc-Tig were grown to stationary phase in LB media containing 100 µg/ml of ampicillin. 1:10 serial dilutions were spotted on LB plates containing 50 µg/ml of ampicillin, 10 µM IPTG and indicated concentrations of SDS/EDTA or vancomycin. Plates were incubated for 16 hours at 37°C.

As shown in Figure S5A, MG1655 cells harboring pNde (empty vector) and the corresponding MG1655 Δ*tig::kan* mutant cells containing pNde, pTig or pTig-TEV-Avi were grown to stationary phase in LB media containing 100 µg/ml of ampicillin. 1:10 serial dilutions were spotted on LB plates containing 50 µg/ml of ampicillin, 70 µM IPTG

and indicated concentrations of SDS/EDTA or vancomycin. Plates were incubated for 16 hours at 37°C.

To measure growth curves (shown in Figures S4 and S5B), stationary cultures were diluted in fresh LB media and grown to an OD_{600} of 0.4. Cells containing plasmids were supplemented with IPTG. Cultures were diluted to an OD_{600} of 0.1 in LB media containing indicated concentrations of SDS/EDTA or vancomycin. Optical density was measured at 600 nm following dilution at indicated time points.

Analysis of chemical genetics screen

Correlation values across various strains were taken from a previously published chemical genetic screen (Nichols et al., 2011) and represented as a histogram.

Analysis of σ^E activity

σ^E activity was determined by measuring β-galactosidase expression using a previously described σ^E-dependent reporter system (Ades et al., 1999; Mecsas et al., 1993) in which φλ(*rpoHP::lacX*) was integrated into MG1655 and MG1655 Δ*tig::kan* cells. Cultures were first grown in LB media starting from an OD_{600} of 0.01 at 30°C and β-galactosidase activity was measured at multiple growth phases.

Isolation of outer membrane proteins for SILAC

Light and heavy M9 minimal media were prepared containing 43 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 10 µg/ml of thiamine. Each amino acid was supplemented to a final concentration of 0.5 mM, except Lysine-8:HCl and Arginine-10:HCl (Silantes, Munich, Germany) were used for heavy media. pH was adjusted to 7.0 using NaOH, and glucose was added to a final concentration of 0.4%. MG1655 and MG1655 Δ*tig::kan* cells were grown to stationary phase in both light and heavy M9 minimal media. Stationary cultures were diluted to an OD_{600} of 0.02 in 50 ml of M9 minimal media and grown to mid-log phase at 37°C. Translation was stopped by adding 100 µg/ml of chloramphenicol. Cultures were chilled on ice for 20 min. Equal amounts (gauged by optical density) of wild-type and Δ*tig::kan* cells grown in different media were mixed and harvested by centrifugation. Cell pellets were subsequently frozen in liquid nitrogen.

Frozen cells were thawed in a 30°C waterbath for 2 min, resuspended in lysis buffer (18 mM Tris pH 7.5, 18 mM KCl, 0.36 mM EDTA pH 8.0, 180 µg/ml of lysozyme, 18 µg/ml of RNase A, 9 µg/ml of DNase I) and lysed by sonication. Unlysed cells were cleared by light centrifugation at 500x*g* for 15 min at 4°C. The supernatant (or total lysate) was collected and spun at 13,200 rpm for 16 min. The resulting pellet (or total membrane fraction) was washed once with 20 mM $NaPO_4$ pH 7.0 and thrice with 20 mM $NaPO_4$ pH 7.0 containing 0.5% sarcosyl, which solubilizes and removes the inner membrane fraction. The final pellet (or outer membrane fraction) was resuspended in 20 mM $NaPO_4$ pH 7.0.

