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Functional overexpression of membrane proteins in *E. coli*: the good, the bad and the ugly

Margot Di Cesare¹, Aissatou Maty Diagne¹, Benjamin Bourgey¹, Jean-Michel Jault^{1,*} and Cédric Orelle^{1,*}

¹Molecular Microbiology and Structural Biochemistry, UMR 5086 CNRS/University of Lyon, 7 passage du Vercors, 69367 Lyon

*To whom correspondence should be addressed: cedric.orelle@ibcp.fr; jean-michel.jault@ibcp.fr

Abstract

Overexpression of properly folded membrane proteins is a mandatory step for their functional and structural characterization. One of the most used expression systems for the production of proteins is *Escherichia coli*. Many advantageous strains combined with T7 expression systems have been developed over the years. Recently, we showed that the choice of the strain is critical for the functionality of membrane proteins, even when the proteins are successfully incorporated in the membrane (Mathieu *et al.*, Sci Rep. 2019; 9(1):2654). Notably, the amount and/or activity of the T7-RNA polymerase, which drives the transcription of the genes of interest, may indirectly affect the folding and functionality of overexpressed membrane proteins. Moreover, we reported a general trend in which mild detergents mainly extract the population of active membrane proteins, whereas a harsher detergent like Fos-choline 12 could solubilize them irrespectively of their functionality. Based on these observations, we provide some guidelines to optimize the quality of membrane proteins overexpressed in *E. coli*.

Keywords

Membrane proteins, overexpression, *E. coli*, detergent, solubilisation, ABC transporter, T7

Running head

Functional overexpression of membrane proteins in *E. coli*

1 Introduction

Membrane proteins represent between 20 and 30% of synthesized proteins in all organisms [1]. They play essential roles and are targeted by more than half of therapeutic drugs. Because native membrane proteins are generally insufficiently abundant to conduct biochemical and structural studies, they are often overexpressed in heterologous systems. The bacterium *Escherichia coli* is the most widely used organism for the overexpression of both soluble and membrane proteins [2,3]. This is due to multiple reasons: (i) development of effective genetic tools, including strains and plasmids, allowing high levels of overexpression in favorable cases; (ii) easy DNA transformation, fast growth and high cell density cultures; (iii) inexpensive culture costs. The *E. coli* BL21(DE3) strain in combination with T7 promoter-based plasmids have been extensively employed to overexpress proteins. In this system, the T7 RNA polymerase gene is inserted in the DE3 prophage of the chromosome under the control of the IPTG-inducible L8-UV5 *lac* promoter, which is a more powerful variant of the wild-type *lac* promoter. The BL21(DE3) strain is also deficient in Lon and OmpT proteases, and the T7 RNA polymerase transcribes ~ 8 times faster than native *E. coli* RNA polymerases [4] to generate high level of mRNAs available for protein synthesis. However, this strategy is not the most appropriate for many proteins, especially those that are toxic. The overexpression of membrane proteins may have detrimental effects due to their intrinsic function, improper folding or by exceeding the capabilities of the machineries involved in membrane protein biogenesis and protein secretion [5-7]. More than twenty years ago, Miroux and Walker isolated BL21(DE3) mutant strains that displayed improved membrane protein overexpression abilities [8]. These strains were named C41(DE3) and C43(DE3) and are now widely used to overexpress membrane proteins, although they do not improve yields for all tested proteins [9,10]. Much later, it was discovered that the C41(DE3) and C43(DE3) strains contained mutations in the *lacUV5* promoter [11]. A reduced transcription rate in these derivative strains likely explains why overexpression of many membrane proteins is less toxic for their growth and results in substantially improved membrane

protein overexpression yields. Wagner and colleagues hypothesized that high transcription levels of membrane proteins is counterproductive because it leads to the saturation of the Sec translocon [11]. As a result, most overexpressed and endogenous membrane proteins fail to insert into the membrane and aggregate, resulting in deleterious effects for the cell. Based on these observations, this team successfully engineered a BL21(DE3) variant strain named Lemo21(DE3), in which the activity of the T7 RNA polymerase can be finely tuned by its natural inhibitor T7 lysozyme, whose gene is under the control of the rhamnose promoter [12,11]. The optimization of membrane protein synthesis and their insertion into the membrane could thus be tuned to strongly minimize the toxic effects associated with membrane protein overexpression, resulting in higher bacterial biomass and protein yield. While misfolded proteins typically aggregate and form inclusions bodies, overexpressing conditions are typically optimized based on the amount of proteins associated with the membrane fraction. A popular strategy to screen experimental conditions and assess the quality of membrane protein expression is to fuse GFP to the C-terminal end of membrane proteins, based on the premise that only properly folded fusion proteins would display GFP fluorescence [13,14]. According to our recent work [15], we advocate that functionality of membrane proteins drastically depends on the *E. coli* expressing strain and cautions against choosing an expression strain based solely on expression levels in membranes. In particular, we showed that several ABC transporters can be expressed and addressed to the membrane equally well in different *E. coli* strains, but they are substantially more functional and easier to solubilize by mild detergents when overexpressed in the appropriate strain [15]. Hence, we observed a general trend in which mild detergents mainly extract the population of active transporters, whereas a harsher detergent like Fos-choline 12 could solubilize transporters irrespective of their functionality. Similar detergent observations were recently described for membrane proteins expressed in eukaryotic systems [16]. Hereafter we provide methodological guidelines and advices to optimize the quality of overexpressed membrane proteins in *E. coli*.

