Transformation and Protein Expression

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Abstract

Transformation is an important step in recombinant DNA technology that allows transfer of genetic material inside cells. Often, this transfer accompanies a change in trait of the cells depending upon the genetic material used. Together, the transfer of genetic material and change in trait of cells is defined as transformation. Since its discovery, this technique is credited with countless profound findings in biology and holds an important position in a biologist's toolbox for manipulating DNA and cells. In this chapter, fundamental information essential for in vitro transformation, together with a series of principles and protocols that are routinely used in transforming bacterial cells, are discussed. Dedicated sections have been provided to preparation of competent cells, in vitro cellular transformation methods, and posttranslational protein modification in bacterial expression systems. Further, acknowledging the popularity of bacteria as "protein-production factories," special sections have been devoted to using different types of bacteria and optimizing gene expression in them. Additionally, discussion on approaches to troubleshoot difficulties and standardize experimental protocols provided comprehensiveness to the chapter. Several fundamental problems related to this topic have been discussed at the end of the chapter for the readers to further enhance their understanding on the topic by actively involving themselves in an experience that resembles the routine experimental protocols pertaining to recombinant protein expression.

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Transformation · Posttranslational modification · Competent cells · Protein expression · Bacterial expression systems

4.1 Introduction

A groundbreaking discovery that revolutionized the field of molecular biology is the ability to modify the genetic material of cells. Genetic alteration requires that the recipient cells allow entry to foreign genetic material, its successful incorporation, and stable expression so as to bring changes in cellular behavior. Let us imagine that a geneticist wants to investigate the functions of a gene that he thinks might be responsible for a particular behavior of a cell or a tissue. In particular, the geneticist is inquisitive about the role of this gene in a human disease. He also wants to understand the design (nucleotide sequence), regulation, and mutations that contribute to the distinct functions of the gene! To address the abovementioned questions, the geneticist should not only find a way to obtain this gene in sufficient amount in the genome, but also be able to artificially introduce it into the cells to express it so as to study and manipulate its functions. Interestingly, the technique of in vitro transformation solves this problem by allowing transfer of naked fragments of foreign genetic material inside target cells by artificially permeabilizing the cell membrane. In most cases, the genetic material is a plasmid harboring complementary DNA (cDNA) or a gene that is inserted in target cells using chemical, physical, or biological methods. As a result of the transformation process, the genotype of the recipient cells is modified.

Historically, the discovery of natural genetic transformation was one of the key events in biology that stems from the work on *Streptococcus pneumoniae* in 1928 by Frederick Griffith. This work laid the foundation for the identification of DNA as the genetic material in most living organisms [1]. As the field advanced, transformation was demonstrated to be a frequently occurring natural process and was soon accepted to be a common mechanism for generating and maintaining genetic diversity in bacteria. With advancement of research in this area, this naturally adept molecular biology tool was soon introduced into biomedical and industrial applications. The scope of this technique for manipulation of genotype was further increased by inspecting eukaryotic cells for transformation. Since the genetic code is fairly conserved across the three domains of life, protein coding genes from eukaryotes can be expressed in prokaryotes and vice versa. The oldest published report on artificial transformation of animal cells comes from the injection of DNA from the tissue of one bird into an individual of the same species by Benoit et al. in 1957 [2]. It should be noted that the term "transformation" in the context of mammalian systems is generally used to describe the transition of a cell from normal to cancerous phenotype [3]. Hence, in mammalian systems the term "transfection" is coined for the addition of exogenous DNA to the cell. However, this chapter will

confine itself toward understanding of "transformation" into the bacterial hosts for recombinant protein purification in a laboratory setup.

Recombinant DNA technology coupled with transformation/transfection provides us the opportunity to express proteins in bacterial, archaeal, yeast, mammalian, and plant cells for a multitude of purposes that include studying the functional implications of proteins in specific diseases, and large-scale production of enzymes for therapeutic and industrial use. In view of the fact that several different organisms have been adopted as hosts for expression of genes, the choice of the expression system depends on multiple factors. Prime considerations while selecting an appropriate host include the quantity of the protein required, size of the protein, and any disulfide bonds or posttranslational modifications of the protein. For example, the use of *E. coli* for protein production is a simple and economical method of producing bulk quantities of proteins that do not require posttranslational modification (PTM) for functioning; however, proteins that require PTM to function should be expressed in a eukaryotic host system. In this chapter, we discuss the different methods of transformation and the types of expression systems that can be used for protein production, however focusing majorly on the bacterial host systems.

4.1.1 Competence and Competent Cell Preparation

Before a cell could undergo transformation, it has to be "competent." Competence in terms of genetic transformation is defined as the inducible trait of cells for both importing and processing foreign genetic material [4]. The second component in the definition of competence is essential to generate new genotype because the internalized foreign DNA faces three fates inside a recipient cell, which decide the success of transformation. Firstly, the DNA might be rejected and degraded by nucleases; secondly, it might be inserted into the chromosome; and lastly, it might co-exist with chromosome. Competence in most naturally transformable organisms is genetically regulated by dedicated proteins that cause the uptake and processing of DNA. These proteins known as *competence-specific proteins* are a collection of membrane-embedded DNA-binding proteins, various nucleases, DNA importer enzymes, methylases, and recombinases [5, 6]. The molecular machinery involved in regulating competence might perhaps not be constitutively active inside bacteria but instead can respond to specific extracellular or intracellular signals in order to develop competence. Such a form of competence is termed as spontaneous transformation. On the other hand, some bacterial species could lack transformation-specific DNA-uptake and processing genes and thus might not be naturally transformable. However, both bacterial and eukaryotic cells can be artificially forced to enter a transient state of competence along with introduction of the desired DNA in the growth medium to enable in vitro genetic transformation [4]. Competence is thus a transient opportunity for gene introduction (Fig. 4.1). Extracellular DNA that is impure, often damaged, and in trace concentrations is not a rare sighting in a cell's natural environment, where it has naturally evolved. The probability of transformation in natural conditions is thus reduced by natural barriers such as adsorption of

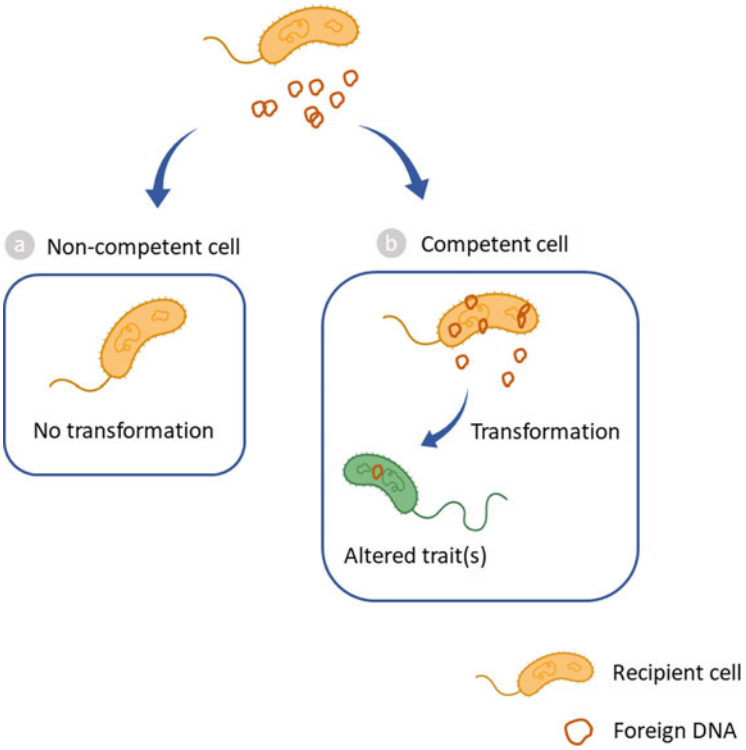

Fig. 4.1 Cellular competence and its role in transformation. (**a**) A cell such as a bacterium might not be naturally able to import and process foreign DNA. Such a cell is termed non-competent. (**b**) DNA could be imported and processed by a competent cell, leading to change in its trait(s). Competence could be artificially generated

DNA by particles in soil and suboptimal temperature, pH, osmolarity, and shear force [7, 8].

In contrast, optimization of the abovementioned parameters in laboratories provides much higher transformation efficiencies. Having appreciated the key importance of cellular competence in transformation, let us now explore how cells could be made competent artificially.

4.1.2 Competent Cell Preparation

In cloning step, the most commonly used bacterial species for transformation is *E. coli*. The history of artificial bacterial transformation began with the thought that *E. coli,* a commonly used laboratory organism, was resistant to transformation. However, in 1970, Mandel and Higa demonstrated that after treatment with calcium chloride ($CaCl_2$), *E. coli* might be induced to take up DNA from bacteriophage λ without the use of a helper phage [4]. Two years later, another group of scientists

showed that $CaCl_2$ treatment is also effective for transformation using plasmid DNA [5]. The original method was further improved by Douglas Hanahan.

