

Chapter 4

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

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4.1 Introduction

Production of biological molecules is a challenge for the next decade in the field of medicinal chemistry. After heterologous production, the biological molecule must be active, well defined homogenous and the cost of its production should remain low. An interesting example is given by the relative success of therapeutic antibodies. Twenty monoclonal therapeutic antibodies are presently on the market (Oldham and Dillman 2008). All of them are produced with the hybridoma technology, which significantly increases the social cost of treating corresponding diseases and prevents the worldwide distribution of these drugs. Smaller-sized antibody peptides, named nanobodies, are being produced in bacteria to circumvent the cost of the hybridoma technology. Although *Escherichia coli* is probably the most versatile and the cheapest host for protein production, several obstacles remain: inclusion bodies formation, LPS contamination, incomplete synthesis, degradation by proteases, and the lack of post-translational modifications.

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In the case of membrane proteins, the situation is even more complex because they are difficult not only to produce but also to keep, in an active state, in solution. In medicinal chemistry, the need for large-scale production of membrane proteins is increasing. For instance, producing the major outer membrane protein (MOMP) of *Chlamydia trachomatis* is a major issue for establishing a robust vaccine against this pathogenic bacterium. Although the protein can easily be produced in bacteria and refolded in several detergents, only the native protein can be used to generate protective antibodies. Indeed, its quaternary structure must be preserved to generate an efficient B-cell response. Despite recent progress in maintaining the MOMP quaternary structure in solution (Tifrea et al. 2011), large-scale production of the protein is still a challenge. Bioproduction is a challenge not only for producing biological drugs or drug targets but also for the development of new drugs. Membrane proteins represent up to 50% of human drug targets (Overington et al. 2006). Several milligrams to grams of proteins are required to screen and validate drugs, which is a major limitation in pharmaceutical research.

Beyond its impact in medicinal chemistry and in the pharmaceutical industry, bioproduction is also a bottleneck for biologists and biophysicists. For instance, there are 424 unique membrane protein structures in the Protein Data Bank (PDB; see <http://blanco.biomol.uci.edu/mpstruc/>), which corresponds to only 2% of the total number of solved protein structures. Despite the exponential growth of membrane protein structures, they are still 20 years behind soluble protein, in terms of number of structures solved. Over the past decades, a tremendous effort has been invested in developing alternative expression systems and new surfactants (see Zoonens et al., Chap. 7 of this book for review; Chae et al. 2010; Popot et al. 2011) to purify and refold membrane proteins in an active state (Catoire et al. 2010). However, it becomes clear that determining the atomic structure of membrane proteins isolated in detergent might not answer fundamental biological questions. Membrane proteins may also need to be studied in native-like lipid membranes, which is even more challenging (Abdine et al. 2010; Park et al. 2012).

There is a need to develop robust expression systems for producing membrane proteins in native membranes. Although mammalian cell-based expression systems have been very successful for crystallization of G protein-coupled receptors (GPCR; Tate 2012), microorganisms, mainly bacteria and yeast, are still subject to intense studies and technological developments. For instance, Le Maire and colleagues have obtained in 2005 the first X-ray structure of a eukaryotic membrane protein after overexpression in the yeast *Saccharomyces cerevisiae*, by fusing the rabbit sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA ATPase) with a biotin acceptor domain peptide (Jidenko et al. 2005). In parallel, 16 membrane protein structures have been obtained using the *Pichia pastoris* yeast expression system (for review, see Alkhalfioui et al. 2011), including two GPCRs. In bacteria, the lactobacillus expression system is highly promising because it has the main advantage of avoiding inclusion bodies formation (see Frelet-Barrand et al., Chap. 5 of this book and Frelet-Barrand et al. 2010 for review). However, the yield of membrane protein production remains low and, to our knowledge, this expression system has not generated any membrane protein structure.

The bacteria *E. coli* today is still the most widely used host for protein overexpression. Most prokaryotic membrane protein structures found in the PDB have been obtained after production of the corresponding protein in *E. coli*. Extending the production of membrane proteins in *E. coli* to eukaryotic sequences is facing two major problems: the formation of inclusion bodies (see the review from Banères, Chap. 3 of this book) and the toxicity associated with the induction of the target gene expression, which frequently results in cell death. This chapter will focus on the second aspect because overcoming the toxicity of expression has proven to be extremely useful and productive. A good example is given by bacterial mutants isolated using the T7 RNA polymerase-based expression system (see below for a full description). In this expression system, induction of the expression of the target gene by addition of the inducer Isopropyl- β -D-1-thiogalactopyranoside (IPTG) kills the cells, usually the BL21(DE3) bacterial host. This phenotype was used to screen for spontaneous mutants on IPTG-containing plates. Starting with the expression of the mitochondrial oxoglutarate carrier protein (OGCP) in the BL21(DE3) bacterial host, a first mutant was isolated, named C41(DE3), in which OGCP protein levels were strongly increased despite a tenfold reduction of corresponding mRNA levels (Miroux and Walker 1996). A second round of selection was conducted expressing *uncF*, which encodes AtpF, the *E. coli* b-subunit of the F1Fo ATP synthase, in C41(DE3) bacterial host. A second mutant C43(DE3) was isolated.

Overproduction of AtpF in its adapted bacterial host C43(DE3) resulted in the development of a large network of internal membranes. The bacterial host C43(DE3) reacted to the overproduction of a membrane protein by synthesizing lipids and by converting phosphatidyl glycerol into cardiolipids at the stationary phase (Weiner et al. 1984; Arechaga et al. 2000). Whereas de novo lipid synthesis may serve to maintain the lipid/protein ratio constant, the function of the increased cardiolipid content is unclear. Although the mutation in the C43(DE3) genome remains unknown, a delay in the transcription of the *uncF* gene (60 min) was observed, allowing membrane synthesis and proper folding of the b-subunit. Indeed, although AtpF forms inclusion bodies in C41(DE3) cells, it is readily inserted and folded in the membranes of C43(DE3) (Arechaga et al. 2000). Thus, slowing down the expression of *uncF* improved coupling between transcription, translation folding-insertion processes and consequently the storage of the b-subunit into internal proliferating membranes (Miroux and Walker 1996).

