Journal of Experimental Microbiology and Immunology (JEMI)

Differential Transformation Efficiencies Observed for pUC19 and pBR322 in *E. coli* May Be Related to Calcium Chloride Concentration

Gavin Lim, Daisy Lum, Betina Ng, Christine Sam
Department of Microbiology and Immunology, University of British Columbia

Calcium chloride is commonly included in buffers used to generate chemocompetent bacterial cells. The mechanism that underlies the uptake of exogenous DNA into bacteria is not completely understood. Previous studies have suggested that calcium ions may function to neutralize electrostatic repulsion between DNA and membrane proteins, thereby allowing exogenous DNA to enter membrane pores. Other studies have shown that transformation efficiency decreases as plasmid size increases. We hypothesized that increasing calcium chloride concentrations during the transformation may increase transformation efficiency of larger sizes. To test whether or not calcium chloride concentration is related to differential transformation efficiency of plasmids of different sizes, we compared the transformation efficiency of pUC19 (2686 base pairs) and pBR322 (4361 base pairs) at varying calcium chloride concentrations of 0.05 M, 0.1 M, 0.15 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 1.0 M. We observed maximum transformation efficiencies of pUC19 and pBR322 at 0.15 M $CaCl_2$ and 0.1 M $CaCl_2$, respectively. Further increases in calcium chloride concentrations resulted in a decrease in transformation efficiency for both plasmids. Above 0.2 M $CaCl_2$, no transformants were observed with either plasmid. A decreasing trend in cell viability was observed in the absence of antibiotic suggesting that the increasing hypertonic environment may affect cell viability. Based on our experiments, maximal transformation efficiency for pUC19 was found to be $4.8x10^4$ colony forming units per ug at 0.15 M of calcium chloride while pBR322 had a maximum transformation efficiency of $1.8x10^4$ colony forming units per ug at 0.1 M of calcium chloride. Taken together, our results do not show a clear relationship between calcium chloride concentration and plasmid size with respect to transformation efficiency.

Genetic transformation is the uptake of exogenous DNA by competent cells. Transformation contributes to genetic evolution in naturally competent bacteria and is used as a tool for molecular cloning. *E. coli* cells are commonly transformed with plasmid DNA using electroporation or the calcium chloride heat-shock method. Genetic transformation is the uptake of exogenous DNA by competent cells. Transformation contributes to genetic evolution in naturally competent bacteria and is used as a tool for molecular cloning. *E. coli* cells are commonly transformed with plasmid DNA using electroporation or the calcium chloride heat-shock method. Transformation efficiency of *E. coli* has been shown to vary between plasmids pUC19 and pBR322, which are commonly, used as cloning vectors (3, 7, 16). In a co-transformation experiment, pUC19 was isolated at a 5:1 ratio compared to pBR322 from transformed cells (16). The difference in transformation efficiency correlated with plasmid size pUC19 consists of 2686 base pairs whereas pBR322 encodes 4361 base pairs. This is consistent with other studies where bacterial cells transformed with larger sized plasmids show lower transformation efficiency and maintenance of the foreign DNA (3, 7, 14). The difference in plasmid size may be responsible for the transformation efficiency between pUC19 and pBR322. More specifically, larger sized plasmids may negatively affect the ability for *E. coli* cells to be transformed. However, the exact mechanism has not been elucidated.

Differences in plasmid size may specifically affect the uptake of DNA during transformation via the calcium chloride heat shock method. Kang *et al*. have proposed that negatively charged exogenous foreign DNA may be electrostatically repelled by the negatively charged bacterial membrane (10). Dissolved calcium ions in the calcium chloride heat-shock method may neutralize the repulsive negative charges in order to allow the plasmid to interact with the cell membrane (4, 6, 13). Specifically, calcium cations form strong covalent bonds with the phosphate groups in the double stranded DNA (10). Once neutralized, the DNA is no longer repelled from the membrane, and then during the heat-shock step at 42° C, the fluidity of the membrane is altered which may release membrane lipids resulting in generation of membrane pores through which the plasmid enters (6, 15).

