Chapter 3

Membrane Protein Production in *Escherichia coli*: Protocols and Rules

Federica Angius, Oana Ilioaia, Marc Uzan, and Bruno Miroux

Abstract

Functional and structural studies on membrane proteins are limited by the difficulty to produce them in large amount and in a functional state. In this review, we provide protocols to achieve high-level expression of membrane proteins in *Escherichia coli*. The T7 RNA polymerase-based expression system is presented in detail and protocols to assess and improve its efficiency are discussed. Protocols to isolate either membrane or inclusion bodies and to perform an initial qualitative test to assess the solubility of the recombinant protein are also included.

Key words Production of recombinant proteins, *E. coli*, T7 RNA polymerase

1 Introduction

Membrane protein (MP) production is still a challenge for biochemists and biophysicists. Over the last decade, eukaryotic expression systems have emerged and have proven to be very useful for structural studies of eukaryotic MP such as G-protein-coupled receptors [1]. However bacterial expression systems remain widely used. We have recently conducted a global survey of the protein data bank (PDB) and found that half of unique MP structures deposited in the PDB have been produced in *E. coli* [2]. Provided that the recombinant MP is well folded within the membrane of the host, bacteria can produce, at very low cost, sufficient amount of the target MP for X-ray crystallization or NMR studies. *E. coli* is also the most versatile host for specific isotopic labeling of proteins required for NMR studies. In this review, we focus on the T7 RNA polymerase (T7 RNAP) bacterial expression system which is, so far, the most efficient in producing large amount of membrane proteins for structural studies [2]. Figure 1 provides an overview of how the expression system works in the bacterial host BL21λ(DE3). The gene encoding the T7 RNAP is inserted in the lambda DE3 under the control of the *lacUV5* promoter. Upon addition of

Isabelle Mus-Veteau (ed.), *Heterologous Expression of Membrane Proteins: Methods and Protocols,* Methods in Molecular Biology, vol. 1432, DOI 10.1007/978-1-4939-3637-3_3,

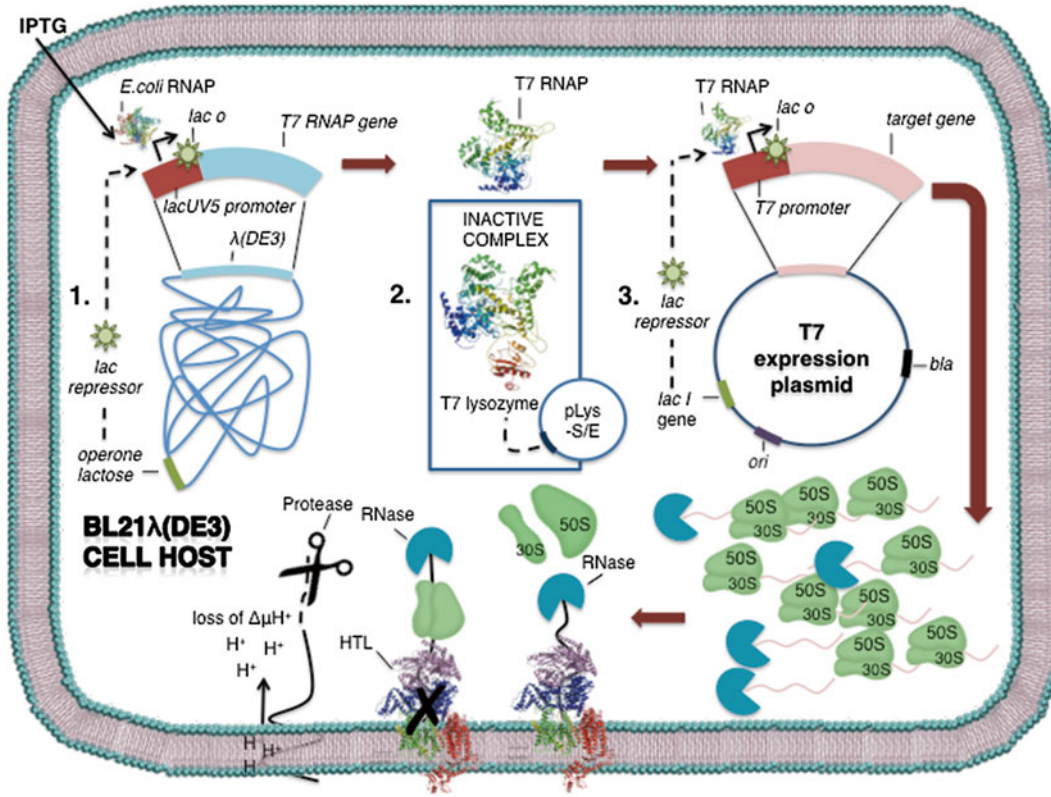

Fig. 1 Global view of the T7-based expression system in BL21λ(DE3). The T7 RNA polymerase gene is lysogenic in the genome and its expression is under the control of the IPTG-inducible *lacUV5* promoter. Upon addition of IPTG, the T7RNAP will specifically transcribe the target gene inserted in the T7 expression plasmid and the target MP might be produced at very high levels. However, overexpression of the target mRNA is, most of the time, toxic to the cell because it overloads the translation machinery and uncouple transcription from translation. The newly synthetized membrane protein might also overload the folding and secretion machineries causing mistargeting of the overproduced MP, protein aggregation, and ultimately proton leak and loss of energy homeostasis. To circumvent these difficulties, the expression system can be regulated by several ways: *1*. Repressing the *lacUV5* promoter using a *lac* repressor; *2*. expressing the T7 lysozyme from a pLys-S/E plasmid, which will inhibit its activity; *3*. inserting the *lac* repressor in the T7 multi-copy expression plasmid