Since then, numerous methods involving chemicals have been used to make cells competent such as by using other monovalent and divalent cations, polyethylene glycol (PEG) or dimethyl sulfoxide (DMSO) [9]. Other than these chemical methods, electroporation has also been extensively developed and used to induce competency [10–12]. However, the requirement of special equipment has limited the popularity of this method. Nonetheless, the use of cations has proven to be the most effective chemical treatment to bring about bacterial transformation. Among various cations, use of Mn^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, and Mg^{2+} has proven to be effective [13].

Although the factors that regulate competence differ among various genera and remain as the key for the process, a simple procedure to acquire cells of reasonable competency using the chemical method is described below.

4.1.3 Chemical Method

Principle: High polarity and an overall negative charge of DNA pose a barrier for its transfer across the cell membrane. Hence, to facilitate the entry of DNA inside bacteria, its charge must be neutralized. Additionally, the cell membrane of bacteria must be transiently modified to neutralize charges as well as create pores using cations followed by a brief pulse of heat. This method is known as the heat-shock method of transformation [9]. Therefore, prior to exposure of cells to DNA and varying temperatures, they must be made competent using chemicals such as calcium chloride. Other key component in this protocol is Glycerol. The process of calcium chloride-based competence generation encourages rapidly growing bacterial cells to uptake DNA from the surrounding environment. The exact mechanism of how this process works is still largely unknown, but there are hypotheses on the different aspects of the procedure. The main role of calcium ions in the cell suspension is hypothesized to be a cation bridge that reduces repulsion between the phosphate backbone of foreign DNA and phosphorylated lipid A in lipopolysaccharide (LPS) of bacterial cell, owing to the negative charges on both. While the calcium ions neutralize the charge, they can also serve to cause folding of DNA molecule into a ball-like structure that enters cells easily. Glycerol brings DNA close to the surface of the cell due to molecular crowding, and also protects cells when stored at freezing temperatures. During the subsequent heat-shock transformation procedure, the heat pulse at 42 °C is believed to cause temporary pores in lipid membrane through which foreign DNA enters a cell. 10^5 to 10^7 colonies of transformed *E. coli* per μg of DNA generated by this method is more than enough for routine work such as mass-producing plasmid DNA or protein production [14]. However, for special purpose of cDNA library creation that includes environmental DNA sample, a higher number of transformed colonies are desirable.

Based on this method, we shall now discuss the protocol for generating competency in bacteria using *E. coli* as an example.

Protocol

This protocol for competence generation in bacteria is based on the original protocol published by Hanhan et al. [14]

Materials

- 2 mL of *E. coli* starter culture grown overnight
- 100 mL of sterile fresh Lysogeny Broth (LB) medium, pre-warmed to 37 °C
- 500 mL of sterile ice-cold Transformation Buffer 1 (TfB1)
- 100 mL of sterile ice-cold Transformation Buffer 2 (TfB2)

Equipment

1. 37 °C shaking incubator
2. Spectrophotometer/Colorimeter at 600 nm wavelength light
3. Centrifuge

Methods

1. $CaCl_2$ Buffer preparation
   (a) Transformation buffer 1 (TfB1)
      - Potassium Acetate 30 mM
      - Potassium Chloride 100 mM
      - Calcium Chloride 10 mM
      - Glycerol 15% (v/v)
      - Manganous Chloride 50 mM

      Make up the volume to 500 mL using deionized water (dH_2O) and autoclave.
   (b) Transformation buffer 2 (TfB2)
      - MOPS 10 mM
      - Potassium Chloride 10 mM
      - Calcium Chloride 75 mM
      - Glycerol 15% (v/v)

      Make up the volume to 100 mL using dH_2O and autoclave.
2. Growing cultures overnight
   Inoculate 2 mL of LB with a single colony of *E. coli* and incubate at 37 °C and 200 rpm in a shaker incubator for 12–16 h.
3. Subculturing overnight culture
   - Add 2 mL of overnight grown culture to 100 mL of fresh LB with no antibiotics.
   - Shake incubate at 37 °C and 200 rpm for 3–4 h until the optical density (OD) at 600 nm wavelength light reaches 0.5–0.7.
4. $CaCl_2$ wash (generating competency)
   - Stop bacterial growth by gently swirling the flask in an ice-water bath for 10–15 min.
   - Transfer the culture to appropriate container and centrifuge at 3000 × *g* for 15 min at 4 °C.
   - Discard the supernatant.

- Resuspend the cell pellet with gentle pipetting in 20 mL of ice-cold TfB1 and centrifuge at 3000 × *g* for 10 min at 4 °C.
- Discard the supernatant.
- Resuspend the cell pellet with gentle pipetting in 2 mL of ice-cold TfB2.
- Aliquot 100 μL cell suspension in each chilled sterile 0.5 mL microcentrifuge tube.

These competent cells can be used immediately for transformation or stored for future use. For future use, immediately snap freeze the tightly closed tubes by immersing them in liquid nitrogen followed by storing at −80 °C. The stored cells can remain competent for several months with minimal loss in transformation efficiency.

Important Notes

1. It is important to not let the Optical Density at 600 nm (OD_{600}) go higher than 0.6 for *E. coli* since maximum transformation efficiency is achieved when cells are in their log phase of growth. Therefore, the OD should be frequently monitored accurately using a spectrophotometer or a colorimeter, especially when it gets above 0.3, as bacterial cells grow exponentially. A 100 mL subculture medium inoculated with 2 mL starter culture usually takes 2.5 h to reach an OD of 0.6.
2. It is also crucial to keep the cells, buffers, and vessels at 4 °C for the rest of the procedure.
3. For storage, the prepared cells should be aliquoted in small volumes for single use since each freeze/thaw cycle reduces transformation efficiency. 10^5 to 10^7 colonies of cells transformed with 1 μg of plasmid DNA can be obtained using this simple and robust procedure to generate competent cells [15]. Such efficiency is usually enough for routine purposes such as extracting large amount of plasmid DNA, gene expression for protein production, and functional studies. However, much higher efficiencies are required when obtaining every possible clone is of utmost importance, for example, generating cDNA library from low concentrated DNA sample. Therefore, several modifications and optimizations of this basic procedure have been described in the literature [15, 16]. These improved procedures have generated competent cells with transformation efficiencies between 10^8 and 10^9 transformed colonies per μg of supercoiled plasmid DNA.

4.1.4 Preparing Electrocompetent Cells

Principle: The procedure for generating competent bacterial cells for transformation using the electroporation method is the easiest, fastest, most efficient, and highly reproducible compared to chemical methods. Just like the chemical method, bacteria are cultured till they reach the exponential phase, characterized by faster growth [17], chilled, centrifuged, washed extensively using dH_2O or a suitable buffer of extremely low ionic strength, and then suspended in 10% (v/v) glycerol. Cell viability is reduced due to arcing of electric current in the electroporation cuvette

during DNA transfer by means of electroporation. Therefore, the ionic strength of electroporation buffer and DNA solution should be kept as low as possible to achieve high transformation efficiencies [18, 19]. Let us now discuss the protocol for generating competency in *E. coli* by using the method of electroporation.

Protocol

Materials

- 50 mL of *E. coli* culture grown overnight
- 500 mL of sterile fresh Lysogeny Broth (LB) medium, pre-warmed to 37 °C
- Sterile ice-cold dH_2O
- 250 mL sterile ice-cold 10% (v/v) Glycerol
- 10 mL of sterile fresh Glycerol Yeast extract Tryptone medium

Equipment

1. 37 °C shaking incubator
2. Spectrophotometer/Colorimeter at 600 nm wavelength light
3. Centrifuge

Methods

1. Preparing GYT medium ([20])
   - 0.25% (w/v) Tryptone
   - 0.125% (w/v) yeast extract
   - 10% (v/v) glycerol
2. Growing cultures overnight
   - Inoculate 50 mL of LB with a single colony of *E. coli* and incubate at 37 °C and 200 rpm in a shaker incubator for 12–16 h.
3. Subculturing overnight culture
   - Add 25 mL of overnight grown culture to 500 mL of fresh LB with no antibiotics.
   - Shake incubate at 37 °C and 200 rpm for 3–4 h until the OD at 600 nm wavelength reaches 0.5–0.7.
4. Generating competency.

   For maximum transformation efficiency, the temperature of bacteria should not rise above 4 °C at any step in the protocol given below:

- Stop bacterial growth by gently swirling the flask in an ice-water bath for 10–15 min. In preparation for the next step, place centrifuge bottles in an ice-water bath.
- Transfer the culture to the cold bottles and centrifuge at 3000 × *g* for 15 min at 4 °C.
- Discard the supernatant and resuspend the cell pellet with gentle pipetting in 50 mL of ice-cold dH_2O.
- Centrifuge at 3000 × *g* for 10 min at 4 °C.
- Discard the supernatant and resuspend the cell pellet with gentle pipetting in 250 mL of ice-cold 10% (v/v) Glycerol.

