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Natural Competency and Electrotransformation Efficiencies in *Escherichia coli* DH5α Spheroplasts

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Escherichia coli **are non-competent, gram-negative cells possessing an outer membrane (OM), in addition to a cell wall and cytoplasmic membrane, as a barrier to transformation by the uptake of environmental DNA. As it has been reported that spheroplasts may be transformable at 100-fold higher efficiency than control *E. coli* DH5α using a modified version of the Hanahan protocol, this study was aimed at determining the effect of the cell wall and OM in transformation, in the absence of competence inducing divalent cations. Spheroplasts induced from an exponentially growing culture were subjected to both heat-shock and electrotransformation methods using pUC8 plasmid DNA (conferring ampicillin resistance to transformed cells), and were assessed for survival rates and transformation efficiencies. Here, we report that while heat-shocked spheroplasts had survival rates 46 to 71% of control cells, divalent cations play a critical role in transformation, and thus no transformants were produced. We also found that spheroplasts survived electroporation at rates 36 to 51% of non-spheroplasted cells, but the transformation efficiency was questionable.**

Deoxyribonucleic acid (DNA) is a negatively charged, hydrophilic macromolecule, which cannot pass freely through the hydrophobic interior of the phospholipid membranes (6). Phospholipids, containing negatively charged phosphate groups as their name suggests, confer a net negative charge on cells, particularly gram-negatives containing lipopolysaccharide (LPS) in the OM (12). As a result, there exists a repulsive force between the cell and DNA molecules in the environment. Certain organisms have developed mechanisms to overcome this obstacle to DNA uptake, and have thus been designated 'naturally competent' (2).

Escherichia coli is a gram-negative bacterium not known to be naturally competent, and hence does not readily take up exogenous DNA from its environment (7). In order for transformation of these cells to occur, it is necessary to artificially induce a competent state through physicochemical treatments with divalent cations, such as $CaCl_2$ (10).

The outer and inner membranes act as physical barriers to the environment in gram-negative cells (4). DNA uptake must therefore involve crossing both of these membranes to allow transformation. The processes involved following the development of competency including binding of the DNA to the cell membrane, its uptake, processing, and expression (2).

In addition to chemical methods, electroporation has become a widely adopted technique to obtain greater transformation efficiencies (13). Electroporating, as the name suggests, involves the transient formation of holes in the cell membrane by a strong pulse of electricity, allowing the uptake of DNA. However, increased efficiency comes with decreased cell survival due to the creation of larger holes, which may potentially lead to areas of rapid membrane rupture (14).

A modified method was used to produce viable *E. coli* spheroplasts using ampicillin and sucrose (8,9). This study uses the same technique to determine whether the loss of the cell wall and most of the OM, which causes the characteristic spherical morphology, has any effect on the natural competency of *E. coli*. Moreover, we investigated the feasibility and survival of transforming spheroplasts by electroporation.

MATERIALS AND METHODS

Cells and plasmids. *E. coli* DH5α cells (MICB 421 teaching lab, frozen stocks) were grown on Luria-Bertani (LB) agar plates (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl and 1.5 % agar), pH 7.0 at 37°C and stored at 4°C. Overnight broth cultures of these cells were grown in LB medium, shaking at 100 rpm at 37°C, and were used for spheroplasting and transformation experiments. The pUC8 plasmid was expressed in *E. coli* (John Smit, University of British Columbia), and is a 2665 bp plasmid that encodes a gene for ampicillin resistance (3).

Plasmid extraction. Plasmid DNA was extracted by the alkaline-lysis method (1) from an overnight culture of *E. coli* cells containing pUC8. The overnight culture was grown in LB with 50 µg/ml ampicillin (Sigma, catalogue number A9518), 37°C. Successful plasmid extraction was confirmed by electrophoresis in 0.7 % agarose (Invitrogen, 15510-027) gel at 8 V/cm for 45 minutes. The gel was stained with ethidium bromide (0.2 µg/ml) for 15 minutes and visualized using Alpha Imager Software (Alpha Innotech Corporation). A mini-prep plasmid extraction kit (Invitrogen, catalogue number K2100-10) was used isolate plasmid from an overnight culture of electroporated spheroplasts recovered following inconclusive growth on selective plates (LB and ampicillin), in order to confirm their transformation with pUC8 plasmid DNA (growth conditions and visualization as above).