IPTG, isopropyl β-D-1-thiogalactopyranoside, a non-metabolized derivative of lactose, the T7 RNAP is produced and will specifically transcribe the target gene inserted in a T7 expression vector downstream of the T7 promoter. The mRNA of the target MP is highly expressed because the T7 RNAP transcriptional elongation rate is ten times faster than the *E. coli* enzyme. In addition, the T7 expression vector is present in multiple copies. In many cases, the target mRNA overloads the translation machinery triggering ribosome destruction and growth arrest [3]. Naked un-translated mRNA are rapidly degraded by RNases and, in some cases, the RNA degradation is faster than the transcriptional activity of the T7 RNAP leading to lower yield of the target than expected [4]. For this

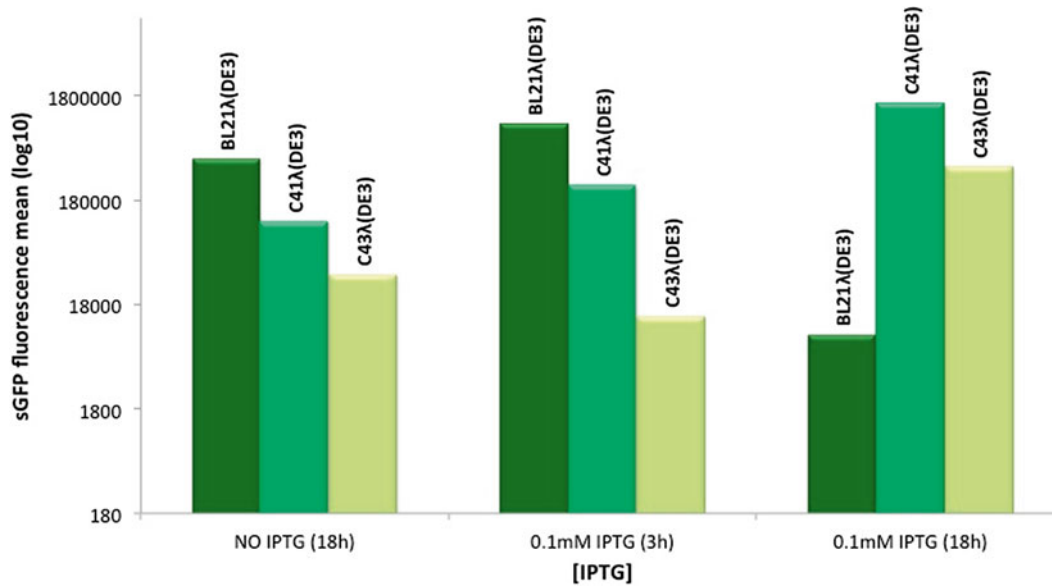

Fig. 2 Analysis of GFP fluorescence by flow cytometry. The pHis17-sGFP T7 expression plasmid has been transformed in the BL21λ(DE3) (*green*), C41λ(DE3) (*light green*), and C43λ(DE3) (*yellow*) bacterial hosts. The fluorescence has been recorded with the flow cytometer Accuri C6 3 h and 18 h after induction with 0.1 mM of IPTG. To assess the basal level of expression of sGFP in each host, cells were grown for 18 h with no addition of IPTG

reason, we provide here a rapid protocol to assess the levels of your target mRNA when the yield of the corresponding MP is low. Insertion of the target MP at the *E. coli* membrane can also overload the translocation and secretion machineries. The recombinant MP will then be not only misfolded and produced at low level but proton permeability of the bacterial membrane might be compromised leading to cell death. Over the last 20 years, the T7 expression system has been optimized to improve its regulation and extend its ability to produce large amount of MP. For instance the use of lysozyme has been shown to strongly inhibit the activity of the T7 RNAP, thus providing a means to decrease the basal activity of the LacUV5 promoter and to tune the activity of the T7-RNAP upon induction [5, 6]. Other groups have isolated mutant hosts from the parental strain BL21λ(DE3) [7, 8]. Some of them namely C41λ(DE3) and C43λ(DE3) have proven to be extremely useful for structural biologists; these mutant hosts contributed to 28% of non-*E. coli* unique MP structures and 19% of *E. coli* unique MP structures deposited into the PDB [2]. To illustrate that the mutant hosts are better regulated than the parental strain, we used the green fluorescent protein (superfold version, sGFP) as gene reporter. After transformation with the pRSET-sGFP expression plasmid, cells have been induced at $OD_{600nm} = 0.6$ with 0.1 mM IPTG. Figure 2 shows the mean green fluorescent intensity analyzed by flow cytometry. In all three bacterial hosts, the basal level of sGFP fluorescence after an overnight culture in 2*TY-rich

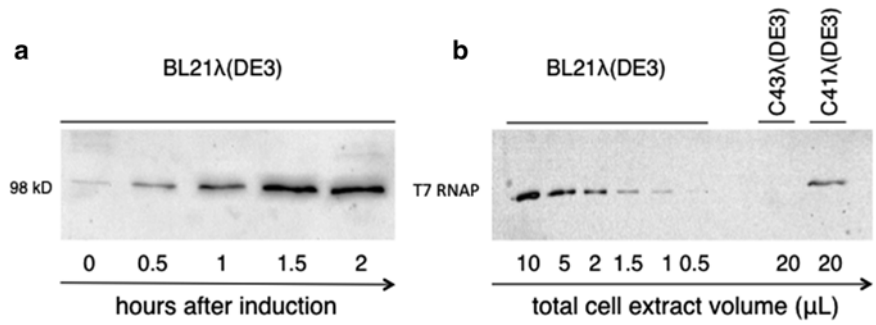

Fig. 3 Immunodetection of the T7 RNAP enzyme in T7 expression hosts. Total cell extracts were loaded on SDS-PAGE and proteins were transferred on nitrocellulose membrane. The T7 RNAP protein was revealed using the anti-T7RNAP from Novagene and a second antibody coupled to peroxidase. Peroxidase activity was detected by chemiluminescence. (**a**) Time course of the T7 RNAP protein expression in BL21λ(DE3) upon addition of 0.7 mM IPTG. (**b**) Expression levels of T7 RNAP 2 h after 0.7 mM IPTG induction in BL21λ(DE3), C43λ(DE3), C41λ(DE3). For BL21λ(DE3) host, decreasing amounts of total cell extract have been loaded to compare the intensity of the signal with the mutant hosts

