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Relative variability in transformational efficiency of $CaCl_2$, $MgCl_2$, Tris-HCl, and Tris EDTA in E coli and Agrobacterium tumefaciens

KEYWORDS: E. coli transformation; Agrobacterium tumefaciens transformation; CaCl2; transformation efficiency

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

Mr. P. Rathinasabapathi
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.

ABSTRACT *Bacterial transformation is an inevitable tool for gene manipulation and plant transformation. Ca2+ 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 CaCl2, MgCl2, Tris-HCl, Tris EDTA, NaOAc and NH4OAc 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.*

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 $CaCl_2$[1] gram negative bacteria were artificially transformed using plasmids[2, 3], 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[6]. It is suggested that cations form coordination complex with DNA and lipopolysaccharides (LPS) [7-9]. In addition DMSO, DTT, mercaptoethanol and 10% ethanol have enhancing effects on transformation efficiency[4].

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_4OAc 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.

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.

Particulars	Concentration in mM	pH
$MgCl_2$	10, 50 and 100	8
Tris EDTA	10, 50 and 100	8
$CaCl_2$	10, 50 and 100	8
Tris HCl	10, 50 and 100	8
NaOAc	10	8
NH4OAc	10	8

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. Resuspension 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_4OAc). 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

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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 placed 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' TCAGAAGAACTCGTCAAGAAG 3' and 5' ATGGGGATTGAACAAGATG 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 $CaCl_2$ in E coli and Agrobacterium transformation
The efficiency of transformation in *E coli* was maximum at 100mM concentration with 1.31 X 10^7 CFU. The efficiency was 1.37 X10^6 CFU in 10 mM with cell viability 1.25 X10^{10} CFU. The transformation efficiency increased with increase in the concentration of $CaCl_2$ and had maximum at 100 mM (fig. 3). The efficiency in agrobacterium transformation was 2.7 X10^3 CFU with a cell viability of 1.35 X10^{10} at 10mM concentration (fig. 2). The efficiency of transformation decreased with increase in the concentration (fig. 4). Further, $CaCl_2$ 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 X10^5 CFU at 10mM concentration (fig. 1.) with cell viability of 8.4 X10^9 while the efficiency in agrobacterium was 5.8 X10^3 CFU and cell viability was 1.31 X10^{10} (fig. 2). The efficiency in *E coli* decreased with increase in the concentration of tris-HCl (1.3 X10^5 at 10mM, 8.4 X10^4 at 50mM, and 4.2 X10^4 at 100mM) (fig.3) while it increased with increase in concentration in agrobacterium (5.8 X10^3 at 10mM, 6.8 X10^3 at 50mM and 7.7 X10^3 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 $MgCl_2$ in E coli and Agrobacterium transformation
Tris EDTA and $MgCl_2$ produced about 7.0 X10^5 CFU and 1.08 X10^6 CFU in *E coli* at 10mM concentration and 2.9 X10^3 CFU and 2.1 X10^3 CFU in agrobacterium. As the concentration increased the efficiency reduced in both the bacteria. Tris EDTA produced 19% and 20% transformants while $MgCl_2$ produced 7% and 30% 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 NH_4OAc in E coli and Agrobacterium transformation
NaOAc and NH_4OAc showed lower transformation efficiency with *E coli* and agrobacterium. They produced 2.7 X10^5 CFU and 2.0 X10^4 CFU in *E coli* and 4.0 X10^2 CFU and 1.2 X 10^3 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 Ca^{2+} ions at 0°C, and independent heat shock in Ca^{2+} induced bacterial transformation is established. DNA in solution bind strongly to $CaCl^{2+}$ and with increased extracellular concentration of Ca^{2+} the DNA uptake is induced[10]. In our study, Ca^{2+} 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 phosphatidylethanolamine[15]. $MgCl_2$ was preferred by *E coli* rather than agrobacterium. It is known that $MgCl_2$ is required for the stabilization of LPS present on the outer membrane of *E coli*. Tris EDTA also chelates Mg^{2+} ions required for stabilization of the LPS on the outer membrane of the bacteria [14, 15].

This confirms the toxic effect of NH_4 ions on cell and inhibiting activity of Na^+ ions in DNA LPS binding [16].

Conclusion
Ca^{2+} 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 $CaCl_2$ > $MgCl_2$ > tris EDTA > Tris-HCl and Tris-HCl > Tris EDTA > $CaCl_2$ > $MgCl_2$ 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.

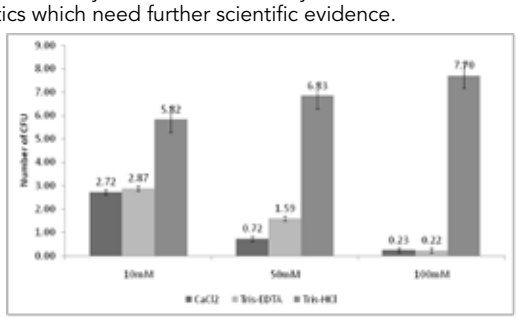

Fig 4 Effect of 10mM, 50mM and 100mM of CaCl2, Tris-HCl and Tris EDTA in *Agrobacterium tumefaciens* transformation (CFU X 10^3)

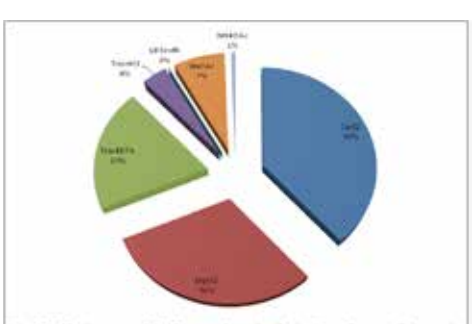
Fig 5 Efficiency of different chemicals in *E. coli* transformation

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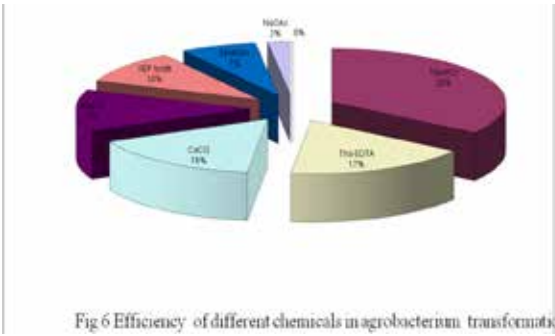

Fig 6 Efficiency of different chemicals in agrobacterium transformation

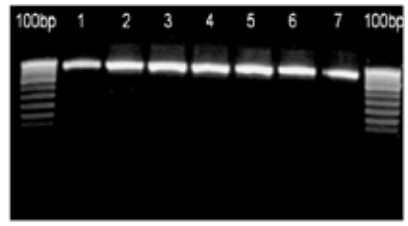

Fig 7 Confirmation of agrobacterium recombinant colonies by amplification of 800bp fragment of *nptII* by colony PCR

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