Mass spectrometry analysis

Proteins from the outer membrane fraction were separated by SDS-PAGE. The entire lane was cut in 3 mm segments. Excised gels were reduced by DTT, alkylated by iodoacetamide and digested by trypsin (Catrein et al., 2005) using a Digest Pro MS liquid handling system (Intavis). Following digestion, the tryptic peptides were extracted from the gel slices with 50% acetonitrile/0.1% trifluoroacetic acid (TFA), concentrated in a SpeedVac vacuum centrifuge until nearly dried and diluted to a max volume of 30 µl with 0.1% TFA. 25 µl of sample was analyzed by a nanoHPLC system (Tempo nanoMDLC, Applied Biosystems) coupled to an ESI LTQ Orbitrap mass spectrometer (Thermo Fisher). Samples were next loaded on a C18 trapping column (Reprosil-pur, C18, Dr Maisch) with a flow rate of 10 µl/min in 0.1% TFA. Peptides were eluted and separated on an analytical column (75 µm x 150 mm packed with Reprosil-pur, C18, Dr Maisch) with a flow rate of 200 nl/min in a gradient of buffers A (0.1% formic acid) and B (0.1% formic acid and acetonitrile): 0 to 6 min, 3% of B; 6 to 60 min, 3-40% of B; 60 to 65 min, 60-90% of B. The column was connected to a nano-ESI emitter (New Objectives). 1500 V were applied by liquid junction. One survey scan (resolution at 60000) was followed by five information dependent product ion scans in the LTQ. Only doubly and triply charged ions were selected for fragmentation. Data analysis was performed using MaxQuant (1.2.0.11) containing the search engine Andromeda (Cox et al., 2011). ncbi.Ecoli_K12_substrDh10B.25-Jan-2010, provided by MaxQuant, was used as the database. Trypsin specificity (one missed cleavage), fixed modification of cysteines (carbamidomethyl) and variable modification of methionines (oxidation) were used. The false discovery rate of peptides and proteins was set to 0.01. Proteins with less than three peptides detected were omitted in this analysis. The outer membrane fraction was normalized to the total fraction, which in turn was normalized by MaxQuant. Outer membrane proteins were identified based on their GO annotation.

Pulse-labeling and two-dimensional gel analysis

Cultures of MG1655 and MG1655 Δ*tig::kan* were grown to mid-log phase in M63 minimal media containing 0.2% maltose and an 18 amino acid mix lacking cysteine and methionine. Cultures were incubated with ^{35}S-labeled methionine for 30 sec. Labeling was terminated by adding cold trichloroacetic acid (TCA) to a final concentration of 5%. Immunopreciptation and two-dimensional gel analyses were performed as previously described (Randall, 1983).

SUPPLEMENTAL REFERENCES

Ades, S.E., Connolly, L.E., Alba, B.M., and Gross, C.A. (1999). The Escherichia coli sigma(E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-sigma factor. Genes Dev *13*, 2449-2461.

Amann, E., Ochs, B., and Abel, K.J. (1988). Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in Escherichia coli. Gene *69*, 301-315.

Catrein, I., Herrmann, R., Bosserhoff, A., and Ruppert, T. (2005). Experimental proof for a signal peptidase I like activity in Mycoplasma pneumoniae, but absence of a gene encoding a conserved bacterial type I SPase. FEBS J *272*, 2892-2900.

Chaba, R., Grigorova, I.L., Flynn, J.M., Baker, T.A., and Gross, C.A. (2007). Design principles of the proteolytic cascade governing the sigmaE-mediated envelope stress response in Escherichia coli: keys to graded, buffered, and rapid signal transduction. Genes Dev *21*, 124-136.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., and Mann, M. (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res *10*, 1794-1805.

Datta, A.K., and Burma, D.P. (1972). Association of ribonuclease I with ribosomes and their subunits. J Biol Chem *247*, 6795-6801.

Guo, H., Ingolia, N.T., Weissman, J.S., and Bartel, D.P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature *466*, 835-840.

Homann, O.R., and Johnson, A.D. (2010). MochiView: versatile software for genome browsing and DNA motif analysis. BMC Biol *8*, 49.

Ingolia, N.T. (2010). Genome-wide translational profiling by ribosome footprinting. Meth Enzymol *470*, 119-142.

Ingolia, N.T., Ghaemmaghami, S., Newman, J.R.S., and Weissman, J.S. (2009). Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science *324*, 218-223.