Membrane proliferation upon overexpression of a membrane protein has been observed before in *E. coli* (Weiner et al. 1984; von Meyenburg et al. 1984; Wilkison et al. 1986; Arechaga et al. 2000; Eriksson et al. 2009) and in the yeast (Wright et al. 1988). For instance, overproduction of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the yeast *Saccharomyces cerevisiae* resulted in the formation of paired membranes closely associated with the nuclear envelope called “Karmellae” (Wright et al. 1988). Proliferation of endoplasmic reticulum structures has also been observed upon the regulated overexpression of the P-type H(+) ATPase (Supply et al. 1993). However, in the case of AtpF, the stronger intensity of membrane proliferation opens a way to the study of AtpF in situ in its native membrane environment (see Chap. 12 from Catoire et al. of this book). Co-expression of AtpF with other membrane proteins of interest is also a promising avenue (Zoonens and Miroux 2010).

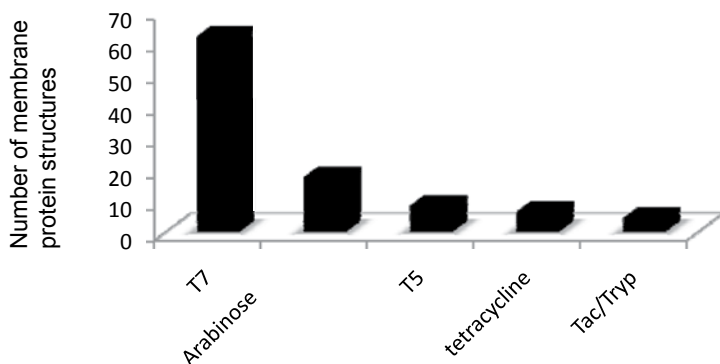


Fig. 4.1 Distribution of bacterial promoter usage in structural biology of membrane proteins (adapted from Hattab et al. 2014). A Hundred and fifty one unique membrane protein structures were extracted from the Protein Data Bank (Warschawski 2013; White 2013) on the basis of heterologous production of the protein in *Escherichia coli* (homologous production in *Escherichia coli* was excluded). The chart shows the number of solved membrane protein structures for each promoter used to produce the corresponding protein.

In order to assess the impact of these mutant hosts on structural biology of membrane proteins, we have conducted, 20 years after their discovery, a large-scale analysis of membrane protein structure databases (<http://www.drorlist.com/nmr/MPNMR.html> and <http://blanco.biomol.uci.edu/mpstruc/>; Hattab et al. 2014). Figure 4.1, adapted from Hattab et al. (2014), summarizes the PDB search and shows that the T7 RNA polymerase-based expression system (Novagen) accounts for more than 60% of membrane protein structures obtained after heterologous production in *E. coli*. The arabinose promoter-based expression system (Guzman et al. 1995) comes second, followed by T5 (Quiagen) and tetracycline promoter-based expression systems (IBA). Within the T7 expression system, the bacterial mutant hosts C41(DE3) and C43(DE3), commercially available from Lucigen, have been used for 50% of solved prokaryotic integral membrane protein structures so far. In this chapter, we will therefore focus on the T7 expression system, which, thanks to its multiple levels of regulation, has been the most successful in structural biology of membrane proteins.

4.2 Overview of the T7-Based Expression System

4.2.1 Regulation Levels of the T7 Expression System

In its most usual configuration, the T7 RNA polymerase gene is inserted in the genome of a lambda DE3 bacteriophage that is maintained into the lysogenic *E. coli* BL21(DE3) host. The T7 RNA polymerase gene is under the control of a *lacUV5*

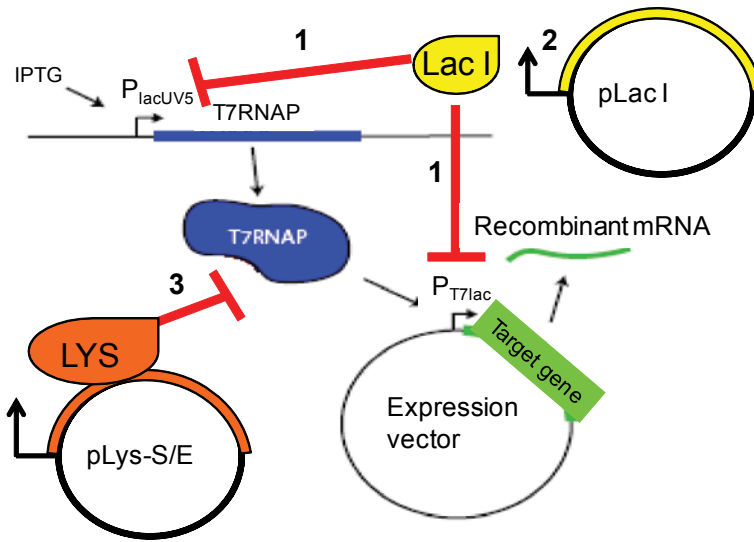


Fig. 4.2 Levels of regulation of the T7-based expression system. The amount of active T7 RNA polymerase is controlled in several ways: 1. repressing the *lacUV5* and *T7lac* promoters using the *lacI* repressor which binds to the *lacO* operating sequence; 2. expression of *lacI* gene from the expression plasmid or from a companion plasmid; and 3. expression of lysozyme, which inhibits the T7 RNA polymerase, from a companion plasmid.

promoter, which is weakly sensitive to cAMP regulation, associated with a *lacO*-regulating sequence (Fig. 4.2). The DE3 insert also contains *lacI* gene, which product represses the *lacUV5* promoter upon binding to the *lacO* sequence. The second level of regulation is the expression vector itself. In its simplest version, the vector only contains the T7 promoter (pRSET, Invitrogen; pMW7/pHIS; Way et al. 1990; Orriss et al. 1996), but many of the pET derivatives (Novagen) also contain a *T7lac* promoter that is fully repressed by the LacI repressor. In addition, the *lacI* gene is often expressed separately in a companion expression plasmid to ensure a multi-copy expression of the LacI repressor. A third level of regulation relies on the expression of lysozyme, either constitutively expressed or inducible by rhamnose (Wagner et al. 2008). Lysozyme specifically inhibits the T7 RNA polymerase, thus further attenuating the expression system. Two other parameters also influence the final strength and stability of the system: the plasmid copy number and antibiotic resistance genes. Multiple other versions of this expression system are still under development. For instance, in the BL21AI bacterial host (Invitrogen), the arabinose promoter replaces the *lacUV5* promoter. Expression of the T7 RNA polymerase can be titrated using increasing concentrations of arabinose. In the *Lemo* bacterial host, expression of the lysozyme is under the control of rhamnose promoter, which indirectly titrates the amount of active RNA polymerase via the expression of lysozyme (Wagner et al. 2008).