medium is very high showing that the expression system is leaky. However, basal fluorescence intensities are 4 and 13 times decreased in C41λ(DE3) and C43λ(DE3), respectively. Upon addition of IPTG, fluorescence intensity increased twice in BL21λ(DE3) host 3 h after induction and decreased strongly after overnight induction. This is due to loss of the expression plasmid, cell death, and lysis. In contrast, sGFP production reached a maximal value after overnight induction in both C41λ(DE3) and C43λ(DE3) hosts (13- and 11-fold induction, respectively). At the molecular level, Wagner et al. have shown that, in C41λ(DE3) and C43λ(DE3) hosts, the strong *lacUV5* promoter recombined with the wild-type genomic copy of the *lac* promoter. Consequently, the amount of T7 RNAP enzyme produced upon addition of IPTG is ten times reduced in C41λ(DE3) and undetectable in C43λ(DE3) using the commercially available anti-T7 RNAP antibody from Novagene (Fig. 3). In this chapter we provide protocols to design your construct and choose the appropriate host/vector combination, isolate new bacterial hosts, set up growth conditions, assess your expression system by flow cytometry, fractionate bacterial cells, and perform a first biochemical analysis.

2 Materials

2.1 Materials for RNA Isolation and Sucrose Gradient

1. Tips, plastic tubes, glass, gloves, water must be RNase free.
2. A dry bath to warm up the samples to 65 °C.
3. A laboratory fume hood.
4. A spectrophotometer.

5. QIAGEN RNase-Free DNase I Set.
6. Lysis solution for 700 µL of culture: 35.5 µL 20% SDS + 7 µL of 200 mM Na-EDTA + 500 µL water-saturated phenol.
7. Water-saturated phenol.
8. Phenol/chloroform solution v/v 1:1. The chloroform should contain isoamyl alcohol in a proportion v/v 24:1.
9. 3 M Na-acetate pH 5.
10. Ethanol 100% RNase free.
11. Ethanol 70% RNase free.
12. Gradient maker.
13. 10 mM Tris–HCl, pH 8.
14. Sucrose solutions: 50% and 5% (w/v) prepared in 10 mM Tris–HCl, pH 8.
15. Beckman Coulter Ultra-Clear™ centrifuge tubes.

2.2 Media, Buffers, and Chemicals

1. 1*LB Medium: 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, and 5 g NaCl. Add ultrapure water to 900 mL. Adjust the pH to 7.2 with NaOH. Add water at a final volume of 1 L and autoclave for 20 min at 121 °C.
2. 2*TY Medium: 16 g Bacto Tryptone, 10 g Bacto Yeast Extract, and 5 g NaCl. Add ultrapure water to 800 mL. Adjust the pH to 7.2 with NaOH, adjust the final volume to 900 mL, and autoclave for 20 min at 121 °C.
3. 2*TY with glucose: 2 g Glucose in 100 mL of final volume of water, filter sterilize. Add the glucose solution in the autoclaved medium. Adding glucose could be useful if you wish to repress further the expression vector before induction.
4. Isopropyl-beta-D-galactoside (IPTG): Prepare 100 mM, 500 mM, 700 mM, and 1 M stock solutions in ultrapure water, sterilize with 0.22 µm filter, aliquot, and store at –20 °C.
5. Antibiotics: Prepare 1000 times stock solutions of antibiotics. Ampicillin (100 mg/mL) can be prepared in ultrapure water and stored at –20 °C. Tetracycline (12,5 mg/mL) and kanamycin (30 mg/mL) are freely soluble in water but in time the solutions can turn turbid due to precipitation. It is thus recommended to prepare it in 95% ethanol. Dilute 1000 times the stock solution in medium prior to use.
6. Phosphate-buffered saline (PBS): 10 mM Phosphate, 150 mM NaCl, pH 7.4 (tablets are commercially available).
7. TEP buffer: 10 mM Tris–HCl, pH 8, 1 mM EDTA, and 0.001% PMSF.
8. Triton X-100.
9. Dodecyl-maltoside (DDM).

10. Phosphododecylcholine (Fc12).
11. Sodium dodecyl sulfate (SDS).

2.3 Web Resources

2.3.1 Sequence Analysis and Molecular Biology Tools

1. **SMART** (protein domain database): http://smart.embl-heidelberg.de/
2. **Jpred** (secondary structure prediction): http://www.compbio.dundee.ac.uk/jpred/index.html
3. **ExPasy**: http://www.expasy.ch/
4. **Amplify** (PCR simulation, oligonucleotide design) http://engels.genetics.wisc.edu/amplify/

Vector design:

5. **Dna20:** https://www.dna20.com/resources/bioinformatics-tools
6. **Serial-Cloner**: http://serialbasics.free.fr/Serial_Cloner-Download.html
7. **APE**: http://biologylabs.utah.edu/jorgensen/wayned/ape/

