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Review

Bacterial-based membrane protein production ☆

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ABSTRACT

Escherichia coli is by far the most widely used bacterial host for the production of membrane proteins. Usually, different strains, culture conditions and production regimes are screened for to design the optimal production process. However, these *E. coli*-based screening approaches often do not result in satisfactory membrane protein production yields. Recently, it has been shown that (i) *E. coli* strains with strongly improved membrane protein production characteristics can be engineered or selected for, (ii) many membrane proteins can be efficiently produced in *E. coli*-based cell-free systems, (iii) bacteria other than *E. coli* can be used for the efficient production of membrane proteins, and, (iv) membrane protein variants that retain functionality but are produced at higher yields than the wild-type protein can be engineered or selected for. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.
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1. Introduction

In both pro- and eukaryotes 20–30% of all genes encode membrane proteins, which usually form supra-molecular complexes acting in many different and often essential capacities and are fundamental in maintaining the function of a healthy cell/organism [1]. Membrane proteins also play key roles in many diseases and around 70% of all drugs act on membrane proteins [2] (http://www.drugs.com/top200.html). The natural abundance of membrane proteins is often too low to isolate sufficient material for *in vitro* functional and structural studies. Furthermore, the use of natural sources excludes the possibility of genetically modifying proteins to facilitate their detection and/or purification, and efficiently labeling them for nuclear magnetic resonance (NMR) and crystallographic studies.

There are two classes of membrane proteins: β-barrel and helical bundle membrane proteins [3]. Usually, β-barrel membrane proteins can be produced in *E. coli* as inclusion bodies from which they are readily isolated and refolded in their native conformation [4]. Nevertheless, to determine the structure of a number of bacterial β-barrel membrane proteins they were produced in the outer membrane rather than in inclusion bodies (*e.g.*, [5,6]). There are only few examples of helical bundle membrane proteins that could be isolated from inclusion bodies in their native conformation (see *e.g.*, [7]). Usually, the isolation of helical bundle membrane proteins from inclusion bodies is not successful. Therefore, helical bundle membrane proteins must be produced in such a way that they properly insert into the membrane from which they can be purified after detergent extraction or wherein they can be studied directly. This makes that in general the production of helical bundle membrane proteins is more complex than the production of β-barrel membrane proteins. In this review, we will deal only with the production of helical bundle membrane proteins, hereafter referred to as membrane proteins.

E. coli is the most widely used bacterial host when attempting to produce membrane proteins. Usually, a variety of strains, culture conditions and production regimes are screened for to design the optimal membrane protein production process (*e.g.*, [8,9]). However, yields – especially of eukaryotic membrane proteins – are usually not sufficient for functional and structural studies. Therefore, eukaryotic protein production hosts are increasingly used for the production of eukaryotic membrane proteins [10,11]. However, the high costs involved in using eukaryotic hosts for producing eukaryotic membrane proteins make that there is a growing interest in developing cost-effective bacterial-based production alternatives for these proteins.

Recently, considerable progress has been made in producing both pro- and eukaryotic membrane proteins using bacterial-based systems. It has been reported that (i) *E. coli* strains with strongly improved membrane protein production characteristics can be engineered or selected for, (ii) many membrane proteins can be efficiently produced in *E. coli*-based cell-free systems, (iii) bacteria other than *E. coli* can be used for the efficient production of membrane proteins, and (iv) membrane protein variants that retain functionality but are produced at higher yields than the wild-type protein can be engineered or selected for. The aim of this review is to give a comprehensive overview of these exciting

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developments. Our knowledge of the biogenesis of membrane proteins and newly developed analytical methods for monitoring membrane protein production has been key to many of these developments. Therefore, we will start with a brief introduction to these two topics.

2. Membrane protein biogenesis

Membrane protein production and biogenesis are intimately linked as membrane proteins must be produced in such a way that they insert into the membrane and fold properly. The biogenesis of membrane proteins in the cytoplasmic or inner membrane of the widely used bacterial membrane production host *E. coli* has been extensively studied (see [12] and references herein). Below, an overview is presented of what is known about the biogenesis of membrane proteins in the inner membrane of *E. coli* (this overview is supported by Fig. 1).

In *E. coli*, ribosome-inner membrane protein nascent chain complexes (RNCs) are targeted cotranslationally to the membrane *via* the SRP pathway (comprising the signal recognition particle (SRP) and its receptor FtsY) [13,14]. At the membrane, the RNC docks at the Sec-translocon — a protein-conducting channel that facilitates both the translocation of hydrophilic polypeptide chains across the membrane and the insertion of transmembrane segments into the lipid bilayer [15,16]. The translocation of sizeable periplasmic loops requires the ATPase SecA [17,18]. YidC has been proposed to mediate the transfer of transmembrane segments from the Sec-translocon into the lipid bilayer and can assist the folding of membrane proteins [19]. Some membrane proteins are targeted *via* the SRP pathway or directly to YidC [19]. YidD functions in the biogenesis of both YidC and Sec-YidC dependent membrane proteins [20]. The SecDFYajC complex can play a role in the biogenesis of membrane proteins [19]. Notably, it has been shown that mRNAs encoding membrane proteins localize to the cytoplasmic membrane in a translation-independent mechanism [21]. This may indicate that mRNAs encoding membrane proteins are targeted to the membrane.

Folding of soluble cytoplasmic domains of membrane proteins might be supported by cytoplasmic chaperones such as DnaK, whereas that of periplasmic domains of membrane proteins might be supported by periplasmic chaperones such as DegP (which can also act as a protease). The FtsH complex is involved in quality control and degradation of membrane proteins [22,23].

Targeting and insertion of tail anchored (TA) proteins into the cytoplasmic membrane may occur in a post-translational manner *via* the SRP/Sec-translocon system or an alternative pathway.

Secretory proteins are targeted by the chaperone SecB in a mostly posttranslational manner to the Sec-translocon [24]. The translocation of secretory proteins is SecA dependent. The signal sequence of secretory protein is removed by leaderpeptidase (Lep). The SecDFYajC complex can also play a role in the translocation and folding of secreted proteins.

Recently, we have addressed in great detail how the heterologous production of membrane proteins may be affected by differences in the membrane protein biogenesis machinery of the different membrane protein production platforms used (see [9] and references herein). Here, we only briefly mention the most important ones.

There are differences in polypeptide elongation and protein folding rates between different prokaryotes and these rates are considerably higher than the ones in eukaryotes. These different rates may cause mistargeting and misfolding of heterologously produced membrane proteins. Although there is a high homology between most of the key components involved in membrane protein biogenesis in pro- and eukaryotic systems, there is some circumstantial evidence indicating that this does not necessarily mean they are also well suited for assisting the targeting, insertion and folding of heterologously produced membrane proteins into the membrane [25–27]. Some membrane proteins require membrane-protein-specific chaperones for proper folding, (see *e.g.*, [28]). Thus, it is possible that chaperones essential for the heterologous production of membrane proteins may be absent in the production host used.

