

Reprogramming chaperone pathways to improve membrane protein expression in *Escherichia coli*

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Abstract: Because membrane proteins are difficult to express, our understanding of their structure and function is lagging. In *Escherichia coli*, α -helical membrane protein biogenesis usually involves binding of a nascent transmembrane segment (TMS) by the signal recognition particle (SRP), delivery of the SRP-ribosome nascent chain complexes (RNC) to FtsY, a protein that serves as SRP receptor and docks to the SecYEG translocon, cotranslational insertion of the growing chain into the translocon, and lateral transfer, packing and folding of TMS in the lipid bilayer in a process that may involve chaperone YidC. Here, we explored the feasibility of reprogramming this pathway to improve the production of recombinant membrane proteins in exponentially growing *E. coli* with a focus on: (i) eliminating competition between SRP and chaperone trigger factor (TF) at the ribosome through gene deletion; (ii) improving RNC delivery to the inner membrane via SRP overexpression; and (iii) promoting substrate insertion and folding in the lipid bilayer by increasing YidC levels. Using a bitopic histidine kinase and two heptahelical rhodopsins as model systems, we show that the use of TF-deficient cells improves the yields of membrane-integrated material threefold to sevenfold relative to the wild type, and that whereas YidC coexpression is beneficial to the production of polytopic proteins, higher levels of SRP have the opposite effect. The implications of our results on the interplay of TF, SRP, YidC, and SecYEG in membrane protein biogenesis are discussed.

Keywords: trigger factor; TF; signal recognition particle; SRP; YidC; membrane proteins

Introduction

Integral membrane proteins account for 20–30% of open reading frames in sequenced genomes and play essential roles in cell survival.¹ Despite their importance as pharmacological and therapeutic targets,^{2,3} and their promise in the emerging field of nanobiotechnology,⁴ less than 300 membrane protein struc-

tures have been solved to date. Aside from difficulties associated with the production of high quality crystals for X-ray or electron crystallography, one of the major obstacles to high throughput membrane protein structural biology is the need to obtain sufficient amounts of properly folded material for crystallization trials or isotopic labeling in NMR studies.

Although the elucidation of the structure of certain membrane proteins (e.g., bacteriorhodopsin) has been facilitated by their natural abundance, overproduction in bacteria, yeasts or insect cells is generally required. Among these expression hosts, *Escherichia coli* remains the most popular because of its rapid growth, extensive characterization, and the availability of a wide array of powerful genetic tools.^{5,6} Unfortunately, membrane protein overexpression is often accompanied by misfolding and cellular

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toxicity, resulting in poor yields of functional material. Common approaches to alleviate these problems include accumulating membrane proteins into inclusion bodies,⁷ which shifts the problem to one of refolding, expressing them at low temperatures or under the control of weak promoters,^{8–10} which reduces overall productivity, and using fusion partners to promote membrane insertion,^{11–13} which requires controlled proteolysis steps to yield a native protein. Clearly, strategies allowing for enhanced accumulation of authentic and functional membrane proteins in the inner membrane of exponentially growing *E. coli* cells would be highly desirable.¹⁴

In *E. coli*, most inner membrane proteins are inserted into the lipid bilayer in a cotranslational process orchestrated by the signal recognition particle (SRP).¹⁵ Bacterial SRP, which is composed of a 48-kDa GTPase (Ffh) and a 4.5S RNA (*ffs*), docks to the L23 and L29 ribosomal proteins near the peptide exit site and binds with high affinity to the hydrophobic α -helical transmembrane segment (TMS) of inner membrane proteins emerging from the ribosome.¹⁶ Once a nascent chain is engaged, the SRP–ribosome–nascent chain complex (RNC) is targeted to the SRP receptor, FtsY, which delivers the RNC to the SecYEG translocon.¹⁷ Cotranslational insertion of membrane proteins into the plasma membrane is believed to involve the opening of a gate in SecY that allows for direct interactions between the TMS and the lipid bilayer.¹⁸ YidC, a 61-kDa integral membrane protein, facilitates the insertion process,^{19,20} and functions as a bona fide chaperone that promotes correct membrane protein folding within the lipid bilayer.²¹ Interestingly, YidC also serves as a SecYEG-independent insertase for a subset of membrane proteins.^{22,23}

Trigger factor (TF) is a molecular chaperone that shields hydrophobic regions in nascent and newly synthesized polypeptides to prevent aggregation and encourage proper folding in the cytoplasm.²⁴ TF binds with moderate affinity to the L23 ribosomal protein,^{25,26} and strong biochemical evidence indicates that TF and SRP can simultaneously localize near the mouth of the peptide exit tunnel, where they compete for the binding of nascent chains.^{27,28} However, although TF is present at a twofold to threefold molar excess over ribosomes and associates with most 70S particles,^{26,29} the concentration of SRP is substoichiometric (1–25%) relative to ribosomes.^{30,31} To compensate for its low abundance, SRP is preferentially recruited to ribosomal particles that are in the early stages of the translation.³² Whether a nascent chain is bound by TF or SRP is determined by its chemical (and possibly structural) characteristics: TF prefers linear stretches of amino acids enriched in basic and aromatic residues that are commonly found in protein cores,³³ whereas SRP selectively captures highly hydrophobic segments that are typical of TMS or of

the signal sequence of SRP-dependent secretory proteins.¹⁶ Thus, the TF versus SRP binding decision ultimately determines whether a newly synthesized protein will be released post-translationally in the cytoplasm or targeted for SRP-dependent cotranslational inner membrane insertion or export.

