Relative variability in transformational efficiency of CaCl$_2$, MgCl$_2$, Tris-HCl, and Tris EDTA in E coli and Agrobacterium tumefaciens

**ABSTRACT** Bacterial transformation is an inevitable tool for gene manipulation and plant transformation. Ca$^{2+}$ divalent cations are indispensable for the induction of competency in E coli transformation. Different types of ions have been reported to induce and enhance transformation in E coli with varied efficiency. In the current study we investigated the effect of CaCl$_2$, MgCl$_2$, Tris-HCl, Tris EDTA, NaOAc and NH$_4$OAc in Escherichia coli and Agrobacterium tumefaciens. The results indicate that the effect of chemicals on E coli and agrobacterium may depend on the electrochemical physiology of the cell and the membrane characteristics. It is reported in the current study that the efficiency of mediator chemicals are appreciably varying when related to the two samples.

**Materials and methods**

E coli strain Top 10, A tumefaciens strain LBA4404 were the bacterial strains used.

**Media preparation**

YEP - 10 g of yeast extract powder, 10 g of peptone, 5 g of NaCl, 1.5% agar, pH 7.0 for 1 litre.

LB – 10 g of tryptone, 5 g of yeast extract, 10 g NaCl, 1.5% agar, pH 7.0 for 1 litre.

**Key Words**

E. coli transformation, Agrobacterium tumefaciens transformation, CaCl$_2$, transformation efficiency

| Ms. Mrinalini | Mr. P. Rathinasabapathi |
|--------------|-------------------------|
| Department of Genetic Engineering, SRM University, Kattankulathur – 603 203, Tamil Nadu, India. | Department of Genetic Engineering, SRM University, Kattankulathur – 603 203 Tamil Nadu, India. |

**Mr. Rex Arunraj**

Department of Genetic Engineering, SRM University, Kattankulathur – 603 203 Tamil Nadu, India.

**Mr. A. Muralidharan**

Department of Zoology, Presidency College Chennai 600 005, Tamil Nadu, India.

**Introduction**

Transformation is the process of uptake of exogenous DNA or plasmid by competent E coli cells.

This involves two stages, firstly the E coli cells become competent to intake DNA from the environment and secondly the entry of DNA across the bacterial membrane. After the first artificial transformation of E coli using CaCl$_2$, gram negative bacteria were artificially transformed using plasmids, but it was Hanahan$^{4-5}$ who established that cation induced competency in E coli was the criterion for transformation.

Ability of CaCl$_2$ to induce competency in bacterial cells for transformation has proven to be very efficient and is the most commonly used chemical method. Competency is the physiological condition of the cell wherein the electrostatic repulsive forces between DNA and membrane is reduced. It is suggested that cations form coordination complex with DNA and lipopolysaccharides (LPS). In addition DMSO, DTT, mercaptoethanol and 10% ethanol have enhancing effects on transformation efficiency.

There is a direct impact of efficiency of transformation in cloning works involving genomic DNA, cDNA or EST library preparation. Efficiency in transformation is the number of CFU produced per microgram of plasmid used. It would be appropriate to have the best conditions to achieve efficient transformation. As a consequence, this study was done to evaluate the potential of various chemicals like Tris HCl, Tris EDTA, NaOAc and NH$_4$OAc alongside CaCl$_2$ and MgCl$_2$ in E coli and Agrobacterium tumefaciens transformation. Transformation of E coli and A tumefaciens were carried out with plasmids pUC18 and pCAMBIA 2300 respectively.

**Antibiotic preparation**

Rifampicin was prepared by dissolving 10 mg of the antibiotic in 1ml methanol.

Kanamycin was prepared by dissolving 50 mg of the antibiotic in 1ml sterile water.

Streptomycin was prepared by dissolving 50 mg of the antibiotic in 1ml sterile water.

**Competent cell preparation**

E. coli and A tumefaciens were cultured in LB and YEP broth with ampicillin 100 mg/l and rifampicin 10 mg/l respectively. 1% inoculum was used for competent cell preparation. Cells were harvested at the physiological active stage (O.D 0.3-0.5 at A$_{600}$) and pellet made at 5000rpm for 5 minutes at 4˚C. Re-suspension of pellet was done in 1 ml of ice cold filter sterilized competent solution (various concentrations of CaCl$_2$, MgCl$_2$, Tris-EDTA, Tris-HCl, YEP, NaOAc, NH$_4$OAc). The cells were placed on ice for 30 minutes and pelleted out at 5000rpm for 5 minutes at 4˚C. The pellet was re-suspended in 80µl of respective competent solution and 20µl of 98% glycerol and was stored in -70°C till transformation.

**Cell viability assay**

Cell viability test for E coli and A tumefaciens competent cells were performed alongside transformation without plasmids. 100 µl of culture at 10$^8$ dilution was cultured on petriplate without selection. The number of colonies was counted to calculate the colony forming units. All experiments were performed in triplicates.