- Centrifuge at 3000 × *g* for 10 min at 4 °C. Supernatant should be discarded carefully since cells adhere loosely in 10% Glycerol.
- Discard the supernatant and resuspend the cell pellet with gentle pipetting in 10 mL of ice-cold 10% (v/v) Glycerol.
- Centrifuge at 3000 × *g* for 10 min at 4 °C.
- Discard the supernatant and resuspend the cell pellet with gentle swirling in 1 mL of ice-cold GYT medium.
- Take a small volume of the cell suspension, dilute it 1:100 with ice-cold GYT medium and measure its OD_{600}. Further, dilute the cell suspension appropriately to obtain a concentration of ~2.5×10^{10} cells/mL. Roughly, 1.0 OD_{600} = ~2.5×10^{10} cells/mL for most *E. coli* strains.
- To test whether arcing (electrical shorting that leads to burning of cells) occurs when electric current is applied to the cuvette, transfer a small volume of cell suspension to an ice-cold electroporation cuvette and apply a voltage of 13–15 kV/cm. If arcing occurs, remove excess salts from cell suspension by washing cells several times with ice-cold GYT.
- Aliquot 100 μL of ~2.5×10^{10} cells/mL concentration cell suspension in each chilled sterile 0.5 mL microcentrifuge tube.

These electrocompetent cells can be used immediately for transformation or stored for future use. For future use, immediately snap freeze the tightly closed tubes by immersing them in liquid nitrogen followed by storing at −80 °C. The stored cells can remain competent for several months with minimal loss in transformation efficiency.

Important Notes

1. It is crucial to keep the cells, buffers, and vessels at 4 °C for the rest of the procedure.
2. The dH_2O used should have low electric conductivity to avoid arcing during electroporation.
3. For storage, the prepared cells should be aliquoted in small volumes for single use since each freeze/thaw cycle reduces transformation efficiency.

4.1.5 Transformation Methods

Due to the constant requirement for introducing exogenous genetic material into bacteria, the scope of methods available for artificial transformation has broadened over time. The accessibility of such a broad scope has endowed several downstream applications of molecular cloning technology. This section attempts to describe the principles of various methods available for introducing DNA into bacterial cells and the factors that govern their efficiencies. A short comparison between the various methods has been made, which can serve as a starting point to determine the best method that could fulfill the specific requirements of a cloning application. Furthermore, in continuation of the previous section on competent cell preparation, the

procedures for the two most commonly used methods of bacterial transformation such as the *Heat-shock* and *Electroporation* have been elaborated in the following sections.

The subsequent paragraphs describe various methods available for the transfer of exogenous DNA into a suitable host.

4.2 Heat-Shock Method

E. coli cells made competent by treating with a mixture of salts can be subjected to alternating high and low temperatures to facilitate the entry of DNA molecules through the outer and inner cell membranes. This method is based on the initial observation of Mandel and Higa, who demonstrated that log-phase bacterial cells treated with a solution of cations when briefly subjected to 37 °C or 42 °C could be easily transfected with λ bacteriophage DNA [15]. The exact mechanism of how the process of calcium chloride-based competence generation and heat-shock treatment encourages bacterial cells to uptake DNA is still largely unknown. However, there are a few hypotheses on the different features of the procedure. Mechanistically, in the heat-shock method, the role of calcium-rich environment and other divalent cations used for generating competence in bacterial cells is hypothesized to be a cation bridge that reduces electrostatic repulsion between the phosphate backbone of foreign DNA and phosphorylated lipid A in lipopolysaccharide (LPS) of bacterial cell. This neutralization of charge is necessary owing to the negative charges on both the DNA and LPS layer of the bacterial cell. While the calcium ions neutralize the charge, they can also serve to cause folding of DNA molecule into a ball-like structure. Condensation of DNA is necessary to cause its entry inside cell. This is consistent with the observation that super helical plasmid DNA is transferred more efficiently than linear DNA. Glycerol brings DNA close to the surface of the cell due to molecular crowding, and also protects cells when stored at freezing temperatures. During the subsequent heat-shock transformation procedure, the brief heat pulse at 37 °C or 42 °C is believed to cause temporary pores in lipid membrane through which foreign DNA enters a cell [21] (Fig. 4.2). The heat is also believed to enhance Brownian motion in cell suspension that further facilitates motion of DNA through cell membranes [21]. As per the hypothesized mechanism, owing to their thick cell wall, Gram-positive bacteria are transformed with low efficiency compared to Gram-negative bacteria using the heat-shock method. In this method, DNA transfer and therefore transformation efficiency is inversely proportional to the size and topological form of DNA [14]. 10^5 to 10^7 colonies of transformed *E. coli* per μg of DNA generated by this method is more than enough for routine cloning work such as mass-producing plasmid DNA or protein production. However, for special purpose of cDNA library creation, wherein each unique cDNA is important, a higher number of transformed colonies is desired. The procedure for heat-shock method of transformation is described in the succeeding paragraphs.

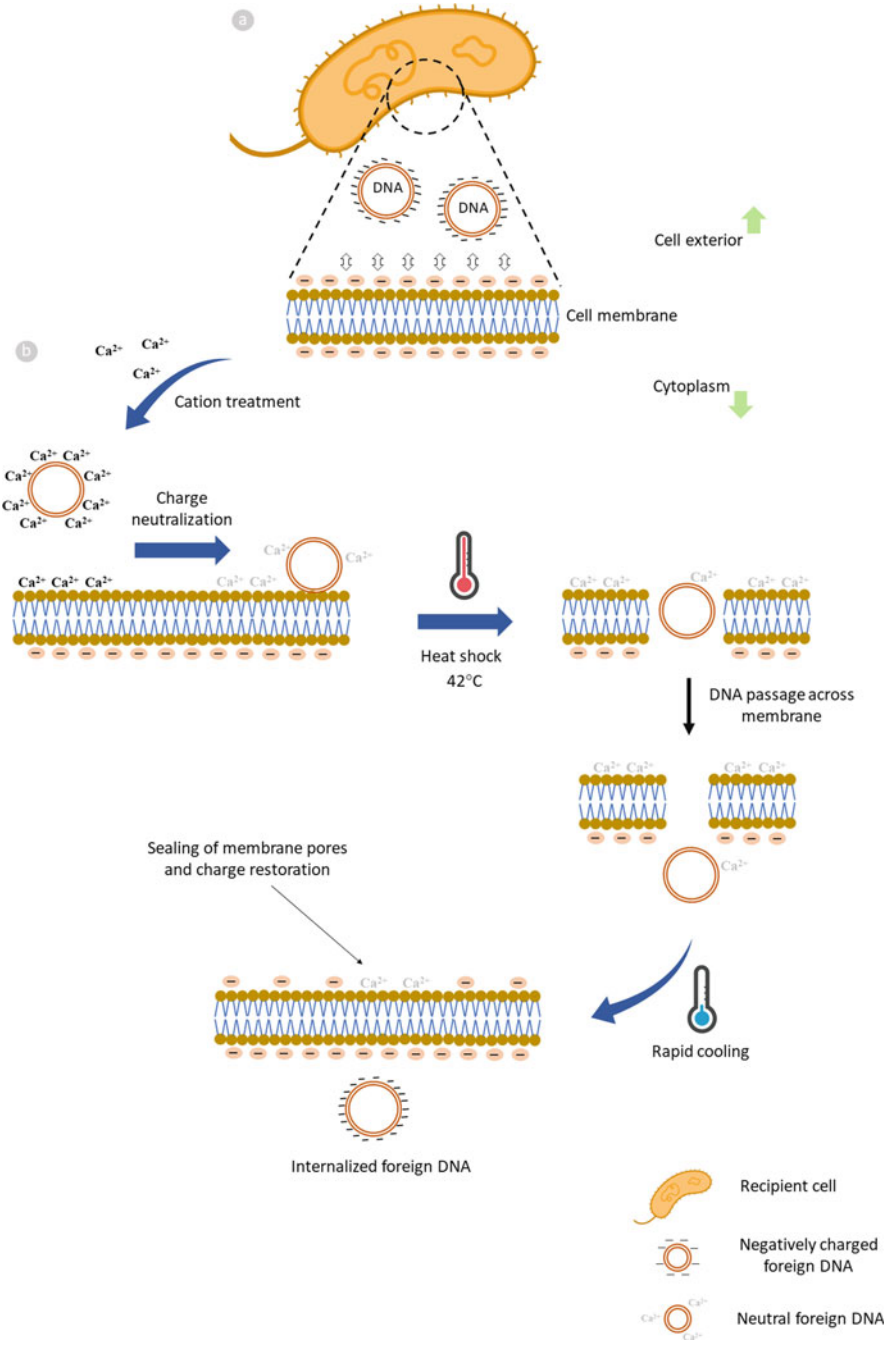

Fig. 4.2 Heat-shock method. (**a**) Charge repulsion between foreign DNA and cell membrane, together with low membrane porosity, pose a barrier for the entry of DNA inside recipient cells, as depicted in the magnified view. (**b**) While presence of cations such as Ca^{2+} reduces repulsion between the foreign DNA and cell membrane, a brief period of heating and cooling transiently