Table 4.1 Toxicity of T7 expression vectors

Plasmid name	Tag	Size of colonies on 2 × TY plate		
		BL21(DE3)	BL21(DE3)	C41(DE3)
		–IPTG	+IPTG	+IPTG
pMW7 ^a	None	Normal	None	Normal
pHis17 ^b	C-ter (6*) His	Normal	None	Normal
pET17b ^c	N-ter T7	Normal	Very small	Normal
pET29a ^d	N-ter S	Normal	None	Normal
pGEMEX-1	N-ter T7gene10	Small	None	Small

^aHigh copy number T7 plasmid from (Way et al. 1990)

^bDerivative of pMW7(Orriss et al. 1996)

^cpET series vector are low copy number derivatives of pBR322

^dContains T7/*lac*, *lacI* and Kan genes

4.2.2 Toxicity Associated with Membrane Protein Expression

Very early in the construction of the T7-based expression system, Studier and Moffatt noticed that the size of bacterial colonies on plate was dependent on the genomic insertion site of the lambda DE3 (Studier et al. 1990). Actually, the BL21(DE3) host was selected on its ability to form normal-sized colonies in the presence of expression plasmids but only in the absence of IPTG inducer. In the presence of IPTG, most expression plasmids, even without an inserted coding sequence behind their T7 promoter (“empty plasmid”), prevent the formation of colonies on plate. Table 4.1 gives an overview of different types of plasmids toxicities in the BL21(DE3) host. Very high copy number plasmids (>200 copies) that do not contain a *lacO* sequence, such as pMW7 or pHis vectors, do not allow colony formation on an IPTG-containing plate, even when they are empty. Low copy number plasmids (50 copies), i.e. deriving from pBR322, are slightly less toxic to BL21(DE3), showing that the expression plasmid copy number is an important parameter (Table 4.1, see pET17b phenotype). However, the addition of a coding sequence, even a small tag sequence such as S-Tag, completely prevents the growth on an IPTG plate. In contrast, none of the empty expression plasmids are toxic to the bacterial host C41(DE3), in which the production of T7 RNA polymerase is ten times decreased (Wagner et al. 2008). This suggests that a first level of toxicity occurs at the transcriptional level, when the T7 RNA polymerase is produced in excess. This basic level of toxicity does not necessarily compromise the successful expression of a target protein. Actually, in some cases where the target protein is produced at high levels, it can be advantageous to stop bacterial growth while expressing the target protein, in order to increase its concentration per cell and therefore to facilitate its purification. In isotope-labelling experiments, it can also be useful to specifically label the expressed protein so that, after purification, the remaining contaminants will be invisible on nuclear magnetic resonance (NMR) spectra. For this reason, we provide in the protocol section of this chapter some pragmatic tips to express proteins in toxic conditions.

1 Overload of the target mRNA



2 Unfolding stress and loss of periplasm integrity

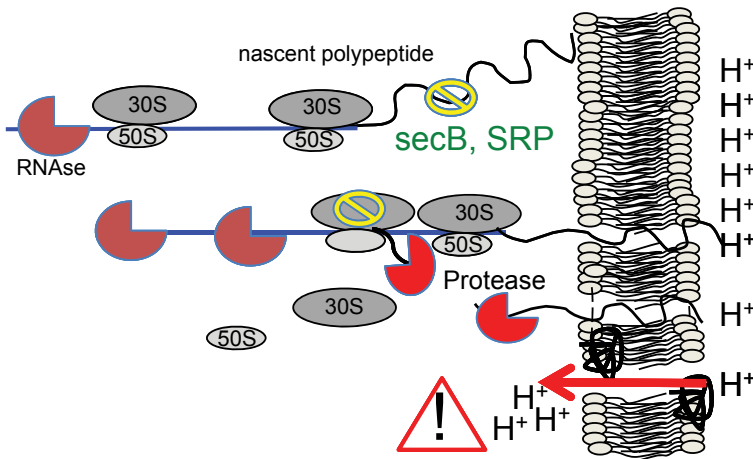


Fig. 4.3 Origins of toxicity during overexpression of membrane proteins in *Escherichia coli*. Overproduction of the target mRNA is toxic to the cell because it overloads the translation machinery at the expense of the cell's intrinsic protein synthesis (Dong et al. 1995). A second level of toxicity is linked to the folding and insertion of the newly synthesized protein. Co-translational folding of secondary structures is non-optimal and can lead to either inclusion bodies formation or mistargeting of the protein. Mistargeting occurs due to the lack of membrane-targeting signalling sequences and failure of chaperones to recognize the foreign protein sequence. A side effect of mistargeting the protein is the destabilization of the membranes upon aggregation of the proteins. Local disruption of the membrane triggers proton leak and loss of energy homeostasis.

A second level of toxicity occurs when the target protein, for instance a membrane protein, needs and therefore recruits and overloads the *E. coli* folding or insertion machinery (Wagner et al. 2007). In the best-case scenario, the chaperones recognize the foreign membrane protein but cannot synchronize its insertion into *E. coli* membranes because the T7 RNA polymerase is working too fast (Fig. 4.3). Consequently, an increasing fraction of the target membrane protein is partially inserted and folded in *E. coli* membranes, which in turn compromises ion gradient homeostasis and ultimately adenosine triphosphate (ATP) synthesis (Fig. 4.3).