Other organismal factors are lipid composition and glycosylation. Differences in lipid composition between membrane systems can have a significant effect on the insertion, folding and functioning of a heterologously produced membrane proteins [29,30]. Glycosylation of eukaryotic membrane proteins can be essential for proper folding, stability and also function [31]. The most commonly used prokaryotic membrane protein production vehicles, *E. coli* and the Gram-positive bacterium *Lactococcus lactis* (see Section 5.1.), cannot glycosylate proteins.

Finally, it is of note that the high-level production of membrane proteins, including homologous ones, may saturate the membrane protein biogenesis machinery of the production host. Indeed, for *E. coli* it has been shown that the production of both homologous and heterologous membrane proteins can lead to the saturation of the Sec-translocon capacity, which negatively affects the fitness of the *E. coli* cell as well as membrane protein production yields [26,32,33].

3. Monitoring membrane protein production

Monitoring the localization, quantity and quality of produced membrane proteins is important to allow assessment and optimization of

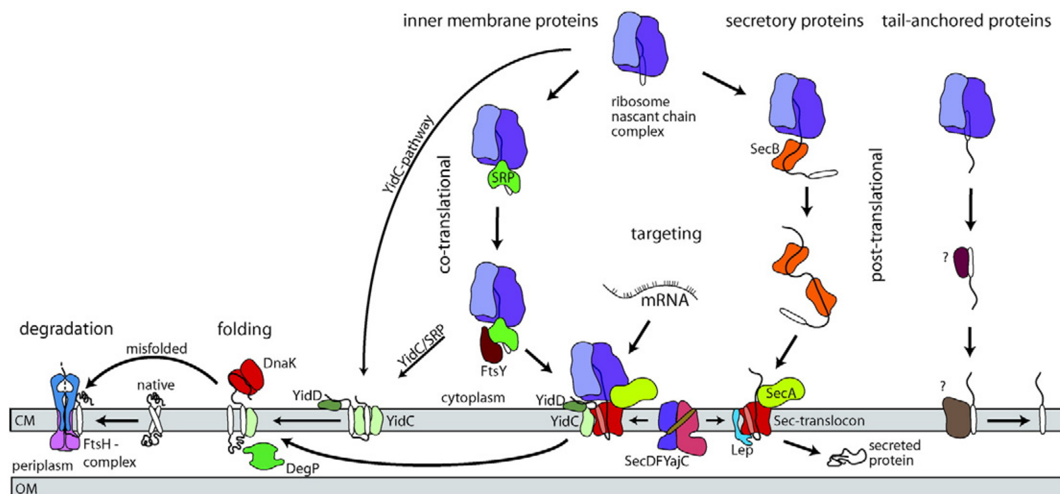


Fig. 1. Membrane protein biogenesis in *E. coli*. Overview of the different components involved in membrane protein biogenesis and membrane protein biogenesis pathways operational in *E. coli* (see Section 2 for more information). Picture was taken from [9].

production yields. It is unpredictable whether produced membrane proteins end up in the lipid bilayer or in inclusion bodies. Therefore, the first step in monitoring membrane protein production is usually the fractionation of the production host into an insoluble fraction (inclusion bodies), a soluble fraction and a membrane fraction [34]. From here on if not stated otherwise we mean with membrane the bacterial cytoplasmic membrane.

Coomassie/silver stained SDS-polyacrylamide gels have been routinely used to detect produced membrane proteins in (sub-fractionated) production hosts. They allow assessing the integrity and the quantity of produced membrane proteins. Immuno-blotting, using antibodies against *e.g.*, a production or purification tag, is also widely used to detect proteins. Due to the hydrophobic nature of membrane proteins their transfer from a gel to blotting membrane can be troublesome, making immuno-blotting less suitable for quantitative purposes. Size exclusion chromatography (SEC), often combined with static light scattering, is usually used to assess the quality of purified membrane protein material (see *e.g.*, [35,36]). These 'classical' approaches to monitor membrane protein production are very laborious, time-consuming and the gel-based approaches often not very accurate. Therefore alternative methods have been developed.

To easily and rapidly monitor the production of large numbers of soluble proteins in *E. coli* at low costs, the so-called colony filtration (CoFi) blotting method was developed [37]. In this method colonies are transferred from an agar plate to a filter membrane where protein production is induced and cells subsequently lysed. Upon cell lysis, the soluble proteins diffuse through the filter membrane and are captured on a nitrocellulose membrane, whereas inclusion bodies cannot pass through the filter. Subsequently, the nitrocellulose membrane is incubated with antibodies or probes that specifically recognize the protein of interest. The CoFi blotting method was modified so that it can also be used to monitor the production of detergent solubilized membrane proteins [38].

C-terminal selectable markers that confer a drug resistance phenotype have been used to assess membrane protein production yields in *E. coli* and the Gram-positive bacterium *L. lactis* [39–41]. The idea behind using these selectable markers is very simple, the higher the yields of a fusion produced in the membrane the higher amounts of drugs cells can cope with. Notably, just like the CoFi blotting method also the use of selectable markers that confer a drug resistance to assess membrane protein production yields does not require any special infrastructure.

Fusing Green Fluorescent Protein (GFP) to the C-terminus of a membrane protein enables monitoring the levels of membrane-integrated material in whole cells [42–45]. The GFP-moiety does not fold properly (and is therefore non-fluorescent) if the membrane protein GFP-fusion ends up in inclusion bodies, but it becomes fluorescent if the membrane protein is inserted into the membrane. Correctly folded GFP is not denatured in SDS-polyacrylamide gel solubilization (SB) buffer at temperatures below 37 °C. Using whole cells as starting material, the membrane protein GFP-fusion can thus be visualized directly through in-gel fluorescence in SDS-polyacrylamide gels [43]. This enables to rapidly assess the integrity of the produced material quantitatively, and is a welcome alternative for immuno-blotting. Since the GFP moiety of a membrane protein–GFP fusion that has been inserted in the membrane remains properly folded in SB buffer it will migrate faster in a gel than a non-inserted fusion whose GFP moiety is denatured in SB buffer. This allows using a simple SDS-polyacrylamide gel/immuno-blotting based assay to get an idea about the fraction of the total produced protein that is integrated in the membrane [46,47].

Membrane protein GFP-fusions allow using minute amounts of detergent solubilized whole cells/membranes to rapidly evaluate both their production levels and degree of monodispersity using fluorescence-detection size-exclusion chromatography (FSEC) [48]. Using different fluorescent fusion partners membrane multiprotein complexes have been characterized using multicolour FSEC [49]. Rather than fusing fluorescent proteins to membrane proteins a fluorescent multivalent NTA probe that interacts with polyhistidine-tags on target proteins can be used for FSEC [50].

The thiol-specific fluorochrome N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) is used for stability profiling of purified membrane proteins [51]. The CPM-based stability assay uses the chemical reactivity of the frequently in transmembrane segments occurring native cysteines as an indicator for the overall integrity of the folded state. FSEC and the CPM-based stability assay have been combined to rapidly assess the suitability of membrane proteins for crystallography [52].