Keseler, I.M., Bonavides-Martínez, C., Collado-Vides, J., Gama-Castro, S., Gunsalus, R.P., Johnson, D.A., Krummenacker, M., Nolan, L.M., Paley, S., Paulsen, I.T.*, et al.* (2009). EcoCyc: a comprehensive view of Escherichia coli biology. Nucleic Acids Res *37*, D464-470.

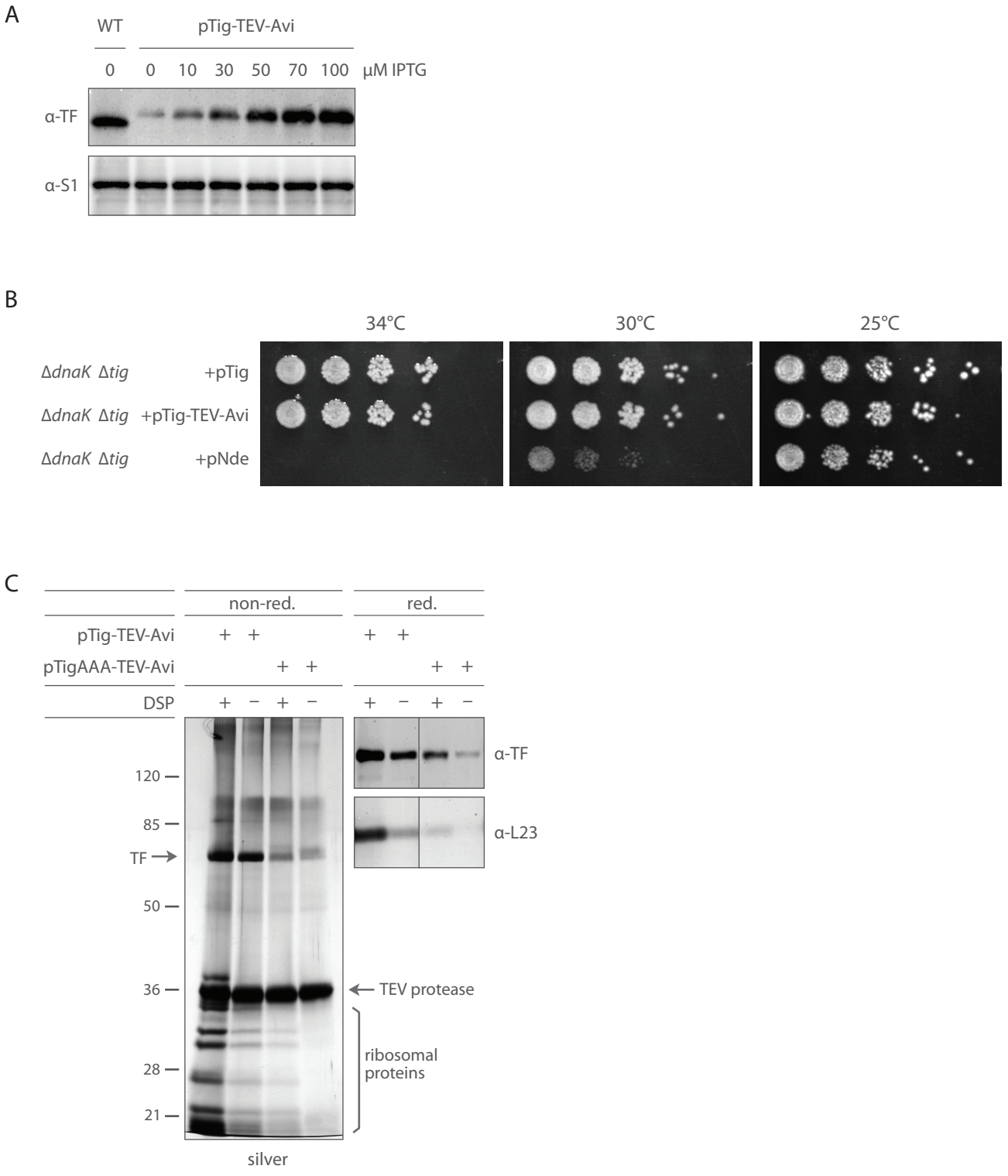
Mecsas, J., Rouviere, P.E., Erickson, J.W., Donohue, T.J., and Gross, C.A. (1993). The activity of sigma E, an Escherichia coli heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. Genes Dev *7*, 2618-2628.