Overexpression of membrane proteins has the potential for upsetting the TF/SRP balancing act by greatly increasing the number of ribosomes translating SRP-dependent substrates. It is further expected to exert a disproportionate demand on the SecYEG–YidC insertase machinery.³⁴ Here, we capitalize on a growing understanding of α -helical membrane protein biogenesis to show that TF inactivation and YidC coexpression can significantly improve the production of properly folded bitopic and 7 TMS membrane proteins in the inner membrane of *E. coli*, leading to productivity gains as high as sevenfold. This simple approach to enhance functional membrane protein production is likely to prove useful in addressing a major bottleneck in structural biology.

Results

TF inactivation triples the yields of a bitopic kinase receptor

To investigate how the interplay of TF, SRP, and YidC affects the fate of overproduced membrane proteins, we made use of isogenic cells containing (*tig*⁺) or lacking the structural gene for TF (*tig*), and constructed plasmids encoding the *ffh* and *ffs* genes (pSRP) or the *yidC* gene (pYidC) under control of their native promoters that lead to a fourfold to fivefold increase in the intracellular levels of these chaperones. As an initial model, we selected *E. coli* ZraS, a histidine kinase that functions as the Zn²⁺/Pb²⁺ sensing element of the ZraS–ZraR two-component system.³⁵ ZraS is a 51-kDa protein that is predicted to contain two transmembrane helices defining a 167-residue-long periplasmic segment and a 22-kDa C-terminal cytoplasmic domain responsible for the autocatalytic phosphorylation of His-254 [Fig. 1(A)]. The *zraS* gene was PCR-amplified from the *E. coli* chromosome, fused to a sequence specifying a C-terminal hexahistidine tag for easy immunodetection, placed under transcriptional control of the *Para*_{BAD} promoter to prevent leaky expression in the absence of inducer, and the resulting plasmid was introduced into isogenic *tig*⁺ of Δ *tig* cells along with pSRP, pYidC or the control vector pMM102.

Consistent with the behavior typically observed with overproduced membrane proteins, arabinose induction of mid-exponential phase cultures growing at 37°C led to pronounced inhibition of cell growth [Fig. 1(B)]. Neither TF inactivation (open symbols) nor YidC overproduction (triangles) affected this phenotype, although there was a small but

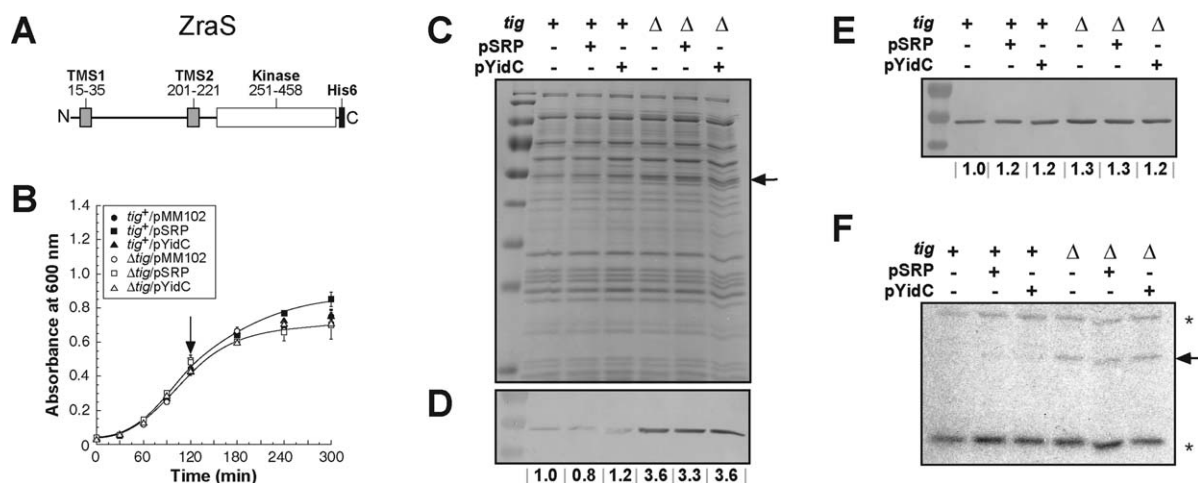


Figure 1. Influence of TF, SRP, and YidC on ZraS expression. (A) Predicted topology of ZraS. Boxes show the location of transmembrane segments (TMS, gray), kinase domain (white), and hexahistidine tag (black). (B) Growth curves of wild type (tig^+ ; closed symbols) and TF-deficient (Δtig ; open symbols) cells harboring the indicated plasmids following arabinose induction of ZraS expression in mid-exponential phase (arrow). SDS-PAGE (C) and Western analysis (D) of membrane fractions harvested 3 h postinduction from the indicated strains and corresponding to identical amounts of cells. The arrow shows the migration position of ZraS. Numbers under the blot quantify the intensity of each band relative to an arbitrary value of 1 for control cultures. (E) Western analysis of ZraS inclusion bodies in the insoluble fractions of the indicated strains. (F) Autoradiogram of membrane fractions isolated from the indicated strains following incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The position of ZraS (arrow) and two native kinases (*) are indicated.

