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The Effect of Spheroplast Formation on the Transformation Efficiency in *Escherichia coli* DH5α

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Based on the observation that transformation in *Escherichia coli* is an inefficient process, it was hypothesized that transformation may be hindered by the presence of the outer membrane and the cell wall. To test this hypothesis, cells which lack both a cell wall and most of its outer membrane, termed spheroplasts, were created and transformed with pUC8 plasmid. Spheroplast formation was induced by incubating exponentially growing *E. coli* DH5α in ampicillin and sucrose. To transform these spheroplasts, the cells were subjected to a modified version of the Hanahan protocol, which involves treating the cells with calcium chloride. The results of this experiment suggest that spheroplasts can transform at 4.9×10^{-4}. This is 100 fold more efficient than using the modified Hanahan protocol on wildtype *E. coli* DH5α cells.

Competence is the ability of a bacterium to take up foreign DNA from the environment and the recombination or incorporation of this DNA is known as transformation (14). Although *Escherichia coli* is not considered to be a naturally competent organism, competence can be developed through various procedures. These methods include glycerol (2) and calcium chloride ($CaCl_2$) treatment (6). However, these processes are highly inefficient, because most cells do not take up any DNA (14).

The outer membrane of *E. coli* cells is a barrier for DNA uptake, an impediment that is relieved somewhat by the presence of the divalent cation, Ca^{2+} that is used during the Hanahan protocol. Although the general mechanism of how $CaCl_2$ assists transformation is unclear, different proposed mechanisms of the effect of calcium (Ca^{2+}) on cells have shed light on how the outer membrane hinders transformation. For example, calcium may facilitate the interaction of DNA with the LPS molecules that are on the outer membrane of *E. coli* (14). In the absence of Ca^{2+}, the negatively charged LPS molecules on the outer membrane repel the negatively charged DNA molecule (14). In the presence of Ca^{2+}, however, the ion may suppress this repulsive force by acting as a cation bridge (14). Secondly, calcium treatment has also been shown to disrupt the membrane, thereby increasing its permeability and facilitating DNA uptake (14). Thirdly, there seems to be a correlation between transformation and the presence of poly-b-hydroxybutyrate/calciumpolyphosphate ($PHB/Ca^{2+}/PP_i$) membrane complexes (7). These complexes form channels or defects in the cytoplasmic membrane to allow DNA transfer. However, in order for DNA to bind to these complexes, DNA must still be able to penetrate the outer membrane to reach the periplasm (7). From these hypotheses it is clear that electrostatic and physical aspects of the outer membrane hinders DNA uptake. If this is true, then removing the outer membrane should increase transformation efficiency.

In the past, many groups have created strains of *E. coli*, such as ML-308, which have lost most their cell wall and only have remnants of an outer membrane (1, 17). Several groups have developed various ways to create potentially viable spheroplasts by using penicillin, lysozyme and ampicillin methods (5, 11). These methods allow the spheroplasts to revert back to their original growth competent rod-shaped morphology once the stress is relieved (8). Although one group had induced competence in spheroplasts, the results were not verified (11). In this study, the ability and the efficacy of spheroplasts to transform were explored. Results indicated that spheroplast transformation frequency is higher than wildtype cells when a modified version of the Hanahan protocol is used.

MATERIALS AND METHODS

Cells and Plasmids. *E. coli* DH5α cells (MICB 421 Teaching lab, frozen stocks) were used for spheroplast formation and transformation. These cells were grown on Luria-Bertani (LB) agar plates (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl and 1.5 % agar) at 37ºC and stored at 4ºC. Before use, cells were inoculated overnight into LB medium and shaken in a 37ºC water bath at 100 rpm. The *E. coli* strain expressing the pUC8 plasmid was a gift from the Smit Laboratory (UBC). pUC8 is a 2665bp plasmid that encodes an ampicillin resistance gene (4).