2 Materials

2.1 Growth Media

All media must be autoclaved at 121 °C for 20 min.

1. LB medium: 10 g/L Tryptone, 5 g/L Yeast Extract, and 5 g/L NaCl (Lennox Broth recipe)
2. 2xYT medium: 16 g/L Pancreatic Digest of Casein, 10 g/L Yeast Extract, and 5 g/L NaCl
3. TB medium: 24 g/L Yeast Extract, 12 g/L Tryptone, 2.2 g/L KH_2PO_4 and 9.4 g/L K_2HPO_4 , 4 mL/L glycerol (to be added after autoclaving).
4. LB-agar: LB medium + 1.5% agar

2.2 Chemicals, reagents and equipment

1. Isopropyl- β -D-thiogalactoside (IPTG) stock solution: 1 M in water, filtered at 0.22 μm and stored at -20 °C
2. L-Rhamnose monohydrate (Rhamnose) stock solution: 0.8 M in water, filtered at 0.22 μm and stored at 4 °C
3. 1000X antibiotic stock solutions: 100 mg/mL ampicillin and 50 mg/mL kanamycin in water, 25 mg/mL chloramphenicol in pure ethanol. The antibiotic stock solutions are stored at -20 °C
4. 5X Laemmli buffer: 312 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 3.5 M beta-mercaptoethanol, 0.01% bromophenol blue
5. 10% N-Dodecyl- β -D-maltoside (DDM) in water and stored at -20 °C
6. 10% lauryl maltose neopentyl glycol (LMNG) in water and stored at -20 °C

7. 10% Fos-Choline 12 (FC12) in water and stored at -20 °C
8. ATP stock solution: 400 mM in water. Adjust the pH around 7.2 by successive additions of NaOH (stock at 2 M) and check the pH with pH papers. Calculate the final ATP concentration by measuring the absorbance of 10 000X dilution triplicates at 259 nm ($\epsilon = 15\,400\text{ M}^{-1}\text{cm}^{-1}$ at 259 nm)
9. Centrifuge
10. Ultracentrifuge
11. Cell disruptor: Microfluidizer, French Press or Sonicator
12. Fluorimeter (e.g. Photon Technology International Quanta Master I fluorimeter)

2.3 Buffers used for the multidrug ABC transporter BmrA (see Note 1).

1. **Lysis buffer:** 50 mM Tris-HCl pH 8, 5 mM MgCl₂, 1 mM DTT and 5 µg/mL DNase I (optional: antiproteases)
2. **Resuspension Buffer:** 50 mM Tris-HCl pH 8, 1.5 mM EDTA
3. **Membrane storage buffer:** 50 mM Tris-HCl pH 8, 1 mM EDTA, 300 mM Sucrose
4. **Transport buffer:** 50 mM HEPES-KOH pH 8, 8.5 mM NaCl, 4 mM phosphoenolpyruvate, 60 µg/mL pyruvate kinase, 3 mM MgCl₂
5. **Solubilization buffer:** 50 mM Tris-HCl pH 8, 10% glycerol, 100 mM NaCl, 1 mM DTT, 1% detergent

3 Methods

3.1 Expression vectors

1. The gene encoding the protein of interest must be cloned into an expression vector compatible with the bacterial host. In this chapter, we will focus on *E. coli*, which remains the most popular host [3], although *L. lactis* [17] and *B. subtilis* [18] can be alternative choices.
2. In *E. coli* expression vectors, different promoters with various strength and properties are available, such as the commonly used *lacUV5*, *tac*, *trc*, pBAD and T7 promoters [2,19]. Due to its strength [20], the pTac system (hybrid of *Trp* and *Lac* promoters) is for instance more appropriate for the production of nontoxic proteins. The pBAD systems, which rely on catabolite repression and positive induction, are tightly regulated by arabinose. The T7 systems have the ability to produce higher levels of expression than pBAD systems, but they are prone to leaky expression [21].
3. Finally, the presence of an encoded affinity tag in the vector should also be considered [2]. The choice of the tag and its position (N- or C-terminal of the produced protein) can be important for downhill applications.