reproducible improvement in the fitness of wild-type cultures coexpressing SRP (filled squares). When membranes isolated from identical amounts of cells [Fig. 1(C)] were analyzed by immunoblotting with anti-His₆ antibodies [Fig. 1(D)], we observed approximately a 20% drop in ZraS accumulation in tig^+ cells coexpressing SRP, although there was a comparable increase in ZraS production in pYidC transformants. More remarkably, inactivation of TF (Δtig lanes) led to an over threefold increase in the levels of membrane-associated ZraS, again with a small negative impact associated with SRP coexpression [Fig. 1(D)]. This large improvement in yields did not correlate with reduced aggregation, because the amount of ZraS inclusion bodies—about 75% of the total ZraS present in wild-type cells and 50% that in Δtig cells—remained essentially constant on a per cell basis under all strain/plasmid combinations tested [Fig. 1(E)].

To verify that the protein was properly folded, we conducted autophosphorylation assays by incubating membrane fractions with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and visualizing radioactively labeled proteins by autoradiography. In agreement with immunoanalysis data [Fig. 1(D)], phosphorylated ZraS was barely detectable in the wild type, although a much stronger signal was observed in Δtig cells [Fig. 1(F)]. By contrast, the intensity of nonoverproduced inner membrane kinases (stars) remained comparable in tig^+ and tig null cells. We conclude that TF inactivation more than triples the production of bioactive ZraS in exponentially growing *E. coli* by facilitating delivery or insertion of the protein into the membrane.

TF inactivation and YidC coexpression improve the accumulation of two rhodopsins in the inner membrane

To assess the influence of TF, SRP, and YidC on the expression of more topologically challenging substrates, we selected two archaeal rhodopsins as representative of the G protein-coupled receptor (GPCR) family, *Haloterrigena turkmenica* deltarhodopsin (HtdR) and *Naatronobacterium pharonis* sensory rhodopsin II (pSRII). Both of these 7 TMS proteins [Fig. 2(A,B)] have been expressed in a functional form in *E. coli*^{36,37} and use an all-*trans* retinal chromophore to harvest light. However, they perform distinct functions: HtdR is a light-driven outward proton pump, whose photocycle is similar to that of *Halobacterium salinarum* bacteriorhodopsin,³⁶ whereas pSRII (also called phoborhodopsin) is a sensor for negative phototaxis that is used by *N. pharonis* to move away from blue-green light.³⁸

Not surprisingly, expression of both rhodopsins proved toxic to *E. coli* with pSRII exerting the most deleterious effect on the growth of control cells [Fig. 2(C,D), filled circles]. In contrast to the results obtained with ZraS, coexpression of SRP had a strong negative impact on the growth of both tig^+ and Δtig cells (squares), whereas TF inactivation (open symbols) or YidC coexpression (triangles) independently improved cell growth. In the case of pSRII, the effects were additive leading to a twofold increase in the density of Δtig /pYidC cultures [Fig. 2(D), open triangles]. On the other hand, although YidC overproduction improved the growth of HtdR-