Müller, M., and Blobel, G. (1984). Protein export in Escherichia coli requires a soluble activity. Proc Natl Acad Sci USA *81*, 7737-7741.

Nichols, R.J., Sen, S., Choo, Y.J., Beltrao, P., Zietek, M., Chaba, R., Lee, S., Kazmierczak, K.M., Lee, K.J., Wong, A.*, et al.* (2011). Phenotypic landscape of a bacterial cell. Cell *144*, 143-156.

Randall, L.L. (1983). Translocation of domains of nascent periplasmic proteins across the cytoplasmic membrane is independent of elongation. Cell *33*, 231-240.

Vorderwülbecke, S., Kramer, G., Merz, F., Kurz, T.A., Rauch, T., Zachmann-Brand, B., Bukau, B., and Deuerling, E. (2004). Low temperature or GroEL/ES overproduction permits growth of Escherichia coli cells lacking trigger factor and DnaK. FEBS Lett *559*, 181-187.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50
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100
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α-TF
α-S1
B
34°C
30°C
25°C
ΔdnaK Δtig
+pTig
ΔdnaK Δtig
+pTig-TEV-Avi
ΔdnaK Δtig
+pNde
C
non-red.
red.
pTig-TEV-Avi
pTigAAA-TEV-Avi
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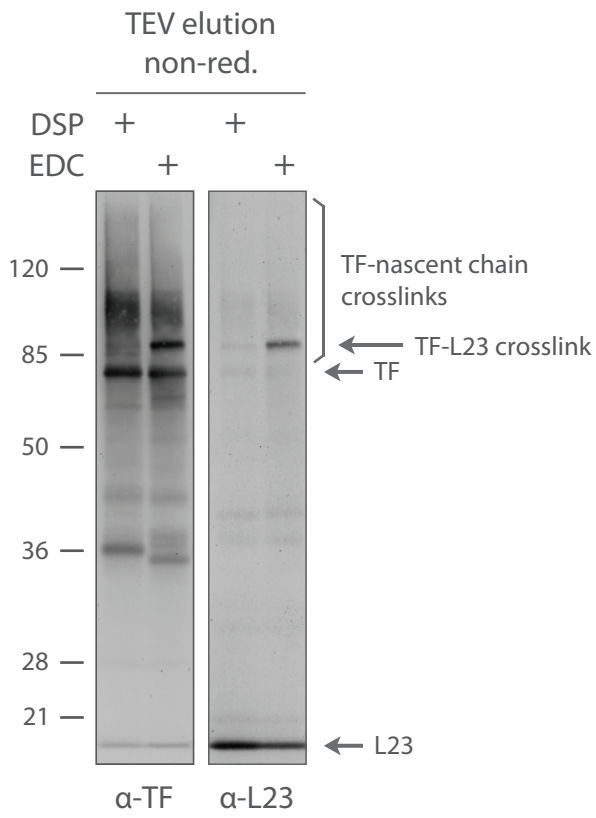
α-L23
120
85
TF
50
36
28
21
TEV protease
ribosomal proteins
silver


TEV elution
non-red.
DSP + +
EDC + +
120
85
50
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28
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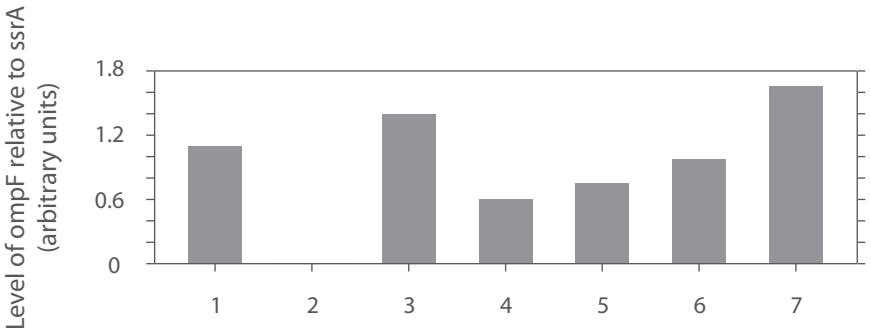
TF-nascent chain
crosslinks
TF-L23 crosslink
TF
L23
α-TF
α-L23