3.2 *E. coli* strains and induction conditions

Finding the appropriate bacterial strain and growth conditions are essential steps to achieve the optimal production of membrane proteins. For that purpose, we recommend optimizing the production by analyzing multiple expression strains and induction conditions.

3.2.1 Selection of *E. coli* strains

We present here below a selection of useful strains for protein overexpression in *E. coli*. Recent advances in the development and characterization of a number of T7-based strains offer a variety of opportunities to facilitate the functional overexpression of membrane proteins.

1. **BL21(DE3)**: BL21(DE3) is an early-developed *E. coli* strain suitable for the overexpression of proteins [22]. It contains the λ DE3 lysogen prophage that carries the gene encoding T7 RNA polymerase, under the control of a *lacUV5* promoter for specific IPTG induction. Thanks to 3 mutations located in the -10 promoter sequence and inside the CAP/cAMP binding site, the *lacUV5* promoter is a strong variant of the lac promoter and is insensitive to catabolic repression. Moreover, this strain is deficient in Lon protease located in the cytoplasm and OmpT protease located in the outer membrane, reducing degradation of heterologously expressed proteins.
2. **C41(DE3)**: C41(DE3) is a famous strain derived from BL21(DE3) [8]. In 1996, Miroux and Walker selected this mutant strain for improved expression of toxic membrane proteins. This strain was isolated on plates containing IPTG and showed mutations in the *lacUV5* promoter: two in the -10 promoter region of T7 RNA polymerase and one in the lac operator [11]. These mutations reduced the promoter strength. As a result, smaller amounts of membrane proteins are produced, thereby preventing saturation of the Sec translocon and improving their insertion and proper folding in the membranes.

3. **C43(DE3)**: C43(DE3) is derived from C41(DE3) [8]. This strain has an additional mutation in the *lacI* gene of the DE3 region that may weaken the IPTG inducer affinity for the LacI repressor and therefore reduces the transcription initiation of the *lacUV5* promoter [23]. C43(DE3) can better express some membrane proteins that remain too toxic for C41(DE3), such as the *E. coli* F-type ATPase subunit b [8].
4. **C44(DE3) et C45(DE3)**: C44(DE3) and C45(DE3) are recent strains also derived from the BL21(DE3) [24]. They have stop codons in the T7 RNA polymerase gene (either located before the B catalytic domains of the polymerase for C45(DE3) or right after the B catalytic domain for C44(DE3)). However, as the BL21(DE3) strain exhibits a weak suppressive stop codon activity, the strains are still able to express sufficient but lower amounts of T7 RNA polymerase. As a result, less proteins will be produced but they may be better inserted and folded into the membranes.
5. **C41(DE3) Δ ompF Δ acrAB**: C41(DE3) Δ ompF Δ acrAB is derived from C41(DE3) [25]. This strain is deleted of the genes encoding OmpF and AcrB proteins, which are major membrane protein contaminants during purification [26]. Surprisingly, these gene deletions altered the phospholipid composition of the membrane and its lipid to protein ratio. Consequently, this strain showed improved expression for a number of membrane proteins.
6. **Lemo21(DE3)**: Lemo21(DE3) is derived from BL21(DE3) [11]. This strain contains an additional plasmid called pLemo that expresses, under the control of a rhamnose-dependent promoter, the T7 Lysozyme. Because the T7 lysozyme is a natural inhibitor of T7 RNA polymerase, one can finely tune the expression of the protein of interest by varying the rhamnose concentration. Lemo21(DE3) is thus tunable for membrane protein overexpression and may allow optimizing expression by using a single strain rather than a multitude of different strains.
7. **Other BL21 derivatives** : Other BL21 derivatives have been developed with specific advantages. For instance, BL21-AI [27] and BL21-SI [28] are strains in which the expression of the T7 RNA polymerase is induced and tuned by arabinose or salts, respectively.

3.2.2 Combination with useful plasmids

Most of the aforementioned T7-based strains can be combined with useful plasmids. For instance, the Rosetta strain is a BL21(DE3) strain that contains a plasmid encoding rare tRNA codons to improve the expression of eukaryotic proteins. The pLysS or pLysE plasmids can reduce leaky expression, which is advantageous for toxic proteins. Another example is the CyDisCo system, which encoded a sulfhydryl oxidase and a disulfide bond isomerase that facilitate the formation of disulfide bonds in recombinant proteins [29].

3.2.3 Induction conditions

In order to optimize the expression of the target membrane protein, multiple conditions can be tested experimentally. The most important ones are presented below.

1. Culture broth: A wide range of media can be tested. Here we suggest the use of three typical media used for membrane protein production: LB, 2xYT and TB.
2. Temperatures: Before induction, bacteria are commonly grown at 37 °C. After induction, temperature has been shown to play a significant role in expression level and protein folding. For better membrane protein folding, low post-induction temperatures between 18 °C and 25 °C are typically used.
3. IPTG Induction: The concentration used for induction does not fine-tune levels of expression, and it is common to use IPTG around 1 mM. However, if low expression levels are observed,

different concentrations might be tried. In some cases, omitting the IPTG gave the best expression results [30].