The aim of our study was to test the effect of calcium chloride and plasmid size on transformation efficiency of *E. coli* DH5α cells. Given the model in which calcium is required to neutralize the anionic charges on the plasmid DNA (in addition to the bacterial membrane), we hypothesize that larger plasmids, which possess a longer sugar-phosphate backbone, may require a greater amount of calcium to neutralize the molecule. Specifically, low concentrations of calcium chloride may be insufficient to neutralize pBR322 in order to interact with the bacterial membrane to permit efficient uptake (Fig. 1). Conversely, pUC19 is a smaller plasmid and therefore has fewer negative charges. At low concentrations of calcium chloride, it may be possible to neutralize all the negative charges.

To study the effects of calcium ion concentration on transformation efficiency of *E. coli* DH5α cells, pBR322 and pUC19 cloning vectors were used as they have been

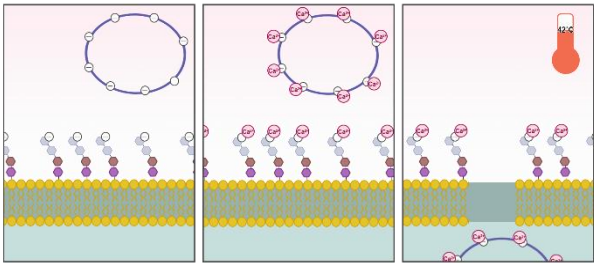

Figure 1. Proposed model of the role of calcium chloride in transformation of *E.coli*. *Left:* LPS on the bacteria cell membrane and the plasmid DNA are both negatively charged and therefore repel each other. *Center:* Calcium ions neutralize all the negative charges on LPS and plasmid. *Right:* Heat shock at 42˚C alters the fluidity of the membrane, resulting in pores that allow the neutralized plasmid to enter the cell.

previously shown to have different transformation efficiencies(9). Szostková and Horáková have shown that pUC19 has up to 3.5 fold greater transformation efficiency when plasmids are delivered at 17 kV/cm using electroporation (18). This is consistent with previous studies in which the relationship between plasmid size and plasmid uptake was analyzed (3, 8, 11, 18). In this study, we compare transformation efficiency of pUC19 and pBR322 in *E. coli* at varying calcium concentrations. The maximum transformation efficiency for pBR322 and pUC19 was observed at 0.1 M and 0.5 M $CaCl_2$. At these calcium concentrations, the transformation efficiency was observed to be 2.5 times higher for pUC19 versus pBR322.

MATERIALS AND METHODS

Growth conditions and bacterial strains. *E. coli* DH5α cells harboring pUC19 and pBR322 constructs were obtained from the Ramey strain collection, UBC Department of Microbiology and Immunology. Bacterial cultures were grown on Luria-Bertani (LB) agar for 16-24 hours at 37℃ overnight and in LB broth at 37℃ at 180 rpm overnight. Transformed *E. coli* DH5α cells containing either pUC19 or pBR322 were selected on LB agar containing 100 µg/ml ampicillin

Plasmid isolation and confirmation verification. pUC19 and pBR322 were purified from overnight liquid cultures of *E. coli* DH5α containing the respective plasmid. The plasmids were isolated using Invitrogen PureLink® Quick Plasmid Miniprep Kit (CAT# K210011). Plasmids were then linearized with EcoRI and resolved on a 1% agarose gel to verify identity based on size.

Restriction enzyme digest of pUC19 and pBR322. pUC19 and pBR322 were linearized by a single digestion with EcoRI. A 25 µl reaction was set up for each plasmid using 0.5 µg of DNA, 1x Invitrogen React3 buffer (CAT# Y9004), and 5U of Invitrogen EcoRI (CAT# 15202-021). Digest reactions were incubated at 37℃ for 1 hour.

Gel electrophoresis to confirm plasmid identity. The identities of digested plasmids were confirmed by comparing the size to an Invitrogen High Mass DNA Ladder (CAT# 10496-016). Both samples and standard were mixed with Fermentas 6X DNA Loading Dye (CAT# R0611) and run on a 1% agarose gel. The gel was run for 50 minutes at 120 V in 1X TAE buffer, then stained in ethidium bromide (0.5 µg/ml) for 20 minutes and visualized on the MultiImage™ Light Cabinet UV gel doc.