Fusion partners are routinely attached to the C-terminus rather than N-terminus of a target membrane protein since attaching them to the N-terminus may hamper targeting to the cytoplasmic membrane. The location of the C-terminus (C_{in}/C_{out}) of the target membrane protein determines in which compartment the fusion partner will end up. The fusion partner should be active in the compartment it ends up in. Thus far, only GFP variants that fold properly in the cytoplasm and not in the periplasm have been used to make membrane protein GFP fusions. For C_{in} membrane proteins this is not a problem. For C_{out} membrane proteins *gfp* fusion expression vectors have been constructed that introduce the genetic information encoding a transmembrane segment between the genetic information encoding the target membrane protein and the genetic information encoding GFP [45]. This strategy can in principle be applied to any fusion partner whose functionality is compartment dependent.

All the above described methods do not provide direct information on the functionality of the produced proteins. To monitor the functionality of a membrane protein, its function should be known and also an activity assay should be available. Depending on the protein *e.g.*, binding assays with fluorescent or radioactive ligands or transport assays have been used (for examples see [8]). Functional assays for membrane proteins are usually carried out using isolated membranes or protein. However, sometimes also cells can be used. For instance, using fluorescently labeled ligands it has been shown that the levels of properly folded GPCRs produced in *E. coli* can be monitored directly in cells with a permeabilized outer membrane [53,54] (see also Section 6.2.).

4. Improving membrane protein production yields in *E. coli*-based platforms

Why has *E. coli* become the most widely used bacterial host for (membrane) protein production? The answer is largely for historical reasons. The organism was isolated over a century ago and turned out to be particularly easy to cultivate. Furthermore, a very large toolbox of genetic, molecular biological and biochemical methods has been developed for *E. coli*. All this has paved the way for *E. coli* to become a favorite model organism and the most widely used bacterial platform for the production of (membrane) proteins.

Obviously, during evolution there has not been selective pressure on *E. coli* towards the production of (heterologous) membrane proteins. However, this does not mean that it is impossible to engineer or select for *E. coli* strains that can efficiently produce membrane proteins. Indeed, there are now a number of examples of the creation of *E. coli* strains with improved membrane protein production characteristics. In addition, to avoid any toxic effects of membrane protein production on the living *E. coli* cell and to facilitate the labeling of proteins for NMR and crystallographic studies, *E. coli*-based cell-free protein production systems can be used to produce membrane proteins.

4.1. Selecting and engineering *E. coli* strains with improved membrane protein production characteristics

4.1.1. Tuning transcription and translation rates

The *E. coli* strain BL21(DE3) and derivatives thereof are widely used for protein production. The BL21(DE3) strain was originally developed

for the production of soluble proteins [55]. In this strain expression of the gene encoding the target is driven by the bacteriophage T7 RNA polymerase (T7 RNAP), which transcribes much faster than *E. coli* RNAP [56]. Expression of the gene encoding T7 RNAP is governed by the *lac*UV5 promoter, which is a mutant that is more powerful than the wild-type *lac* promoter [57]. The rationale behind BL21(DE3) is very simple; the more mRNA synthesized, the more protein can be produced. However, it has been shown that too high expression levels of genes encoding membrane proteins can lead to the saturation of the Sec-translocon capacity (see Section 2).

Almost two decades ago, in the laboratory of John Walker a simple screening approach was used to isolate derivatives of BL21(DE3) with improved membrane protein production characteristics [58]. *E. coli* BL21(DE3) cells producing 'toxic' membrane proteins were plated on solid medium with inducer to select for survivors, *i.e.*, cells that can cope with the toxic effects of membrane protein production. The C41(DE3) and C43(DE3) strains – which have commonly become known as the Walker strains – were isolated in this way and are now widely used to produce membrane proteins. It should be noted that the Walker strains do not show improved yields for all membrane proteins tested [33,58]. It has been shown that mutations in the *lac*UV5 promoter governing expression of the gene encoding T7 RNAP are key to the improved membrane protein production characteristics of the Walker strains [33]. These mutations result in the production of much lower amounts of T7 RNAP upon induction of expression than in BL21(DE3). Subsequent lower production rates of the mRNA for the target membrane protein ensure that the capacity of the Sec-translocon is sufficient to integrate the produced proteins in the membrane [32,33,47].

Based on the characterization of the Walker strains, a derivative strain of *E. coli* BL21(DE3), termed Lemo21(DE3), was engineered in which the activity of the T7 RNAP can be precisely controlled by its natural inhibitor T7 lysozyme (T7 Lys) [33]. In Lemo21(DE3) the gene encoding the T7 Lys is on a plasmid under control of a rhamnose promoter, which is extremely well titratable and covers a broad range of expression intensities [59]. The combination of the *lac*UV5- and the rhamnose promoters governing expression of the gene encoding T7 RNAP from the chromosome and the gene encoding T7 Lys from a plasmid, respectively, guarantees the widest window of expression intensities possible. Therefore, in Lemo21(DE3) the amount of membrane protein produced can be easily harmonized with the Sec-translocon capacity of the cell [33,47]. The development of this strain was sped up tremendously by using GFP-fusions to monitor membrane protein production. Notably, the Lemo21(DE3) strain has been used to produce membrane protein GFP-fusions from which properly folded membrane protein material could be recovered for functional and structural studies [47] (Fig. 2).

The above described findings indicate that tuning the transcription rate is a powerful tool to improve membrane protein production yields. In this respect it should be mentioned that the rhamnose promoter used to govern expression of the gene encoding T7 Lys in Lemo21(DE3) can also be used directly to tune the expression intensity of genes encoding membrane proteins, thereby improving membrane protein production yields [59]. The importance of tuning the expression levels of genes encoding membrane proteins for improving membrane protein production yields is further supported by the isolation of a mutant in a membrane protein expression vector based on the widely used arabinose-inducible pBAD promoter system [60]. A single transversion in a conserved region of the cyclic AMP receptor protein-binding site reduces transcript levels more than twofold. This results in improved cell growth and a twofold increase in membrane protein production yields. Tuning translation rates by using expression vectors with ribosome binding sites of different strength can also be used to improve membrane protein production yields [61]. Finally, expression vectors encoding small N-terminal fusion tags with different translation initiation rates have also been used to improve membrane protein production yields [62].

4.1.2. Drug resistance-based strain selection

A sophisticated selection strategy to isolate mutant strains of *E. coli* with improved protein production characteristics for targeted membrane proteins was recently described [39]. The gene encoding the targeted membrane protein was simultaneously expressed from two separate plasmids. Each plasmid was constructed such that upon expression the membrane protein was C-terminally fused to a different cytoplasmic selection marker; *i.e.*, a kanamycin resistance marker and mouse dihydrofolate reductase, which confers resistance to trimethoprim. Cells containing both plasmids were exposed to treatments that randomly introduce mutations and subsequently selected for growth on selective medium. An increased resistance towards the two selection drugs indicated an increased production of the target protein. Importantly, the use of a dual selection strategy considerably lowers the risk of obtaining mutations that confer resistance to both drugs without increasing membrane protein production levels. Furthermore, a rapid method for curing isolated strains of the plasmids was used during the selection process; the plasmids were removed by *in vivo* digestion with the homing endonuclease I-*Cre*I. It has been shown that in one of the isolated strains the copy number of the expression plasmids is considerably lower, suggesting that in this strain a lowered target gene expression level improves membrane protein production yields (see Section 4.1.1.). For the other strains it is not known why they have improved membrane protein characteristics. Membrane proteins C-terminally fused to the periplasmic drug resistance marker β-lactamase have made it possible to use multi-copy based plasmid libraries to select for strains with improved membrane protein production characteristics by screening for increased ampicillin resistance [40] (see Section 4.1.4.)