**E coli transformation**

E coli competent cells were added with 10 ng of plasmid pUC18 and treated with ice for 30 minutes. The cells were
given a heat shock for 90 seconds at 42°C and placed on ice for 5 minutes. The cells were added with 900µl LB broth and incubated in shaker at 37°C for 1 hour. 100µl of the culture was spread plated on LB medium with ampicillin (100µg/ml). The plates were incubated in a static incubator overnight at 37°C. All experiments were performed in triplicates.

**Agrobacterium transformation**

The Agrobacterium competent cells along with 1µg of plasmid pCAMBIA 2300 were treated with ice for 10 minutes and in -70°C for 1 hour. The cells were thawed on ice for 5 minutes and incubated at 37°C for 10 minutes. The cells were plated on ice for 5 minutes and 900µl of YEP broth was added and incubated in a shaker for 3 hours at 28°C. The culture was pelleted and resuspended in 100µl of YEP broth and spread plated on YEP media containing rifampicin (10µg/ml) and kanamycin (50µg/ml). Theses plates were incubated in static incubator at 28°C for 2 days. All experiments were performed in triplicates.

**Colony PCR**

Three colonies from transformed plates were randomly inoculated in 41µl of sterile water and the PCR master mix (nptII primers 1 µl each 5’ TCAGAAGAACTGCTGAAGAAG 3’ and 5’ ATGGGGGATTGAACAAGATG 3’, 10mM dNTPs 1µl, 10X taq buffer 5 µl, Taq polymerase 1unit ) was added. The samples were amplified with the given condition, an initial hold at 94°C for 5minutes, 35 cycles of 94°C – 40 seconds, 58°C – 40 seconds, 72°C – 40 seconds and final extension of 2 minutes at 72°C. The amplified product was checked using 1% agarose gel.

**Results**

The transformants in Agrobacterium for pCAMBIA was confirmed by colony PCR (fig. 7).

**Effect of CaCl2 in E coli and Agrobacterium transformation**

The efficiency of transformation in E coli was maximum at 100mM concentration with 1.31 X 105 CFU. The efficiency was 1.37 X 105 CFU in 10 mM with cell viability 1.25 X 105 CFU. The transformation efficiency increased with increase in the concentration of CaCl2 and had maximum at 100 mM (fig. 3). The efficiency in agrobacterium transformation was 2.7 X 105 CFU with a cell viability of 1.35 X105 at 10mM concentration (fig. 2). The efficiency of transformation decreased with increase in the concentration (fig. 4). Further, CaCl2 induced competency produced 38% of the transformants in E coli (fig. 5) and only 16% in agrobacterium (fig. 6).

**Effect of Tris-HCl in E coli and Agrobacterium transformation**

Tris-HCl induced competency in E coli produced 1.3 X 105 CFU at 10mM concentration (fig. 1) with cell viability of 9.4 X 104 while the efficiency in agrobacterium was 5.8 X 104 CFU and cell viability was 1.31 X 104 (fig. 2). The efficiency in E coli decreased with increase in the concentration of tris-HCl (1.3 X 105 at 10mM, 8.4 X 104 at 50mM, and 4.2 X 104 at 100mM) (fig.3) while it increased with increase in concentration in agrobacterium (5.8 X 103 at 10mM, 6.8 X 103 at 50mM and 7.7 X 103 at 100mM) (fig. 4). Tris-HCl had greater influence in agrobacterium transformation with 35% (fig. 6) transformants and only 4% transformants in E coli (fig. 5).

**Effect of Tris EDTA and MgCl2 in E coli and Agrobacterium transformation**

Tris EDTA and MgCl2 produced about 7.0 X 104 CFU and 1.08 X 105 CFU in E coli at 10mM concentration and 2.9 X 104 CFU and 2.1 X 103 CFU in agrobacterium. As the concentration increased the efficiency reduced in both the bacteria. Tris EDTA produced 19% and 20% transformants while MgCl2 produced 14% and 13% transformation in agrobacterium and E coli respectively. Tris EDTA at 10mM had positive effect on agrobacterium transformation but repressed E coli transformation at higher concentration.

**Effect of NaOAc and NH4Ac in E coli and Agrobacterium transformation**

NaOAc and NH4Ac showed lower transformation efficiency with E coli and agrobacterium. They produced 2.7 X 104 CFU and 2.0 X 104 CFU in E coli and 4.0 X 104 CFU and 1.2 X 104 CFU in agrobacterium respectively.

**Discussion**

In principle the entry of DNA in to the bacteria is based on the transmembrane electrochemical gradient. The requirement of Ca2+ ions at 0°C, and independent heat shock in Ca2+ induced bacterial transformation is established. DNA in solution bind strongly to CaCl2+ and with increased extracellular concentration of Ca2+ the DNA uptake is induced12. In our study, Ca2+ ions had varied effect on the two bacteria.

The varied effect of tris-HCl on E coli and agrobacterium could be due to difference in the membrane characteristics of the individual bacteria.