Molecular and structural biology websites

8. Steewe White: http://blanco.biomol.uci.edu/mpstruc/
9. Dror Warschawski: http://www.drorlist.com/nmr/MPNMR.html

Academic expression plasmid resources

10. Protein Science Initiative. http://psimr.asu.edu/about.html

3 Methods

3.1 Designing Constructs for Expression

1. Before starting molecular cloning experiments, check if your target MP is already available in an expression vector (*see* Protein Science Initiative Web site) and search the literature to see if your MP target or related proteins have been produced in recombinant systems (*see* **Note 1**).
2. Be aware that *E. coli* cannot produce at high levels proteins larger than 90 kDa. Ribosomes drop off very long mRNA leading to incomplete synthesis products. If possible break up your protein into smaller fragments. Use SMART (protein domain identification) or Jpred (secondary structure prediction) to define the boundaries carefully.
3. Addition of purification Tag: For N-terminal constructs start protein synthesis with three amino acids before the Tag. In pRSET vector (Invitrogen), the N-terminal sequence is **MRGS**-(His)6 which gives a very good yield of recombinant protein. Consider adding more than 6 histidines (up to 12 but then preferentially in C-terminal position) to achieve a stronger

binding on Nickel column. There is no generic rule regarding cleavage sequences after the Tag but TEV cleavage sequence is widely used for MP as the TEV protease is still active in the presence of the most commonly used detergents [9].

4. Your target MP might not spontaneously go to the inner membrane of the bacteria. Consider making a C-terminal fusion with periplasmic maltose-binding protein (MBP), which contains a periplasmic signal sequence, to target your MP to the *E. coli* membrane [10, 11].
5. If possible engineer dua-ribosome-binding site (RBS) expression vectors like pET-Duet (Novagen) so that you can clone a fluorescent protein (FP) gene downstream of your target MP gene. This allows you to follow the cell population of bacteria by flow cytometry, to assess the stability and toxicity of your expression vector quickly, and to establish the optimal induction conditions (*see* Subheading 3.5.1). FP fusion with your target MP is also an option developed successfully by several laboratories [12].

3.2 Selecting the Optimal Expression Vector/ Bacterial Host

Selecting the right combination of vector/bacterial host is an essential step to achieve the optimal production of your MP (*see* **Note 2**). The following rules apply to the T7 RNAP-based expression system:

1. In combination with C41λ(DE3) and C43λ(DE3) bacterial hosts use high-copy-number plasmids like those containing the pMB1 origin of replication (200–600 copies/cell). A non-exhaustive list is pMW7 and derivatives (pHis and pRun) [13, 14], pGEM (Promega), pRSET and pDEST (Invitrogen), pIVEX (5prime), and pPR-IBA (IBA). Avoid *lacI* and *lacO* sequences in the plasmid.
2. In combination with BL21λ(DE3), use preferentially medium-copy-number vectors and those containing *lacI* and *lacO* sequences like pET (3, 9, 14, 17, 20, 23 from Novagen), to reduce the amount of T7 RNAP before induction. Consider using the companion plasmid pLyS to inhibit the T7 RNAP after induction.
3. The BL21AI host, which contains the T7RNA polymerase gene under the control of the arabinose promoter, and the Lemo21 host [6], which contains a companion plasmid expressing the lysozyme under the rhamnose promoter, may also be useful to titrate the amount or activity of T7 RNA polymerase (*see* **Note 3**).

3.3 Viability Test on Agar Plate

1. Preparing the agar plates: Melt 500 mL (or less) 2*YT agar medium in water bath at 100 °C. When the solution is clear switch the temperature to 55 °C. Add 500 μL 0.7 M sterile IPTG and mix vigorously. Add the required antibiotic.

Pour the plates and wait for 1 h till the agar is solid. Incubate the plate upside down O/N at 37 °C.

2. Cell transformation: Take out of the −80 °C freezer a stock of competent cells and thaw them on ice. Add 10 ng of plasmid to 50 μL of cells and leave it on ice for 20 min. Place the microcentrifuge tube on a water bath set at 42 °C for 90 s. Replace the tube on ice for 5 min. Add 500–700 μL of SOC or LB media (without antibiotic) and allow the culture to grow for 45 min at 37 °C. Plate 100 μL of transformation mix on 2*TY agar plate containing only the antibiotic and 100 μL on agar plate with both antibiotic and IPTG. Incubate O/N at 37 °C.
3. Analysis of the cell population: Count the number of colonies on both plates. If you see no colony on IPTG-containing plates then expression of your target MP compromises cell growth. Transform the cells with the empty plasmid to verify if the plasmid is also toxic for the cell. If you have the same number of colonies in both conditions, then expression of your target MP is not toxic. Usually the size of the colonies is reduced in the presence of IPTG.

3.4 Selecting a Host Strain Adapted to the Expression of Your Target MP

The protocol below allows you to genetically isolate low-frequency mutants when your bacterial host/vector expression system compromises cell growth upon induction (*see* **Note 4**). The protocol uses the green fluorescent protein from Aequora Victoria [15] but can be performed without FP marker [7].

3.4.1 Selection Procedure

1. It is essential to work in a sterile environment. Pre-warm five 250 mL flasks containing 50 mL of 2*TY media. Add antibiotic prior to use. Autoclave 40 microcentrifuge tubes and fill them with 900 μL of sterile water or 2*TY media.
2. Transform the parental strain with your target MP expression vector, ideally in co-expression with a fluorescent protein. Inoculate a freshly transformed colony in 50 mL 2*TY medium. Grow cells until OD_{600nm} has reached 0.4–0.6. Add IPTG to 0.7 mM final concentration. One, two, and three hours after induction, take 1 mL of culture. After a low-speed centrifugation (300 × *g* for 2 min), gently resuspend the pellet in 1 mL of sterile water.
3. Perform serial dilutions from 1/10 to $1/10^4$ and immediately plate 100 μL of all dilutions on the IPTG/antibiotic-containing plates. Incubate the plates O/N at 37 °C.
4. Check the presence of green fluorescent colonies (Fig. 4) under UV light (above 300 nm to avoid the mutagenic effect of UV). Select ten small fluorescent colonies (*see* **Note 5**) for each selection experiment and make over-day cultures in tubes with 1 mL 2*TY containing ampicillin. After 2–3 h (when the