Several strategies have been developed to overcome the toxicity associated with membrane protein expression: (1) adjusting the time course of expression of the target membrane protein by selection of bacterial mutants (Miroux and Walker

1996; see protocol in Sect. 4.3.2), (2) optimizing growth conditions (see protocol in Sect. 4.3.3 and Sevastyanovich et al. 2010, for review), (3) co-expressing bacterial chaperones (Chen et al. 2003), (4) inserting signal-targeting sequences to help the recognition of the foreign membrane protein by the *E. coli* machinery (for maltose-binding protein (MBP) fusion, see Miroux et al. 1993; Bocquet et al. 2008; Nury et al. 2011 as examples), (5) preventing misfolding into bacterial membranes by facilitating inclusion bodies formation (see Chap. 3 from JL Banère in this book and Mouillac and Banères 2010, for review), (6) introducing mutations into the target membrane protein to enhance its thermostability and/or its folding in vivo (Sarkar et al. 2008) and (7) cell-free expression of the target membrane protein using bacterial extracts (Rogé and Betton 2005; Miot and Betton 2011).

4.3 Protocol Section

4.3.1 *Choosing the Appropriate Strategy and Host/Vector Combination*

In a previous study (Hattab et al. 2014), we have conducted a systematic analysis of expression protocols in bacteria, based on membrane protein structures solved after heterologous expression of the protein in *E. coli*. Table 4.2 lists genotypes of all bacterial hosts that are used in structural biology of membrane proteins. Figure 4.1 summarizes one of the major outcomes of this study: T7 and arabinose-based promoters account for 80% of membrane protein structures. Therefore, we recommend running both expression systems in parallel to maximize your chances of getting your target membrane protein in sufficient amounts. The arabinose promoter-based expression system is well defined in terms of vector/bacterial host combination (Guzman et al. 1995). However, we have found ten membrane protein structures in the PDB that were solved after overproduction of the protein in the C43(DE3) bacterial host transformed with a pBAD arabinose-inducible vector (Hattab et al. 2014). This is unusual and requires further investigation. In this chapter, we focus on the T7 promoter-based expression system because C41(DE3) and C43(DE3) bacterial hosts account for 50% of heterologous integral membrane protein structures (Hattab et al. 2014).

A large survey on these bacterial host users revealed that high copy number vectors harbouring a wild-type T7 promoter, like the pRSET (Invitrogen), pMW7/pHis (Way et al. 1990; Orriss et al. 1996) or pPR/pPSG (IBA) vectors, are most frequently associated with C41(DE3) and C43(DE3) bacterial hosts. Avoid pET vectors bearing a pBR322 origin of replication and most importantly those carrying T7lac promoter and *lacI* gene. If you need to down-regulate your expression system, use, instead, the bacterial hosts BL21AI (Invitrogen) or C41(DE3) and C43(DE3) (Lucigen). In these hosts, the amount of T7 RNA polymerase is decreased or can be titrated with the inducer. Avoid companion plasmids that express lysozyme (pLyS/E) to inhibit the T7 RNA polymerase activity (Moffatt and Studier 1987) because they

Table 4.2 Genotypes of *Escherichia coli* hosts used for structural determination of membrane proteins

<i>T7-based expression hosts</i>	<i>Genotype</i>
BL21λ(DE3)	F- <i>ompT hsdS</i> (rB- mB-) <i>dcm gal</i> λ(DE3 [<i>lacI lacUV5-T7 gene 1 ind1 sam7 nin5</i>])
C41λ(DE3)	BL21λ(DE3 [<i>lacI lac-T7 gene 1 ind1 sam7 nin5</i>])
C43λ(DE3) ^a	C41λ(DE3) derivative
BL21λ(DE3) pLysS	BL21λ(DE3) pLysS (CamR)
BL21λ(DE3) CodonPlus ^a	BL21 <i>dcm</i> + TetR λ(DE3) <i>endA Hte</i> [<i>argU proL</i> CamR]
BL21 Star λ(DE3)	BL21 <i>rne131</i> λ(DE3)
BL21 Rosetta λ(DE3) pLysS	BL21 λ(DE3) pLysSRARE (CamR)
BL21λ(DE3) Tuner	BL21 <i>lacY1</i> λ(DE3)
BL21(AI)	BL21 <i>lon araB::T7RNAP-tetA</i>
<i>Other expression hosts</i>	<i>Genotype</i>
BL21Rosetta	BL21 RARE (CamR)
BL21-Gold	BL21 <i>dcm</i> + TetR <i>endA Hte</i>
BL21-T1 ^R competent	<i>fhuA2</i> [<i>lon ompT gal</i> [<i>dcm</i>] Δ <i>hsdS</i>
Origami B	BL21 <i>lacY1 aphC gor522::Tn10 trxB</i> (KanR TetR)
B834	F- <i>ompT hsdSB</i> (rB- mB-) <i>gal dcm met</i>
BLR	F- <i>ompT hsdSB</i> (rB- mB-) <i>gal dcm</i> Δ(<i>srl-recA</i>)306::Tn10 (TetR)
DH10B	F- <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i>
TOP10	<i>nupG recA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galE15 galK16 rpsL endA1</i> λ-
	DH10B <i>rpsL</i> (StrR)
KRX	[F', <i>traD36, ΔompP proA</i> + B + <i>lacIq</i> Δ(<i>lacZ</i>)M15] Δ <i>ompT endA1 recA1 gyrA96</i> (Nalr) <i>thi-1 hsdR17</i> (rk-mk +) e14- (McrA-) <i>relA1 supE44</i> Δ(<i>lac-proAB</i>) Δ(<i>rhaBAD</i>):T7 RNA polymerase
XL10-Gold	F' [<i>proAB lacIqZ</i> ΔM15 Tn10(TetR Amy CmR)] <i>recA1 endA1 glnV44 thi-1 gyrA96 relA1 lac Hte</i> Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 TetR
XL1-Blue	F' [<i>proAB, lacIq Z</i> ΔM15 Tn10(TetR)] <i>recA1 endA1 gyrA96 thi-1 relA1 supE44 hsdR17</i> (rk-mk +) l-
DH5α	F- <i>ø80dlacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rk-mk +) <i>phoA supE44</i> λ- <i>thi-1 gyrA96 relA1</i>
SG13009	NaI[s] Str[s] Rif[s] Thi[-] lac[-] Ara[+] Gal[+] Mtl[-] F[-] RecA[+] Uvr[+] Lon[+]
LS6164	Δ <i>fadR</i> Δ <i>fadL</i>
MC4100	F- [<i>araD139</i>]B/r Δ(<i>argF-lac</i>)169* &lambda- <i>e14-flhD5301</i> Δ(<i>fruK-yeiR</i>)725 (<i>fruA25</i>) <i>relA1 rpsL150</i> (strR) <i>rbsR22</i> Δ(<i>fimB-fimE</i>)632(::IS1) <i>deoC1</i>
SCM6	NS (Patented)
MC1061	F- Δ(<i>ara-leu</i>)7697 [<i>araD139</i>]B/r Δ(<i>codB-lacI</i>)3 <i>galK16 galE15</i> λ- e14- <i>mcrA0 relA1 rpsL150</i> (strR) <i>spoT1 mcrB1 hsdR2</i> (r- m+)
JM83	<i>rpsL ara</i> Δ(<i>lac-proAB</i>) Φ80 <i>dlacZ</i> ΔM15
Other	PA(Δ <i>oprH</i>)

