

Transformation of *Escherichia coli* Made Competent by Calcium Chloride Protocol

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Information

History

The ability to transform bacteria can be traced back to 1928 with Frederick Griffith's experiment using *Streptococcus pneumoniae*, a bacterium that causes respiratory disease in mammals (7). In that study, Griffith worked with two strains of *S. pneumoniae*, a pathogenic encapsulated smooth strain (smooth refers to the glistening appearance of colonies on artificial media) and a nonpathogenic nonencapsulated rough strain with no glistening appearance. Mice infected with the smooth strain died whereas mice infected with the rough strain survived. Mice infected with heat-treated smooth strain survived. When Griffith mixed live rough strain with killed heat-treated smooth strain and infected the mice, the animals died. Clearly, some genes from the dead bacteria were transferred to the rough strain causing the harmless bacteria to become encapsulated and therefore pathogenic. Griffith called the phenomenon transformation. Oswald Avery provided a molecular explanation for this process in the 1930s and 1940s. Avery and his colleagues determined the chemical identity of these capsules (2). He and others at the Rockefeller Institute showed that transformation can also occur in a test tube by mixing crude DNA extract with living rough cells. Avery and his colleagues isolated and identified a substance within the bacterial extract that was the "transforming principle" causing a heritable change in bacterial cells. The transforming principle is known today as deoxyribonucleic acid or DNA. Today, chemically induced transformation is commonly used in many laboratories. Some strains of bacteria are not naturally transformable but can be artificially induced to take up DNA by chemical (1, 13) or electrical shock method (1, 11). In the early 1970s, Mandel and Higa demonstrated that bacteriophage DNA can be transferred to *Escherichia coli* K12, a strain that is not naturally transformable, by addition of calcium chloride (13). Three years later, Cohen and Chang showed that circular, nicked circular, or sheared DNA can also be assimilated by bacterial cells and recovered in a covalently circular form (6). Since then, many laboratories have modified the original methods with the goal of increasing transformation efficiency. Factors such as bacterial strain, medium composition, growth phase, divalent cations, and DNA size are important in the transformation process (9, 14). Actively growing cells in early log phase are more susceptible to transformation. The transformation process also differs between gram-negative and gram-

positive bacteria (5). Gram-negative bacteria regulate “competency” internally and incorporate DNA with certain sequences. Gram-positive bacteria produce a competence factor that induces competency in the neighboring cells. Today, transformation has been performed in more than forty species of gram-negative and gram-positive bacteria. Furthermore, it has been shown that transformation can also occur naturally in genera such as *Streptococcus*, *Bacillus*, *Acinetobacter*, *Neisseria*, and *Pseudomonas* (4, 12, 16).

Purpose

Chemically induced competence followed by transformation is a commonly used technique to introduce plasmids or other DNA fragments into *Escherichia coli*. Depending on the genetic information it carries, the incoming DNA can be replicated as an independent entity or integrated into the host chromosome. To select for cells that incorporate the DNA, a plasmid is engineered to carry selectable markers such as antibiotic resistance genes. Plasmid uptake provides the host cell with the ability to survive on a selective media. Today, chemically induced transformation is frequently used to clone and amplify a fragment of a gene, a whole gene, or an entire DNA library. Transformation of cells with DNA is an invaluable technique that provides scientists with a way to introduce and manipulate genes.

Theory

Since *E. coli* is not naturally transformable, the ability to take up DNA or competency must be induced by chemical methods using divalent and multivalent cations (calcium, magnesium, manganese, rubidium, or hexamine cobalt) (9, 14). Alteration in the permeability of the membranes allows DNA to cross the cell envelope of *E. coli* which is composed of an outer membrane, an inner membrane, and a cell wall. The outer membrane of *E. coli* can be understood by application of the fluid mosaic model for membranes and is composed of phospholipids, proteins, and lipopolysaccharides. Many channels or zones of adhesions are formed by the fusion of the outer membrane and the inner membrane through the cell wall layer. Although the transformation mechanism is not known, previous studies indicate that these channels allow for the transport of DNA molecules across the cell membrane (3, 15). The negative charges of the incoming DNA, however, are repelled by the negatively charged portions of the macromolecules on the bacterium’s outer surface. The addition of CaCl_2 serves to neutralize the unfavorable interactions between the DNA and the polyanions of the outer layer. The DNA and competent cells are further incubated on ice for thirty minutes to stabilize the lipid membrane and allow for increased interactions between calcium ions and the negative components of the cell. The reaction mixture is then exposed to a brief period of heat-shock at 42°C . The change in temperature alters the fluidity of the semi-crystalline membrane state achieved at 0°C thus allowing the DNA molecule to enter the cell through the zone of adhesion (for animation go to <http://www.dnai.org/text/mediashowcase/index2.html?id=1009>).