Spheroplasting. A modified protocol of culture incubation in media with ampicillin and high sucrose was used (8, 9). Overnight culture of *E. coli* DH5α was inoculated into fresh LB medium at an OD_{660} of 0.1 and grown until an OD_{660} of approximately 0.3. The culture was then diluted 5-fold into each of the following media (Table 1).

Table 1. Culture components for induction of spheroplast formation in *E. coli* DH5α.

Culture Condition	Reagent	[Initial]	[Final]	Volume (ml)
Control	LB broth	1 X	1 X	8.0
	Culture ($0.3\ OD_{660}$)	-	-	2.0
Spheroplast	LB broth	2 X	1 X	4.0
	Sucrose	2 M	0.4 M	2.0
	$MgSO_4 \cdot 7H_2O$	0.1 M	8 mM	0.8
	Ampicillin	50 mg/ml	50 µg/ml	0.01
	H_2O	-	-	1.2
	Culture ($0.3\ OD_{660}$)	-	-	2.0

Cultures were incubated shaking at 100 rpm at 37°C until spheroplast morphology was observed for greater than 80% of the cells (about two hours) determined by phase contrast microscopy at 40X magnification. The control culture was diluted in LB media to compensate for growth over the time of spheroplasting to give the same OD_{660} of the spheroplast culture prior to experimentation.

Natural competency. Samples of control *E. coli* DH5α and spheroplasted cells (optical densities at 660 nm were 0.099 and 0.152 for each of two respective trials using 1 mL of culture per treatment in duplicate) were harvested by centrifuging at 4000 rpm for 10 min, and re-suspended in either 50 µl of water or 0.4 M sucrose (Table 2). Cells were incubated on ice for 30 minutes prior to the addition of either 2 µl of pUC8 plasmid DNA (500ng) or water (mock transformation) to duplicate samples, and placed back on ice for 10 minutes. All samples were heat shocked for 30 seconds at 42ºC and transferred back onto ice for 2 minutes. Heat-shocked cultures were then diluted in 0.95 ml of LB or LB with 0.4 M sucrose as appropriate, transferred to sterile glass test tubes, and incubated overnight (about 18 hours) at 37ºC. Following recovery and reversion into rod morphology, samples were plated for total survival and transformation efficiency on LB and LB with ampicillin, respectively.

Table 2. Treatment conditions for natural competency experiments.

	Treatments Combinations		
Culture and Solution	**1**	**2**	**3**
Control Culture	✓	✓	-
Spheroplast Culture	-	-	✓
H_2O	✓	-	-
0.4M Sucrose	-	✓	✓

Electroporation. Samples of control *E. coli* DH5α and spheroplasted cells (optical densities at 660nm as for natural competency experiments, using 1.5 mL of culture per treatment in duplicate) were harvested by centrifuging at 7500 rpm for 5 min, and re-suspended in either 1.5 ml of chilled 10% glycerol or 10% glycerol with 0.4 M sucrose (Table 3). The cells were washed twice in 250 µl and re-suspended in 40 µl of the appropriate solution and placed on ice. 2 µl of pUC8 plasmid DNA (500 ng) were mixed in chilled cuvettes (BioRad, catalogue number 165-2086) with 40 µl of each treatment. All treatments were electroporated with the Gene Pulser at 2.5 kV, 25 µF, and 200Ω. Following electroporation, 0.95 ml of recovery media (LB or LB with 0.4 M sucrose) was immediately added to the cuvette, then transferred to sterile glass test tubes, and incubated overnight (about 16 hours) at 37ºC. Following recovery and reversion into rod morphology, samples were plated for total survival and transformation efficiency on LB and LB with ampicillin, respectively.

Table 3. Treatment conditions for electroporation experiments.