Generation of chemocompetent cells. 5 ml of *E. coli* DH5α cells were initially grown overnight at 37℃ at 180 rpm and transferred into 500 ml of LB broth three hours prior to isolation. Once turbid, cells were placed on ice for 30 minutes and centrifuged at 3000 g for 10 minutes. Cell pellets were then divided evenly, centrifuged again and the supernatant was discarded. Cell pellets were resuspended and incubated with 5 ml of $CaCl_2$ 0.05 M, 0.01 M, 0.015 M, 0.02 M, 0.03 M, 0.04 M, 0.05 M and 1 M for 30 minutes. After incubation, cells were pelleted by centrifugation and the supernatant was discarded. All pellets were then re-suspended in $CaCl_2$ at each respective concentration, which was supplemented with 15% glycerol. Competent cells were stored at -80℃.

Heat-shock transformation. Measurement of plasmid was achieved using the Thermo Scientific Nanodrop 2000c. 175 ng of pUC19 and pBR322 were mixed separately into 50 µl of chemocompetent *E. coli* DH5α. Tubes were placed on ice for 10 minutes and then exposed to heat treatment at 42℃ for 30 seconds in a water bath. Tubes were then placed on ice for 10 minutes. Transformed cells were then suspended in 1 ml of pre-warmed LB broth, and subsequently transferred to 15 ml glass tubes by pipetting. Cells were then incubated at 37℃ for two hours at 130 rpm. Three separate transformation reactions with either pUC19, pBR322 or a no plasmid insert were conducted for each experiment. A total of two experiments were completed with plasmids prepared from two separate mini-preps.

Plating of transformed cells. After two hour incubation at 37℃ at 130 rpm, serial dilution was performed for both pUC19- and pBR322-transformed cells at 1/10, 1/50, 1/100 and 1/10, 1/20, 1/100, respectively at a final volume of 50 µl. The undiluted sample contains transformed cells resuspended in 1 ml LB broth. Sterile glass beads were used to plate the 50 µl cell suspension on LB plates supplemented with 100 µg/ml ampicillin. Plates were then incubated at 37℃ overnight. Competent cells to which no plasmid DNA was added were used as negative controls, while transformed cells were also plated on antibiotic-free LB agar as a positive control to ensure the competent cells were viable.

Transformation efficiency calculation. Plates containing 30 to 300 isolated colonies were used to enumerate transformants. The amount of transformants per µg of plasmid represents the transformation efficiencies of the respective plasmids at different concentrations of calcium chloride. Transformation efficiency (cfu/µg) was calculated as Dagert and Ehrlich, 1979(5):

$$\textit{Transformation efficiency} = \frac{\textit{Number of transformants}}{\textit{µg of plasmid x dilution factor}} \quad \text{Equation 1}$$

RESULTS

Calcium chloride concentration affects transformation efficiency. To investigate the relationship between calcium chloride concentration and transformation efficiency, we transformed two differently sized plasmids, pUC19 and pBR322, into chemocompetent *E. coli* DH5α at varying calcium chloride concentrations. An increase in transformation efficiency was observed for both pUC19 and pBR322. Maximum transformation efficiency was observed for pUC19 ($4.8\mathbf{x}10^4$ transformants per µg) and pBR322 ($1.8\mathbf{x}10^4$ transformants per µg) at 0.15 M and 0.1 M CaCl2 respectively. The transformation efficiency of pUC19 and pBR322 was calculated using Equation 1 as described in the previous section. Transformation efficiencies of less than $5\mathbf{x}10^3$ transformants per µg were observed for both pUC19 and pBR322 between calcium concentrations of 0.3 M to 1.0 M. These data suggest that transformation efficiency may be related to calcium chloride concentration. Transformants were not observed for the negative control (i.e. no added plasmid) showing that our chemocompetent