Other factors also affect transformation efficiency. Prior studies showed that some *E. coli* strains are more susceptible to transformation than others due to differences in the composition of the lipopolysaccharide (17, 18). *E. coli* with a lengthy O-linked polysaccharide blocks or hinders DNA from entering the cell. Adding magnesium to the media increases transformation yield by enhancing the ionic interaction of the molecules on the surface and therefore alters the suppleness of the membrane for more efficient transformation.

PROTOCOL

The protocol described here represents a standard protocol. However, other protocols can be used that will increase transformation efficiency.

RECIPES

LB broth (Lysogeny broth, also called Luria-Bertani broth)

Per liter of water:

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Adjust pH to 7.5 with NaOH and autoclave for 20 minutes.

LB plates, add 15 g of agar to LB broth before autoclaving.

For LB plates with antibiotic, add the appropriate amount of the selective antibiotic to sterile LB-agar media that has been precooled to 48°C (see molecular biology textbook (1, 14) for stock and working concentration of a specific antibiotic).

SOC (Super Optimal Catabolite repression) medium

1. Add the following to 900 ml of distilled H₂O
 - 20 g of Bacto tryptone
 - 5 g of Bacto yeast extract
 - 2 ml of 5M NaCl
 - 2.5 ml of 1M KCl
 - 10 ml of 1M MgCl₂
 - 10 ml of 1M MgSO₄
 - 20 ml of 1M glucose
2. Adjust to 1 liter with distilled H₂O.
3. Sterilize by autoclaving.

Chemicals and biological supplies required:

Luria broth (LB)

SOC medium

LB plates containing selective antibiotic

Filter-sterilized antibiotic solution (see molecular biology textbook (1, 14) for stock and working concentration of a specific antibiotic)

E. coli K12 derivatives (TB1, JM109) or other commercially available strains (Carolina Biological, New England Biolab, Fisher Scientific, etc.)

Plasmid vector or other DNA (pUC 19, pBR322, pBLU, other cloning vectors) (Carolina Biological Supply Company, Bio-Rad Laboratories, New England Biolabs)

1x Tris-EDTA (TE) buffer pH 8.0 (10 mM Tris Cl, 1 mM EDTA pH 8.0)

Sterile 60 mM cold CaCl₂ solution (60 mM CaCl₂, 15% glycerol, 10 mM piperazine-*N,N'*-bis(2-hydroxypropanesulfonic acid) (PIPES), pH 7)

Pipettors (1 to 10 µl and 10 to 200 µl range)

Sterile pipette tips

Sterile 50-ml glass or polypropylene tubes with caps

250-ml sterile Erlenmeyer flask with cap

5-ml glass or plastic pipettes precooled in refrigerator

Thermometer (0 to 100°C)

Permanent marker for labeling

Clock or watch for timing

0.2-mm filter (Fisher Scientific)

Styrofoam bucket with crushed ice

Equipment required:

- 37°C incubator
- Water bath shaker set at 37°C
- 42°C water bath (using a thermometer ensure the temperature is exactly 42°C for the 2 minutes of heat shock)
- Centrifuge
- Roller drum in a 37°C incubator

Procedure

1. Inoculate 5 ml of LB media with *E. coli* and grow overnight at 37°C in a roller drum.

2. Inoculate 1 ml of overnight culture into a sterile Erlenmeyer flask containing 100 ml of LB broth.

3. Shake culture in a 37°C water bath until cell density reaches mid-log growth phase (about 5×10^7 cells/ml). This should take 2 to 4 hours. The growth rate of the culture is determined by removing a 1-ml aliquot at various times and reading the optical density at 550 nm wavelength using a spectrophotometer. The relationship between optical density and cell number will vary depending on the bacterial strain. For a strain such as MM294 (*rec*⁺), 1 OD₅₅₀ = 0.2 (5×10^7 cells/ml); for a strain such as HB101 (*rec*⁻), 1 OD₅₅₀ = 0.5 (5×10^7 cells/ml).

4. Chill the culture on ice for 10 minutes.

5. Spin the cell suspension at 4,000 g in a centrifuge for 5 minutes at 4°C.

6. Discard the supernatant.

7. Resuspend the cells in half the volume (50 ml) of the original culture with ice-cold sterile 60 mM CaCl₂.

8. Place the cell suspension in an ice bath for 30 minutes.

9. Centrifuge the suspension at 4,000 g for 5 minutes at 4°C.

10. Discard the supernatant.

11. Gently resuspend the cells in 5 ml of sterile ice-cold CaCl₂ using precooled pipettes. Cells will remain competent for up to 24 hours at 4°C. Transformation efficiency increases four- to six-fold during this time. For long-term storage, dispense 250-ml aliquots into prechilled, sterile microfuge tubes and store at -70°C until needed. Depending on the strain used, some *E. coli* cells will remain competent to take up DNA for as long as 6 months. A competency test (see below) should be performed each time an aliquot is removed from storage and used for transformation. The most recent number of transformants is then compared to the number obtained during the initial preparation.