4.2.1 Procedure for Bacterial Transformation Using the Heat-Shock Method

Materials

- Freshly prepared or frozen competent bacterial cells
- Plasmid DNA solution
- Sterile fresh Lysogeny Broth (LB) medium, pre-warmed to 37 °C (~1 mL of this broth is needed per transformation aliquot)
- Lysogeny agar plates supplemented with appropriate antibiotic(s)

Equipment

1. 37 °C shaking incubator
2. Centrifuge
3. Water bath set to 42 °C
4. 37 °C static incubator

Method

- Using a micropipette add 50–100 ng of plasmid DNA to each 100 μL aliquot of competent cells. Gently mix the contents of the tube using the micropipette. When using frozen competent cells, allow cells to thaw gradually by keeping the tube in ice for 30 min. It is recommended to set both positive and negative controls for every transformation experiment. The positive control tube may contain competent cells that would receive a known amount of plasmid DNA of standard quality. On the other hand, the negative control tube may contain competent cells that shall not receive DNA at all.
- Place all the tubes in ice for 20–30 min to allow DNA-cell interaction. Meanwhile, a water bath with floating tube rack can be set to 42 °C for the next step.
- Transfer the tubes to the floating tube rack inside the preheated water bath. Place the tubes inside water bath for exactly 90 seconds without shaking. This heat-shock step is the major deciding factor of transformation efficiency.
- Immediately take out the tubes from the water bath and place in ice for 15 min. Meanwhile, a 1 mL aliquot of Lysogeny Broth (LB) medium can be warmed to 37 °C for the next step.
- Inside a bacteriological laminar air flow cabinet, add 700 μL of the pre-warmed LB medium to each tube.
- Place all the tubes in a shaker incubator and incubate for 60 min at 37 °C and 180 rpm shaking speed. Addition of antibiotics is not recommended to these small cultures since the cells need sufficient time to recover and express antibiotic resistance gene(s) present in the plasmid DNA.

Fig. 4.2 (continued) increases membrane porosity. The cumulative effect of cation treatment and varying temperatures enables entry of foreign DNA inside cells

- Transfer 100–200 μL of culture from each tube on separate Lysogeny agar plates supplemented with appropriate antibiotic(s), and spread uniformly using a sterile glass spreader. Alternatively, low density cultures can be centrifuged at 3000 × *g* for 5 min, resuspended in 100–200 μL of medium, and spread. When selecting cells that produce extracellular inactivators of antibiotics, cultures should be spread with low densities to prevent sensitive colonies from growing in the proximity of resistant cells.
- Place all the plates in inverted position inside a static incubator and incubate at 37 °C for 12–18 h.

4.2.2 Expected Observations

Test: Colonies of transformed bacteria may or may not appear on plates after appropriate incubation period.

Positive control: Several colonies of transformed bacterial cells should appear after appropriate incubation period.

Negative control: No colonies should appear on the plate. Appearance of any is an indication of the following possibilities:

1. The aliquot of competent cells was contaminated with antibiotic-resistant bacteria during the procedures of competence generation and/or transformation.
2. The plates were contaminated with antibiotic-resistant bacteria during their preparation and/or storage.
3. The antibiotic(s) added to the plate lost their potency either due to prolonged storage or because it was added to the medium when it was too hot.

4.3 Electroporation Method

The problems of low competency due to the genotype of bacterial cells or limited efficiency of the heat-shock method of transformation led to the usage of an alternative method of bacterial transformation. Since bacterial cells are electrically conductive, exposure to high intensity electric current physically distorts the cell membranes by causing their polarization. When a high-voltage electric current in the range 10–15 kV/cm is applied to bacteria, the physical distortion in the cell membranes causes formation of small temporary pores (hence, the name electroporation), which serve as entry routes for exogenous DNA (Fig. 4.3). First demonstrated by Neumann on mouse cancer cells [18], this method called Electroporation was later used to transfer DNA into other eukaryotic cells of fungi, plants, and yeasts and cells of both Gram-positive and Gram-negative bacteria with high efficiency [22]. This method, therefore, is independent of the type of host cell used in the transformation procedure. Since then, electroporation that is also known as electro-injection or electro-transfection has established itself as the fastest, easiest, most efficient, and highly reproducible method for introducing foreign DNA into various cell types [19].

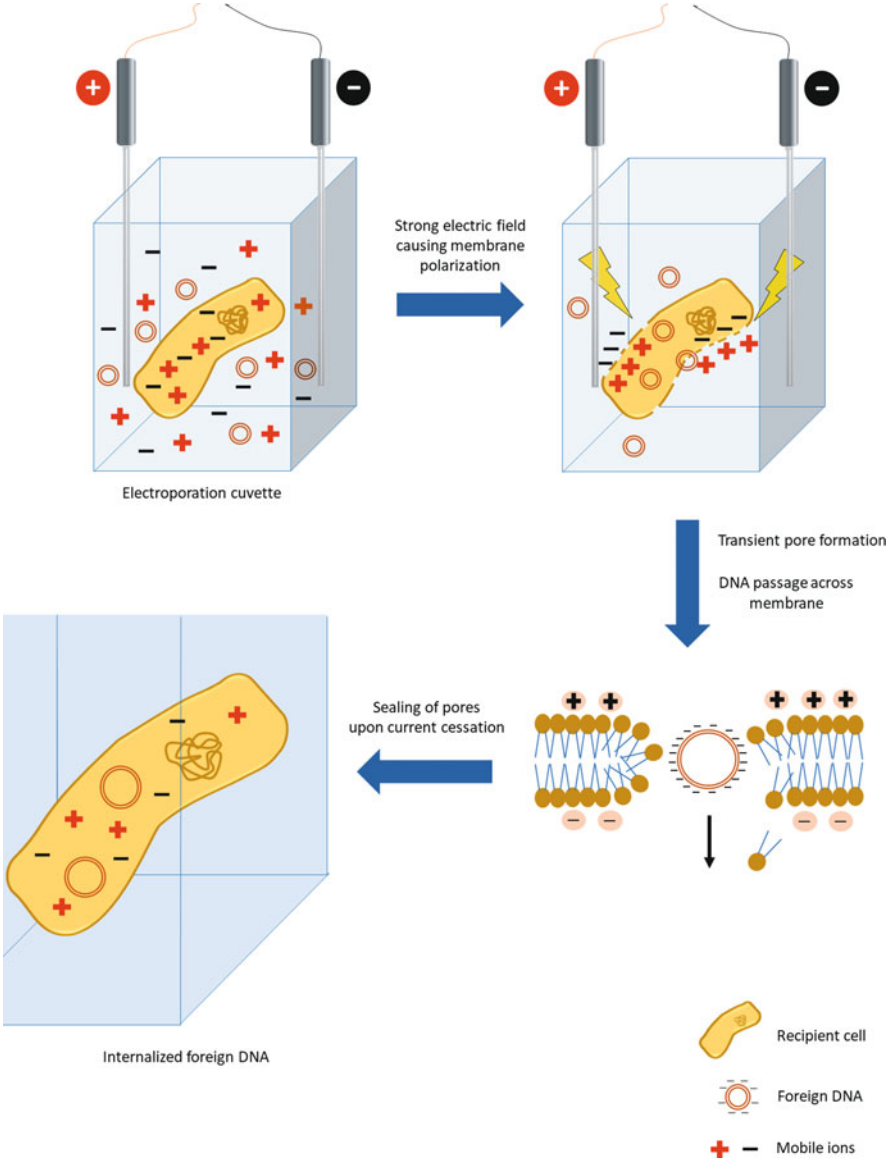

Fig. 4.3 Electroporation method. A suspension of cells and DNA is exposed to high intensity electric current inside an electroporation cuvette. Charge polarization by migration of ions caused as a result of electric field leads to formation of transient microscopic pores in the cell membrane, facilitating the entry of foreign DNA into cells. Subsequently, cessation of current causes the pores to seal, trapping the foreign DNA inside

The many advantages offered by this method over conventional ones are summarized below:

1. Plasmids ranging in size from 3 kb to 85 kb can be introduced in *E. coli* with transformation efficiencies in the order of 10^{10} and 10^{7}, respectively. This is extensive compared to the efficiencies of ~10^{6} obtained by the routinely practiced heat-shock method.
2. Unlike the $CaCl_2$ heat-shock method of transformation, the efficiency of which is inversely related to the size and topology of DNA, the efficiency of electroporation method is related only to the concentration of input DNA. Therefore, a DNA as large as 150Kb has been transferred using electroporation [23].
3. Since the incubation step of host cells with DNA is eliminated, the procedure is rapid. Moreover, with no addition of chemicals such as PEG and DMSO, toxicity is near zero in this method.