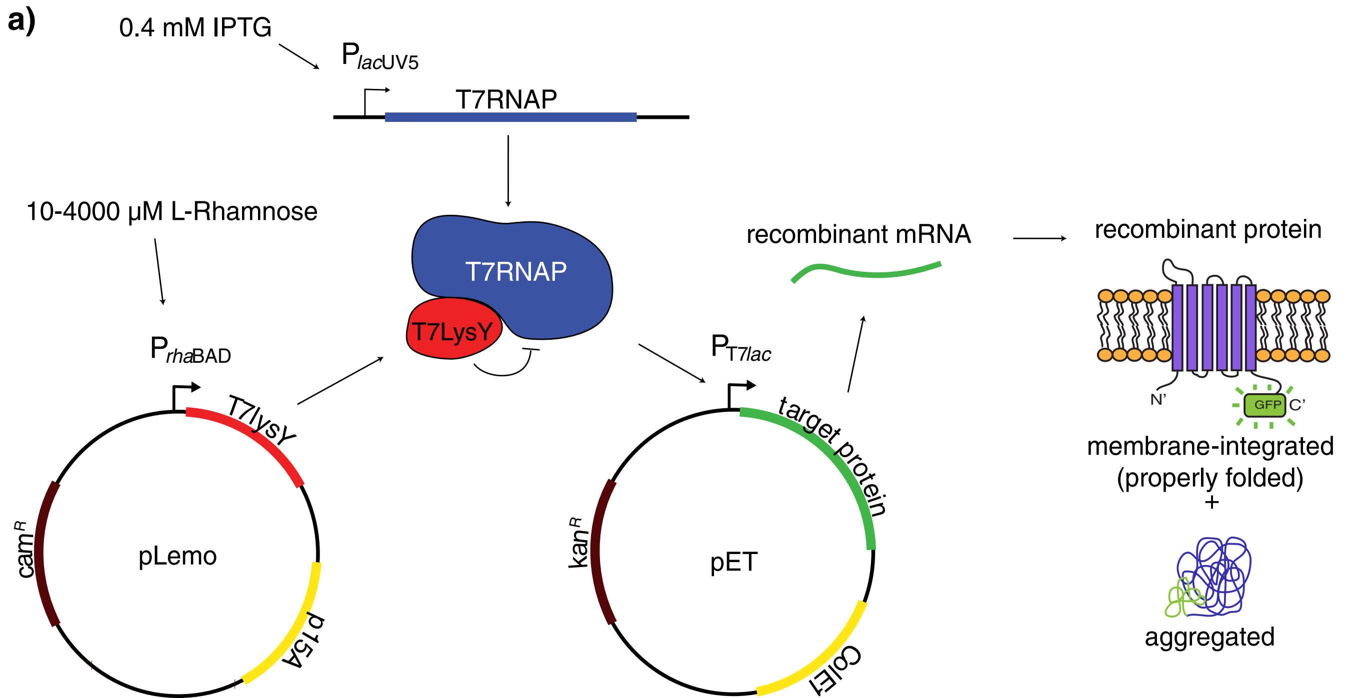
4.1.3. Co-production of biogenesis factors

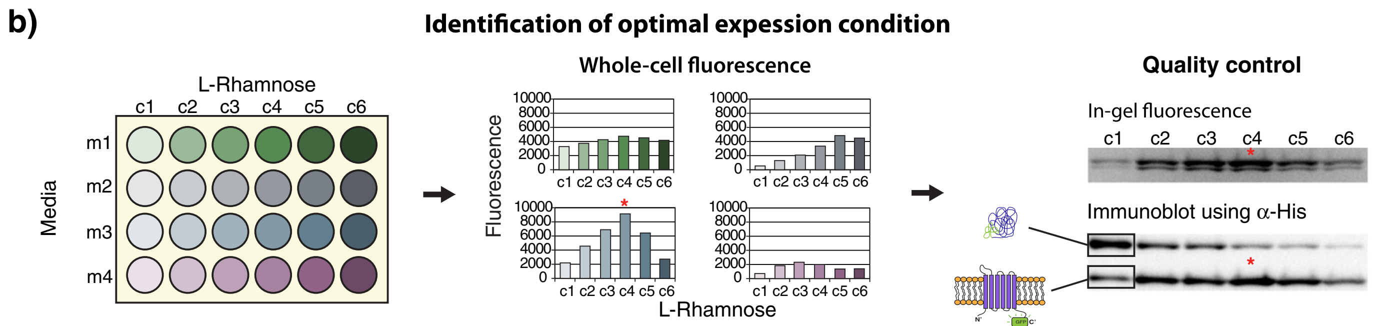
Co-production of chaperones is routinely used to improve production yields of soluble proteins (see *e.g.*, [63]). Chen et al. pioneered this approach for membrane proteins [64]. They tried to improve the production yields of the magnesium transporter CorA in *E. coli* by co-producing various components involved in membrane protein biogenesis (SRP/FtsY, SecA), secretory protein targeting (SecA/B) and protein folding in the cytoplasm (DnaK/J, GroEL/S; see the review by Genevaux *cum suis* in this special issue for more information). CorA production levels could be improved by the co-production of the cytoplasmic DnaK/J chaperone system. The CorA transporter is a homopentamer