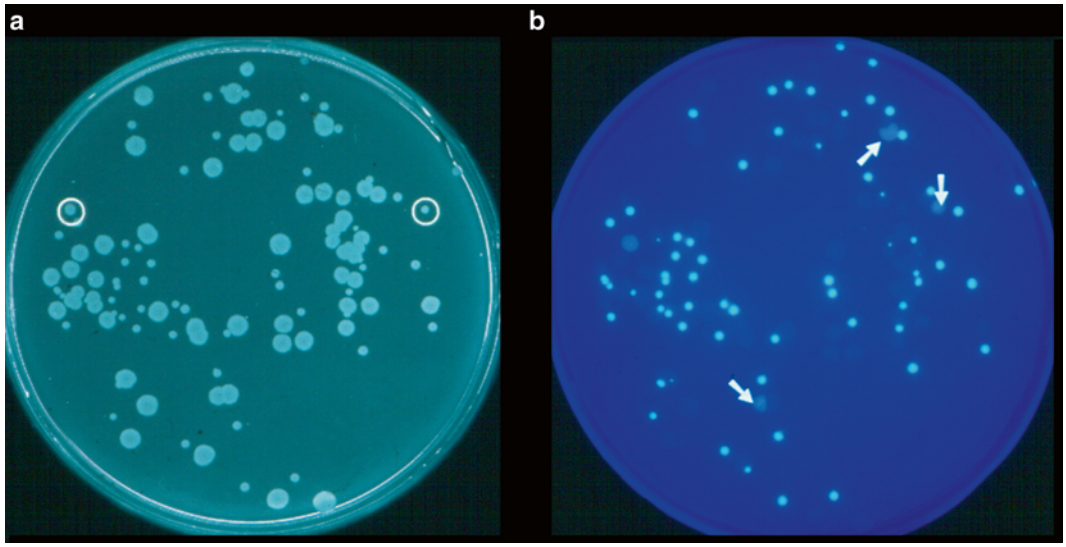

Fig. 4 Selection of bacterial hosts using GFP as gene reporter. After transformation of the pMW7-GFP expression plasmid, cells are grown in 2*TY medium and induced at $OD_{600\ nm} = 0.6$. Two hours after induction, serial dilutions of the culture are plated on IPTG-containing plates. The next day, plates were illuminated under a normal light (**a**) or UV light (**b**)

culture becomes turbid) save the mutants on 2*TY ampicillin agar plates with and without IPTG. Incubate the plates O/N at 37 °C.

5. Check that all mutants now grow on IPTG-containing plate. The size of the colonies in the presence of IPTG can be considered as a characteristic feature for a couple of vector/host.

3.4.2 Localization of the Mutation

You need to check if the mutation is in the bacterial genome or in the plasmid.

1. Plasmid rescue: Purify the plasmid DNA from each clone and transform the initial host, i.e., BL21λ(DE3). Plate 100 μL of the transformation mix on 2*TY plates with antibiotic and with or without IPTG. Incubate O/N at 37 °C. Check the presence of colonies on IPTG-containing agar plates. No colony means that the mutation is not in the expression vector and consequently most likely in the bacterial host genome.
2. Curing the bacterial host from the plasmid: Grow the bacterial mutant host in a 250 mL flask containing 50 mL of 2*TY without antibiotic. Make daily serial 10 times dilutions of the culture and plate 100 μL of the $1/10^8$ and $1/10^7$ dilutions on 2*TY agar plates containing IPTG but no antibiotic. After O/N incubation at 37 °C, check the fluorescence under UV light. Usually, after 3–5 days of culture in the absence of antibiotic, large colonies that have lost their green fluorescence and therefore the expression plasmid will appear.

3. Isolate a large nonfluorescent colony, make a glycerol stock, and prepare calcium-competent cells. Transform the cured host with the original expression vector (not the cured one) and verify that the "colony size phenotype" on an IPTG-containing plate is restored.

3.5 Optimization of Growth Conditions

1. General rules: Choose expression hosts where the T7 RNAP expression of activity is tightly controlled. Systematically test induction temperature below 25 °C. Check the optimal IPTG concentration for your target MP (*see* Subheading 3.5.1).
2. Specific rules for BL21λ(DE3) host: Do not induce the target MP with IPTG or follow Alfasi's protocol [8] by adding extremely low concentration of IPTG (10 μM). Use pLysS as companion plasmid to downregulate the activity of the T7RNAP.
3. Specific condition for C41λ(DE3) and C43λ(DE3) bacterial mutant hosts: Test cell viability on IPTG plates. If the expression of your target MP is not toxic in C41λ(DE3) then keep this mutant host and induce with 0.1 and 0.7 mM IPTG. Test 3-h and O/N induction. Use C43λ(DE3) when expression of your target gene is toxic for C41λ(DE3). Add IPTG at 0.7 mM O/N.

3.5.1 Exploring Induction Conditions and Mutant Hosts by Flow Cytometry

If you co-express a fluorescent protein, superfold GFP for instance, then you can detect GFP fluorescence on FL1 graph (*see* **Note 6**) and also check the size (FSC) and the granularity (SSC) of your cells by using a density plot. As illustrated in Fig. 2, this can be extremely useful to compare rapid expression hosts as well as expression conditions.

1. Native conditions: Collect cells by centrifugation (300 × *g* for 2 min) and remove the supernatant. Resuspend cells in 0.5–1 mL PBS. Repeat the washing step three times. Dilute 1/1000 the cells in PBS before loading the sample on the cytometer.
2. Analysis on fixed cells: Collect cells by centrifugation and aspirate the supernatant. Resuspend cells in 0.5–1 mL PBS. Add formaldehyde to 4%. Fix for 10 min at 37 °C. Wash the cells three times with PBS. Dilute 1/1000 the cells in PBS before loading the sample on the cytometer.