4.3.1 Procedure for Bacterial Transformation Using the Electroporation Method

This procedure of Electroporation is applicable to DNA of size <15 kb and most strains of *E. coli* such as DH5α, DH10B, and XL-1 [23–29] to yield transformation efficiency that is suitable for routine applications of cloning.

Materials

- Freshly prepared or frozen electrocompetent bacterial cells
- Plasmid DNA solution
- Sterile ice-cold dH_2O
- Sterile and fresh Super Optimal broth with Catabolite repression (SOC) medium at room temperature
  (~1 mL of this broth is needed per transformation aliquot)
- Lysogeny agar plates supplemented with appropriate antibiotic(s)

Equipment

1. Sterile electroporation cuvettes
2. Electroporation apparatus
3. 37 °C shaking incubator
4. 37 °C static incubator

Method

- Clean electroporation cuvettes thoroughly using sterile ice-cold dH_2O.
- Pipette 50–100 μL of electrocompetent cells into a clean electroporation cuvette kept in ice. When using frozen competent cells, allow cells to thaw gradually by keeping the tube in ice for 30 min before transferring cells to the cuvette. It is recommended to set two control tubes for every transformation experiment by including an aliquot of competent cells that gets a known amount of super helical plasmid DNA of standard quality and another aliquot of cells that gets no DNA at all.

- Using a micropipette add 10 pg to 25 ng of plasmid DNA in appropriate volume (not exceeding 5 μL) to each aliquot of cells inside the ice-cold cuvettes. Gently mix the contents of the cuvettes using the micropipette and keep the cuvettes in ice for 1 min. The plasmid DNA preparation should be in dH_2O or TE buffer (pH 8.0).
- Set the electroporation apparatus to convey an electrical pulse of 2.5 kV, 200 Ω resistance and 25 μF capacitance.
- Wipe all the cuvettes from the outside to dry moisture. Place all the cuvettes in the apparatus and deliver an electrical pulse to the cells at the settings mentioned above.
- Immediately after the pulse, remove the electroporation cuvettes and add 1 mL of SOC medium to each cuvette.
- Aspirate the entire contents of the cuvettes and transfer to sterile microcentrifuge tubes inside a bacteriological laminar air flow cabinet.
- Place all the tubes in a shaker incubator and incubate for 60 min at 37 °C and 180 rpm shaking speed. Addition of antibiotics is not recommended to these small cultures since the cells need sufficient time to recover and express antibiotic resistance gene(s) present in the plasmid DNA.
- Given the higher transformation efficiency of this method, transfer small volumes (20–50 μL) from each tube on separate Lysogeny agar plates supplemented with appropriate antibiotic(s), and spread uniformly using a sterile glass spreader. Alternatively, a loop-full of culture can be streaked on plates.
- Place all the plates in inverted position inside a static incubator and incubate at 37 °C for 12–18 h.

For information on expected observations, please refer to the Heat-shock transformation method section.

Apart from the two most popular methods of transformation, few others have been developed recently that include electrospray [24, 25], sonoporation [26], and microinjection [26]. However, they are mostly used for transformation in mammalian and plant cell systems and hence beyond the scope of this chapter.

In summary, the description of a wide array of common options for transferring DNA into cells and tissues implies that each one of these was developed based on the identification of certain lacunae in the previous ones. While certain limitations have been overcome with the advent of newer methods, the new ones also have their own constraints. Therefore, it is difficult to make a "score board" of transformation methods with regard to their efficiencies alone. Therefore, the preference for one method over the other should depend primarily on the particular application.

Troubleshooting Guide

Since transformation procedures involve several steps, researchers might experience a number of problems that need to be addressed for downstream applications of cloning. This section on troubleshooting discusses the common problems that arise routinely in bacterial transformation experiments that employ the Heat-shock and Electroporation methods (Table 4.1).

Table 4.1 Troubleshooting of transformation procedure

Problem	Explanation	Recommendation
Few or no transformants	Poor transformation efficiency	1. Ensure care while preparing competent cells. Store cells at −80 °C without fluctuations in temperature. 2. Ensure care while performing the heat-shock step of transformation. 3. Ensure that cells chosen are compatible with the exogenous genetic material used for transformation. 4. Include a positive control using plasmid DNA of known compatibility.
	Poor quality of transforming plasmid DNA	1. If the plasmid DNA sample is product of reactions such as ligation, PCR, DNA probe attachment, etc., adequate amount of sample clarification can be done using mini filtration columns or gel electrophoresis followed by exclusion of DNA from the gel. For products of some reactions involving proteins such as enzymes, heat inactivation can increase transformation efficiency.
	Low concentration of transforming plasmid DNA	1. Use adequate amount of plasmid DNA suitable for the chosen competent cells. 2. Using excessive amount is shown to reduce transformation efficiency in some cell types.
	Inserted DNA or its gene product is toxic to host cell	1. The choice of genetic elements present on the plasmid DNA such as the inducible gene promoter, recombination sites, and the number of replication sites can be reviewed. A tightly regulated inducible promoter can be used to ensure minimal basal-level gene expression. 2. gene products toxic to the host can be avoided.
	Insufficient number of cells were plated	1. After the recovery step in transformation procedure, the culture can be centrifuged to increase cell concentration before plating.
	Wrong antibiotic or its high concentration in plate	1. Review the choice of antibiotic for the plasmid DNA's resistance marker. 2. Ensure that the plates have appropriate concentration of antibiotic(s) tolerable by resistant cells.
	Erroneous use of spreading tool	1. Ensure that the spreading tool used is cooled sufficiently before spreading cells.
Colonies do not contain desired plasmid DNA	Inserted DNA incompatible with host	1. Ensure that cells chosen are compatible with and do not cause exclusion of the exogenous genetic material used for transformation.
	Selected colony belongs to untransformed cells	1. Ensure that the plates do not have too low a concentration of antibiotic(s) to avoid

(continued)

Table 4.1 (continued)

Problem	Explanation	Recommendation
		growth of untransformed cells. 2. Avoid adding antibiotic(s) to agar while it is too hot. 3. Include a negative control. 4. Incubation time can be reduced to avoid growth of satellite colonies that benefit from antibiotic-inactivators secreted by true colonies. 5. Consider picking colonies from the center of the plate.
A lawn of cells appears	Large number of cells plated	1. Use appropriate volume of culture for plating or dilute if necessary.
	Long incubation period	1. Review if the chosen host is genetically modified for fast growth.

4.4 Recombinant Protein Expression in Different Bacterial Systems

With the rapid advancement of recombinant DNA technology, obtaining both prokaryotic and eukaryotic gene products from bacterial cells became plausible. This method simplified the method of protein synthesis for laboratory as well as industrial-scale applications since it eliminated the need of large amounts of animal and plant tissues or fluids. Production and purification of desired proteins in enormous quantity allows their biophysical and biochemical characterization and development of commercial materials. Mass production of recombinant proteins was made feasible mainly due to the constant research on the physiology and genetics of bacteria. Despite the remarkable expansion of the challenging protein-production field, the use of bacterial cells as protein-production mills has remained an indispensible tool. Since no universal protein production host has been found so far, the choice of the best host relies on several parameters such as the cellular source of a gene, type of gene expression vector, and protein production conditions such as temperature and cultivation media. Therefore, several types of bacteria were identified and developed to make them suitable for the varying needs of protein-production programs. The following sections of this chapter will elaborate on different species and strains of bacteria for recombinant protein synthesis.

Escherichia coli The spectacular potential of *Escherichia coli* as a gene expression host was first realized in the early 1970s after DNA of eukaryotic origin was propagated in it ([30]). Since then, *E. coli* has been used widely for protein production owing to its weak pathogenicity, easy genetic manipulation, simple and inexpensive culture media, low maintenance, and fast high-density growth, allowing

proteins to be produced in less than one day. As a result, there are numerous molecular tools at our disposal for mass-production of proteins, such as several modified *E. coli* strains, broad inventory of gene expression vectors, optimized cultivation and transformation strategies. Different types of *E. coli* strains that have been developed and are commercially available have specific advantages and disadvantages, thus making them suitable for specific situations. For most applications the popular strains are described below.