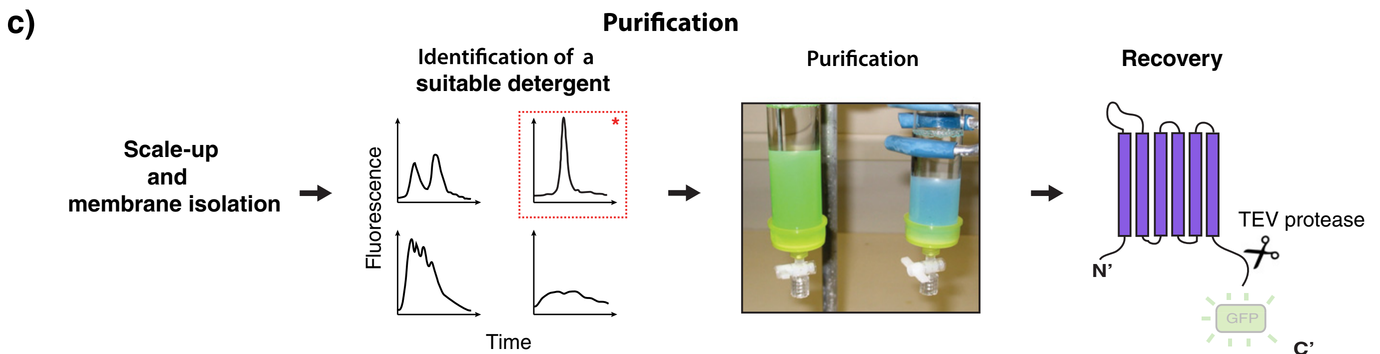
Fig. 2. Optimizing membrane protein production yields in *E. coli* using Lemo21(DE3) and GFP-fusions.A. Schematic representation of the Lemo21(DE3) strain. Expression of the chromosomally located gene encoding the T7 RNA polymerase (T7 RNAP) is governed by the not well titratable, isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible *lac*UV5 promoter. Expression of the gene encoding the natural inhibitor of the T7 RNA polymerase, T7 lysozyme (T7 Lys), is governed by the exceptionally well titratable rhamnose promoter from the pLemo plasmid. The pLemo plasmid has a p15A origin of replication and contains a chloramphenicol resistance marker. The gene encoding the target membrane protein is located on a pET vector and its expression is governed by the T7*lac* promoter. The pET vector has a ColE1 origin of replication. For the production of membrane proteins pET vectors with a kanamycin resistance marker are used. Membrane proteins are expressed as C-terminal GFP fusions. The GFP moiety only folds properly and becomes fluorescent when the membrane protein GFP fusion is inserted in the cytoplasmic membrane (see Section 3). B. Lemo21(DE3) cells are cultured in the presence of different concentrations of rhamnose. The expression of genes encoding membrane protein-GFP fusions is induced with 0.4 mM IPTG if no autoinduction based medium is used. Whole-cell fluorescence is used to monitor the production of membrane protein-GFP fusions in the cytoplasmic membrane. Subsequently, in-gel fluorescence is used to assess the integrity of the produced membrane protein GFP-fusions. The ratio of the cytoplasmic membrane inserted to non-inserted membrane protein is monitored using an SDS-PAGE/immuno-blotting based assay (see Section 3). C. The GFP-moiety facilitates the identification of a detergent to optimally extract the produced membrane protein from the membrane, and to monitor the stability of a membrane protein in a detergent using FSEC. Membrane protein GFP-fusions can also be seen by eye. Finally, membrane proteins can be recovered from the GFP-fusion using a site-specific protease and subsequently be used for functional and structural studies. Results observed for optimal production/solubilization conditions are marked (*).

and each protomer of CorA consists of a large N-terminal cytoplasmic domain and two transmembrane segments at the C-terminus. The architecture of CorA suggests that, unlike most other membrane proteins, it is not targeted co-translationally *via* the SRP/Sec-translocon pathway, but is targeted post-translationally [12]. Thus, it is likely that the DnaK/J chaperone system is somehow involved in the targeting and folding of CorA and could explain why co-production of this system improves CorA yields.

In an effort to improve production yields of GPCRs in *E. coli* various components involved in membrane protein biogenesis (SRP, FtsY, the Sec-translocon components SecY/E, YidC and FtsH), secretory protein targeting/translocation (SecB, DnaK/DnaJ, Trigger Factor (TF) and the Sec-translocon components SecY/E) and protein folding in the cytoplasm (GroEL/GroES, SecB, DnaK/DnaJ and TF) were co-produced when producing different GPCRs (see [65] and the review by Genevaux *cum suis* in this special issue for more







information). The use of GPCR-GFP fusions allowed the rapid assessment of the effect of the co-production of aforementioned components on GPCR production levels using flow cytometry. Notably, expression of the genes encoding the co-produced components was induced well before the genes encoding the GPCRs were induced. Only the co-production of the membrane-bound quality control factor/protease FtsH greatly enhanced the production yields of all four GPCRs. It has been proposed that the co-production of FtsH 'primes' the cells for the toxic effects of producing the GPCRs by inducing the expression of genes that may help the cell cope with membrane protein production stress rather than it plays a more direct role in the biogenesis of the GPCRs [66].

Finally, using a bitopic histidine kinase from *E. coli* and two archaeal rhodopsins the effects of the co-production of the insertion and folding factor YidC on membrane protein production yields were tested [67]. YidC co-production only had a positive effect on the production yields of the two rhodopsins.

4.1.4. Identification of factors improving production yields using multi-copy based plasmid libraries

The effects of multi-copy based plasmid libraries of bacterial chromosomal fragments on membrane protein production yields were screened for using two separate systems that monitor: (i) elevated fluorescence conferred by enhanced production of GPCR–GFP fusions and (ii) increased binding of fluorescent ligand in cells producing more active receptor [68]. Three hits were isolated by both methods: *nagD*, encoding the ribonucleotide phosphatase NagD; a fragment of *nlpD*, encoding a truncation of the predicted lipoprotein NlpD, and the three-gene cluster *ptsN–yhbJ–npr*, encoding three proteins of the nitrogen phosphotransferase system. Expression of these genes resulted in a 3- to 10-fold increase in the production yields of different GPCRs. It has been proposed that the expression of these genes may serve to maintain the integrity of the bacterial periplasm and to provide a favorable environment for proper membrane protein folding, possibly by inducing a fine-tuned stress response and/or *via* modifying the composition of the bacterial cell envelope. There is no experimental evidence supporting this. In another screen using membrane proteins C-terminally fused to the drug resistance marker β-lactamase, co-expression of *ybaB*, a gene encoding a putative DNA-binding protein of unknown function, was found to enhance the production yields of a variety of GPCRs and other membrane proteins in *E. coli* by up to 10-fold [40]. In the same study two more genes, *yciQ* and *glpQ*, were identified, whose co-expression improved membrane protein production yields. There is no ready explanation for the positive effect of the co-expression of these genes on membrane protein production yields.

4.1.5. Components hampering membrane protein production

It is also possible that there are host proteins that hamper membrane protein production. Therefore, the production of the human GPCR CB1 fused to GFP was screened in an *E. coli* transposon library [69]. Fluorescence-activated cell sorting (FACS) was used to identify and isolate cells with improved production yields for further characterization. A transposon insertion in the *dnaJ* gene resulted in an increase in CB1-GFP fluorescence and an enhancement in production of membrane-integrated CB1. Thus, the chaperone/co-chaperone DnaJ seems to inhibit production of CB1. However, it was not shown if improved yields also resulted in more functional material. DnaJ did not inhibit production of another GPCR tested. This suggests that – at least in some cases – the optimal strain background for the production of a membrane protein may be protein-specific. Recently, it has been shown that membrane protein production yields can also be improved in *E. coli* strains lacking the *tig* gene, which encodes the cytoplasmic chaperone TF [67]. It has been suggested that TF can compete with SRP binding and therefore hamper the targeting of produced membrane proteins. Notably, combining the *tig* deletion and the co-production of YidC (see Section 4.1.3.) can have a positive additive effect on membrane protein production yields [67].