	Treatments Combinations		
Culture and Solution	**4**	**5**	**6**
Control Culture	✓	✓	-
Spheroplasts Culture	-	-	✓
10% Glycerol	✓	-	-
10% Glycerol + 0.4 M Sucrose	-	✓	✓

RESULTS

Confirming plasmid isolation. Isolated pUC8 plasmid was assessed for size and purity by agarose gel electrophoresis (Fig. 1). The bright band in lanes 3 and 4 is the undigested, supercoiled plasmid, which ran at the expected size of 2665 bp, indicating successful isolation for use in the following experiments.

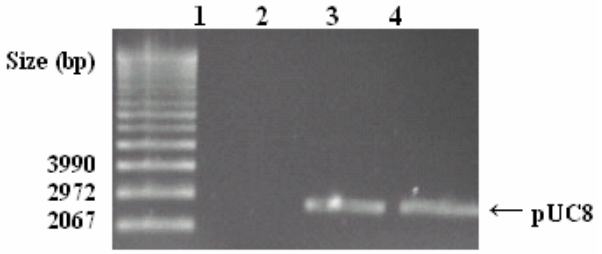

FIG. 1 Confirmation of the purity and size of isolated pUC8 plasmid DNA isolation by 0.7% agarose gel electrophoresis. Lane Key: 1 – 5 µg Supercoiled DNA Ladder (Invitrogen, 15622-012); 2 – empty; 3, 4 – 0.5 µg isolated plasmid DNA (undigested).

Confirming spheroplast formation. The formation of spheroplasts was induced as detailed in the materials and methods section. Transition of cell morphology from rods to spheres was confirmed by phase contrast microscopy, and was complete for more than 80% of the sample after two hours of incubation. Though it was not confirmed following recovery, it has been reported that within 18 hours of incubation in media lacking ampicillin, the morphology of the spheroplasts reverts back into rods (9).

Heat-shock and electrotransformation of spheroplasts. Growth was absent on the selective ampicillin plates for all treatments. Interestingly, replating large volumes following incubation at 4˚C over 24 hours resulted in growth being observed for

treatment 6, that is, the electroporated spheroplasts (data not shown). Plasmid DNA isolated from this sample, run on an agarose gel electrophoresis, as shown in Figure 2, resulted in bright bands in lanes 2, 3, and 4, representing undigested, supercoiled plasmid, which ran at the expected size of 2665 bp for pUC8. This was thus indicative of the successful transformation of spheroplasts by electroporation, whereas no transformants were obtained in any other sample. The strain of *E. coli* used for pUC8 plasmid DNA isolation plated on the selective plates as a positive control confirmed the growth of resistant cells on the medium.

Spheroplast survival rates. Unfortunately, the initial dilutions plated for total survival resulted in colonies that were too numerous to count (TNTC, data not shown). Thus, following storage at 4˚C, cultures were re-plated for total survival (Table 4). As shown in Table 5, the survival of spheroplasts appeared to be about half that of the wild-type cells. However, in another trial of experiments, survival of spheroplasts relative to control were 71% and 36%, for heat-shock and electroporation treatments, respectively (data not shown).

Table 4: Heat-shocked and electroporated *E. coli* DH5α samples plated on Luria-Bertani (LB) plates to assess total survival following 16 hours outgrowth at 37˚C and 24 hours incubation at 4˚C. Gly: glycerol, Suc: sucrose.

Treatment	Culture	Solution	Total Survival (x 10^5 cfu/ml)	
			Transformed (pUC8 plasmid DNA)	Mock Transformed (water)
1	Control	H_2O	289	125
2	Control	0.4 M Sucrose	137	137
3	Spheroplast	0.4 M Sucrose	64	167
4	Control	10% Glycerol	159	8
5	Control	10% Gly /0.4 M Suc	235	177
6	Spheroplast	10% Gly /0.4 M Suc	120	102

Table 5: Relative survival of control and spheroplasted *E. coli* DH5α following transformants by heat-shock and electroporation.

Experiment	Spheroplast survival as % of Control
Heat-shock	46.4
Electroporation	51.0

DISCUSSION

In the present study we investigated the effect of the loss of the cell wall and most of the OM on the transformability of control and spheroplast *E. coli* DH5α cells using both heat-shock and electroporation methods, and pUC8 plasmid DNA. As pUC8 plasmid DNA encodes a gene for ampicillin resistance, transformed cells were quantified by plating on selective plates containing ampicillin.