BL21(DE3) This strain was first described by Studier in 1986 after various modifications of the "B" strain of *E. coli* [31]. Like the parental B strain, BL21 cells lack proteases Lon and OmpT that degrade foreign proteins. While Lon is a cytoplasmic protease, OmpT is an outer membrane protease that degrades extracellular proteins to salvage amino acids [32, 33]. These proteases pose a hurdle for producing foreign proteins in large quantities. For example, after cell lysis, the OmpT might degrade recombinant proteins in cell lysate, thereby reducing the total yield. Additionally, mutations in the Host specificity of DNA Subunit B (*hsd*SB) gene that disrupts DNA methylation and degradation prevents plasmid loss from transformed cells [34]. The DE3 designation means that this strain contains a λDE3 bacteriophage lysogen carrying a T7 phage RNA polymerase gene. The T7 RNA polymerase, which is many times faster than the bacterium's own RNA polymerase, is kept under the control of Lactose Operon repressor protein. Therefore, the phage RNA polymerase gene can be expressed by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) in growth medium. IPTG-induced production of T7 RNA polymerase gene in turn leads to expression of recombinant genes cloned downstream to the T7 promoter in a plasmid. However, the BL21(DE3) strain slightly expresses recombinant protein without the addition of IPTG. This phenomenon known as "leaky expression" becomes problematic for some proteins that are toxic to host cells often leading to protein misfolding. These misfolded proteins remain insoluble in the bacterial cells and termed as *Inclusion bodies* that will be discussed in a later chapter.

BL21(DE3)-pLysS Based on the original BL21(DE3) strain, the BL21(DE3)-pLysS strain contains a plasmid bearing the T7 Lysozyme gene. The T7 Lysozyme helps reduce leaky expression of recombinant proteins by inhibiting T7 RNA polymerase. This inhibition is overcome after adding appropriate amount of IPTG to bacterial growth medium.

Rosetta (DE3)pLysS Rosetta strains based on the BL21(DE3)-pLysS supply tRNAs that recognize codons, which are used more frequently in eukaryotes. Since tRNA population in *E. coli* reflects the codons that are frequently used by the bacterium's genes, translation of heterologous mRNA might be impeded due to lack of one or more tRNAs against eukaryotic codons. In Rosetta(DE3)pLysS cells, the genes encoding such special tRNAs are encoded in the same plasmid that carries the T7 lysozyme gene.

Although the BL21 and its derivatives are used commonly, the K-12 lineage of *E. coli* is also a popular choice for producing recombinant proteins.

4.5 Expression of "Difficult-to-Fold" Proteins in *E. coli*

One of the main strategies undertaken to reduce improper protein folding in bacteria is to slow down the rate of protein production, thereby allowing newly formed polypeptides enough time to fold properly. Growing cells at low temperatures reduces their growth rate that in turn keeps protein concentration low and decreases aggregation to facilitate proper folding. However, bacterial chaperones that assist in protein folding might perhaps not function efficiently at low temperatures. The Artic Express™ strain of *E. coli* that is modified to contain chaperonin Cpn60 and co-chaperonin Cpn10 from the psychrophilic (cold-loving bacterium with optimum growth at 15–20 °C) bacterium *Oleispira* spp. displays improved protein folding ability and *E. coli* growth at low temperatures [35].

Pseudomonas spp. While higher yield is important for a successful recombinant protein production program, the quality of the synthesized protein is equally important. Yielding adequate amount of properly folded, functional proteins that are free from host cellular contaminants, however, is challenging as it involves several complex and expensive gene expression and protein refolding strategies. To avoid the problem and allow easy purification of active proteins, scientists have explored the possibility of using a bacterial expression system with intrinsic ability to produce and secrete soluble recombinant proteins. Out of the multiple bacterial hosts available for recombinant protein production, *Pseudomonas* is specifically sought for production and secretion of proteins having complex folding requirements in high quantities due to its efficient protein secretion system [36]. By this way, the accumulation of inactive protein in cells that remains a major disadvantage is eliminated and recombinant protein can be produced without harvesting cells. Moreover, the non-pathogenic nature of this Gram-negative bacterium allows it to be used for producing pharmaceutically and agriculturally non-toxic proteins [37]. Further, the detailed knowledge of the genome of *Pseudomonas* indicates that the performance and scope of this expression system can be increased by genetic engineering of the bacterium to develop novel *Pseudomonas*-based protein production platforms.

Streptomyces spp. Although inappropriate folding of recombinant proteins and their arduous purification strategies can often be overcome with the use of the secretion system of *Pseudomonas,* other bacterial hosts with better protein secretion systems based on Gram-positive bacterium *Streptomyces* have also been developed. Among many other *Streptomyces* species, the easy acceptability of foreign DNA and weak protease activity of *S. lividans* have made it the most extensively used species for production and secretion of recombinant proteins [38]. With the knowledge of the genome of *Streptomyces*, a broad collection of vector systems have been

constructed. Several of these are based on the plasmid pIJ101, such as pIJ702 and pIJ486, which are compatible with a wide variety of bacterial hosts. Such broad compatibility allows exchanging recombinant DNA between *Streptomyces* and other bacterial species. Moreover, unlike *E. coli* and *B. subtilis, Streptomyces* has shown exceptional proficiency in production of proteins such as Xyloglucanase from Actinobacteria, and Endoglucanase CelA from a thermophilic bacterium [39, 40]. This entails possibilities for many important and novel recombinant protein constructs that were either not studied or were de-prioritized due to strict dependence on conventional bacterial hosts, to be re-explored.

Rhodobacter spp. Proteins that are embedded in the plasma membrane perform crucial functions in both prokaryotic and eukaryotic cells. These proteins, called integral membrane proteins, comprise more than 50% of drug targets [41]. However, studies on these proteins are severely limited because their hydrophobic properties pose extreme difficulties in production and purification of functionally active forms in sufficient quantities. One of the emerging strategies to mass-produce membrane proteins makes use of natural coordination between synthesis of a membrane protein and lipid bilayer jackets in a bacterium's cytoplasm. These lipid bilayer jackets are used as platforms that harbor integral membrane proteins [42], thereby providing a suitable environment to hydrophobic proteins. However, common expression systems based on *E. coli*, for example, do not couple de novo membrane synthesis with protein production. Moreover, a high concentration of proteins can overwhelm the bacterium's secretory pathway leading to protein aggregation and/or cell death. Therefore, the physiological property of *Rhodobacter* species of photosynthetic bacteria to produce large amount of lipid jackets in cytoplasm is being exploited to develop strategies for production and purification of natively folded, functional membrane proteins. Naturally, the *Rhodobacter* species produce cytoplasmic lipid jackets to assemble transmembrane proteins of the photosynthetic apparatus.

4.6 Optimizing Gene Expression

One of the most fruitful applications of recombinant DNA technology is the capability to artificially produce large amounts of proteins in a host cell such as bacteria. Protein production is an indispensible component of the "protein design cycle" that is commonly known as "protein engineering." It involves an array of biochemical, biophysical, computational, and analytical techniques to study proteins and design their variants with desired characteristics for use both in fundamental research and in industry. Therefore, for the preparation of a protein in reasonable amounts and its analysis, the gene encoding the protein is expressed in a transformed host such as bacteria. It is to be noted that around 31 recombinant proteins were approved for use in therapies between year 2003 and 2006 [43] underscoring the importance of recombinant protein production.

However, the success of high-quality recombinant protein production depends on the proficiency with which the gene expression is carried out to complete the protein

design cycle. Manipulation of the host's natural ability to recognize and express a foreign gene to an extraordinary degree is called "gene over-expression." When an over-expressed gene encodes for a protein, the host allows production of foreign proteins in amounts greater than those of the host's native proteins. This is an important advantage as it increases the chance of purification of a desired protein from a pool of host's proteins. While harvesting substantial amount of desired proteins is a prize, the proportion of useable protein depends upon how the host is manipulated for gene over-expression. Therefore, the goal of each protein production procedure is not only to identify conditions under which the host produces large amount of proteins, but also to identify conditions under which high-quality, natively folded, and functionally active proteins could be obtained.

The expression of foreign genes is primarily carried out in the host cell with the help of cloning vectors, as mentioned in the previous chapter. However, it is worth mentioning that the approach to use cell-free transcription and translation system that direct the synthesis of proteins without the need to grow and maintain host cells is also viable. In the following sections, we will learn how *E. coli* cells are manipulated to produce large amounts of proteins while ensuring that their structural integrity and biological activity are maintained.

4.7 Protein Production Protocol for Bacteria

Owing to the complexity of bacterial growth, a gene expression experiment can be improved by optimizing a great number of parameters. This is analogous to tweaking a factory's assembly line to produce foolproof products. With each change in parameters affecting the yield, folding, solubility, and activity of proteins, this task appears baffling. However, great efforts by several protein chemists and biophysicists have led to the following commonly accepted protocol that allows most proteins to be produced in *E. coli* [44].