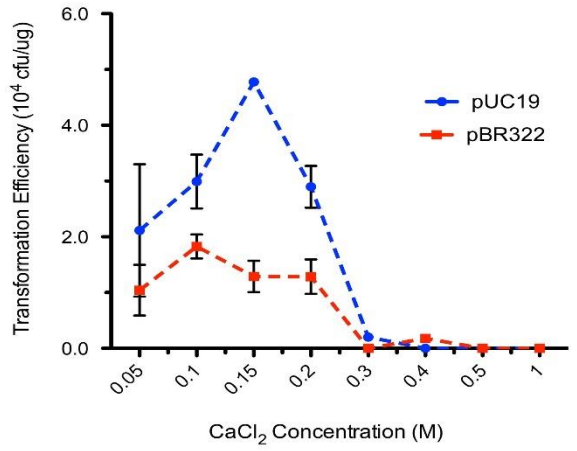

Figure 2. Transformation efficiency of pUC19 and pBR322 with increasing calcium chloride concentration. Chemocompetent *E.coli* DH5α were generated and stored in either 0.05 M, 0.1 M, 0.15 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M or 1.0 M $CaCl_2$ concentrations with 15% glycerol. pUC19 (Blue) and pBR322 (Red) transformants were plated on LB Amp+ agar (n=2). Data presented in this figure is representative of two independent experiments where the plasmids were separately prepared. Each data point represents the average of the two biological replicates in each experiment. The data point at 0.15 M for pUC19 yielded the same numerical results and thus no error bar is shown.

cells were not ampicillin resistant prior to the transformation.

High calcium chloride concentrations inhibit cell viability. To further explain the low transformation efficiency observed from 0.3 M to 1.0 M $CaCl_2$ for both plasmids, we plated untransformed cells in the absence of antibiotic selection. The number of colonies observed decreases as calcium chloride concentration increases (Fig 3). A confluent lawn of *E. coli* DH5α was observed at 0.05 M, 0.1 M and 0.2 M $CaCl_2$. At 0.5 M $CaCl_2$, the appearance of isolated colonies was seen and a significant reduction in colonies is evident at 1.0 M $CaCl_2$. These results suggest that calcium chloride decreases *E. coli* DH5α cell viability at high concentrations.

DISCUSSION

In previous studies, Ca^{2+} has been shown to be the most effective cation in the preparation of competent cells for transformation experiments (17). Our model proposes that during transformation, electrostatic repulsion between the negative charges on the bacterial outer membrane and DNA prevents proximal association, which in turn lowers transformation efficiency. The presence of positively charged calcium ions, aids in transformation activity by neutralizing the negative charge associated with DNA and the cell membrane, and consequently decreases the electrostatic repulsion. As plasmid size increases transformation efficiency decreases (8). In this study we aimed to test whether increasing calcium concentration, would compensate for the lower transformation efficiency in larger plasmids.

The standard concentration of calcium chloride used in many transformation experiments is 0.1 M (1). In our experiments, calcium concentrations ranging from 0.05 M to 1.0 M were used. While the number of moles of calcium required to neutralize all negative charges on the plasmid and cells could be calculated, it is not a feasible approach due to the large number of external factors that cannot be measured, such as the amount and size of the competent cells. Therefore we assume that at the lowest calcium chloride concentration of 0.05 M, the negative charges are not completely saturated with calcium ions. Our results have shown a similar trend for both pUC19 and pBR322 where transformation efficiency increases initially at low and moderate concentrations of calcium chloride before reaching a loss of transformation activity at the maximum $CaCl_2$ concentrations. Although pBR322 transformed cells could be selected on tetracycline, ampicillin was used as the single antibiotic selection factor to maintain consistency between experiments since pUC19 is tetracycline-sensitive.

The pUC19 transformation efficiency reached a maximum at 0.15 M $CaCl_2$ (12). This suggests that the increasing Ca^{2+} concentration could play a role in increasing transformation efficiency. Transformation efficiency of pUC19 decreased with increasing $CaCl_2$ concentration until 0.3 M, above which transformation was not observed. Based on our hypothesis, we expected to observe a proportional relationship between increasing $CaCl_2$ and transformation efficiency. Because the extent of negative charges available for neutralization is limited, we also expected that the increase in transformation efficiency would reach a maximum threshold, where all the negative charges would be neutralized. This expectation would correspond to a plateau in the number of transformants observed. Our preliminary data does not support our proposed model in which transformation efficiency of larger plasmids increases with increasing $CaCl_2$ concentration. pBR322 reached its maximum transformation efficiency at 0.1 M $CaCl_2$. pBR322 transformants were not observed at $CaCl_2$ concentration above 0.3 M. A notable difference between the transformation efficiency of pUC19 and pBR322 is that pBR322 did not show a stepwise decrease before reaching zero, as shown in Figure 2. This may be due to our limited data set.