Plasmid extraction. Plasmid extraction was performed with an overnight culture of *E. coli* cells containing pUC8 using a plasmid mini-prep kit (Invitrogen, catalogue number K2100-01). The overnight culture was grown in LB at 37˚C with 2 mg / ml of ampicillin. To check that the plasmid extraction was successful, 0.5 µg of the plasmid was linearized with *Eco*RI (Life Technologies, catalogue number IS202-013) which cuts pUC8 once. The size of the plasmid was determined by gel electrophoresis using a 0.7% agarose gel at 10V/cm for 2 hours. Afterwards, the gel was stained with ethidium bromide and visualized using the Alpha Imager Software.

Formation of Spheroplasts. A modified protocol (8) utilizing ampicillin was used to induce spheroplast formation. An overnight culture of *E. coli* DH5α was inoculated into fresh LB medium at an OD_{660} of 0.1 and grown until OD_{660} was approximately 0.3. This

culture was then diluted 5 fold in LB, with each treatment containing the following reagents (Table 1). Cultures were then incubated at 37°C at 200 rpm until spheroplasts were formed (approximately 2 hours). The transition from rod to spherical morphology was observed by using a phase contrast microscope at 40X magnification.

Table 1. Induction of spheroplast formation in *E. coli* DH5α. An overnight culture of *E. coli* DH5α was diluted to an OD_{600} of 0.1, grown to OD_{600} of approximately 0.3 at 37°C in a shaking water bath, and diluted into LB supplemented with the following reagents. Reagents were used at the following concentrations to induce spheroplast formation in *E. coli* DH5α: 0.4M sucrose, 8 mM $MgSO_4 \cdot 7H_2O$, and ampicillin at 50 µg / ml.

Reagent	Treatment #			
	1 (Control)	2 (Sucrose only)	3 (Sucrose and ampicillin)	4 (Ampicillin only)
Sucrose	–	✓	✓	–
$MgSO_4 \cdot 7H_2O$	–	–	✓	–
Ampicillin	–	–	✓	✓

Transformation. Once spheroplasts were formed, the cells were then subjected to a modified version of the Hanahan protocol (16). Samples were centrifuged at 1100 g for 10 minutes and were resuspended in the following solutions (Table 2). Treatment numbers in Table 2 correspond to the treatment numbers in Table 1.

Table 2. Reagents used in the transformation of *E. coli* DH5α cells to induce cell competence. Reagents were used at the following concentrations to induce competence in *E. coli* DH5a: 0.4 M sucrose and 50 mM $CaCl_2$

Reagent	Treatment #			
	1	2	3	4
$CaCl_2$	✓	✓	✓	✓
Sucrose	–	✓	✓	–

The resulting solutions were then incubated on ice for 30 minutes and each treatment was then further split into two groups (A and B). Group A (totalling 4 samples) was incubated with 1.5 µg of DNA for 20 minutes on ice and Group B served as the "no DNA" control. Next the cells were incubated at 42°C for 2 minutes followed by an additional incubation on ice for 2 minutes. Cells were recovered by incubating in LB at 37˚C with shaking at 100 rpm. Treatment 2 and 3 (Group A and B) was supplemented with 0.4 M sucrose in order to continue osmotic protection. The next day, the resulting overnight cultures were plated on LB agar with or without ampicillin (100 µg/mL) in order to assay for transformation.

RESULTS

Confirming plasmid isolation. After isolating pUC8, 0.5 µg of the extracted plasmid was digested with *Eco*R1. The size of the plasmid was confirmed by gel electrophoresis (Fig. 1). The bright band in lane 2, representing the digested plasmid, ran at the expected size of 2665 bp (4). The brightest band in lane 3 represents the uncut, supercoiled pUC8 plasmid. The second fainter band in lane 2, which migrated the same distance as the bright band in lane 3, is residual undigested supercoiled plasmid.