The gene encoding desired protein (target gene) is cloned into a bacterial expression vector consisting of the T7 *lac*O promoter system of the Lactose Operon. The T7 promoter system provides strong transcription of adjacent target gene. Next, the expression vector is used to transform derivatives of the BL21(DE3) strain, such as Rosetta™(DE3)pLysS. The larger-volume culture, also called expression culture, is grown until the cells reach mid-log phase (OD_{600} of ~0.6). The temperature of the cultures is subsequently lowered to 30 °C and protein production is initiated using IPTG (Fig. 4.4). The lower expression temperature aids in production of properly folded and soluble protein. Finally, the cells are harvested by centrifugation and used for extracting and purifying proteins.

Let us now explore the factors that have an immense effect on the yield, folding, solubility, and activity of proteins produced in *E. coli*.

Rare Codons in Target Genes Often genes of human proteins fail to express in bacterial hosts due to the inability of the host to recognize certain codons. Such codons are labelled as "*rare codons*" owing to the fact that *E. coli* cells do not use

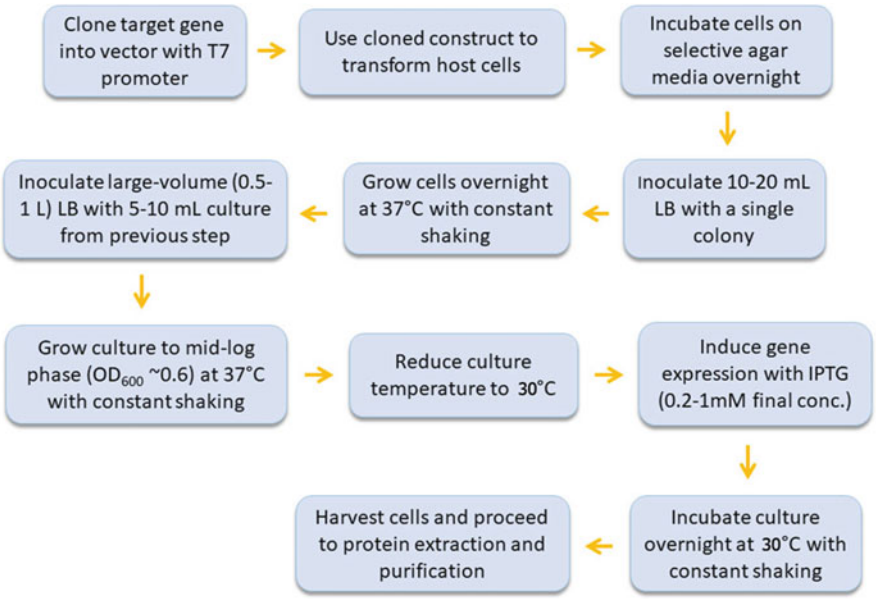

Fig. 4.4 Flowchart of a commonly followed protocol that allows most proteins to be produced in *E. coli* at a large scale

those frequently [45]. Rare codons include codons for Proline, Leucine, Isoleucine, and arginine. When an *E. coli* cannot recognize these codons due to lack of corresponding tRNAs, the process of translation stops midway and thus incomplete proteins are produced. Rarely, proteins might be produced with wrong sequence due to incorporation of wrong amino acids at the position of rare codons. Fortunately, we can now find whether a target gene has rare codons using a web tool (e.g., https://www.genscript.com/tools/rare-codon-analysis). If rare codons occur, the gene can be modified or a special *E. coli* host such as Rosetta(DE3)pLysS can be used. Such hosts co-express genes encoding the rare tRNAs with the unmodified target gene [46]. Both these approaches have overcome the problem successfully.

Leaky Target Gene Expression Production of target protein in most *E. coli* hosts is based on T7 RNA polymerase, which is several-fold faster than the bacterium's own RNA polymerase. The expression of T7 RNA polymerase gene in *E. coli* is often controlled via an inducible chromosomal copy of the gene under the control of the lacUV5 promoter. When the production of polymerase is not induced by an external inducer molecule such as IPTG, the target gene is not expressed. However, T7 promoter being a strong promoter, even minimal basal production of T7 RNA polymerase can cause "leaky" expression of the target gene. This is undesirable if the recombinant protein is prone to misfolding if produced at 37 °C when the growth of *E. coli* is fast. Secondly, leaky expression can lead to cell death if the protein is toxic to the host. Therefore, to overcome leaky expression, special *E. coli* hosts such

as BL21(DE3) pLysS, Rosetta(DE3) pLysS, and Rosetta-gami(DE3) pLysS are used [47]. These strains encode T7 lysozyme that binds and prevents T7 RNA polymerase from initiating transcription elongation. Alternatively, a weaker promoter such as araBAD promoter can be used. This expression system is based on the Arabinose operon under the control of L-arabinose as an inducer ([44]).

Concentration of Inducers Despite its several advantages, there exists a major disadvantage to using *E. coli* as a protein production system. Since transcription and translation are rapid and coupled in *E. coli,* many mammalian proteins partially fold or misfold upon gene induction. In contrast, these proteins would fold properly due to availability of longer folding times and the assistance from chaperones in eukaryotic cells. However, in *E. coli,* reduction in transcription rate can be brought about by choosing the lowest concentration of inducers that yields properly folded proteins. For example, using lower concentrations of the inducers IPTG and L-arabinose has proven to be highly effective in routine recombinant protein production experiments [48]. The routinely used IPTG and L-arabinose concentrations for protein production range from 0.05 to 2 mM and 0.0002 to 2%, respectively. The wider concentration range of L-arabinose-based induction suggests that gene expression can be tuned finely using the araBAD promoter compared to the lacUV5 promoter of Lactose Operon.

Temperature In addition to reducing the concentration of inducers in expression culture, lowering the culture temperature is a routine approach for producing high-quality recombinant proteins. At lower temperature, cellular metabolism slows down leading to diminished rates of transcription and translation, hence reduced protein misfolding and aggregation in the host cell. Additionally, several proteases are less active at lower temperatures consequently minimizing the degradation of proteolytically sensitive recombinant proteins [49]. Due to the profound role of temperature in protein production, it is greatly advocated to grow the expression culture at 18 °C for certain types of proteins (as described above) in *E. coli*.

Using Molecular Chaperones Often, the proper folding of large eukaryotic proteins is assisted by special proteins called molecular chaperones. Even though molecular chaperones are naturally produced in *E. coli*, their contribution is severely limited either due to low concentration or low specificity toward foreign proteins during their over-expression. Thus, chaperones having broad specificity are co-expressed singly or in combination with the target protein in bacterial hosts. During folding of nascent polypeptide chains, exposure and binding of their hydrophobic surfaces to each other accelerate aggregation of partially folded proteins. Such aggregated complexes are tough to reverse and often pose burden to the host cells. Common chaperone systems, for example, the GroEL-GroES system, function by temporarily masking the hydrophobic surfaces of nascent target proteins from each other during the folding process.

4.8 Posttranslational Modifications in Bacterial Expression Systems

Often, successful episode of transcription and translation of a recombinant protein does not ensure that the product will be biologically active. This is because most proteins, especially those of eukaryotic origin, are naturally modified by the cell through posttranslational modifications (PTMs). Only after being modified, these proteins display function. Posttranslational modifications are chemical modifications made naturally to proteins after they are synthesized [50]. Via PTMs, a protein's activity is modulated by covalent modification of its backbone (cleavage of peptide bonds) or of its amino acid side chains. So far, we know more than 500 different types of PTMs in proteins. Commonly used chemical modifiers include phosphoryl, hydroxyl, acetyl, carboxyl, amide, methyl, adenylyl, palmitoyl, myristoyl, uridylyl, prenyl, sulfate, oligosaccharides, and adenosine diphosphate ribosyl groups ([50]). When an amino acid residue is modified, a novel property is introduced in the protein. Therefore, evolutionarily, PTMs have aided extension of the functions of proteins beyond those manifested by unique amino acid sequences. Hence, to unravel the functions of proteins, their PTMs must be mirrored when expressed recombinantly. Often, it is the inability of an expression host like bacteria to apply appropriate PTMs on a recombinant protein that causes it to be inactive, misfold, and become insoluble while being produced. While there are PTMs in prokaryotes like *E. coli*, for example, glycosylation, they are less common and slightly different in nature (Fig. 4.5). Consequently, there arises the need for either not choosing or modifying *E. coli* as an expression host for some proteins of eukaryotic origin.

Although it is interesting to learn about such a diverse range of PTMs in both eukaryotes and bacteria, to cover them all from the standpoint of recombinant protein production in bacteria is beyond the scope of this chapter. We shall therefore discuss one of the most common PTMs, i.e., glycosylation.