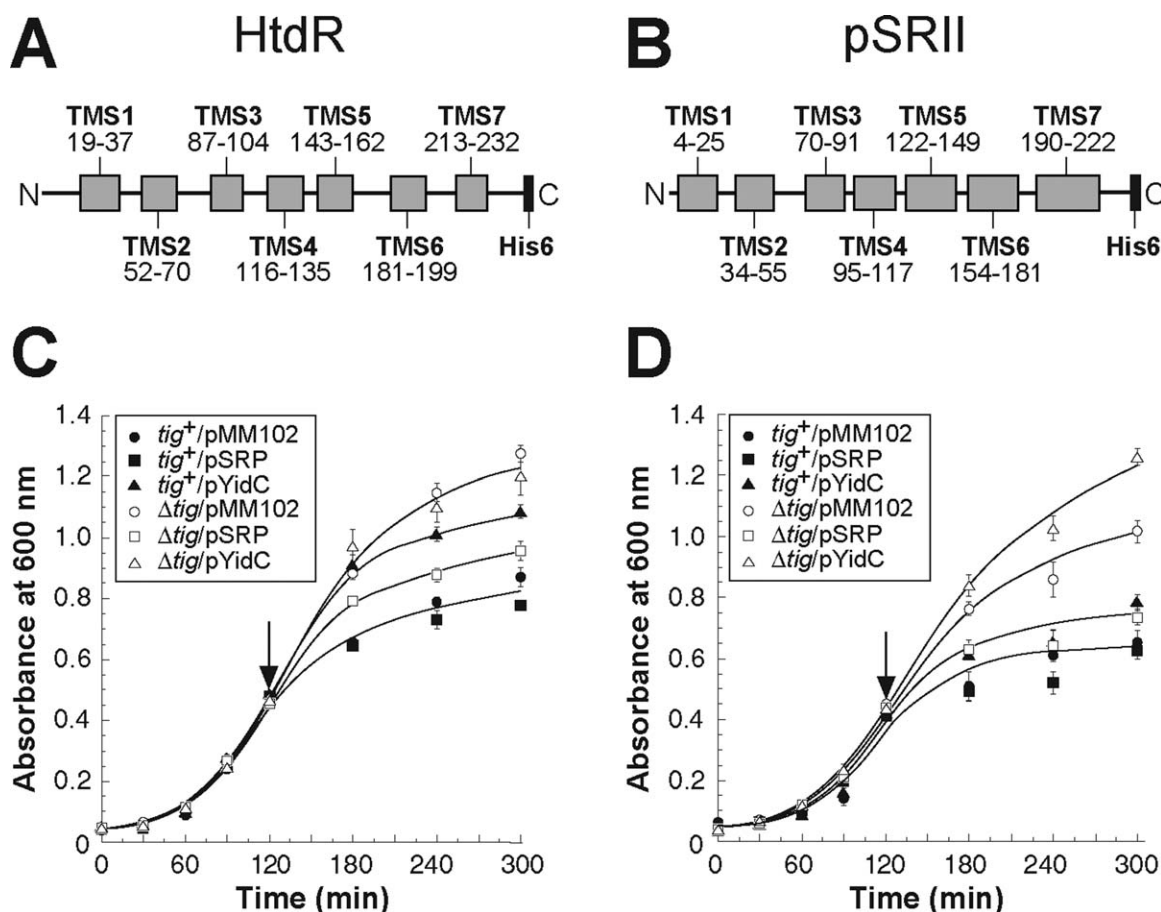


Figure 2. Structure of model rhodopsins and impact of their expression on cell growth. Topology of HtdR (A) and pSRII (B). Boxes show the location of transmembrane segments (TMS, gray) and hexahistidine tags (black). Growth curves of wild type (tig^+ ; closed symbols) and TF-deficient (Δtig ; open symbols) cells harboring the indicated plasmids following arabinose induction of HtdR (C) or pSRII (D) expression in mid-exponential phase (arrow).

expressing tig^+ cells by 25% [Fig. 2(C), filled triangles], it did not confer an additional growth advantage to Δtig cells.

Inspection of membrane fractions adjusted for variations in culture density revealed that although coexpression of SRP in wild-type cells reduced the yields of HtdR and pSRII by about half, higher levels of YidC improved the amount of membrane-associated HtdR by 60% and that of pSRII by over two-fold (Fig. 3). Making use of Δtig cells had an even greater beneficial effect: the amount of membrane-integrated HtdR increased 2.6-fold and those of pSRII 3.4-fold relative to control cultures, and the presence of pYidC led to a slight increase in yields (Fig. 3). As in the case of ZraS, the improvement in membrane-integrated rhodopsins could not be explained by a reduction in the amount of aggregated HtdR [Fig. 3(C)] or pSRII [Fig. 3(F)].

Based on the observation that reduced levels of transcription can improve the yields of over-expressed membrane proteins,¹⁰ we used quantitative PCR to quantify the amount of *htdR* transcripts in all six genetic backgrounds. We found no significant difference in the amount of *htdR* mRNA in the

various strains and conclude that the observed improvement in membrane protein production in Δtig strains and YidC-overexpressing cells are not associated with changes in transcriptional efficiency.

Probing the folding of HtdR and pSRII with retinal

Exogenously added all-*trans* retinal binds to a lysine residue located in the seventh helix of HtdR or pSRII through a protonated Schiff base.³⁹ The unique conformation of the chromophore in the correctly folded rhodopsin structure^{40,41} leads to a characteristic UV-visible absorption spectrum that exhibits a single maximum at ≈ 550 nm in the case HtdR,³⁶ and a maximum at ≈ 500 nm together with a pronounced shoulder at ≈ 470 nm for pSRII.⁴² We took advantage of this sensitive probe of proper folding to determine how our various expression protocols affected the functionality of membrane-integrated material. Figure 4(A) shows that the membrane fractions of cells expressing HtdR exhibited the expected spectrum and that there was a good correlation between the intensity of the 550 nm peak and the amount of membrane-associated