12. Add 10 to 40 ng (10 to 25 ml volume) of DNA to 250 ml of competent cells in step 11. Concentrated stock DNA can be diluted using sterile 1x TE buffer. A higher volume or concentration of DNA will cause a decrease in transformation efficiency.

Besides the DNA tube, additional tubes should be set up as listed below:

(A) A control tube in which DNA is not added to the 250 ml of competent cells. The DNA volume should be substituted by an equal volume of sterile 1x TE buffer. The control tube will be treated the same as the DNA tube for the remainder of the experiment.

(B) If competent cells have been stored at -70°C, a test should be performed to ensure that the cells still remain competent to take up DNA. The thawed cells are incubated with 10 ng of a control plasmid such as pBR322. The number of transformants per microgram of DNA will be calculated and should typically yield

from 10^6 to 10^8 colonies/mg DNA for *E. coli*/MC1061 and DH1 cells.

13. Incubate the mixture on ice for 30 minutes.
14. Transfer the reaction to a 42°C water bath for exactly 2 minutes.
15. Incubate on ice for 5 minutes.
16. Add 1.0 ml of SOC medium to each tube and incubate at 37°C for 1 hour in a roller drum (250 rpm) to allow cells to recover and express the antibiotic resistance marker.
17. Spread the appropriate quantity of cells (50 to 100 µl) on selective media. Store the remaining cells at 4°C.
(A) *E. coli* cells from the control tube without DNA in step 12 above are plated on selective medium and nonselective medium. The first plating ensures that the selective medium is working properly since no growth should be observed. The second plating provides the number of viable cells in the absence of selective medium.
(B) *E. coli* cells being tested for competency are plated on LB agar containing ampicillin (50 mg/ml final concentration) to ensure that the transformation efficiency has not decreased over time due to storage.
18. Incubate all plates overnight at 37°C (agar side up).
19. Count the number of colonies.
20. Calculate transformation efficiency and frequency. Transformed *E. coli* typically yields about 10^6 to 10^8 colonies/mg of DNA.

Transformation efficiency = $\frac{\text{total number of colonies on LB (CFU/ml)}}{\text{concentration of DNA plated (in mg/ml)}}$

Frequency of transformation = $\frac{\text{Number of colonies on plate with DNA added}}{\text{Number of colonies on plate without DNA}}$

CFU/ml (colony forming unit per ml) = $\frac{\text{Number of colonies}}{\text{Volume} \times \text{Dilution factor}}$

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

TIPS AND COMMENTS

All liquid and glassware that come into contact with the *E. coli* cells must be prechilled.

For maximum transformation efficiency, the culture must be collected in early logarithmic phase and cell density should be low (1, 14).

It has been found that growing *E. coli* cells at a lower temperature (18 to 20°C) will enhance DNA uptake (10) and decrease heat shock time (19).

Calcium chloride solution should be sterile, made fresh, and kept cold. Calcium solutions should not be more than 2 months old.

Depending on the strain of *E. coli* used, transformation efficiency can be improved by addition of other chemicals such as rubidium chloride, magnesium, manganese, hexamine cobalt, dimethyl sulfoxide, and dithiothreitol to the calcium chloride buffer (1, 9, 14).

Careful handling is important since cells are fragile due to treatment with calcium chloride.

Transformation efficiency increases four- to six-fold if stored at 4°C for 12 to 24 hours and decreases after 24 hours.

DNA or plasmid size also has an effect on transformation efficiency. The number of transformants decreases with increasing DNA size (8).

For a quick and easy method which gives rise to 1,000 fold more transformants (10^9 to 10^{10} transformants per mg DNA), an alternative method called electroporation (1, 10) is used. This method may be the preferred choice when maximum transformation efficiency is required, for instance in making a DNA library.

When performing transformation procedure, it is important to understand that some strains of *E. coli* have restriction systems that protect the organism from foreign DNA. If genes are transferred from another organism with a different methylation system, an *E. coli* host deficient in the restriction modification system needs to be used to protect the transferred DNA from the restriction system of the host cells. Transformation efficiency increases at least 10-fold when a host such as *E. coli* C is used since this strain lacks most of the methylation-dependent restriction systems (9, 20).

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