Glycosylation It is estimated that about two-thirds of proteins in eukaryotes are glycosylated [51]. Therefore, it could be assumed that most eukaryotic proteins of therapeutic importance might also require proper glycosylation for full functionality [52, 53]. This is why the majority of approved therapeutic proteins are expressed in mammalian cells. However, as mentioned earlier, mammalian systems have disadvantages such as high cost, low yield, product heterogeneity, slow growth, and complex manipulation of their characteristics. These disadvantages have a great effect on healthcare research since the high capital investment gives rise to products that are expensive and limited in stock during incidences like disease outbreaks. In view of this fact, numerous studies are currently focused on expressing affordable and high-quality complex human glycoproteins in simple systems such as *E. coli*. It is worth mentioning that the continuing efforts have been fruitful owing to the significant progress in regard to use of genetically engineered *E. coli* for production of antibodies [54].

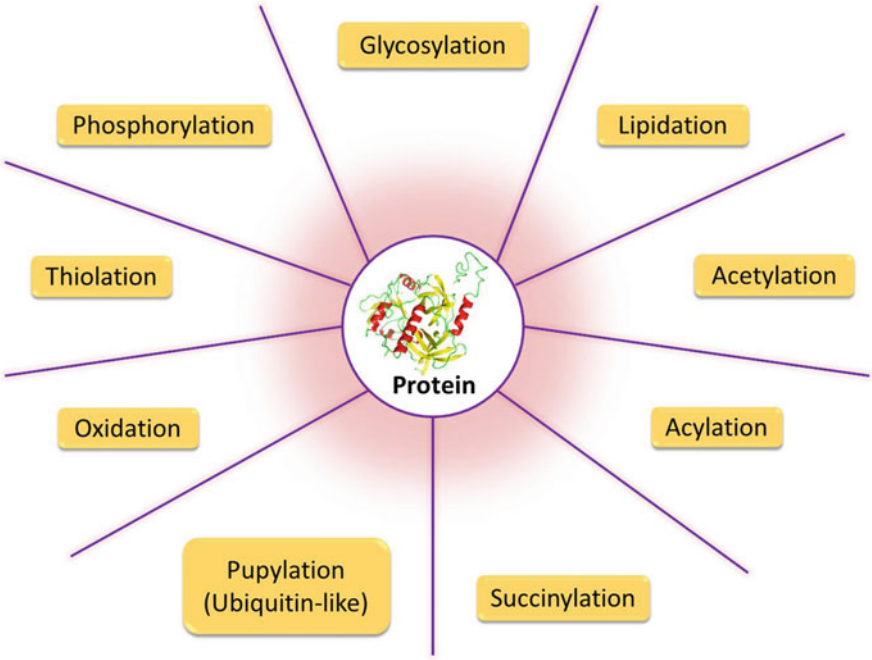

Fig. 4.5 Posttranslational Modifications (PTMs) of *E. coli* proteins expressed in *E. coli*

First thought to be a property of only eukaryotes, it is now well established that natural glycosylation of proteins also occurs in bacteria [55]. From the time of its discovery in *Campylobacter jejuni*, a bacterium that causes diarrhea in humans, the bacterial protein glycosylation machinery has been artificially transferred into *E. coli* [56]. This successful attempt gave rise to an area in biotechnology called "Bacterial Glycoengineering," and is on the rise since then.

Despite the patterns of protein glycosylation in bacteria differing slightly from the eukaryotic counterparts, transferring a broad glycosylation system in *E. coli* has allowed researchers to take advantage of this popular expression host for composing novel products such as vaccines and therapeutic enzymes besides using Glycoengineering for research [57].

4.9 Expression in Yeast Cells

While natural transformation is commonly found among various prokaryotes, there are very few reports which clearly show that eukaryotic microorganisms such as yeasts are naturally capable of taking up genetic material. According to one report, DNA uptake in yeast is active when ample sugar is metabolized in the absence of other nutrients, which suggests that such a condition is likely to occur under normal conditions in a cell [58]. Yeasts are single-celled eukaryotes that have gained appeal

for studies of basic processes in molecular and cellular biology. This is due to the similarities between yeast and human enzymes making the study of human proteins in yeast systems more biologically relevant [59]. Therefore, there are increasing numbers of examples of human proteins that function properly when artificially expressed in yeast cells. This is mainly due to the fact that being eukaryotes, they provide chemical habitat for posttranslational modifications and secretion of proteins, resulting in a product that is similar or identical to the native protein [60]. Like bacteria, yeasts are simple to cultivate at industrial scales on inexpensive growth media, and there is an array of techniques already available for its genetic manipulation. Another important benefit of using yeast for recombinant protein expression is the safety of yeast-derived pharmaceutical preparations. Most yeast cell walls lack toxic pyrogens whereas mammalian cells might contain viral or oncogenic DNA and antigens. Moreover, the genome of the commonly used yeast *Saccharomyces* is rigorously characterized, which permits manipulation of specific regions of its chromosome to better understand eukaryotic biology [61, 62]. Taking into consideration the ease and practicality of the transformation technique of gene manipulation, the importance of yeasts as model organisms and industrial preparations has therefore been uplifted by application of the transformation technique to yeast biology. A number of other yeasts have often been used in preference to *Saccharomyces cerevisiae* for mammalian gene expression owing to the advantages in protein secretion efficiency, accurate posttranslational modifications, sensitive gene regulatory elements, and high yields. Therefore, a substantial section of the literature has been devoted to discussing other yeasts such as *Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis, Yarrowia lipolytica*, and *Schizosaccharomyces pombe* [63]. Despite having several yeasts in the toolbox of yeast recombinant technology, there are still problems that arise due to incompatibility between the yeast expression systems and the proteins that are being expressed. One such problem pertains to human proteins that are processed within cellular organelles (e.g., those taking part in the secretory pathway). Expression of those proteins in yeast cells might lead to incorrectly folded and/or glycosylated proteins. To overcome these problems, the secretory pathway of yeast *P. pastoris* has been genetically modified so as to mimic protein glycosylation in humans [64, 65].

Though important under certain specific conditions, bacterial system still outweighs the yeast expression system due to several factors that include homogeneity, ease of growth and purification, expression and finally the yield. Furthermore, proteins expressed in insect and mammalian systems are primarily used for cell biology studies where posttranslational modification gets priority over yield and homogeneity. Table 4.2 provides a simple comparison of different types of protein expression systems.

Table 4.2 Comparison of different protein expression systems

	Bacteria (*E. coli*)	Yeast	Mammalian cells	Insect cells
Advantages	• Easy manipulation • Low cost • Less cell doubling time (~30 min) • Robust, easy • High yield • Easy radiolabelling for structural studies	• Easy manipulation • Low cost	• Natural protein folding • Posttranslational modifications	• Posttranslational modifications
Disadvantages	• Most posttranslational modifications absent • Highly reducing cytoplasm thus production of disulfide-bonded proteins difficult • Production of membrane proteins difficult	• Minimum posttranslational modifications • Production of membrane proteins difficult	• Large cell doubling time (~24 h) • High cost, lower yield	• Large cell doubling time (~18 h) • High cost • Production of membrane proteins difficult

4.10 Conclusions

This chapter discusses two important components of recombinant DNA technology:

(a) Transformation methods.
(b) Bacterial Protein Expression systems.

It provides a detailed discussion on competent cell preparation, different methods of transformation including protocols and troubleshooting guides. Furthermore, it elaborates on different protein expression systems in bacteria along with protocols and their use for specific requirements. This chapter will therefore guide a researcher in choosing the appropriate transformation method and protein expression system to obtain an optimum amount of functional protein.

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Problems

Multiple Choice Questions

1. Which factor among the following is important to ensure proper folding of the heterologous proteins:
   (a) Expression vector
   (b) RNA polymerase
   (c) Nutrient media
   (d) Temperature and inducer concentration
2. In the chemical method for competent cell preparation, $CaCl_2$ acts as:
   (a) Nutrient component
   (b) Cation bridge
   (c) Anion bridge
   (d) Buffering agent
3. The property of DNA that is a hindrance to its uptake by bacterial cells in the process of transformation is:
   (a) Double helical structure
   (b) Negative charge
   (c) Nitrogen bases
   (d) Use of chemicals during transformation

Subjective Questions

1. A gene of interest was successfully cloned in an expression vector; its sequence was confirmed and was transformed into E. coli DH5α cells for plasmid preparation. Sufficient amount of plasmid was isolated and the quality of the DNA was checked using agarose gel electrophoresis. The same construct was subsequently transformed into BL21 (DE3) cells at a concentration of 10 ng/μL. Although control plates showed colonies, no colonies were observed on the test ampicillin plates. What could possibly explain the observation?
2. A protein coding gene was successfully cloned in an expression vector that was confirmed by sequencing. This construct containing the gene was transformed into a protein expression host like BL21 (DE3) and colonies were obtained. After growing a large-scale culture and harvesting the cells, it was found that no protein was expressed. What could be the probable reason for such an observation?

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