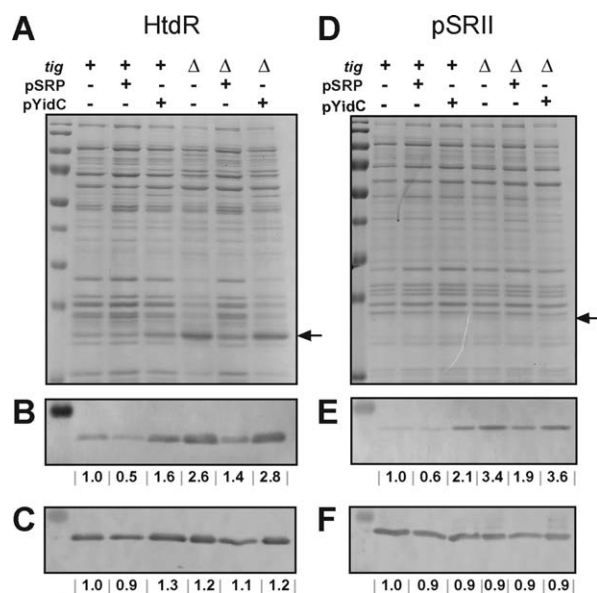


Figure 3. Influence of TF, SRP, and YidC on HtdR and pSRII expression. Cells expressing HtdR (left panels) or pSRII (right panels) were harvested 3 h postinduction. Membrane fractions corresponding to identical amounts of cells were analyzed by SDS-PAGE (A,D) or Western blotting (B,E). Insoluble fractions from the same samples were subjected to Western blotting to quantify HtdR (C) or pSRII (F) inclusion bodies. Arrows show the migration position of each protein. Numbers under blots quantify the intensity of each band relative to an arbitrary value of 1 for control cultures. Note that half as much material was used in the HtdR blots compared with the pSRII blots.

protein estimated from Western blots [Fig. 3(B)]. Notably, coexpression of SRP reduced maximum intensity by about half, higher levels of YidC had the opposite effect, and the use of Δ *tig* cells led to two-fold to threefold increase in the intensity of the 550-nm peak. In fact, the improvement in active HtdR production could be readily visualized by the increased purple pigmentation of cultures grown in the presence of retinal [Fig. 4(A), inset].

Because pSRII is produced at much lower levels than HtdR in wild-type cells [cf. Fig. 3(B) and (E)], the anticipated absorption features were almost undetectable in control cells (filled circles) and only slightly more obvious in the YidC-overproducing wild type (filled triangles). The use of *tig* mutants (open circles) sharpened both minor ($\approx$ 465 nm) and major ($\approx$ 495 nm) absorption features, and both peaks became clearly visible in Δ *tig* cells cotransformed with pYidC (open triangles). In all of these cases, there was good agreement with the trends observed by immunoblotting [Fig. 3(E)]. However, and for reasons that remain unclear, SRP overproduction in *tig*⁺ or Δ *tig* cells yielded membrane fractions that exhibited strong absorption and no distinct features in the sub-500 nm range. Considering that comparable amounts of pSRII accumulate in

the membranes of *tig*⁺/pYidC and Δ *tig*/pSRP cells [Fig. 3(E)], the difference in absorption spectra strongly suggests that at least a fraction of membrane-integrated pSRII is misfolded in strains overexpressing SRP.

Productivity and scale-up considerations

To more accurately compare how the various strategies impacted yields, we took into account variations in growth [Figs. 1(B) and 2(C,D)] and scaled the amount of membrane-associated material per cell to the culture density at the time of harvest (Fig. 6). These data indicate that: (i) SRP coexpression has no effect (ZraS) or is deleterious (HtdR, pSRII) to membrane protein productivity irrespective of the presence or absence of TF; (ii) YidC

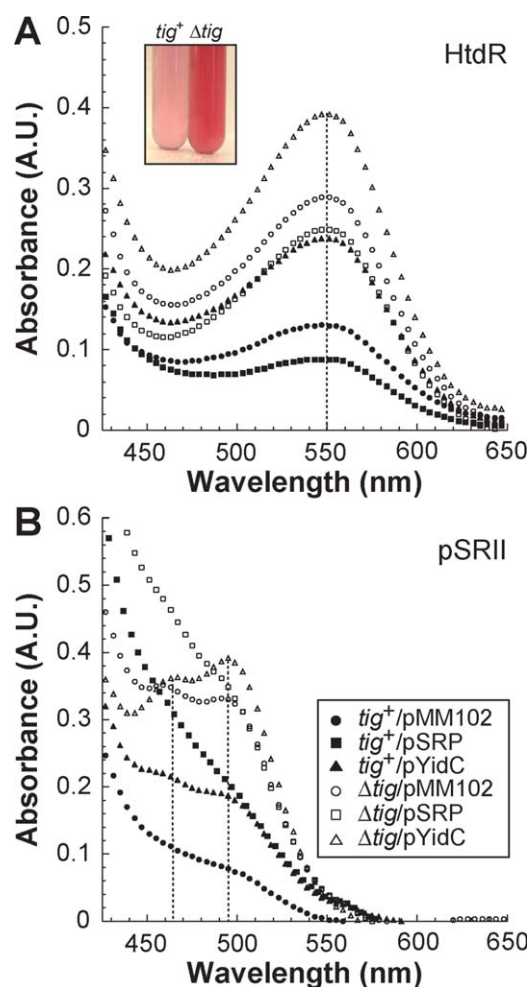


Figure 4. Absorption spectra of retinal-bound rhodopsins. Membrane fractions were collected 3 h postinduction from indicated cells expressing HtdR (A) or pSRII (B). Samples were adjusted to the same culture density, solubilized in DDM, and absorption spectra were collected. The inset shows the color difference between membrane fractions collected from the same amount of wild type (*tig*⁺) or TF-deficient (Δ *tig*) cells expressing HtdR. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