The isolated plasmid DNA was confirmed to be pUC8 by agarose gel electrophoresis. The sample was run undigested, and thus predicted to be mostly in the supercoiled conformation with other conformations (linear, open-nicked, relaxed circular, etc.) present in relatively small proportions. A band was observed at the expected molecular weight of pUC8, 2665 bp, indicating that we had isolated the pUC8 plasmid successfully for use in the following experiments. As expected, bands representing the other conformations were undetectable (Fig. 1).

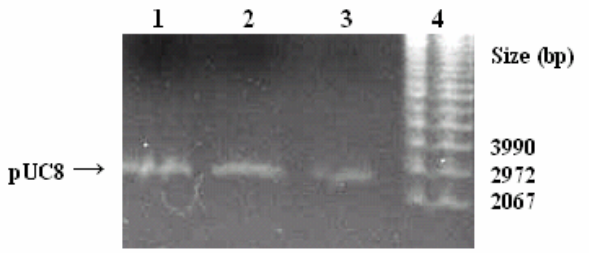

FIG. 2 Confirmation of the recovered pUC8 plasmid DNA in electroporated *E. coli* DH5α spheroplasts by electroporating isolated DNA in 0.7% agarose gel electrophoresis. Lane Key: 1, 2, 3 – 25, 20 and 10 µl, respectively of isolated plasmid DNA (undigested); 4 – 5 µg Supercoiled DNA Ladder (Invitrogen, catalogue number 15622-012).

Spheroplasting was accomplished as previously described by Liu *et al.* (2006), using media containing high sucrose (0.4 M) and ampicillin (50 µg/ml). The formation of spheroplasts was monitored over time by phase-contrast microscopy prior to proceeding with transformation experiments. Cell morphologies began to change within an hour of incubation due to the action of ampicillin on the cell wall formation, affecting the action of transpeptidase, carboxypeptidase, and endopeptidase (11), and after about two hours, approximately 80% of the cells were observed to be spherical (data not shown).

As *E. coli* cells are not naturally competent (7), we hypothesized that spheroplasts, lacking the cell wall and most of the outer membrane, may have an increased ability to take up DNA from the environment due to the loss of these physical barriers. However, contrary to our hypothesis, our results suggest that *E. coli* spheroplasts remain in a non-competent state and cannot be successfully transformed in the absence of competence inducing conditions. This was apparent as no selectable transformants were obtained from either of two trials of heat-shock treatment in the absence of divalent cations (data not shown).

In terms of survival, we predicted spheroplasts to be inherently more fragile than the control cells owing to their lack of cell wall, and thus a requirement for a hypertonic environment to counteract the osmotic

pressure on the cell. As shown in Table 5, we did observe a reduction in spheroplast survival relative to the control cells in the two trials, which may be attributed to the physical manipulations of these cells in the washing and re-suspending steps. However, it appears that the survival of spheroplasts is somewhat variable, our results showing 46% and 71% of control survival rates (Table 5 and data not shown).

Another method, electroporation, has been extensively used in the transformation of *E. coli* (13). Thus, we elected to determine the feasibility of carrying out this procedure on spheroplasts, expecting increased transformation efficiency, should they survive the procedure. Our results indicate that the spheroplasts were able to survive the electric shock. Transformation was carried out using a sample of 80% spheroplasts and 20% remaining rods, thus, if only the rods survived the treatment, the resultant colony forming units plated should have remained about 20% of the control numbers. As shown in Table 5, the total surviving spheroplasts were approximately 50% of the control, indicating the survival of spheroplasts.

To ensure that the media containing a high concentration of sucrose did not affect our experiments, we carried out the control *E. coli* transformations in both water and sucrose solution separately (for natural transformation experiments), and in glycerol and glycerol with sucrose (for electroporation transformation experiments). Table 4 shows the results of these trials. Natural transformation in water and sucrose solution produced a total of 2.89×10^{10} and 1.37×10^{10} cfu/ml, respectively, whereas electroporation transformation in glycerol and glycerol with sucrose resulted in a total of 1.59×10^{10} and 2.35×10^{10} cfu/ml, respectively. Given that these results are comparable within an order of magnitude, we have shown that sucrose did not have any effect, adverse or otherwise, on our experiments.