Comparing between pBR322 and pUC19, the effect of calcium chloride on increasing transformation activity is more prevalent for pUC19 than pBR322. This effect is evident in Figure 2, where the overall transformation efficiency is higher for pUC19. Specifically, the maximum transformation efficiency is 2.5 times higher in pUC19 than pBR322. This observation does not fit our hypothesis, which expected pBR322 to have comparable transformation efficiencies with pUC19 as calcium chloride concentration increases. Transformation efficiency was based on the amount of plasmid used to

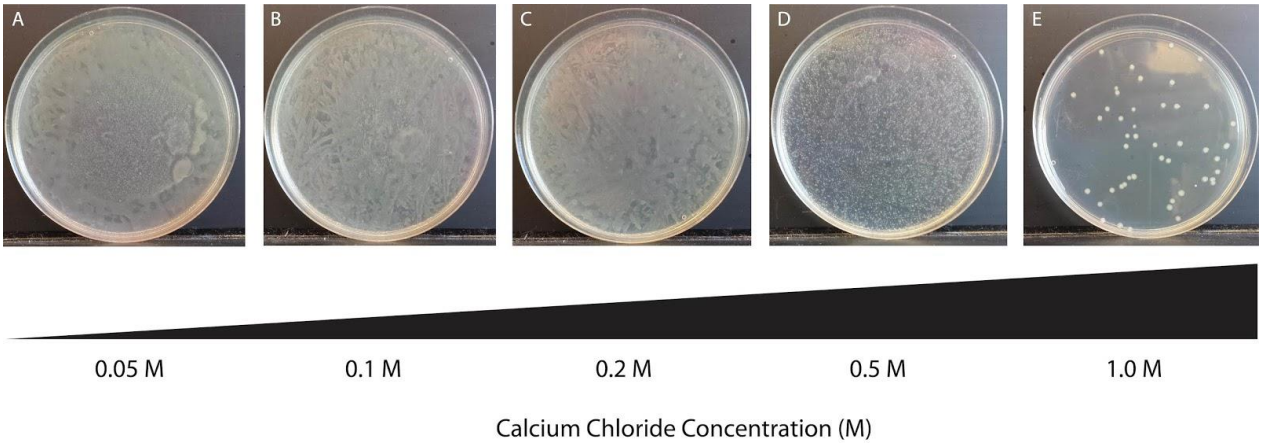

Figure 3. Cell viability decreases with increasing levels of calcium chloride. Chemocompetent *E.coli* DH5α were stored in 0.05 M, 0.1 M, 0.2 M, 0.5 M, or 1.0 M $CaCl_2$ concentrations with 15% glycerol. Cells (lacking plasmid) were then plated on LB agar in the absence of antibiotic selection.

ensure our transformation efficiency results can be compared to other similar papers. Our model suggested that a higher calcium chloride concentration is required for pBR322 to reach maximum transformation efficiency than pUC19. This is because pBR322 is a larger plasmid and therefore would require more Ca^{2+} ions to neutralize the negative charges.

It is noted that the maximum observed transformation efficiency of pUC19 and pBR322 are considerably lower compared to previous studies, which have shown to have efficiencies at magnitudes of $10^6 – 10^9$ (5, 11). The cause for the low transformation efficiencies could be due the methodology in which the cells were handled, or the differences in conditions when creating the competent cells.

An important observation seen for both pBR322 and pUC19 is that transformation efficiency decreases to near undetectable levels at calcium chloride incubations above 0.3 M. A comparison between the results obtained in the transformation efficiency and cell viability experiments indicate a similar trend in decreasing colony counts. The number of transformants cannot be clearly determined at 0.3 M and 0.5 M $CaCl_2$ concentrations (Fig 2), which may coincide with the reduced cell viability observed at 0.5 M and 1.0 M $CaCl_2$ concentrations (Fig 3). Our results suggests that cell viability decreases significantly when chemocompetent cells are generated using levels of calcium chloride beyond 0.3 M. Previous studies have shown that prolonged incubation of $CaCl_2$ results in an unfavorable effect on cell viability (5). From this, we suggest that at concentrations above 0.3 M, the solution becomes hypertonic to the cells. The increase in osmotic pressure of the solution causes the cells to shrink and eventually die.