Supplemental Figure 03

Supplemental Figure 3



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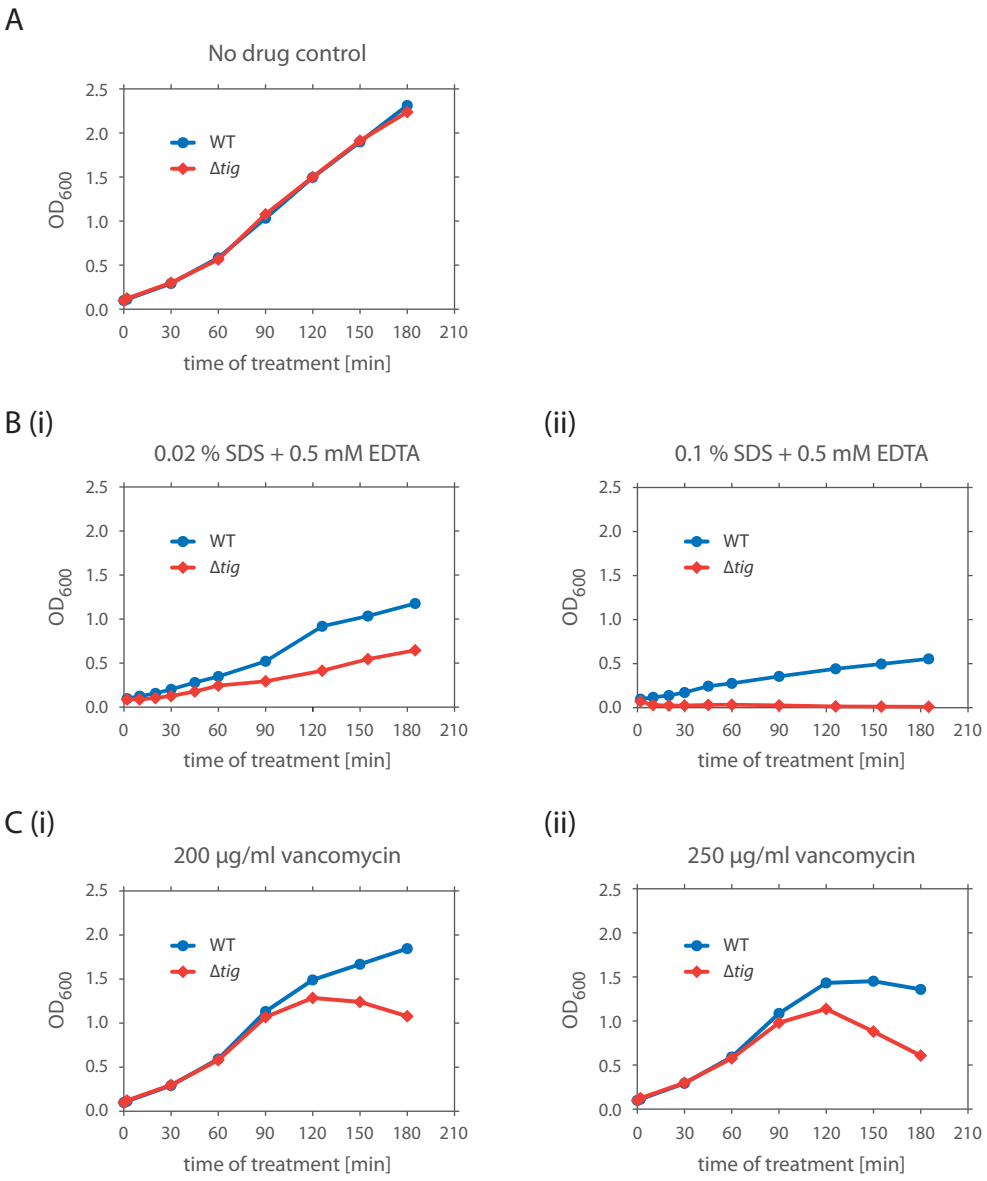
7	Δ*ompF*	myo insert	yes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Δtig
OD600
time of treatment [min]
B (i)
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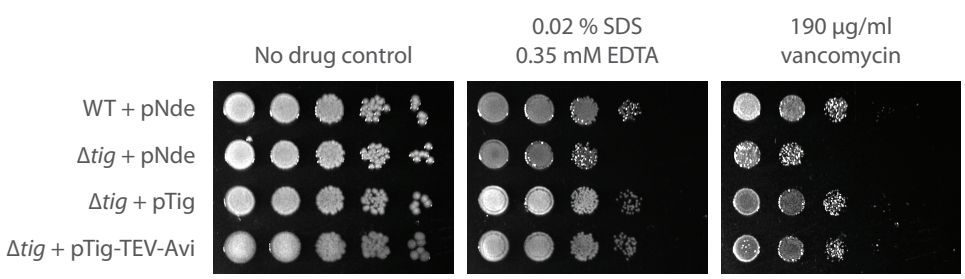
250 µg/ml vancomycin