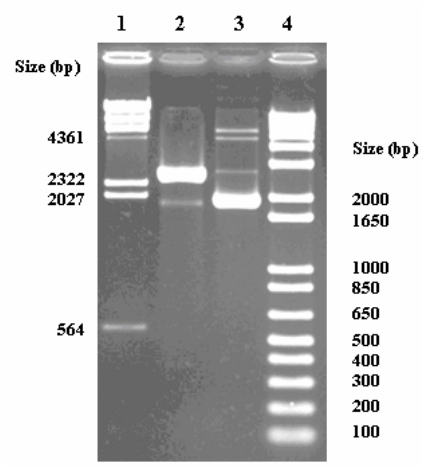

Figure 1: Agarose gel (0.7%) of *Eco*RI digested pUC8 (Lane 2) and undigested pUC8 (Lane 3) plasmid. Voltage was applied at 10 V / cm for 2 hours. Lane 1- λ DNA/HindIII ladder (1 µg as instructed by Invitrogen, catalogue number 10382-018); Lane 2- pUC8 digested with *Eco*R1 (Life Technologies, catalogue number IS202-013) (0.5 µg); Lane 3- undigested pUC8 (0.5µg); Lane 4- 1 kb plus DNA ladder (1 µg as instructed by Invitrogen, catalogue number12308-011).

Spheroplast formation and transformation. In this study, spheroplast formation was induced in treatment 3 when the exponentially growing culture was incubated with ampicillin and sucrose. After two hours of incubation, treatment 3 had changed from rods to spheres, as observed by phase contrast microscopy, whereas the other treatments remained as rods. Visual observation of these treatments indicated that treatment 4 had a notable decrease in turbidity. Cultures were incubated with DNA and these recovered in LB broth overnight. Within 18 hours, all of the treatments became turbid and the cells had converted back to their rod morphology.

Table 3. *E. coli* DH5α was grown with 1 of the 4 treatments and then treated with $CaCl_2$ or sucrose or both to induce competence. Cells were transformed with or without pUC8 and recovered overnight in LB. CFU: Colony forming units.

Treatment	DH5α grown on selected media (cfu/ml)			
	A. 1.5 µg pUC8 added		B. No pUC8 added	
	LB	LB + Ampicillin	LB	LB + Ampicillin
1. Luria Broth only	6.7×10^9	1.3×10^4	1.9×10^9	0
2. Luria Broth + 0.4 M Sucrose	4.7×10^8	5.2×10^4	1.0×10^9	0
3. Luria Broth + 0.4 M Sucrose + 50 µg/ml Ampicillin + 8 mM $MgSO_4 \cdot 7H_2O$	3.5×10^8	1.7×10^5	5.5×10^8	0
4. Luria Broth + 50 µg/ml Ampicillin	2.9×10^9	9.2×10^3	2.2×10^9	0

Table 3 details the number of transformants and the number of spontaneous mutants in each treatment group as well as the viability of the culture. When pUC8, a plasmid that expresses an ampicillin resistance gene, was added to the treatment, cells (Group A) gained antibiotic resistance. When the plasmid was omitted (Group B), however, cells did not gain antibiotic resistance. This suggests that ampicillin resistance does not occur spontaneously and that the transformants in Group A gained their antibiotic resistance due to the uptake and expression of pUC8 (Table 3).

Transformation efficiency with spheroplasts. All treatments resulted in the formation of transformants (Table 3). Treatment 3 not only had the highest number of transformants (Table 3), but it also had the highest transformation frequency (Table 4).

Table 4: Transformation efficiency of *E. coli* DH5α with pUC8 under different treatments.

Treatment	Efficiency (# of transformants/ total # of viable cells)
1. Luria Broth	1.9×10^{-6}
2. Luria Broth + 0.4 M Sucrose	1.1×10^{-4}
3. Luria Broth + 0.4 M Sucrose + 50 µg/ml Ampicillin	4.9×10^{-4}
4. Luria Broth + 50 µg/ml Ampicillin	3.1×10^{-6}

DISCUSSION

Transformation efficiency of wildtype and cell wall deficient *E. coli* DH5α was determined to study the effect of the cell wall on inhibition of transformation. In order to accomplish this, we isolated pUC8 DNA for transformation. Next, we induced *E. coli* DH5α to lose their cell walls and parts of their outer membrane. These cells, termed spheroplasts were transformed with pUC8. The resulting transformation frequency was compared with wildtype *E. coli* DH5α.