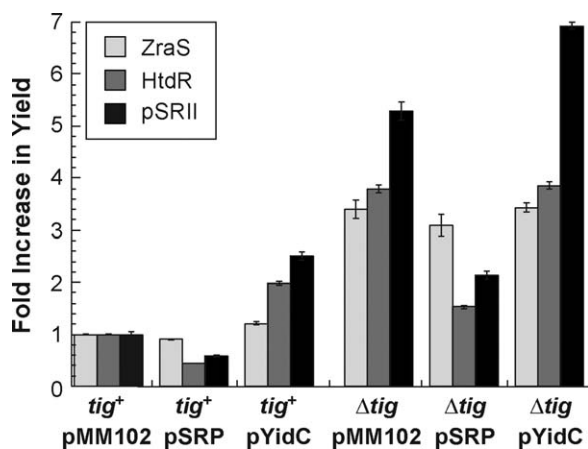


Figure 5. Influence of TF, SRP and YidC on the overall yields of ZraS, HtdR, and pSRII 3 h postinduction.

coexpression is most beneficial to topologically complex membrane proteins expressed in wild-type cells; and (iii) the use of Δ *tig* cells alone is sufficient to confer most productivity gains. Optimal yields obtained in small shake flasks with 3 h postinduction harvest were 0.5 mg/L for ZraS, 4 mg/L for pSRII, and 16 mg/L for HtdR.

To evaluate the potential of TF-deficient strains for preparative membrane protein production, we grew Δ *tig* cells harboring either the HtdR or the pSRII expression plasmid in 500 mL of Luria Bertani (LB) medium supplemented with 10 μ M retinal at 37°C and harvested cell membranes 3 h postinduction. In the absence of a compatible plasmid (e.g., pMM102, pSRP, or pYidC), cells grew to a density that was approximately twice that of the cultures of Figure 5. Such improved growth, which presumably results from a reduced metabolic burden and the use of a single selective pressure, translated into yields of nearly 10 mg/L for pSRII and 35 mg/L for HtdR. Membrane-integrated recombinant proteins could be solubilized with *n*-dodecyl β -D-maltoside and obtained in a fairly pure form through a single round of nickel-nitrilotriacetic acid (Ni-NTA) chromatography [Fig. 6(A)]. More importantly, the absorption spectra of affinity-purified proteins exhibited were indistinguishable from those of functional HtdR and pSRII,^{36,42} suggesting that all membrane-integrated material was properly folded [Fig. 6(B)].

Discussion

The biogenesis of α -helical membrane proteins in *E. coli* involves a complex series of chaperone-assisted steps that must occur with high efficiency within a background of cytoplasmic protein synthesis and extracytoplasmic protein export. Because translation does not stop when bacterial SRP binds to the first TMS of membrane proteins, there is only a narrow time window for delivering RNCs to the SecYEG translocon, inserting newly synthesized α -helical

segments within the lipid bilayer, and folding and packing domains that lie both within and outside the cytoplasmic membrane. Therefore, it is not surprising that overproduction of membrane proteins interfere with this finely tuned ecosystem and lead to misfolding, toxicity and low yields of functional material. Here, we explored the feasibility of reprogramming chaperone pathways to improve the production of recombinant membrane proteins in exponentially growing *E. coli* with a focus on eliminating TF/SRP competition through TF inactivation, improving targeting and/or delivery to the inner membrane through SRP overexpression, and promoting insertion and folding in the lipid bilayer by YidC overproduction.

For all proteins examined, we found that strains lacking TF (Δ *tig* cells) accumulate significantly larger amounts of properly folded, membrane-associated material than their wild-type counterparts. Because TF and SRP bind nonexclusively to the same location of the ribosome,^{27,28} SRP should have unimpeded access to the TMS of nascent membrane proteins in Δ *tig* strains, guaranteeing efficient capture of membrane proteins and proper targeting to the SRP pathway. In addition, docking of SRP-RNC complexes to SecY-bound FtsY,⁴³ and timely transfer of the growing polypeptide to the SecYEG pore, should be facilitated in the absence of interfering TF considering that SecY and TF share the L23 protein as a common attachment site on the ribosome.^{44–46} In agreement with these views, we previously reported that the cotranslational secretion of leech carboxypeptidase inhibitor (LCI) fused to the SRP-dependent DsbA signal sequence is twice higher in Δ *tig* than in wild-type cells and that the improvement in mature protein yields correlates with a decrease in the amount of precursor LCI in the cytoplasm.⁴⁷ We also found that higher levels of YidC increased the yields of both HtdR and pSRII in *tig*⁺ cells. This is fully consistent