4. Cell Density: Exponential growth phase (between 0.4 to 0.8 OD_{600nm}) is commonly used to start induction. OD_{600nm} at 0.6 is often chosen. It is also possible to induce at late OD (>1) or even stationary phase (>2) to improve the yield of toxic proteins.
5. Induction Time: It should be determined by analyzing expression of the proteins by SDS-PAGE. Induction time can vary between few hours to overnight.
6. When using Lemo21(DE3) cells, a range of rhamnose concentrations must be tested to find the best expression conditions (e.g. 0, 100, 250, 500, 750, 1000, 2000 μM)

3.3 Overexpression of membrane proteins: general guidelines

It is essential to work in sterile conditions during the overexpression.

1. **Transformation.** We strongly recommend to perform fresh transformations for each overexpression. In our experience, we often lost expression when starting from glycerol stocks saved at -80 °C. Transformation of chemically-competent cells (calcium chloride) using heat shock protocol is a convenient method, but other standard methodologies can be used (electroporation etc.). Cells need to be plated on LB-agar supplemented with the antibiotic used for selection. *Importantly, if the Lemo21(DE3) cells are used, further supplement the LB-agar plates with 25 μg/mL chloramphenicol for pLemo selection and 2 mM rhamnose for promoter repression.* Incubate the plates overnight at 37 °C.
2. **Preculture and culture.**

(i) The safest way to proceed is to avoid a preculture step. Thus, inoculate a single or several colonies into fresh medium supplemented with antibiotic(s) (see **Note 2**). The culture can be incubated at 37 °C with shaking at 180 rpm.

(ii) Alternatively, if it works, a preculture step can be employed to facilitate the timescale management of the cultures. A single fresh colony is then taken from the agar plate and inoculated in LB medium supplemented with antibiotic(s) for an overnight preculture at 37 °C with shaking. *If using Lemo21(DE3) cells, further supplement the preculture with 25 µg/mL chloramphenicol for pLemo selection and 2 mM rhamnose for promoter repression.* The next morning, dilute the preculture into the desired broth (LB, 2YT, TB or another medium) supplemented with antibiotic(s) at a low OD_{600nm} (usually between 0.05-0.1) and incubate at 37 °C with shaking at 180 rpm.

3. **Induction.** When the OD_{600nm} reaches the desired phase (e.g. ~0.6), proceed with the induction step by adding IPTG (e.g. 1 mM) and by switching to the chosen temperature for the desired incubation time.
4. **Induction analysis.** Harvest 1 mL aliquot of the cultures before and after IPTG addition for protein expression analysis by SDS-PAGE and/or Western Blot (see **Note 3**) by centrifugation at 10,000 x g for 1 min at 4 °C. Discard supernatants. Bacteria pellets are either frozen at -80 °C or used directly. Resuspend the pellets in Laemmli buffer 1X (~10 µL per 0.1 OD_{600nm}). Run a SDS-PAGE with the equivalent of 0.1 OD_{600nm} of resuspended bacteria.
5. **Collecting the cells.** Pellet the cells at 5 000 g for 20 min at 4 °C. Discard the supernatant. Pellets can be frozen at -80 °C for subsequent use.

3.4 Preparation of the membrane fraction

Here we present a mechanical cell disruption based on high pressure lysis using a microfluidizer processor, but other methods are possible (French Press, sonication etc.). Then regular differential centrifugation is performed in order to isolate the membrane fraction.

1. Bacterial pellets are resuspended in the desired lysis buffer (e.g. 30 mL per L of culture). Lyse the cells by three successive passages through Microfluidizer™ at 18 000 psi (see **Note 4**).
2. Remove unbroken cells, cellular debris and insoluble proteins by centrifuging at 15 000 x g for 30 min at 4 °C. Keep the supernatant
3. Harvest membrane vesicles by an ultracentrifugation at 150 000 x g for 1 h (or possibly 200 000 x g for 30 min).
4. Discard the supernatant and resuspend membrane pellets in the desired resuspension buffer (at least 10 mL per L of culture). A potter pestle can be used to resuspend the membranes. Ultracentrifuge again at 150 000 x g for 1 h.
5. Resuspend and homogenize the membranes with a potter in the storage buffer (~2 mL per L of culture).
6. Flash-freeze membrane aliquots in liquid nitrogen and store them at -80 °C.

7. Assess membrane vesicles concentration using a BCA assay and check overexpression by SDS-PAGE gel (load 20 μg of total membrane proteins).
8. If necessary, a western blot analysis can be performed to check the overexpression of the protein of interest. In this case, a lower amount of proteins is usually loaded onto the gel (e.g. 0.5-5 μg).