^aAlso used in the arabinose expression system

require the addition of a second antibiotic (chloramphenicol), which quite substantially decreases cell growth. Moreover, excess of lysozyme impairs cell growth.

Once you have chosen your T7 vector, you need to decide whether to make a fusion to either direct your target gene to the *E. coli* membrane (for MBP fusion, see Bocquet et al. 2008 and Nury et al. 2011 as examples) or form inclusion bodies (for $\alpha 5$ integrin fusion, see Mouillac and Banères 2010, for review). If your protein is of prokaryotic origin, avoid fusion protein constructs or use a green fluorescent protein (GFP) fusion to monitor the yield and aggregation state of your protein on crude extract before any purification step (Drew et al. 2006). GFP fusions also offer the great advantage either to directly assess the production of your protein (Sarkar et al. 2008) or to select new bacterial hosts (Walker and Miroux 1999; Alfasi et al. 2011). If you wish to express an eukaryotic protein, be aware that there are almost no solved integral eukaryotic membrane protein structure after production in *E. coli*. There is one noticeable exception where the author succeeded in refolding and transferring directly the CXCR receptor into liposomes and solved the structure of the receptor by solid-state NMR analysis (Park et al. 2012). Thus, refolding of inclusion bodies from integral eukaryotic membrane proteins is an emerging promising avenue (see Chap. 3 from Banères and Chap. 12 from Catoire et al. of this book and references herein; Catoire et al. 2010; Banères et al. 2011; Park et al. 2012).

4.3.2 Selection of Bacterial Mutant Hosts

Transformation Transform your expression plasmid into BL21(DE3), which is the best host to start with because its induction power is maximal and easy to down-regulate. Prepare 2×Tryptone Yeast (TY) plates with antibiotic and IPTG. Two concentrations of IPTG may be used, i.e., 0.4 and 1 mM (Hattab et al. 2014). Use calcium chloride transformation with 10 ng of plasmid. After 1-h incubation at 37°C of the 1-ml transformation culture, plate 100 μ l on 2×TY plate with antibiotic and 100 μ l on 2×TY plates with antibiotic and both 0.4 and 1-mM IPTG concentrations. If you do not get any colony on any 2×TY plates even in the absence of IPTG, then switch to an electroporation protocol. In all cases, check that you do not have any colony on any IPTG plate. If you have the same number of colonies in the presence and in the absence of IPTG, the expression of your protein is not toxic or is partially toxic but you cannot select mutants. If you get hundreds of colonies in the absence of IPTG but very few in the presence of IPTG, some mutants may appear at high frequency. To make sure that you do not carry any contamination, repeat the experiment from a single colony culture after transformation of your bacterial host with a freshly prepared plasmid.

Culture and Mutant Isolation You should have 50 ready-to-use plates, supplemented with IPTG and antibiotic. Make sure the plates are not wet (incubate them for 16 h at 37°C). Prewarm five 250-ml flasks containing 50 ml 2×TY medium with antibiotic and inoculate each flask with one bacterial colony to perform five independent selection experiments the same day. Measure the optical density at 600 nm

every 30 min starting 2 h after inoculation. Meanwhile, in sterile conditions, label 40 clean and autoclaved Eppendorf tubes and add 900 μl of sterile water in each tube to perform 1/10 serial dilutions of each culture. Water is preferable to $2\times\text{TY}$ to avoid external contamination. Once the culture has reached 0.4–0.6 $\text{OD}_{600\text{ nm}}$, induce the expression of the target gene by adding IPTG at 1 mM final concentration. One hour after induction, transfer 1 ml of the culture into a new clean and sterile Eppendorf tube and gently spin it down for 2 min at 300 g. Discard the supernatant (secreted β -lactamase often gives false positive colonies when the culture is plated without dilution) and resuspend the pellet in 1 ml sterile water. Perform serial 1/10 dilutions until 10^{-4} and immediately plate 100 μl of all dilutions on IPTG and antibiotic-containing plates. Repeat the experiment 2 h after induction. The purpose of diluting the culture is that it is critical to have less than 200 colonies on a plate so that individual colonies can easily be sub-cloned and isolated. Given that the number of mutant hosts appearing on the plate is difficult to predict, it is safer to have extended dilutions. The frequency of appearance of mutant hosts varies from $1/10^{-4}$ to $1/10^{-6}$ (Miroux and Walker 1996). A 1/100 dilution is often the best compromise.

After an overnight incubation at 37°C (or at a lower temperature for thermo-sensitive mutants), estimate the number of colonies of different sizes. Typically, you should see a majority of large colonies, which, in most of cases, have lost the ability to express the target gene. Small colonies arise at a frequency of 1–20%. If you do not see any obvious difference in sizes between colonies, there are two plausible explanations: (1) The cells need to grow for a longer period of time; leave the plates for 8 additional hours at 37°C to reveal mutant clones of smaller sizes. (2) The cells that have lost the expression of the target gene divide faster, rapidly overgrow the culture and outcompete bacterial mutants that form small colonies. In this case, repeat the selection experiment and plate the culture shortly after induction (20–30 min) to avoid “dilution” of small colonies on the plates.