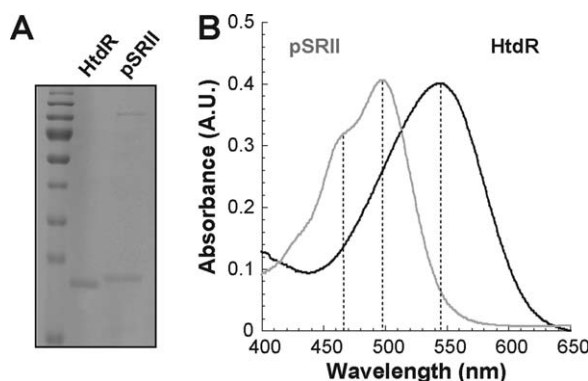


Figure 6. Purification and spectral characteristics of HtdR and pSRII. (A) SDS minigel of DDM-solubilized HtdR and pSRII following their affinity purification from the membranes of Δ *tig* cells. (B) Absorbance spectra of the purified rhodopsins.

with YidC's proven ability to improve the insertion and/or folding of topologically complex substrates (e.g., LacY) in the lipid bilayer.²¹ Why YidC coexpression had a much smaller beneficial effect on the yields of pSRII and no influence on those of HtdR in *tig* null cells (Fig. 5) remains unclear. From a practical standpoint, however, our results indicate that YidC coexpression should be helpful to increase the yields of polytopic membrane proteins in *tig*⁺ cells, and that Δ *tig* strains are superior hosts for the production of recombinant membrane proteins. Like all chaperone-assisted processes,⁴⁸ it is likely that the effectiveness of these approaches will ultimately depend on the identity and folding pathway of the target substrate.

Interestingly, whereas SRP coexpression improved the export of pre-LCI in both *tig*⁺ and Δ *tig* backgrounds,⁴⁷ it reduced the accumulation of membrane-associated material independently of the genetic background and with an impact that worsened with the number of TMS (40–50% decrease in yields for HtdR and pSRII compared with 10–20% for ZraS). A possible explanation for these seemingly contradictory results is that small secretory proteins such as LCI should rapidly transit through the SecYEG pore, although polytopic membrane proteins likely occupy the translocon for longer times to bundle and transfer multiple α -helices into the lipid bilayer. Thus, an increase in the flux of SRP-bound RNCs brought about by SRP overproduction might be well tolerated for a secretory substrate, but could become problematic with membrane proteins because of nonstoichiometric FtsY levels or limited availability of unoccupied SecYEG complexes at the membrane. We favor the translocon bottleneck scenario for two reasons. First, SecY-bound FtsY appears to be displaced upon docking of the SRP–RNC complex to the translocon^{43,49} and is therefore unlikely to be a limiting component. And second, proteomics experiments suggest that the translocation of Sec-dependent proteins through the SecYEG complex becomes inefficient under conditions of membrane protein overexpression.³⁴

In concluding, it is worth noting that, on a per cell basis, the amount of aggregated ZraS, HtdR, or pSRII remained mostly unchanged under conditions, where the amount of membrane-integrated material varied between fourfold more (Δ *tig* cells) and twofold less (SRP coexpression) than in control cells. Thus, the mechanisms responsible for improving the yields of our membrane proteins likely involve resistance to proteolytic degradation associated with better insertion and folding in the membrane.

Materials and Methods

Plasmid constructions

Plasmid pSRP has previously been described.⁴⁷ To build pYidC, the *yidC* gene together with its pro-

motor was amplified from MC4100 genomic DNA using primers 5'-GTTCAAGCTTCGGAATTGAGGC ATTGC-3' and 5'-TCGCTCTAGATGTTCTGTTGCT TTG-3', which introduce *Hind*III and *Xba*I sites at the 5' and 3' ends of the amplified fragment, respectively, and the *Hind*III and *Xba*I digested PCR product was cloned into the same sites of pMM102,⁴⁷ a chloramphenicol-resistant pACYC184 derivative. To construct the expression plasmid pBLN200, pBAD33⁵⁰ was first mutagenized to introduce an *Hpa*I site downstream of the *araC* gene using the primer pair 5'-CCGTCAATTGTCTGATTCGTTAACA ATTATGACAACCTTGACGGC-3' and 5'-GCCGTCAA GTTGTCATAATTGTTAACGAATCAGACAATTGAG G-3'. The resulting plasmid (pBAD33MutHpa) was digested with *Xba*I and *Hpa*I and the fragment containing the *araC* gene and the *P*_{BAD} promoter was cloned into the same sites of pET-24a(+), replacing the T7 promoter and yielding pBLN200. The *zraS* gene was amplified from MC4100 genomic DNA using 5'-TATGGCATATGCGTTTTATGCAACG-3' and 5'-TATATCTCGAGTCCTTGTTGGGTCC-3'. Both sets of primers introduce *Nde*I and *Xho*I sites at the 5' and 3' ends, respectively. PCR products were digested with these enzymes and cloned into the same sites of pBLN200 to create pZraS200. The genes encoding HtdR and pSRII were excised from pET-HtdRHis³⁶ and pET-ppRHis,⁵¹ respectively using *Nde*I and *Xho*I, and cloned into the same sites of pBLN200 to yield pHtdR200 and pPPR200, respectively. All model proteins contain a C-terminal hexahistidine tag. Expression plasmids were cotransformed with pMM102, pSRP, or pYidC into the *tig*⁺ strain BW25113 [Δ (*araD-araB*)567 Δ *lacZ*4787 (::rrnB-3) λ^- *rph-1* D(*rhaD-rhaB*)568, *hsdR*514]⁵² or its isogenic Δ *tig* derivative KTD101.⁴⁷