3.5 Quality assessment of membrane protein expression

Although it is tempting to select the experimental conditions (bacterial strain, induction conditions) that produce the best expression levels in the membrane fraction, we strongly advise caution at this step. For instance, while the multidrug ABC transporter PatA/PatB from *S. pneumoniae* [31] could be overexpressed equally well in the membrane of different *E. coli* strains, the transporter was >3 times more active in the C41(DE3) strain as compared to other strains, and this difference was even more drastic in other induction conditions [15]. Similar conclusions were reached with the multidrug ABC transporter BmrA from *Bacillus subtilis* [32], since most of the transporters overexpressed in the membrane of some strains were inactive [15]. In other words, a good expression in the membrane can be associated with bad or even ugly functionality of membrane proteins. Here below we present three different methodologies to evaluate the functionality of membrane proteins. If you must choose a strain and induction conditions, choose wisely!

3.5.1 GFP-fusion based systems

Methods employing GFP fusion have been very useful to assess the overexpression and quality of membrane proteins [13,33,34]. This strategy presumes that proper folding and fluorescence of GFP

fused to the C-terminus of a target protein depends on the appropriate folding of the latter. When the fusion protein is present in inclusion bodies, the GFP does not fold properly and thus does not fluoresce. Although we observed a substantial amount of GFP fluorescence associated with poorly functional membrane proteins [15], this methodology remains valuable for high-throughput screening of expression conditions.

3.5.2 Functional characterization of membrane proteins

Some membrane proteins display specific functional activities that are obviously the best way to assess the quality of their overexpression. For example, here we describe a transport assay that is applicable for various bacterial multidrug ABC transporters expressed in *E. coli* [35,32]. Membrane vesicles with different conditions of expression may show similar levels of proteins of interest but strong differences in drug transport activities.

1. Use a fluorimeter (e.g. Photon Technology International Quanta Master I fluorimeter) and set the excitation and emission wavelengths according to the fluorescent drug used (e.g. 480 nm and 590 nm for doxorubicin, respectively).
2. In a 2 mL-Quartz cuvette containing a small magnet at the bottom, add 1 mL of transport buffer, and 100 μg of *E. coli* vesicles (amount to be adapted according to the level of expression). Next, place the cuvette inside the fluorimeter and record for 2 min before adding the fluorescent drug.

3. After 2 more min with the fluorescent drug, induce transport with 2 mM ATP and record the fluorescence for 10 min (until stabilization).

3.5.3 Solubilization of membrane proteins by mild detergents

When comparing the transport activities of multidrug ABC transporters expressed in the membrane of various strains, we observed a positive correlation between the activity of ABC transporters in the membrane and the propensity of mild detergents to solubilize them [15]. Furthermore, mild detergents such as dodecyl-maltoside (DDM) and lauryl maltose neopentyl glycol (LMNG) [36,37] mainly, if not exclusively, extracted the population of active membrane proteins (Mathieu *et al.*, 2019). On the other hand, harsh detergents like Fos-choline 12 were able to extract all proteins, including the inactive population. Similar detergent trends were described for membrane proteins expressed in eukaryotic systems [16]. Therefore, in the absence of available functional assays, the efficacy of DDM or LMNG detergents to solubilize the membrane protein of interest is a good indicator of its proper folding and functionality. If the target membrane protein is more solubilized by mild detergents in one expression condition as compared to others, such condition should be favored for subsequent applications.

1. Solubilization of the target membrane protein: Prepare a 2 mL-Eppendorf tube with a small magnet bar and dilute the membranes at 2 to 4 mg/mL in solubilization buffer. Add 0.5 to 1% final concentration of mild detergent (LMNG or DDM) or strong detergent (e.g. FC12). Incubate 2 h at 4 °C with agitation.
2. After 2 h, ultracentrifuge at 150 000 x g for 1 h at 4 °C or >200 000 x g for 30 min at 4 °C.

3. Collect supernatant and resuspend insoluble pellets in equal volume of the same buffer.
4. Run a SDS-PAGE with 5 to 10 μL of each fraction to check if the target membrane protein is in the supernatant (solubilized) or in the pellet (non-solubilized).

The choice of the best expression conditions has to be tuned up together with the ability of mild detergent to efficiently solubilize membrane proteins in a functional state. Gold standards are activity assays (in detergent and after reconstitution in lipid bilayers) (e.g. [38-41] for ABC transporters) and the homogeneous profile in size-exclusion chromatography [34,41]. In addition, high-throughput thermal stability screening can be useful to find detergents that efficiently stabilize membrane proteins [42].