Figure 4.4 provides examples of selection experiments with the GFP as a reporter gene. Panel a shows the typical size difference between mutant hosts. Panel b shows the same plate under UV exposure. Almost all the small colonies are green and therefore express high amounts of GFP. Large colonies exhibit no or weak fluorescence. Panel c shows a selection experiment where all colonies are small. Among them, some exhibit very high fluorescence intensity. Panel d shows that, in this experiment, medium colonies are fluorescent while the very small ones are not. If you do not have GFP co-expressed or in fusion with your target membrane protein, then incubate 50 2-ml $2\times\text{TY-Amp}$ tubes, each containing one small colony (ten small colonies per selection experiment), and make an over-day culture. When the culture is turbid (2–3 h after inoculation), add IPTG (1 mM final concentration) and induce synthesis of your target protein overnight. The next morning, run 10 μl of the overnight culture on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and check the expression of your membrane protein either by immuno-detection with a specific antibody against your protein (or against a tag) or simply by staining the gel with Coomassie blue.

Once you have isolated interesting mutant hosts, you have to cure the strain from the plasmid and check if the mutation is within the bacterial or the plas-

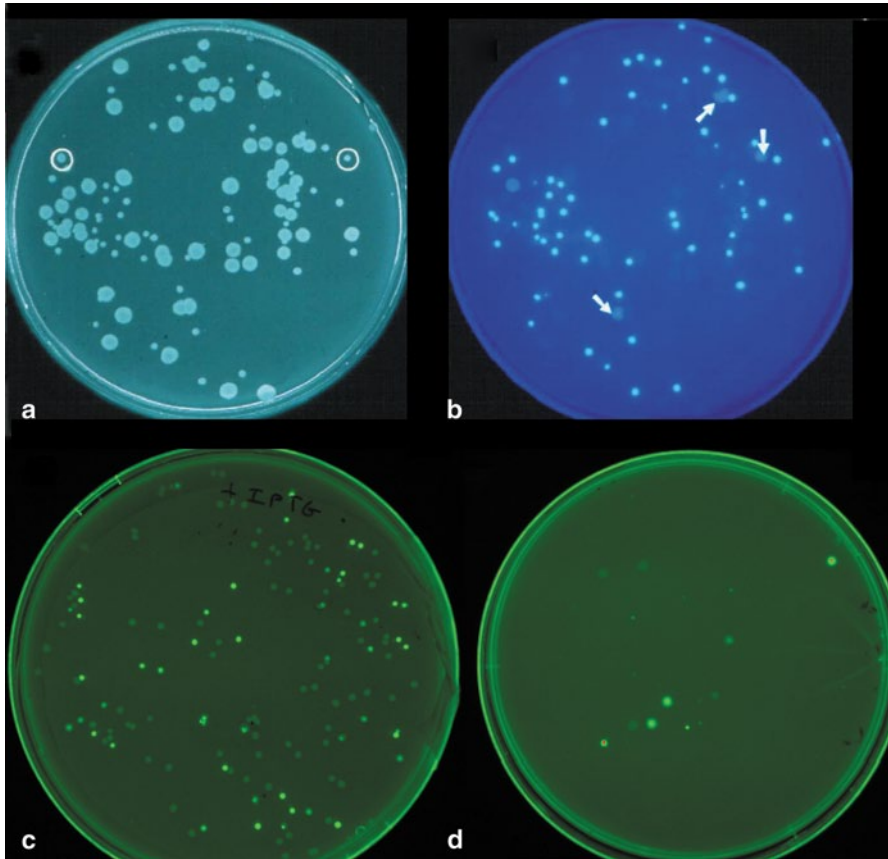


Fig. 4.4 Selection of bacterial hosts using GFP as gene reporter. Isolation of bacterial mutant hosts was performed according to the protocol in Sect. 4.3.2 and to Miroux and Walker (1996) and Walker and Miroux (1999). Briefly, pMW7-GFP-Xa expression plasmid was transformed into BL21(DE3) host (a, b and c) or into C41(DE3) host (d) and a single colony was inoculated in 50-ml $2 \times$ TY medium. At $OD_{600\text{ nm}}=0.4$, cells were diluted in water and 100 μ l of the 1/10 dilutions were plated on IPTG-containing plates. The plates are illuminated under normal light (a) or UV light (b, c and d).

mid DNA (Fig. 4.5). To do so, prepare a miniprep of plasmids from each clone, transform them into the BL21(DE3) reference host and check colony formation on IPTG-containing plates (left panel). If you obtain colonies, then the mutation is within the plasmid; if not, then the bacterial host carries the mutation. To cure the strain from the plasmid, the easiest method is to wait for spontaneous loss of the plasmid (right panel). Inoculate a 50-ml $2 \times$ TY culture without antibiotic with one single colony and maintain the culture for a week by transferring every day 100 μ l of the culture into a new Erlenmeyer containing 50 ml fresh medium. Every day make serial dilutions of the new culture until 10^{-8} and plate 100 μ l of the 10^{-6} , 10^{-7} and 10^{-8} dilutions on IPTG-containing $2 \times$ TY plates without antibiotic. Since your