Supplemental Figure 5

A



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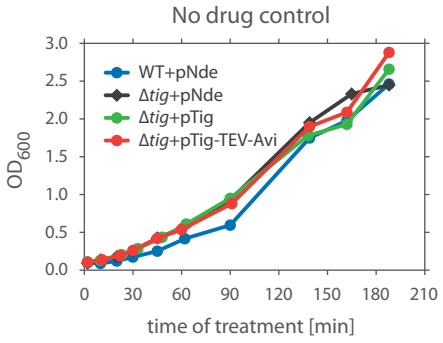

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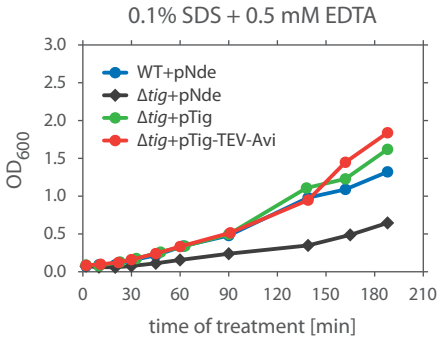

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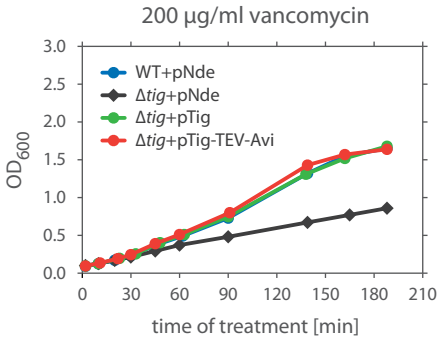