3.6 Case studies

3.6.1 BmrA, a multidrug ABC transporter from *Bacillus subtilis*

The production of the multidrug ABC transporter BmrA [32] has been historically and successfully performed in C41(DE3) strain [43,44]. Recent work suggested that BmrA could be overexpressed even more efficiently in Lemo21(DE3) strain [15]. In section 3.3, we strongly recommended to prevent T7 promoter leakage by growing the Lemo21(DE3) cells in the presence of a high concentration of rhamnose (such as 2 mM) during transformation and preculture of cells. For membrane proteins or toxic proteins in general, this step is critical. The objective is to completely repress the expression of the protein of interest during these steps and consequently prevent toxicity effects that can severely impair the production at the induction step.

As seen in Fig. 1, the use of 2 mM rhamnose was indeed essential during transformation and preculture for the effective induction of BmrA. The optimal concentration of rhamnose during the induction stage has to be determined for each protein of interest and 0.5 mM was appropriate for BmrA.

Using this procedure, we observed similar expressions in the membrane fraction of C41(DE3) and Lemo21(DE3) strains (Fig. 2A and 2B). However, even though these levels of expression were similar, the transport activity of the protein in the membranes of Lemo21(DE3) strain was substantially improved (Fig. 2C) and a higher fraction of BmrA could be solubilized by DDM from these membranes (Fig. 2D).

3.6.2 A BceAB-type transporter from *Streptococcus pneumoniae*

The C41(DE3) strain proved to be excellent for overexpression of ABC transporters [15] and a large range of other membrane proteins [10]. Due to the strength of induction in BL21(DE3), overexpression of membrane proteins in this strain was often toxic [6,7]. However, this strain can still be helpful in some circumstances. In the case of an ABC transporter that confer resistance to bacitracin [45], only BL21(DE3) expressed the protein (Fig. 3A and 3B) and LMNG successfully extracted a substantial fraction of the membrane protein (Fig. 3C).

3.7 Expression of soluble proteins

Although this chapter is focused on membrane proteins, the choice of the strain may also affect the expression and folding of soluble proteins. We compared the expression of a soluble kinase, UbK [46], using the BL21(DE3) and the Lemo21(DE3). As shown in Fig. 4, the leaky expression seen in the absence of IPTG in the BL21(DE3) is strongly reduced in the Lemo21(DE3) strain either in the absence or presence of rhamnose. Therefore, if the protein of interest is toxic, the latter strain could prove to be

advantageous but this should be further verified by functional assays. Although preliminary work with UbK suggest differences in functionality depending on the strain and induction conditions, additional work on a variety of soluble proteins should be conducted to confirm these observations.

4 Notes

1. The buffers indicated are those used for the multidrug ABC transporter BmrA. However, the appropriate buffers need to be chosen according to the membrane protein of interest.
2. For proper oxygenation, the culture broth should ideally represent 1/5 of the total volume of the glass flask. If baffled flasks are used, this volume can be increased.
3. Depending on the levels of expression, it may be possible to see the protein of interest in the total fraction. If not, assess protein expression in the membrane fraction.
4. The high pressure used here favors the formation of inverted (i.e. inside-out) membrane vesicles [47,48]. It is advantageous to use for transport assays. In the case of the multidrug exporter BmrA, the nucleotide-binding domains are exposed outside of the inverted membrane vesicles and transport of fluorescent drugs occurs toward the lumen of vesicles.

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Figures.