As our preliminary data do not fully support our model, we hypothesize that there may be other mechanisms responsible for the relationship of transformation efficiency with plasmid size and calcium chloride concentration. Previous studies have shown that by inducing osmotic stress on cells, there is an upregulation of outer membrane proteins (2). We propose that high calcium concentrations create an environment of high osmotic stress, which leads to the upregulation of non-specific outer membrane porin proteins. Outer membrane protein C (OmpC) is an example of a non-specific solute porin that has been shown to be overexpressed as a result of calcium chloride treatment (1), which in turn may facilitate DNA uptake. We propose that as osmotic stress for the cells is increased, they begin to produce more OmpC to regulate the salt concentrations. OmpC production increases until the membrane is saturated and can no longer compensate for the excessively high osmotic stress. After this point, non-essential cellular processes are halted to maintain survival until the calcium chloride concentration reaches a toxic level where cell death occurs. Applying this model to our data, transformation efficiency increases from 0.05 M to 0.15 M and 0.20 M $CaCl_2$ due to an increase in transcription of OmpC porins. At calcium chloride concentrations above 0.15 M and 0.20 M, transformation efficiency declines due to high osmotic stress, which causes the cells to limit the amount of DNA uptake since it is not essential for cell survival. Further increase of calcium chloride concentrations to levels over 0.3 M then leads to plasmolysis from osmotic pressure.

In summary, our experiments show that pUC19 has higher maximal transformation efficiency than pBR322. The maximum transformation efficiency for pUC19 was $4.8\mathbf{x}10^4$ transformants per µg DNA at 0.5 M $CaCl_2$ and $1.8x10^4$ transformants per µg DNA at 0.1 M $CaCl_2$. Due to project constraints we present a limited data set here. We suggest caution when drawing conclusions from these data until additional experiments are performed.

FUTURE DIRECTIONS

The broad range of data points attributes to the inconsistent trend observed between pUC19 and pBR322. If this experiment were to be repeated, more concentrations between the range of 0.05 M and 0.30 M $CaCl_2$ should be tested. Additionally, cells treated with 0.0 M $CaCl_2$ should be used as a negative transformation control to demonstrate that $CaCl_2$ is required for transformation. Apart from the inconsistent trend, the experimental data showed notable variability and thus additional replicates should be performed.

It is possible that the size of pUC19 and pBR322 is not sufficiently different to observe an effect due to calcium ion neutralization. A larger plasmid, such as pET-21a (5.4 kb), could be tested against pUC19 and pBR322. pUC19 and pBR322 differ in size by 1.6 kb whereas pUC19 and pET-21a differ in size by 2.7 kb.

As an alternative experiment to test our proposed model, calcium chloride could be added to the plasmid prior to transformation of non-competent *E. coli* DH5α cells. This experiment serves to investigate if successful plasmid uptake is achieved by neutralization of negative charges on the DNA itself, or by alteration in membrane permeability.

Finally, further investigation of alternative models should be explored. As discussed above, calcium chloride has been shown to upregulate outer membrane proteins such as OmpC (2) . To test this model, protein samples could be taken before and after transformation at different calcium chloride concentrations and observed by SDS PAGE to assess changes in protein expression related to $CaCl_2$ concentration.

Our work supports previous observations that calcium chloride concentration affects transformation efficiency of plasmids. Furthermore, high calcium chloride concentrations have adverse effects on cellular viability of chemocompetent cells. We suggest that calcium chloride may alter the ability for outer membrane porins to be formed, which permits the uptake of foreign DNA. Our experiment provides a deeper understanding on one of the potential mechanisms of transformation. However, future experiments are still required to assess the validity of our proposed models.

ACKNOWLEDGEMENTS

This work was funded by the Department of Microbiology and Immunology at the University of British Columbia. We thank Dr. David Oliver for his time, support, and guidance to our project. We also thank our teaching assistant, Chris Deeg, for his advice and technical assistance. Finally, we extend our thanks to the media room staff for sterilizing and providing glassware and equipment for our project.