3.5.2 Testing the mRNA Stability: Phenol/Chloroform RNA Extraction from E. coli

If your target membrane protein is not produced in several vector/host combinations then you should check the mRNA stability of the target gene either by quantitative real-time PCR or by "Northern blot" analysis. Obtaining high-quality RNA is the first and often most critical step. Phenol/chloroform extraction is an easy way to remove proteins from nucleic acid samples: nucleic acids remain in the aqueous phase while proteins separate into the

organic phase or lie at the phase interface. This protocol can be used also to extract RNA from bacteria grown in a rich medium. Phenol is a dangerous poison that burns the skin and the lungs upon inhalation. You must wear gloves and manipulate carefully under fume hood. A solution of PEG400 is recommended for first aid.

1. First phenol extraction: Add 700 μL of cell culture into the lysis solution maintained at 65 °C. Keep at 65 °C and vortex vigorously intermittently about ten times for 10 s. Cool the tubes on ice and centrifuge for 2 min, 15,000 × *g*, at 4 °C. Transfer the aqueous phase in a new Eppendorf tube, being careful not to contaminate with the interface phase.
2. Second phenol extraction: Add an equal volume of water-saturated phenol. Place the tubes at 65 °C and vortex vigorously intermittently about ten times for 10 s. Cool the tubes on ice and centrifuge for 2 min, 15,000 × *g*, at 4 °C. Transfer the aqueous phase in a new Eppendorf tube, being careful not to contaminate with the interface phase.
3. First phenol/chloroform extraction: Add an equal volume of phenol/chloroform and vortex vigorously intermittently about ten times for 10 s; this step can be done at room temperature. Centrifuge for 2 min, 15,000 × *g*, at 4 °C. Transfer the aqueous phase in a new Eppendorf tube, being careful not to contaminate with the interface phase.
4. First RNA precipitation: Add 1/10 the volume of 3 M Na-acetate, pH 5 (or 5 M NaCl) and 2.5 volume ethanol. Mix and place at −20 °C for 1–2 h (*see* **Note 7**). Centrifuge at 15,000 × *g* for 30–60 min at 4 °C. Remove carefully the supernatant and wash the pellet with 1 mL of 70% ethanol. Centrifuge at 15,000 × *g* for 30 min at 4 °C. Dry pellet in air and resuspend in 400 μL of sterile water.
5. Treatment with RNase-free DNase I (*see* **Note 8**; protocol provided from QIAGEN).
6. Second phenol/chloroform extraction as described in **step 3**.
7. Second RNA precipitation as described in **step 4**.
8. Measure the RNA concentration with a spectrophotometer: 1 A_{260} corresponds to 40 μg/mL RNA. Check the purity of the RNA by estimating the ratio 260/280 nm which must be 2.0.

3.6 Collecting Membranes or Inclusion Bodies from E. coli

This section provides protocols for isolation of inclusion bodies and bacterial membranes when internal membrane proliferation occurs within the cell (*see* **Note 10**).

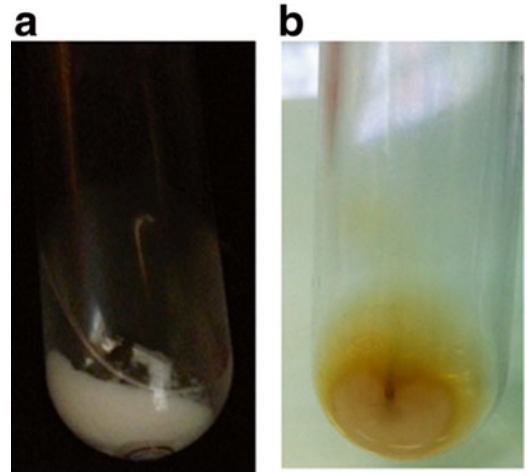

Fig. 5 Pictures of inclusion bodies and intracellular membrane pellets. (**a**) Inclusion bodies of OmpF protein 10,000 × *g* pellet, (**b**) intracellular membrane 100,000 × *g* pellet containing the b subunit of the ATP-synthase

3.6.1 Check the Presence of Inclusion Bodies

1. Breaking the cells: Harvest the culture by centrifugation at 7000 × *g*, 10 min, at 4 °C. Resuspend the pellet in 25 mL of TEP buffer. Disrupt the bacteria by passing the suspension twice in a French Press or cell disruptor.
2. Differential centrifugation: Pellet the cell debris at 600 × *g* for 10 min. Keep the supernatant. Collect the putative inclusion bodies by centrifuging the supernatant at 10,000 × *g* for 15 min at 4 °C. You should see a white brawny pellet. Collect the bacterial membranes by centrifugation of the 10,000 × *g* supernatant at 100,000 × *g* for 1 h.
3. Wash of the inclusion bodies: Wash the first pellet obtained at 10,000 × *g* with 25 mL TEP buffer supplemented with 2% Triton X-100. Centrifuge at 10,000 × *g* for 30 min. Inclusion body pellet is usually white (*see* Fig. 5a). Repeat the wash. Resuspend the pellet in 25 mL TEP buffer without detergent and centrifuge at 10,000 × *g*. Repeat the wash in order to remove all traces of detergent.
4. Resuspend the pellet in 2 mL TEP buffer and proceed to protein assay.

3.6.2 Collecting E. coli Membranes in the Absence of Inclusion Bodies

1. Breaking the cells: Harvest the culture by centrifugation at 7000 × *g*, 10 min, at 4 °C. Resuspend the pellet in 25 mL of TEP buffer. Disrupt the bacteria by passing the suspension twice in a French Press or cell disruptor.
2. Differential centrifugation: Collect the P1 pellet of internal membranes by low-speed centrifugation: 2500 × *g* for 10 min (*see* **Note 11**). Centrifuge the supernatant (S1) at 100,000 × *g* for 1 h to recover the inner and outer membranes.