This may be due to EDTA which has two types of activity on the cell membrane. At low concentration it leaches out the LPS in bacteria enhancing transformation and at higher concentration deforms the cell by leaching out up to 40% LPS, outer membrane proteins, OmpA, OmpF/C, and lipoprotein, periplasmic proteins, and phosphatidylethanolamine15. MgCl2 was preferred by E coli rather than agrobacterium. It is known that MgCl2 is required for the stabilization of LPS present on the outer membrane of E coli. Tris EDTA also chelates Mg2+ ions required for stabilization of the LPS on the outer membrane of the bacteria 14, 15.

This confirms the toxic effect of NH4 ions on cell and inhibiting activity of Na+ ions in DNA LPS binding 16.

**Conclusion**

Ca2+ ions at 100mM show highest efficiency in E coli and Tris-HCl at 100mM show highest efficiency in agrobacterium. Based on our study, the efficiency of the chemicals are of the order CaCl2 > MgCl2 > tris EDTA > Tris-HCl and Tris-HCl > Tris EDTA > CaCl2 > MgCl2 in E coli and Agrobacterium tumefaciens respectively. The efficiency of transformation in bacteria may also be influenced by the membrane characteristics which need further scientific evidence.
REFERENCE

1. Mandel, M. and Higa, A., Calcium-dependent bacteriophage DNA infection. J Mol Biol., 1970, 53, 1, 159-62. | 2. Cohen, S. N., Annie, C. Y., Chang, Hsu, L., Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. Proc. Nat. Acad. Sci. USA, 1972, 69, 2110-2114. | 3. Lederberg, E. M. and Cohen, S. N., Transformation of Salmonella typhimurium by Plasmid Deoxyribonucleic Acid. Journal of Bacteriology, 1974, 119, 3, 1072-1074. | 4. Hanahan, D., Studies on transformation of Escherichia coli with plasmids. J Mol Biol., 1983, 166, 4, 557-80. | 5. Hanahan, D., Jesse, J. and Bloom, F. R., Plasmid trasformation of E. coli and other bacteria. Meth. Enzymol., 1991, 204, 63-113. | 6. Weston, A., Brown, M. G. M., Perkins, H. R., Saunders, J. R. and Humphreys, G. O., Transformation of Escherichia coli with Plasmid Deoxyribonucleic Acid. Calcium-Induced Binding of Deoxyribonucleic Acid to Whole Cells and to Isolated Membrane Fractions. Journal of bacteriology, 1981, 145, 2, 780-787. | 7. Sarkar, S., Choudhuri, S. and Basu, T., Ethanol-induced enhancement of the transformation of E. coli by plasmid DNA. Ind. J. Biotechnol., 2002a, 1, 209-211. | 8. Sarkar, S., Choudhuri, S. and Basu, T., Mechanism of artificial transformation of E. coli with plasmid DNA—clues from the influence of ethanol. Curr. Sci., 2002b, 83, 1376-1380. | 9. Panja, S., Aich, P., Jana, B. and Basu, T., Plasmid DNA binds to the core oligosaccharide domain of LPS molecules of E. coli cell surface in the CaCl2 mediated transformation process. Biomacromolecules, 2008, 9, 9, 2501-09. | 10. Silver, S., Microorganisms and Minerals (Weinberg, E. D. ed.), 1977, pp. 49-103, Dekker, New York. | 11. Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R., Studies on membrane fusion. III. The role of calcium-induced phase changes. Biochim. Biophys. Acta., 1977, 17, 465, 3, 579-98. | 12. Irvin, R. T., Mac Alister, T. J. and Costerton, J. W., Tris (hydroxymethyl) aminomethane buffer modifications of Escherichia coli outer membrane permeability. Journal of Bacteriology, 1981, 145, 3, 1397-1403. | 13. Nogami, T. and Mizushima, S., Outer membrane porins are important in maintenance of the surface structure of Escherichia coli cells. Journal of Bacteriology, 1983, 156, 1, 402-408. | 14. Tsucho, T., Katsu, N., Takeuchi, A., Takano, M. and Shibasaki, I., Destruction of the outer membrane permeability barrier of Escherichia coli by heat treatment. Appl. Environ. Microbiol., 1985, 50, 298-303. | 15. Hans, J. P., Marvin, Martin B. A. Ter Beest and Bernard Witholt, Release of outer membrane fragments from wild type Escherichia coli and from several E. coli lipopolysaccharide mutants by EDTA and heat shock treatments. Journal of Bacteriology, 1989, 171, 5262-5267. | 16. Leonas Grinius Nucleic acid transport driven by ion gradient across cell membrane. FEBS letters, 1980, 114, 1, 1-10. | 17. Panja, S., Saha, S., Jana, B. and Basu, T., Role of membrane potential on artificial transformation of E. coli with plasmid DNA. Journal of Biotechnology, 2006, 127, 1, 14-20. |