REFERENCES

1. **Aich, P, Patra, M, Chatterjee, AK, Roy, SS, Basu, T.** 2012. Calcium Chloride Made E. coli Competent for Uptake of Extraneous DNA Through Overproduction of OmpC Protein. Protein Journal. **31:**366-373. doi: 10.1007/s10930-012-9411-z ER.
2. **Angeles, A, Chang, T, Coombe, L, Rogers, T.** 2013. Regulation of Osmotic Stress by OmpC but not OmpF Increases Kanamycin Resistance in Escherichia coli. J. Exp. Microbiol. Immunol. **17:**73-77.
3. **Chan, V, Dreolini, LF, Flintoff, KA, Lloyd, SJ, Mattenley, AA.** 2002. The Effect of Increasing Plasmid Size on Transformation Efficiency in *Escherichia coli*. J. Exp. Microbiol. Immunol. **2:**207-223.
4. **Clark, J, Hudson, J, Mak, R, McPherson, C, Tsin, C.** 2002. A Look at Transformation Efficiencies in *E. coli*: An Investigation into the Relative Efficiency of *E. coli* to Take up Plasmid DNA Treated with the Complex Molecular Trivalent Cations Spermine or Spermidine within the Context of the Hanahan Protocol for Transformation. J. Exp. Microbiol. Immunol. **2:**68-80.
5. **Dagert, M, Ehrlich, SD.** 1979. Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. Gene. **6:**23-28. doi: 10.1016/0378-1119(79)90082-9.
6. **Geyik, C, Evran, S, Timur, S, Telefoncu, A.** 2014. The covalent bioconjugate of multiwalled carbon nanotube and amino-modified linearized plasmid DNA for gene delivery. Biotechnol. Prog. **30:**224-232. doi: 10.1002/btpr.1836.
7. **Hanahan, D.** 1983. Studies on Transformation of Escherichia-Coli with Plasmids. J. Mol. Biol. **166:**557-580. doi: 10.1016/S0022-2836(83)80284-8.
8. **Kang, F, Wang, H, Gao, Y, Long, J, Wang, Q.** 2013. Ca2+ promoted the low transformation efficiency of plasmid DNA exposed to PAH contaminants. PloS One. **8:**e58238. doi: 10.1371/journal.pone.0058238.
9. **Li, X, Sui, X, Zhang, Y, Sun, Y, Zhao, Y, Zhai, Y, Wang, Q.** 2010. An improved calcium chloride method preparation and transformation of competent cells. Afr. J. Biotechnol. **9:**8549-8554.
10. **Mandel, M, Higa, A.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53:**159-162. doi: 10.1016/0022-2836(70)90051-3.
11. **Neumann, E, Kakorin, S, Tsoneva, I, Nikolova, B, Tomov, T.** 1996. Calcium-mediated DNA adsorption to yeast cells and kinetics of cell transformation by electroporation. Biophys. J. **71:**868-877. doi: 10.1016/S0006-3495(96)79288-3.
12. **Ohse, M, Takahashi, K, Kadowaki, Y, Kusaoke, H.** 1995. Effects of Plasmid DNA Sizes and several Other Factors on Transformation of Bacillus-Subtilis Isw1214 with Plasmid Dna by Electroporation. Biosci. Biotechnol. Biochem. **59:**1433-1437.
13. **Panja, S, Aich, P, Jana, B, Basu, T.** 2008. How does plasmid DNA penetrate cell membranes in artificial transformation process of Escherichia coli? Mol. Membr. Biol. **25:**411-422. doi: 10.1080/09687680802187765.
14. **Plaa, N.** 2010. Determining the Importance of a Specific *rom* Mutation in Plasmid Exclusion of pBR322 During pUC19 Co-Transformation. J. Exp. Microbiol. Immunol. **14:**112-115.
15. **Roychoudhury, A, Basu, S, Sengupta, DN.** 2009. Analysis of comparative efficiencies of different transformation methods of E. coli using two common plasmid vectors. Indian J. Biochem. Biophys. **46:**395.