3. Wash P1 with 25 mL of TEP buffer and centrifuge at 2500 × *g* for 10 min at 4 °C to remove unbroken cells (P2).
4. The supernatant (S2) contains the washed internal membranes.
5. Centrifuge for 1 h at 100,000 × *g* in order to pellet the internal membranes. You should see a brown pellet (*see* Fig. 5b).
6. Resuspend the pellet in 2 mL of buffer and assay the protein concentration.

3.6.3 Sucrose Gradient Protocol

The purpose of sucrose gradient is to concentrate and separate membrane vesicles according to their specific density. For high-purity requirements, continuous gradients are used. If you do not have access to gradient maker, then use step gradients.

1. Setting up the gradient maker: Attach capillary tubes to the end of the tubing emerging from the gradient maker. Gradient maker tubing must be clean; otherwise the sucrose gradient will not flow correctly. Close the mixer between the two compartments of the maker. Add the higher percentage sucrose solution to the outlet side of the maker. Start the stirring and add the lower percentage sucrose solution to the other compartment. Place the capillary tube on the top of the ultracentrifuge tube. Switch on the peristaltic pump and open the mixer between the two sucrose solutions. Check the flow rate of the pump to ensure that the gradient is poured drop by drop. When the gradient is completed, stop the stirrer and carefully remove the capillary tube.
2. Sample loading and centrifugation: Gently load 1 mL of 2 mg/mL protein sample on the top of the gradient paying attention not to mix the sample with the gradient. Make sure to fill up the ultracentrifuge tube (12 mL) and balance tubes with 10 mM Tris–HCl, pH 8. Centrifuge for at least 18 h at 100,000 × *g* and 4 °C. Collect 1 mL fraction in Eppendorf tubes from top to bottom.
3. Run an SDS-PAGE gel with all the fractions to detect your target MP in the different types of membranes.

3.6.4 Testing the Solubility of Your Target MP

Usually, folded MP in native membranes can be solubilized with detergent. However after production in heterologous membranes, it is frequently occurring that the target MP is difficult to solubilize. Although it is associated to the membrane fraction it might be misfolded and therefore behaves like inclusion bodies. A simple test is to compare the solubility of your target MP in three different detergents: dodecyl-maltoside (DDM), phosphododecylcholine (Fc12), and SDS.

1. Solubilization of the target MP: Prepare three Eppendorf tubes with 100 µg of your target MP in TEP buffer supplemented with 150 mM NaCl. In each tube add separately one

detergent to 1% final concentration for DDM and Fc12 and 2% for SDS (at least ten times above the critical micelle concentration). Adjust the final volume to 100 μL with buffer to perform the solubilization at 1 mg/mL. Incubate for 1 h at 4 °C and ultracentrifuge at 100,000 × *g* for 30 min.

2. Run an SDS-PAGE to check if the target MP is in the supernatant (solubilized) or in the pellet.
3. If the target MP is solubilized only by SDS, then you have inclusion bodies. If it is solubilized by all three detergents then it is likely to be well folded. If DDM cannot solubilize your target MP then try other detergents but it is likely that your target MP is misfolded.

4 Notes

1. Expression protocols are usually poorly described and you will have to go to several previous publications to find out the exact expression vector or host that was used. Try to find out the exact final yield of purified MP target per liter of culture. Below 1 mg/L you may spend 90% of your time growing cells to perform a single biophysical analysis.
2. There is a plethora of vectors and expression systems commercially available. A systematic analysis of expression protocols in bacteria [2] showed that for 80% of membrane protein structures, the two main expression systems use the T7 and arabinose promoter-based expression plasmids. The distribution of secondary structures among the different expression system is asymmetrical. For instance beta-barrel membrane protein structures were preferentially obtained using the arabinose promoter-based expression system or the T7 system with BL21λ(DE3) as expression host. In contrast, alpha helical integral membrane proteins (IMP) were almost all produced in the T7 system. The C41λ(DE3) and C43λ(DE3) bacterial hosts succeeded in producing 50% of heterologous IMP.
3. Lysozyme is a natural inhibitor of T7 RNA polymerase. If you chose to downregulate your expression system by using companion plasmids that express lysozyme (pLyS/E), take into consideration that it requires the addition of a second antibiotic which could affect considerably the cell growth.
4. When the production of the target MP is toxic, the bacteria are unable to form colonies on plates containing the inducer. The selection of new bacterial hosts (mutation in the expression vector is rare) is based upon their ability to form colonies on plate in the presence of inducer, here IPTG. By analyzing their presence, number, and size you can determine the degree of toxicity of the expression of the target protein.