Protein expression, membranes isolation and electrophoresis techniques

Shake flasks (125 mL) containing 25 mL of LB media supplemented with 34 μ g/mL chloramphenicol and 50 μ g/mL kanamycin were inoculated to an OD₆₀₀ of $\sim$ 0.05 and grown at 37°C to mid-exponential phase. At OD₆₀₀ $\approx$ 0.45, membrane protein synthesis was induced by the addition of 0.2% L-arabinose to the growth medium; 10 μ M of all-*trans* retinal (Sigma; from a 10 mM stock in methanol) was also added at this time to cultures expressing HtdR or pSRII. Membrane proteins were allowed to accumulate for 3 h at 37°C and samples (5 mL) were collected. Cells were sedimented by centrifugation at 2000g for 10 min at 4°C, resuspended in 3 mL of 50 mM potassium phosphate monobasic pH 7.4, and disrupted in a French press operated at 10,000 psi. Samples were centrifuged at 10,000 rpm for 10 min at 4°C and pellets corresponding to inclusion body material were resuspended in sample buffer (75 mM Tris–HCl, pH 6.8, 0.002% bromophenol blue, 15 mg/

mL dithiothreitol, 5% sodium dodecyl sulfate (SDS), 8% glycerol). The supernatant was centrifuged at 150,000g for 1 h and 4°C, and pellets corresponding to membrane fractions were resuspended in sample buffer and incubated for 5 min at 45°C. In all cases, the volume of sample buffer added was normalized to the OD₆₀₀ value at the time of harvest, so that each sample corresponded to the same amount of cells. Duplicate aliquots were loaded on duplicate 12.5% SDS minigels, one of which was used for immunoblotting and the other stained with Coomassie blue. In the case of HtdR, the gel used for blotting was loaded at half of the concentration used for Coomassie staining. For Western blots, gels were incubated in transfer solution (25 mM Tris-HCl, pH 8.3, 0.2 M glycine, 3 mM SDS, 20% methanol) for 30 min, and proteins were transferred to nitrocellulose overnight at 15 V and 4°C. Nitrocellulose membranes were probed with mouse anti-6-His antibody (Covance) at 1:2000 dilution, incubated with goat antimouse IgG Alkaline Phosphatase (Sigma) at a 1:3000 dilution, and detected by colorimetry with 5-bromo-4-chloro-3-indolyl phosphate and nitro tetrazolium blue. Independent replicates were performed a minimum of three times for all model proteins. Results were similar and representative blots are shown. Yields were determined by loading known amounts of purified Hsp31-His₆⁵³ alongside membrane fractions and constructing a calibration curve via videodensitometric analysis.

Phosphorylation assays and spectroscopy

For autophosphorylation assays, membrane fractions were resuspended in 75 mM Tris-HCl, pH 7.0, 0.1 M NaCl in volumes that were one half of those used for SDS-PAGE. Five μ Ci of [γ -³²P]ATP was added to 10 μ L of sample, and the mixture was incubated at room temperature for 30 min. The reaction was stopped by addition of 4 μ L of 4 $\times$ sample buffer. Samples were electrophoresed on 12.5% SDS minigels that were soaked three times in 30% methanol, 10% acetic acid for 5 min, dried onto blotting filter paper and exposed to X-ray film at -80°C. To collect the absorption spectra of Figure 4, cultures (125 mL) were grown and induced as above and identical amounts of cells (based on 100 mL for the lowest OD₆₀₀ culture) were collected 3 h postinduction. Membranes isolated as above were solubilized in 50 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 6.5, 300 mM NaCl, 5 mM imidazole, and 1.0% *n*-dodecyl β -D-maltoside (DDM) with gentle shaking for 2 h at room temperature. Samples were centrifuged at 150,000g for 1 h at 4°C to remove unsolubilized material, and absorption spectra were collected on a Beckman Coulter DU640 spectrophotometer. For the experiments of Figure 6, membrane fractions collected from 500 mL cultures were processed as above and loaded onto a Ni-NTA equilibrated in 50 mM MES, pH 6.5, 300 mM NaCl,

20 mM imidazole, 0.1% DDM. Following extensive washing, HtdR and pSRII were eluted in the same buffer supplemented with 250 mM imidazole. Spectra were collected after centrifugation at 15,000g for 5 min to remove any aggregated material.

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