In Section 4.1.3., the positive effects of the co-production of components involved in membrane protein biogenesis were discussed. However, their co-production can also have a negative effect on membrane protein production yields. It has been shown that co-production of SRP can have a negative effect on membrane protein production yields [67]. This was not expected in the light of the observations that membrane protein production titrates out SRP, and that co-production of SRP can improve the production of secretory heterologous proteins equipped with an SRP-dependent signal sequence [70,71]. It has been suggested that the different effects of the co-production of SRP on production yields is somehow linked to the time these different proteins occupy the Sec-translocon [67].

Taken together, different strategies have been used to create *E. coli* strains with improved membrane protein production characteristics. Unfortunately, the different strain backgrounds, expression plasmids, membrane protein targets, culture and production regimes, and set-ups to monitor membrane production yields used make it impossible to compare all the studies described in this section. Anyways, it is very likely that what we have seen so far is just the beginning of the creation of *E. coli* strains with improved membrane protein production characteristics.

4.2. Producing membrane proteins using E. coli based, cell-free systems

Cell-free protein production systems have been developed to circumvent the toxic effects of protein production on the production host and to label proteins for NMR and crystallographic studies [72]. Since cell-free systems are 'open systems', ongoing protein synthesis reactions can be easily manipulated, which has made it possible not only to improve production yields considerably, but also to greatly facilitate the labeling of proteins. So far, wheat germ- and *E. coli*-based cell-free systems have been used to produce membrane proteins [72]. The number of membrane proteins produced in cell-free systems that have been used for NMR and X-ray studies is growing steadily (see table 1 in [72]).

E. coli-based cell free systems can be based on extracts and purified components [73,74]. Membrane proteins from both pro- and eukaryotic origin have been produced in *E. coli* based cell-free systems [72]. They have been produced as precipitates that were solubilized afterwards using detergents. They also have been produced in a soluble form in the presence of scaffolds like detergents, lipids, and nanolipoprotein particles. Surprisingly, membrane proteins can assemble properly in the presence of a scaffold in the absence of many of the factors that assist their biogenesis into the membrane *in vivo*. Compared to the *in vivo* situation, membrane protein biogenesis in cell-free based system is a very slow process. This may make it possible for membrane proteins to fold properly in a more or less unassisted fashion. Notably, monitoring the production of membrane proteins using cell-free based systems is greatly facilitated by using membrane protein GFP-fusions (see *e.g.*, [75] and Section 3).

For quite a number of membrane proteins produced in *E. coli* based cell-free systems it has been shown that they are fully functional [74]. Recently, membrane protein complexes, including one containing cofactors, have been produced using *E. coli* cell free systems (see *e.g.*, [76,77]).

Taken together, *E. coli*-based cell free systems have become an important platform for the production of membrane proteins.

5. Bacteria other than *E. coli* as membrane protein production hosts

A priori there is no reason why *E. coli* should be more suitable for the heterologous production of membrane proteins than any other bacterium. In contrast, there may be good arguments why other bacteria could be more suitable for the production of certain membrane proteins. *E.g.*, bacteria with slower translation rates than *E. coli* may be able to deal

better with 'difficult folders' (see Section 2). Similarly, bacteria which have different (extended) repertoires of chaperones than *E. coli*, like Gram-positive bacteria which express two copies of the integral membrane chaperone YidC (*e.g.*, *Bacillus subtilis*/*L. lactis* [78–81]), may perform better at insertion and assembly of heterologous membrane proteins. Also, differences in the lipid composition of different bacterial membranes may affect the insertion and assembly of heterologous membrane proteins. Therefore, instead of optimizing *E. coli* for the production of 'difficult' membrane proteins, a viable alternative strategy is to look for different bacterial production hosts that have distinct properties and may perform better for the protein of interest. The Gram-positive bacteria *L. lactis* and *B. subtilis* have both been used for the production of membrane proteins.

5.1. *L. lactis* as a membrane protein production host

One promising bacterial host for the production of membrane proteins is *L. lactis*. It is a Gram-positive lactic acid bacterium, which is used in the dairy industry. Because of the industrial interest in the organism, its physiology has been studied in great detail. The organism is genetically accessible and a variety of expression plasmids; both high and low copy number plasmids and inducible promoters are available [82]. The widely used nisin-A-based expression system has been used for the production of membrane proteins in *L. lactis* [83,84]. There are several notable cases in which functional production of eukaryotic as well as bacterial membrane proteins (*e.g.*, the human KDEL receptor and the Na^+/tyrosine transporter (Tyt1) of *Fusobacterium nucleatum*) could be achieved in *L. lactis*, but not in *E. coli* [83–87]. Among the potential advantages of *L. lactis* are a slower growth rate than *E. coli* (~1 doubling h^{-1} *vs.* ~1 doubling 0.5 h^{-1} for *E. coli*), which could be beneficial for the production of proteins that do not fold easily; the presence of a single membrane only, which facilitates functional characterization; and the lack of excessive proteolytic activity, which may help to prevent breakdown of produced proteins. Just like in *E. coli*, co-production of multiple proteins from different plasmids is possible in *L. lactis* and can be used for chaperone production alongside the membrane protein of interest [88]. Two recent studies have shown that *L. lactis* also can be used for efficient incorporation of amino acid derivatives in produced proteins [89,90]. This makes the organism a more complete alternative to *E. coli* for production of proteins for X-ray crystallography, where Seleno-methionine incorporation is routinely used for phase determination.

Similar to *E. coli*, the consequences of membrane protein production in *L. lactis* have been studied and used as a starting point for strain engineering [91–93]. Membrane protein production in *L. lactis* leads to heat-shock and cell envelope stress, and impairs growth. The cell envelope stress response, controlled by the two-component regulatory CesSR system, seems to play a key role in the adaption of *L. lactis* to the toxic effects of membrane protein production. In keeping with these observations, the capability of *L. lactis* to produce a membrane protein was severely hampered when the genes encoding the CesSR system itself or genes encoding particular members of the CesSR regulon were knocked out [93]. On the other hand, overexpressing *cesSR* reduced the growth defect, thereby improving membrane protein production yields [93]. Interestingly, genes encoding homologs of *E. coli* FtsH (involved in quality control and degradation of membrane proteins) and YidC (involved in the insertion and folding of membrane proteins) are part of the CesSR regulon. In contrast to *E. coli,* there are no indications that in *L. lactis* – at least under the conditions used – the Sec-translocon capacity is the main bottleneck hampering the production of membrane proteins. Thus, the bottlenecks hampering the production of membrane proteins in *E. coli* and *L. lactis* seem to be – at least partially – different.

Finally, *L. lactis* strains with improved membrane protein production characteristics have been isolated using a screening approach [41]. By fusing GFP and an erythromycin resistance marker in tandem to the C-terminus of a target protein, it is possible to simultaneously select for strains with enhanced membrane protein production characteristics (increased erythromycin resistance) and insertion in the membrane (GFP fluorescence) (see Section 3). All characterized *L. lactis* strains that were isolated in this screen carried single-site mutations in the *nisK* gene. NisK is the sensor protein of a two-component regulatory system that directs nisin-A-mediated expression of the genes encoding the target proteins. The mutations seem to lead to higher expression levels of the genes encoding the membrane proteins of interest, thereby improving production yields. All this is in keeping with the Sec-translocon capacity not being a factor limiting the production of membrane proteins in *L. lactis* under the condition used.