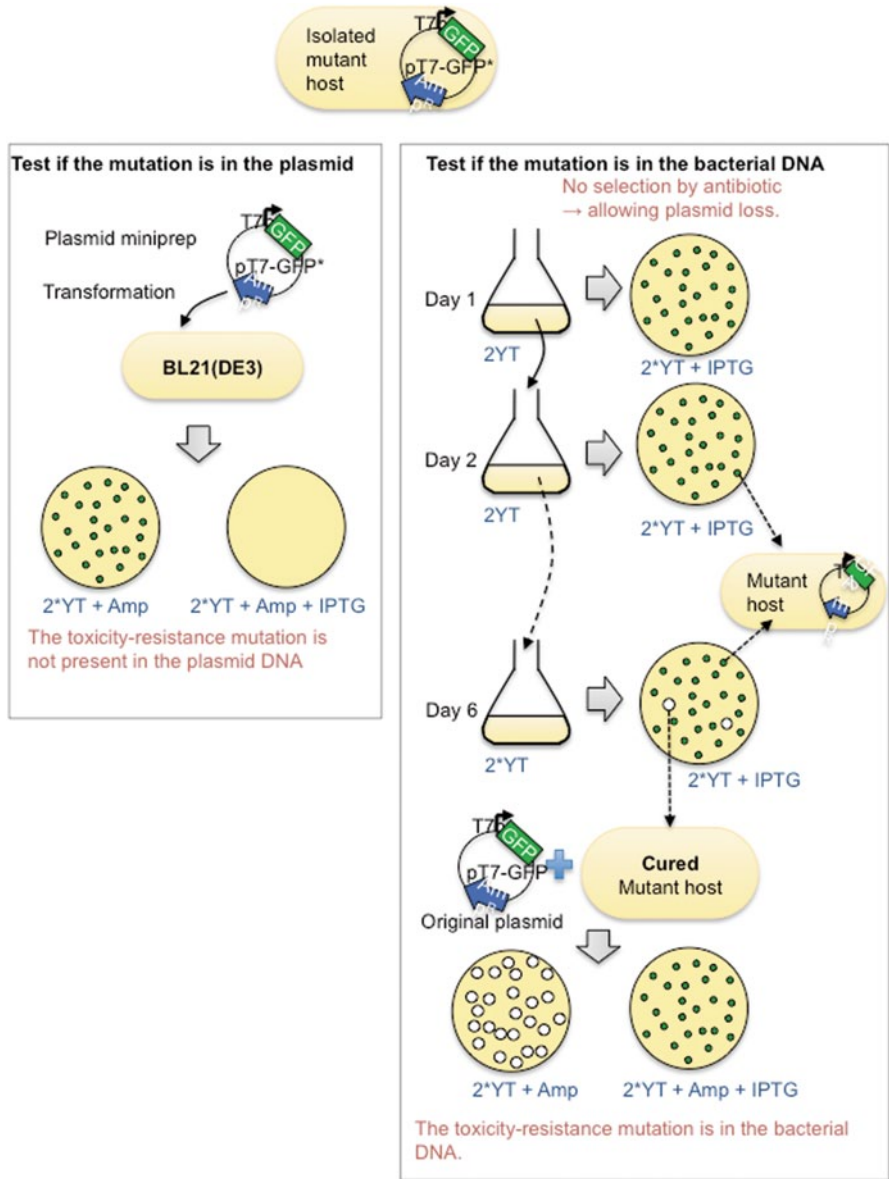


Fig. 4.5 Localization of the mutation in the isolated bacterial mutant host. This step is performed according to the protocol in Sect. 4.3.2, Miroux and Walker (1996) and Walker and Miroux (1999). Basically, your isolated mutant strain has to be cured from the expression plasmid (here, pT7-GFP*) to check that the mutation is present in its genomic DNA (and not in the plasmid DNA). *Left panel:* the plasmid pT7-GFP* is rescued from the mutant strain and transformed into the original BL21(DE3) host. The transformation is plated onto 2 × YT plates with antibiotic, with and without IPTG. If no colonies are formed in the presence of IPTG, this means that the expression of the target gene from this plasmid is still toxic to BL21(DE3) and, therefore, that the mutation that removed the toxicity is absent from the plasmid. *Right panel:* in parallel, the mutant strain is cured

mutant forms small colonies on these plates, cells that have lost the plasmid over the 1-week-time culture period should form large colonies (Fig. 4.5). Isolate two of those colonies and check that they are antibiotic sensitive. Prepare competent cells from these isolated mutant hosts, transform your reference expression plasmid and plate half of the competent cells on a $2 \times$ TY plate with antibiotic and the other half on a $2 \times$ TY plate supplemented with both antibiotic and IPTG. You should see the same number of colonies on both plates, the IPTG-containing plates carrying only small ones.

4.3.3 Tuning Growth Conditions

This guideline is adapted from previous reviews (Shaw and Miroux 2003; Zoonens and Miroux 2010) and enriched with rules from a large-scale bibliographic analysis of the T7-based expression system that we have recently conducted (Hattab et al. 2014). The protocol is divided into two parts, depending on the toxicity of the expression system. For simplicity, we only refer to the T7-based expression system but most advices that are given below can be applied to expression systems other than T7 based.

4.3.3.1 Expression of the Target Gene is Toxic

Despite the toxicity of the target gene, it is possible to optimise the expression level of the corresponding protein by adjusting growth conditions.

Plasmid Stability Transform your expression plasmid on a $2 \times$ TY plate with antibiotic and prepare five individual 2-ml precultures from independent colonies. After overnight growth, make serial 1/10 dilutions and plate 100 μ l of 10^{-6} , 10^{-7} and 10^{-8} dilutions on $2 \times$ TY plates with and without antibiotic. If you get the same number of colonies with or without the presence of antibiotic, then the plasmid is stable and you can proceed with larger cultures. If the number of colonies is increased on $2 \times$ TY plate without antibiotic, then it is unsafe to prepare a large culture from a preculture.

Large-Scale Experiment Start from freshly transformed bacterial cells. Some authors do not plate cells after heat shock but use the whole transformation medium as a preculture (Rogé and Betton 2005). By doing this, they avoid strong

from the plasmid by dropping the selective pressure by antibiotic. Serial cultures are performed for a week, during which each is plated on $2 \times$ YT plates with IPTG, after serial dilutions. Mutants that have lost the plasmid will form large colonies that are no longer GFP positive. These cured mutants are then transformed again with the original expression plasmid pT7-GFP and plated on $2 \times$ YT plates, with and without IPTG. If small colonies are retrieved on both plates, this will mean that the strain contains a mutation in its DNA that allows it to overcome the toxicity associated with the expression of the target gene.