5. In order to isolate and select the new mutant hosts, it is critical to have no more than 200 colonies on the plate. The frequency of occurrence of mutant hosts varies from 10^{-4} to 10^{-6}, hence the importance of diluting the culture. Figure 4 shows a fairly good correlation between the size of the colonies and the intensity of fluorescence. Most of the normal-size colonies do not exhibit fluorescence; they have lost the ability to express the gene. The smaller colonies on the other hand are almost all highly fluorescent.
6. Flow cytometers measure a variety of cellular characteristics such as relative cell size, internal complexity/granularity, cell surface properties/refractive indices, levels of autofluorescence, presence or absence of an exogenous fluorescent probe, and relative fluorescence intensities. Here we used the BD Accuri™ C6 Cytometer. The C6 cytometer is equipped with a blue and a red laser (488 and 640 nm, respectively) and four fluorescent detectors. Standard optical filters are FL1 533/30 nm (e.g., FITC/GFP), FL2 585/40 nm (e.g., PE/PI), FL3 > 670 nm (e.g., PerCP, PerCP-Cy™5.5, PE-Cy7), and FL4 675/25 nm (e.g., APC). Follow the wash procedure of the cytometer before and after the analysis to avoid cells aggregating within the cytometer.
7. If you are in hurry, you can stop the protocol at this step and store the samples at −20 °C. You can also shorten the protocol by omitting the first phenol/chloroform extraction but this is possible only if you have not grown your cells in a rich medium.
8. The treatment with DNase I-RNase free is necessary because samples from high-density *E. coli* culture may be contaminated with DNA.
9. After the 4 °C centrifugation, leave the samples at room temperature for few seconds. This clarifies the solution and helps to transfer the aqueous phase in a new clean tube.
10. On some occasions, upon overexpression of a membrane protein in *E. coli* membrane proliferation has been observed [16–20]. For instance, the overproduction of the *E. coli* b-subunit of the $F1F_o$ ATP synthase resulted in the development of a large network of intracytoplasmic membranes (ICM, Fig. 5b). The bacterial host responds to the overproduction of a membrane protein by synthesizing lipids and by converting phosphatidyl glycerol into cardiolipids at the stationary phase [18, 19].
11. It is highly unusual to collect membranes at 2500 × *g*. It might be due to the high number of cells (1 L culture at $OD_{600nm} = 8$ concentrated in 25 mL), or the high density of the membranes and their association with DNA and cell debris. However, after washing of the 2500 × *g* pellet, membranes will not anymore pellet at 2500 × *g* but, as expected, at 100,000 × *g*.

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique, INSERM, and by the "Initiative d'Excellence" program from the French State (Grant "DYNAMO," ANR-11-LABEX-0011-01). FA is supported by a DYNAMO PhD fellowship.

References

1. Tate CG (2012) A crystal clear solution for determining G-protein-coupled receptor structures. Trends Biochem Sci 37:343–352. doi:10.1016/j.tibs.2012.06.003
2. Hattab G, Warschawski DE, Moncoq K, Miroux B (2015) Escherichia coli as host for membrane protein structure determination: a global analysis. Sci Rep 5:12097
3. Dong H, Nilsson L, Kurland CG (1995) Gratuitous overexpression of genes in Escherichia coli leads to growth inhibition and ribosome destruction. J Bacteriol 177:1497–1504
4. Arechaga I, Miroux B, Runswick MJ, Walker JE (2003) Over-expression of Escherichia coli F1F(o)-ATPase subunit a is inhibited by instability of the uncB gene transcript. FEBS Lett 547:97–100
5. Moffatt BA, Studier FW (1987) T7 lysozyme inhibits transcription by T7 RNA polymerase. Cell 49:221–227
6. Wagner S, Klepsch MM, Schlegel S et al (2008) Tuning Escherichia coli for membrane protein overexpression. Proc Natl Acad Sci 105:14371–14376. doi:10.1073/pnas.0804090105
7. Miroux B, Walker JE (1996) Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 260:289–298. doi:10.1006/jmbi.1996.0399
8. Alfasi S, Sevastsyanovich Y, Zaffaroni L et al (2011) Use of GFP fusions for the isolation of Escherichia coli strains for improved production of different target recombinant proteins. J Biotechnol 156:11–21. doi:10.1016/j.jbiotec.2011.08.016
9. Gräslund S, Nordlund P, Weigelt J et al (2008) Protein production and purification. Nat Methods 5:135–146. doi:10.1038/nmeth.f.202
10. Shibata Y, White JF, Serrano-Vega MJ et al (2009) Thermostabilization of the neurotensin receptor NTS1. J Mol Biol 390:262–277. doi:10.1016/j.jmb.2009.04.068
11. Egloff P, Hillenbrand M, Klenk C et al (2014) Structure of signaling-competent neurotensin receptor 1 obtained by directed evolution in Escherichia coli. Proc Natl Acad Sci U S A 111:E655–E662. doi:10.1073/pnas.1317903111
12. Drew D, Lerch M, Kunji E et al (2006) Optimization of membrane protein overexpression and purification using GFP fusions. Nat Methods 3:303–313. doi:10.1038/nmeth0406-303
13. Way M, Pope B, Gooch J et al (1990) Identification of a region in segment 1 of gelsolin critical for actin binding. EMBO J 9:4103–4109
14. Orriss GL, Runswick MJ, Collinson IR et al (1996) The delta- and epsilon-subunits of bovine F1-ATPase interact to form a heterodimeric subcomplex. Biochem J 314(Pt 2):695–700
15. Walker J, Miroux B (1999) Selection of Escherichia coli hosts that are optimized for the overexpression of proteins. Man. Ind. Microbiol. Biotechnol. 2nd Ed. MIMB2.
16. von Meyenburg K, Jørgensen BB, van Deurs B (1984) Physiological and morphological effects of overproduction of membrane-bound ATP synthase in Escherichia coli K-12. EMBO J 3:1791–1797
17. Wilkison WO, Walsh JP, Corless JM, Bell RM (1986) Crystalline arrays of the Escherichia coli sn-glycerol-3-phosphate acyltransferase, an integral membrane protein. J Biol Chem 261:9951–9958
18. Weiner JH, Lemire BD, Elmes ML et al (1984) Overproduction of fumarate reductase in Escherichia coli induces a novel intracellular lipid-protein organelle. J Bacteriol 158:590–596
19. Arechaga I, Miroux B, Karrasch S et al (2000) Characterisation of new intracellular membranes in Escherichia coli accompanying large scale over-production of the b subunit of F(1)F(o) ATP synthase. FEBS Lett 482:215–219
20. Eriksson HM, Wessman P, Ge C et al (2009) Massive formation of intracellular membrane vesicles in Escherichia coli by a monotopic membrane-bound lipid glycosyltransferase. J Biol Chem 284:33904–33914. doi:10.1074/jbc.M109.021618