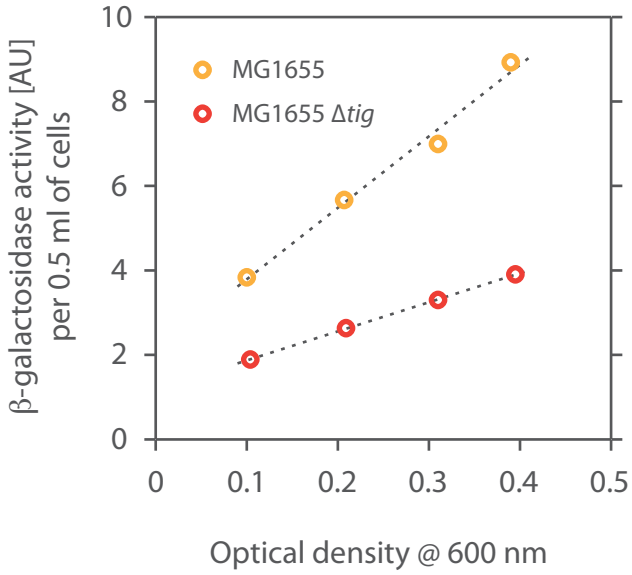
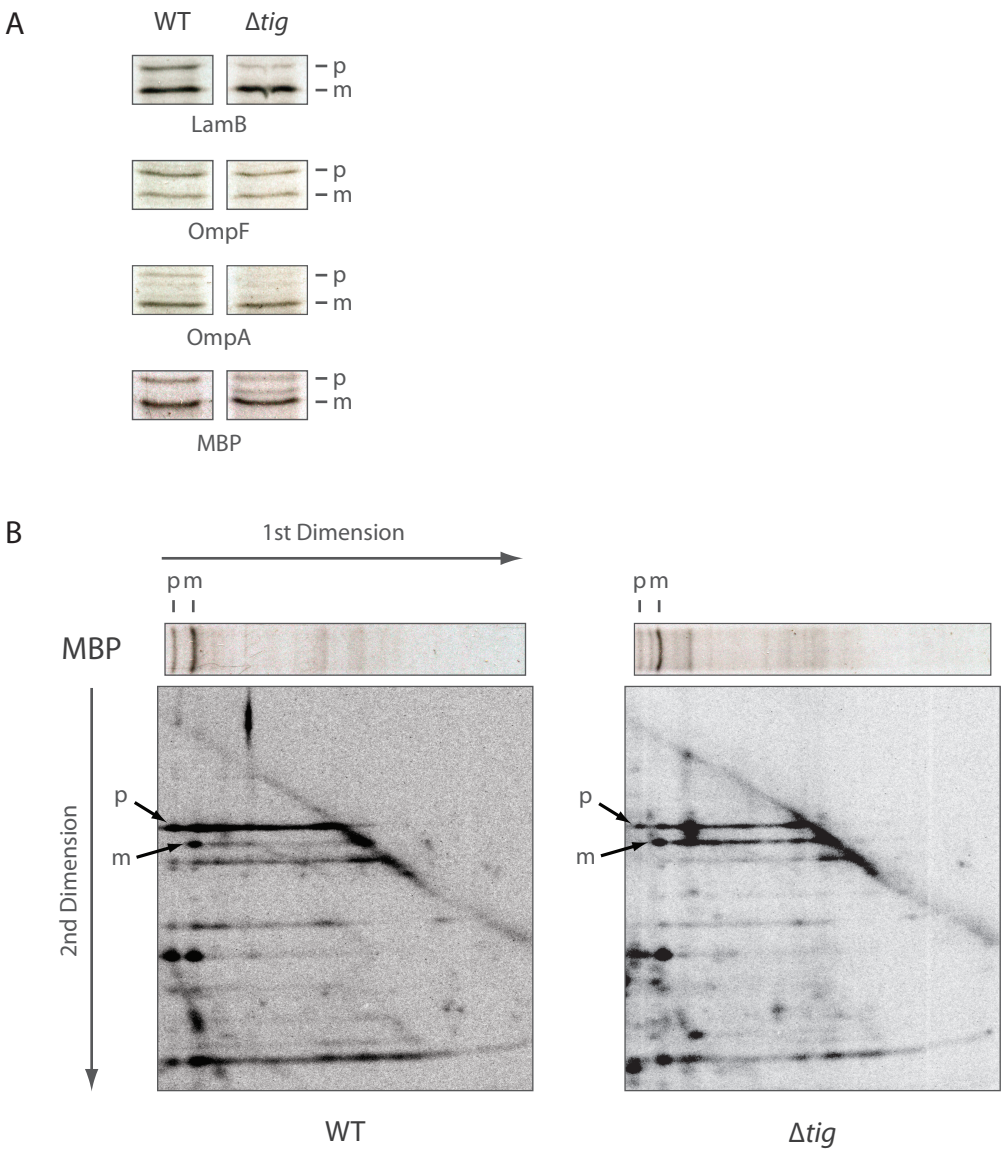


Supplemental Figure 06

Supplemental Figure 6

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LamB
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B
1st Dimension
2nd Dimension
MBP
WT
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