As expected, no transformants of the control cultures were observed on the selective plates. However, initially, electroporated spheroplasts did not produce any colonies when plated on the selective media either. Interestingly, plating three times the volume of the original culture following outgrowth for 16 hours at 37°C and incubation at 4°C for 24 hours, expecting a low colony count, actually produced growth that was TNTC on the selective media. Thus, the actual transformation efficiency by electroporation could not be accurately determined. The presence of the approximately 2665 bp band (Fig. 2) suggest that these cells were transformants carrying the pUC8 plasmid and not contamination. It appears that the additional incubation at 4°C may have somehow increased the survival of transformed spheroplasts, however, we have no definitive explanation for this observation. Growth of the original pUC8 containing *E. coli* DH5α on these selective plates confirmed that the concentration of ampicillin was not too high, and therefore did not initially prevent the growth of resistant cells.

The transformation efficiency of electroporated spheroplasts was unclear, and appears to be either negligible, or possibly quite high if the isolated plasmid is assumed to be pUC8. To consider the latter possibility would require further characterization of the plasmid DNA in order to verify its identity. However, due to the fact that the high growth was obtained following refrigeration overnight, which may have had unknown effects on the cultures, and growth was expected to be seen on the initial plates, direct comparison of these results should not be made. For this reason, we suspect that the transformation efficiency of electroporated spheroplasts was negligible, but not necessarily zero as only a small volume of the culture was plated. Therefore, we argue that the case of negligible efficiency may have been due to the presence of charge on the inner membrane, causing repulsion of environmental DNA molecules. Since the outer membrane of *E. coli* is composed of a high proportion of negatively charged LPS phospholipids (12), by extension, we propose that the inner membrane, also composed of phospholipids would be net negatively charged. This negative charge would repel DNA in the environment, preventing its binding and uptake. In addition, remnants of the OM on the spheroplasts could also provide a negative charge, preventing transformation in the absence of divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, etc.).

Divalent cations play a critical role in membrane stability by shielding the repulsive forces between phosphates on the outer surfaces (5). With respect to transformation and DNA uptake, diffusion of LPS in the outer membrane is thought to shield proteins required for DNA binding. Cations have been shown to both crystallize portions of the OM and reorient LPS molecules into different conformations, away from DNA binding proteins (5). However, Liu *et al.* (2006) were able to show that the use of a modified Hanahan protocol, and thus divalent cations, increased the transformation efficiency of spheroplasts over those of control cells by approximately 100 fold. Thus, we consider the role of abundant divalent cations in destabilizing membranes to be of increased significance over that of reorienting LPS, in this case.

In conclusion, it appears from our study that the transformation of spheroplasts in the absence of competence inducing conditions, specifically the presence of divalent cations, does not significantly increase efficiency over that of control cells. Heat-shock transformation resulted in no change in the state of competency, whereas electroporation appeared to produce negligible transformants. These results stress

the importance of the role of divalent cations in overcoming the repulsive force inherently present between environmental DNA molecules and cell membranes.

FUTURE EXPERIMENTS

It would be useful to repeat the experiments of this study with the addition of a positive control for transformation of competent cells to ensure that the electroporation and heat-shock procedures are carried out properly. Also, the reversion of spheroplast cultures into the rod morphology should be monitored by phase-contrast microscopy to ensure completion prior to plating in case the plasmid was in osmotically sensitive cells, and the optical densities of spheroplast and control cultures of the same number of cells should be compared in case the morphologies introduce a discrepancy.

The recovery period for control cells should not exceed one hour, as these samples will continue to grow. However, as spheroplast reversion to rod morphology must reach completion, it is necessary to determine their optimal recovery period. Being aware of these variables will limit the growth of samples following recovery, and thus result in a more accurate survival rates as well as numbers of transformants.

Other experiments could be performed to investigate the effect of lower temperature on spheroplast recovery, thereby explaining the increased growth of the electroporated spheroplasts on selective plates after the storage at 4^{o}C over 24 hours.

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