5.2. *B. subtilis* as a membrane protein production host

The Gram-positive bacterium *B. subtilis* is widely used in industry for the production of secretory proteins [79]. The physiology of *B. subtilis* has been studied in great detail, it is genetically very well accessible and a variety of expression systems is available. Recently, *B. subtilis* was also used to produce membrane proteins [94]. It was found that membrane-associated stress-responsive systems set major limits to the production of membrane proteins in *B. subtilis.* It was shown that the removal of these systems can significantly improve the membrane protein production yields. However, it is not known if these improved yields also represent properly folded material. The removal of membrane-associated stress-responsive systems can negatively affect the quality control of produced membrane proteins and as a consequence the degradation of misfolded material.

Besides *E. coli*, *L. lactis* and *B. subtilis* other bacteria may also be good production hosts for certain membrane proteins. An important question is: which organism to choose for the protein of interest? There is no general answer to this question, because it is impossible to predict how heterologously produced proteins will behave in different hosts. However, when producing prokaryotic proteins it is likely best to choose a host that is as closely related to the natural host of the protein to be produced, so as to mimic homologous expression as closely as possible. For example, when the *E. coli* and *L. lactis* were compared for the production of membrane proteins from *Salmonella Typhimurium*, *E. coli* – not surprisingly – performed much better than *L. lactis*, because *E. coli* is a member of the closest known genus to *Salmonella* [95,96].

6. Engineering and isolating membrane protein variants with improved production characteristics

Rather than engineering or changing the production host an alternative strategy to improve membrane protein production yields in bacteria, is to engineer the membrane protein of interest. It has been known for a long time that modifying N- and C-termini can improve production yields significantly. Recently, also mutagenesis approaches have been used to isolate genes encoding membrane protein variants that are produced at higher yields than the wild-type protein.

6.1. Modifying N- and C-termini

Many pro- and eukaryotic membrane proteins have N-terminal tails that have to be translocated across the membrane [97–99]. Translocation of an N-terminal tail depends on the ability of the N-terminus to remain in a translocation competent conformation, the number of positively charged residues in the tail region, and the 'strength' of the first transmembrane segment, *i.e.*, the charge difference, the length, and the overall hydrophobicity of the reverse-signal anchor [85]. The inability to efficiently translocate the N-terminal tail of a membrane protein may severely hamper its production. Indeed, the functional production of the yeast mitochondrial carrier AAC2 (ADP/ATP exchanger) can be increased in *L. lactis* if the N-terminus is shortened, or the N-terminal tail is swapped with a shorter one taken from the isoform AAC3 [85]. Likewise, functional production of *e.g.*, the rat neurotension receptor (NTR), a GPCR, could be

improved in *E. coli* by fusing the maltose binding protein (MBP) with its secretory signal sequence to its N-terminus [100]. An SRP-dependent variant of M13 pro-coat protein (P8CBD) has also been used as an N-terminal fusion partner that promotes the efficient production of membrane proteins in *E. coli* [61]. Practical features like a flag-tag (for protein detection) and a chitine binding domain (for protein purification) were incorporated into the P8CBD fusion partner. The N-terminal addition of Mistic, a small protein that is unique to *B. subtilis*, has been reported to improve the production of various pro- and eukaryotic membrane proteins in *E. coli* and *L. lactis* [101–106]. The mechanism by which Mistic improves membrane protein production yields is not clear.

The ability of various additions and combinations of C-terminal tags (*e.g.*, biotinylation-, poly histidine-, flag- and strep-tags), or single additions of either a polyhistidine/c-Myc epitope or *E. coli* thioredoxin (aa 2–109) fusion, to improve the production of the aforementioned MBP-NTR fusion was tested [100]. The most significant improvement of production was obtained with thioredoxin, which was attributed to the remarkable stability of the globular protein. The combined use of an N-terminal MBP and a His-tagged C-terminal thioredoxin fusion, has also been successfully employed for the production of other GPCRs in *E. coli* [107–109]. To remove N- and C-terminal fusion-partners from target membrane proteins site-specific proteases can be used (see *e.g.*, [43,44,61,109]).

Can fusion tags somehow affect the quality of produced membrane protein material? It has been shown that in *E. coli* not properly folded membrane proteins that are *bona fide* FtsH substrates can be stabilized when they are C-terminally fused to a properly folded periplasmic PhoA moiety [110]. PhoA has two intramolecular disulfide bridges, which are critical for its stability, and disulfide bond formation in the periplasm is mediated by the disulfide bond formation (Dsb) system [111]. However, in a strain background lacking the Dsb system PhoA cannot fold properly and as a consequence the membrane protein PhoA fusions are readily degraded by FtsH. Thus, properly folded PhoA makes that the processive protease FtsH cannot pull the membrane protein PhoA fusions out of the membrane [22]. Therefore, when using stable fusion tags to enhance membrane protein production yields it should be kept in mind that they may promote the accumulation of not properly folded material.

6.2. Mutagenesis

Production levels of membrane proteins in bacteria can be improved not only by modifying N- and C-termini, but also by random and directed mutagenesis of the genes encoding membrane proteins can be used as a starting point to isolate variants that have improved production characteristics. Recently, random mutant libraries of genes encoding for nine membrane proteins were screened for production in *E. coli* using the CoFi blotting method [38] (see Section 3). For five out of the nine proteins tested, one cycle of random mutagenesis of the genes encoding these proteins resulted in significant improvements of yields of detergent solubilized membrane protein. Among the five proteins was the human microsomal glutathione S-transferase 2. Notably, the mutations that improved production of this variant of the protein did not interfere with its activity. It should be noted that it is not known how the various mutations contribute to the higher membrane protein production yields.

A gene mutant library approach was also used to isolate NTR variants from rat with improved production characteristics in *E. coli* [53]. The gene encoding NTR was randomly mutagenized and the NTR derivatives were produced as an N-terminal MBP and C-terminal thioredoxin fusions (see Section 6.1.). NTR accumulation levels were monitored directly using a fluorescently labeled ligand that only properly folded protein can bind. Cells producing the largest number of functional receptors exhibited the greatest fluorescence and were isolated using FACS. This way a GPCR variant, DO3, that shows much higher production yields in *E. coli* but is still fully functional was isolated. The beauty of this approach is that the fluorescently labeled ligand allows screening for variants that not only are better produced, but also are still properly folded in a single experiment. For DO3 it has been shown that the protein is also more stable when solubilized and purified than wild-type NTR. Using *E. coli* as a production platform, fluorescently labeled ligands and extensive gene mutant libraries, the production yields and detergent stability of DO3 and also other GPCRs have been further improved [112–114].