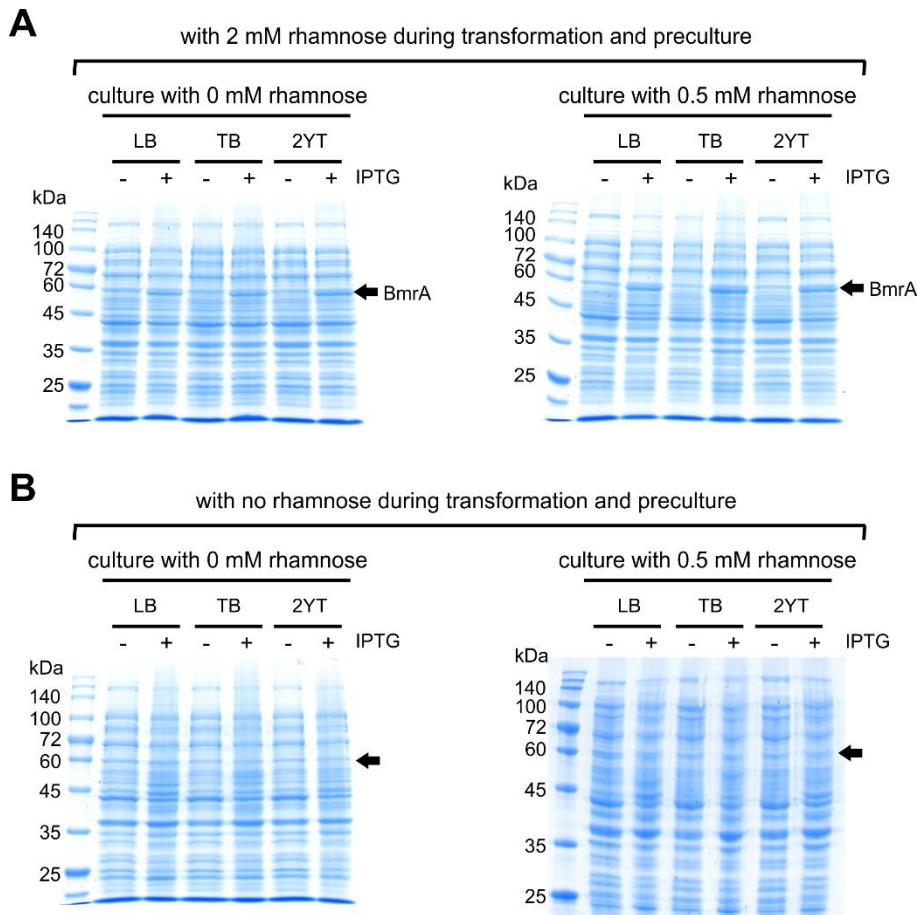


Figure 1. Induction of BmrA expression at the cellular level in Lemo21(DE3) strain. The strain was grown in the presence (A) or in the absence (B) of 2 mM rhamnose during the transformation of pET23-BmrA and the preculture steps, and expression was monitored by SDS-PAGE; in addition, the culture and induction steps were performed in the presence or absence of 0.5 mM rhamnose. On these gels (B), almost no expression of BmrA is observable when rhamnose was not added on the transformation plate and during the preculture.

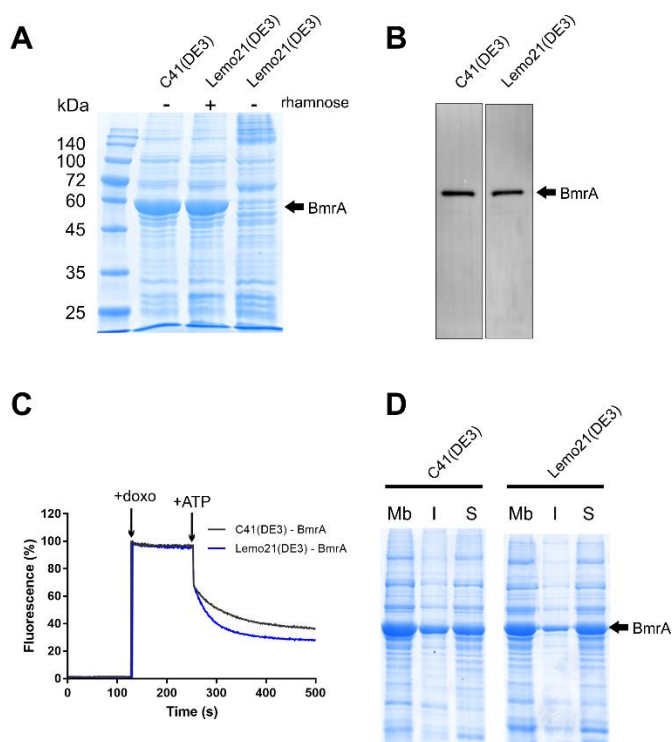


Figure 2. Overexpression of BmrA in membranes of C41(DE3) or Lemo21(DE3) strains. **A**, Expression of BmrA is highly dependent upon the strain and growth conditions. Here, membrane fractions were prepared from different strains grown in TB medium for 4 h at 30 °C either in the presence or the absence of rhamnose. To obtain the same level of expression in Lemo21(DE3) as in C41(DE3), rhamnose was required at each step (2 mM at transformation and preculture; 0.5 mM in main culture). The lack of rhamnose repression induces very low expression for BmrA in Lemo21(DE3), as seen on this SDS-PAGE. **B**, Western Blot analysis showed similar levels of BmrA expression in the membranes of C41(DE3) and Lemo21(DE3) strains (in the presence of rhamnose, as seen in A). HRP-conjugated goat anti-rabbit IgG antibody was used for BmrA revelation. **C**, doxorubicin transport by inverted membrane vesicles containing overexpressed BmrA and prepared from Lemo21(DE3) or C41(DE3) strains. The Lemo21 (DE3) membranes displayed a higher BmrA transport activity as compared to the C41(DE3) membranes. For the assay, 100 µg of total membrane proteins of C41(DE3) or Lemo21(DE3) were used in presence of 10 µM of doxorubicin. **D**, solubilization of BmrA by 1% of DDM. The analysis of soluble (S) and insoluble (I) fractions shows that a higher fraction of BmrA can be solubilized from the Lemo21(DE3) membranes.