Table 4.3 Optimisation of growth conditions in the IPTG inducible T7 expression system

Size of colonies on IPTG plate ^a	Inoculation	Induction	IPTG concentration	Temperature after induction (°C)
No colony	No preculture ^b	No induction ^c OD _{600 nm} = 1	None 10 µM ^d , 0.1 µM	30 or below
Small (> 10% reduction)	Preculture ^c	OD _{600 nm} < 0.6	0.4 or 1 mM	37 or 25
Minor reduction (< 10%)	Preculture	OD _{600 nm} < 0.4	1 mM	37 or 25

^aTry 1 mM and 0.4 mM of IPTG and check phenotype on plates at 37°C and room temperature

^bIf you need a preculture to grow large volumes or to inoculate a fermenter then check plasmid stability

^cSee (Walse et al. 2008; Fairman et al. 2012)

^dSee (Alfasi et al. 2011) for a complete description of the procedure

^ePre-warm the medium and use the preculture at 1/100 dilution; when the plasmid is stable antibiotic is no longer required in the large culture

variability in the target protein expression level from one colony to the other. Prewarm 500 ml of 2 × TY medium in a 2.5-L Erlenmeyer. This is a critical step if you wish to perform the experiment over a day. An alternate option is to incubate the flasks overnight in a 37°C incubator and to add antibiotic the next morning just before use. Inoculate the warm medium with one single colony and follow the optical density at 600 nm. The culture should reach an optical density of 0.6 in less than 5 h; if not, then the basal level of expression of your target gene is sufficient to severely impair cell growth. In addition to the classical protocol of induction (1-mM IPTG at OD_{600 nm} = 0.6), there are two alternative protocols worth trying (Table 4.3): (1) Do not add IPTG; let the culture grow overnight at 30 or 37°C. This protocol works well when your high copy number plasmid is not regulated (no *T7lac* promoter or *lacI* gene) and in combination with the regular BL21(DE3) bacterial host without a companion plasmid (pLysS/E). We have found two membrane protein structures where the authors specifically mentioned this protocol (Walse et al. 2008; Fairman et al. 2012). (2) Add IPTG at the beginning of the stationary phase either in trace amount (10 µM) following the “improved protocol” from Alfasi and colleagues (Alfasi et al. 2011) or at a high concentration (1 mM).

4.3.3.2 Expression of the Target Gene is Non-toxic or Moderately Toxic

In this configuration, the induction of the expression of the target gene slows down cell growth but does not compromise colony formation on IPTG plate. The expression plasmid is usually highly stable and, in most cases, you can use a preculture to inoculate large flasks. This is also an ideal configuration for using bioreactor, as you do not have to worry about plasmid loss at high cell density. In several occasions, we have observed that antibiotic use is not required anymore in the culture, provided that you have added antibiotic to the preculture (Shaw and Miroux 2003).

The induction protocol must be adjusted depending on the size of the colonies you get on IPTG plate. If the size reduction is marginal ($<10\%$), it is not necessarily a good sign because it could simply mean that the production of your target membrane protein is very low. To maximize your chances of having high level of expression of the target gene, add 1 mM IPTG at the early stage of the exponential phase ($\leq 0.4 \text{ OD}_{600 \text{ nm}}$). If the size of the colonies is decreased by 10% or more, then add IPTG at $\text{OD}_{600 \text{ nm}}=0.6$ and test the two concentrations that are most frequently used (Hattab et al. 2014): 0.4 and 1 mM (Table 4.3).

4.4 Frequently Asked Questions

1. What are the main differences between C41(DE3) and BL21(DE3) bacterial hosts?

C41(DE3) is a derivative of BL21(DE3), isolated on an IPTG plate upon the expression of oxoglutarate mitochondrial carrier, expressed as inclusion bodies. We initially observed that the level of the oxoglutarate mRNA was ten times decreased in this host, 3 h after the addition of IPTG (Miroux and Walker 1996). The group of de Gier has recently shown that expression of the T7 RNA polymerase is strongly decreased in both C41(DE3) and C43(DE3) hosts, thus explaining the reduction in target mRNA levels. The mutation in C41(DE3) is most likely the replacement of the *lacUV5* promoter located upstream of the T7 RNA polymerase by the genomic wild-type copy of the *lac* promoter (Wagner et al. 2008).

2. Does the C43(DE3) bacterial host produce constitutively internal membranes?

No. Internal membrane proliferation occurs upon overexpression of the b-subunit of the *E. coli* F_1F_o ATP synthase, which was used to isolate the mutant host from C41(DE3). Membrane proliferation has been observed by electron microscopy on bacteria cross section 3 h after induction by IPTG at 37 or 25 °C in $2 \times \text{TY}$ medium. The best pictures were taken after an overnight induction at 25 °C (Arechaga et al. 2000).

3. What is the purpose of decreasing culture temperature upon induction by IPTG?

The main advantage of decreasing the temperature is to slow down the activity of the transcription/translation machinery and consequently cell growth. At 20 °C, *E. coli* does not initiate translation, which helps reducing translational stress. For soluble proteins, it has been shown to increase target protein solubility. It also helps the insertion of MBP fusion proteins into the bacterial membrane. Another reason to reduce culture temperature is to avoid overgrowth of the culture by cells that have lost the expression plasmid. Therefore, decreasing the temperature is highly recommended when the expression of the target membrane protein is toxic. Using C41(DE3) at 25–20 °C is often optimal while overexpression of the target gene below 37 °C in C43(DE3) is unpredictable and gene dependent.

4. Does removing the toxicity by selection of bacterial mutant hosts always increase the yield of expression of the protein?

No, unfortunately. A good example is given by the a-subunit of the *E. coli* F₁F₀ ATP synthase. The *uncB* gene, encoding the a-subunit, is regulated by RNA degradation and, consequently, its expression under the T7 promoter does not increase the amount of *uncB* mRNA or the a-subunit peptide (Arechaga et al. 2003). If your target mRNA is not overexpressed, then try breaking mRNA secondary structures by using silent mutations. A complete synthetic gene might help although internal mRNA degradation sites are difficult to predict. Protein degradation is also frequent but can be overcome by making fusion proteins.

5. Is supplementing tRNA for rare codons useful?

We have found that 10% of membrane protein structures have been solved following expression of the protein in the BL21(DE3)CodonPlus bacterial host. On the one hand, it is not negligible and certainly worth trying. In addition, the group of von Heijne has recently demonstrated that codon optimization is critical in the N-terminal sequence of the protein to ensure a proper initiation of translation (Nørholm et al. 2013). It has also been suggested that rare codons are useful for co-translational folding of the nascent polypeptide (Pechmann and Frydman 2013).

6. Are culture media important for protein expression in C41(DE3)/C43(DE3)?

The bacterial mutant hosts C41(DE3) and C43(DE3) support all classical media (minimal medium, Luria Bertani, LB, 2×TY, terrific broth, TB). However, as a general rule, we have observed that toxicity of expression plasmids is increased in LB medium and decreased in 2×TY medium. As expected, levels of expression of the target gene are increased in 2×TY or TB medium.

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