Recently, it was shown that single *E. coli* cells of a library, each producing a different GPCR variant, can be encapsulated to form detergent-resistant, semipermeable nano-containers or capsules, which can be used for the identification of well produced and detergent stable variants [115,116]. This method is called CHESS, which stands for cellular high-throughput encapsulation, solubilization and screening (Fig. 3). Importantly, in contrast to the *E. coli* cells the capsules used in the CHESS method are not dissolved by detergents. This makes it possible to solubilize the GPCRs in the capsules while maintaining an association with the genetic information encoding the protein. The pore size of the capsules can be controlled such that fluorescently labeled ligands can go in the container but the solubilized receptor cannot go out. Fluorescently labeled ligands are used to bind to those GPCR variants inside the nano-containers that remain active in detergent. With the use of FACS the capsules containing genes encoding well-produced and detergent-stable GPCR variants can be isolated based on their fluorescence.

Finally, using *E. coli* as a production platform GPCR variants with improved thermostability has been isolated using Ala/Leu-scanning and some of the created variants have been subsequently crystallized (see *e.g.*, [117–120]).

A prerequisite for using the above described mutagenesis approaches is that a relatively easy folding/activity assay is available. It also should be noted that even though the by mutagenesis created variants are functional, there are concerns if these variants really represent the wild-type protein.

7. Future perspectives

In recent years, considerable progress has been made with the development of bacterial-based systems for the production of both pro- and eukaryotic membrane proteins. However, there appears to be ample room for improvements.

For soluble proteins it has been shown that chaperones with increased folding efficiency towards specific target proteins can be created [121]. Thus, it should also be possible to optimize bacterial components involved in membrane protein biogenesis for the production of (heterologous) membrane proteins. Some membrane proteins require membrane-protein-specific chaperones for their biogenesis. To improve the heterologous production yields of these membrane proteins, co-production of these specific chaperones may be used.

It has been shown that production yields of soluble eukaryotic proteins can be enhanced considerably in an *E. coli* strain carrying mutations in the ribosome that slow down the rate of translation [122]. It would be interesting to study the effect of these ribosomal mutations on (heterologous) membrane protein production yields. Using a very limited test set it has been shown that lowering the growth rate and acetate production of *E. coli* can enhance membrane protein production yields [123]. It would be very interesting to extend this study by testing the production of more (heterologous) membrane proteins and monitor the functionality of the produced material.

The lipid composition of the membrane can be important for proper folding, stability and function of produced membrane proteins (see *e.g.*, [124]). Our rapidly growing knowledge of the biosynthesis of lipids/membranes may make it possible to also engineer bacterial strains with a lipid composition that is better suited for the production of specific membrane proteins and create more 'membrane space' to accommodate produced membrane proteins. Recently, in the yeast *Saccharomyces cerevisiae* the composition of the membrane has been

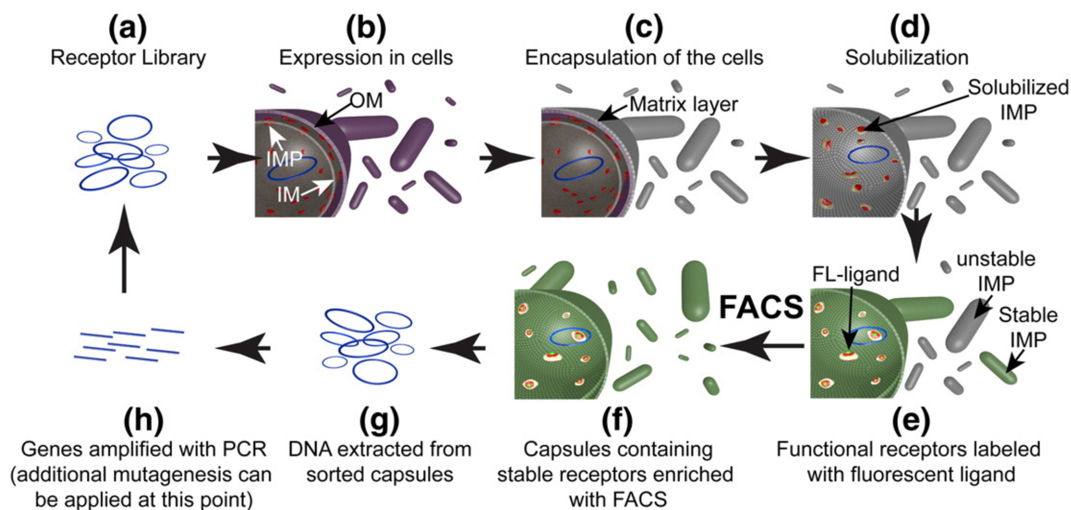


Fig. 3. The CHESS method. A library of genes encoding receptor mutants (a) is transformed and produced in the inner membrane of *E. coli* (b). Subsequently, cells are encapsulated (c) and the cell envelope is permeabilized/solubilized with detergent (d), leading to a solubilization of the receptor. The encapsulation layer serves as a semipermeable barrier, retaining the solubilized receptor and its genetic information within the capsule but allowing fluorescently labeled ligand into the capsule, where it can bind to functional receptors (e). Capsules containing detergent-stable receptor mutants are more fluorescent and can be sorted from the population with FACS (f). Genetic material is recovered from the sorted capsules (g) and used to either identify desired receptor mutants or serve as a template for further rounds of mutation or selection (h). Picture was taken from [115].

engineered such that it is better suited for the production of GPCRs [125]. The cholesterol biosynthetic pathway of mammalian cells was reconstituted in this yeast enabling it to make cholesterol-like sterols, which increases the capacity to make GPCRs in yeast.

Most eukaryotic membrane proteins are glycosylated when inserted into the endoplasmic reticulum (ER) membrane, and for a considerable number of them this is essential for proper folding, stability and also function [31]. The bacteria used thus far to produce membrane proteins are not able to glycosylate them at all. However, efforts are being made to engineer bacteria that can glycosylate proteins in a way resembling the kinds of glycosylation that occur in eukaryotic systems [126]. For the yeast *Pichia pastoris* it has been shown that it is possible to engineer strains that can glycosylate proteins similar to humans [127].

Cell-based membrane protein production is usually done in batch cultures. Recently, a fed-batch (*i.e.*, high cell density) cultivation set-up was used for the efficient *E. coli*-based production of a GPCR for NMR experiments [128]. It has been shown that for *L. lactis* modifying the composition of the culture medium such that key nutrients are not limiting can also help to improve membrane protein production yields significantly [129]. Thus, taking a more 'industrial' approach to culturing cells used for the production of membrane proteins may be a simple but very effective manner to improve production yields.

E. coli cell-free based systems have been successfully used to produce membrane proteins. It has now been shown that these systems can be scaled up and they are becoming less costly [130]. These developments will most likely help to further strengthen the position of these systems for the production of membrane proteins.

Combining different technologies and methodologies can have an additive effect on bacterial-based membrane protein production yields as *e.g.*, nicely shown by the CHESS method. Combining different strain and protein engineering approaches is an obvious thing to try next.

It is beyond any doubt that further developing bacterial-based membrane protein production platforms will greatly stimulate functional and structural studies of this important class of proteins.

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