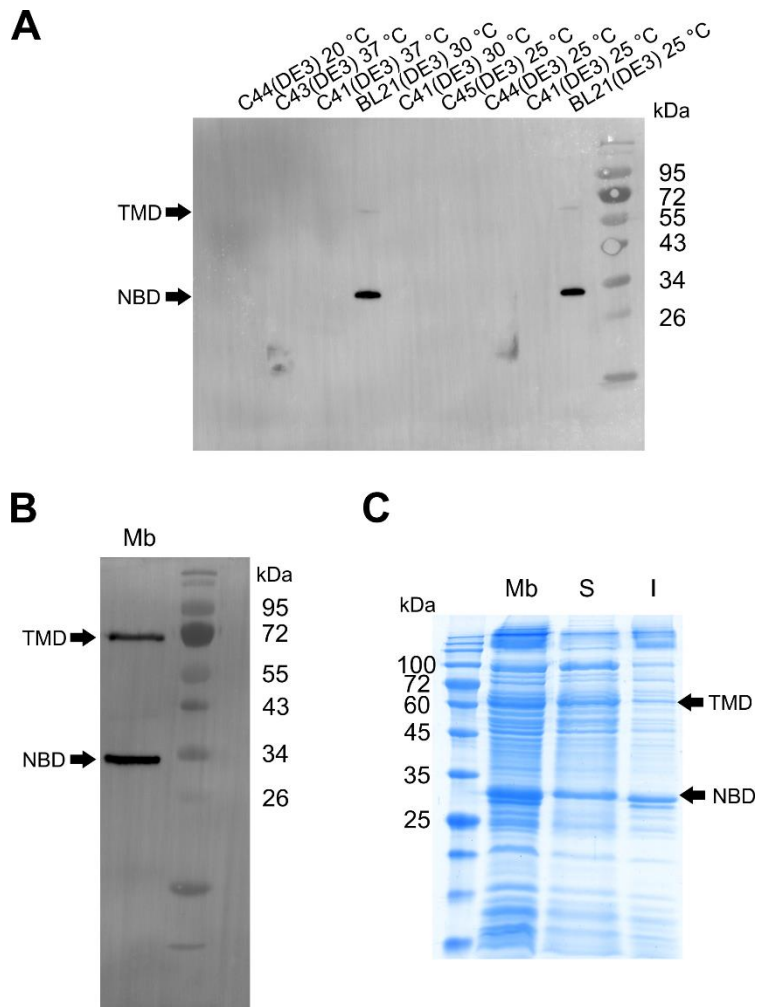


Figure 3. Western blot analysis of SpABC expression in several *E. coli* strains and solubilization from the membrane fraction. A 6His tag was fused to the transmembrane domain (TMD) and the nucleotide-binding domain (NBD) of a BceAB-type transporter from *S. pneumoniae* (SpABC). **A**, Western Blot analysis of SpABC expression in whole cells was performed by using an anti-6His antibody. Several *E. coli* strains (C41, C43, C44, C45 and BL21 (DE3)) and different induction temperatures (20, 25, 30 and 37 °C) were used to express SpABC during an overnight induction. **B**, Western blot analysis of SpABC overexpression in BL21(DE3) membranes. Expression of the transporter was induced by addition of IPTG at exponential phase during an overnight culture grown at 25 °C. Two µg of membrane proteins were loaded. **C**, LMNG solubilization (0,5% for 2 h at 4 °C) of SpABC from membranes isolated from BL21(DE3). After ultracentrifugation, the soluble (S) and insoluble (I) fractions were loaded onto a 15% SDS-PAGE (40 µg of membrane proteins).

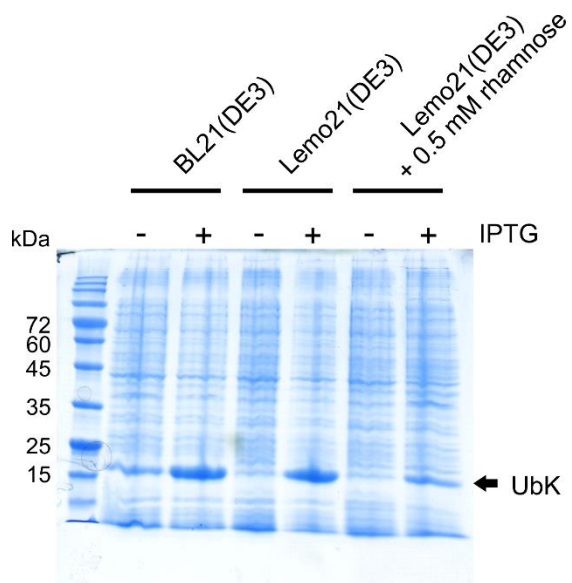


Figure 4. Expression of UbK kinase from *Bacillus subtilis* in BL21(DE3) and Lemo21(DE3) strains. The strains were grown in LB and the induction was performed at 25 °C for 4 h in the presence of 1 mM IPTG. The addition of rhamnose during the culture and induction represses the expression of UbK by the Lemo21(DE3) strain. The expression of UbK was monitored by 15% SDS-PAGE.