Gel electrophoresis was performed to ensure that the plasmid extraction was successful (Fig. 1). As expected, plasmids which were cut with *Eco*RI (Fig 1, lane 2) had 2665 bp (4). Because supercoiled plasmids (lane 3) are more compact, they migrate further than linearized plasmids (lane 2) (W. Ramey, personal communication). This indicates that some plasmids were not cut during the restriction enzyme digest. This inefficiency was probably caused by the non-optimal digest conditions; the optimal temperature for *Eco*RI activity is 37°C (W. Ramey, personal communication), however, due to a non-functional thermometer, the digestion was carried out between 55-60°C.

The next part of this study was to create viable spheroplasts with the ability to transform. Ampicillin degrades the peptidoglycan layers of the cell wall; therefore an osmotically protective environment, such as a high concentration of sucrose, was used in order to prevent the cell from undergoing lysis (8). The presence of both sucrose and ampicillin is needed to induce spheroplast formation. Since spheroplasts do not divide, and therefore do not grow on LB agar (8), transformed cells in treatment 3, along with the other treatments, were incubated in LB broth overnight to allow the cells to regenerate their cell wall.

It is important to note that spheroplasts, when subjected to the modified Hanahan protocol, transformed at a higher frequency than wildtype *E. coli* DH5α. Previously, some reports have stated that spheroplasts are too fragile and cannot survive the transformation procedure (8). Moreover, other studies have reported that the transformation frequency of peptidoglycan defective mutants resulted in transformation frequencies that were similar to wildtype cells (15). In this study however, the spheroplasts (treatment 3; Table 4) were able to transform and the transformation frequency was approximately 100 fold higher than that of the control (treatment 1; Table 4). One possible reason for this is that spheroplasts lack a cell wall and have only remnants of an outer membrane. Therefore, the removal of these physical barriers allowed DNA uptake to be more efficient.

Another important point from this experiment is that *E. coli* cells treated with only sucrose (treatment 2; Table 3) had more transformants than wildtype *E. coli* cells (treatment 1; Table 3). One possible reason for this is that the presence of sucrose alone weakened the integrity of the outer membrane (8). By removing this physical barrier, it assisted transformation. Thus, these sucrose treated cells (treatment 2; Table 4) had a higher transformation frequency when compared to wildtype, untreated cells.

Incidentally, transformants were also observed in cells treated with ampicillin but without sucrose (treatment 4; Table 3). Although the transformation frequency was not as high as the frequency for spheroplasts (treatment 3; Table 4), it was higher than that of wildtype, untreated *E. coli* cells (treatment 1; Table 4). One possible reason for this phenomenon is that the presence of ampicillin destroyed the cell wall causing many of the cells to lyse. However, a few of these cells did not lyse. Instead, they were now more

permeable for DNA uptake. Furthermore, because this process killed many of the cells, the competition for DNA decreased. In any case, the low transformation frequency and the low survival rate of cells treated with ampicillin only (treatment 4; Table 3) illustrate the necessity of using osmotic protectants, such as sucrose, to prevent lysis.

One cause of concern is that the control (treatment 1; Table 4) had a five-fold lower transformation frequency than was expected compared to previous findings (16). Normally, treatment of DH5α with the Hanahan protocol should result in a 10^{-5} transformation frequency (16). One possible explanation is that the control had time to reach stationary phase whereas the cultures treated with ampicillin and/or sucrose did not grow during spheroplast formation (12). As cultures age, cells undergo a morphological change, resulting in a less permeable cell wall (13). Thus, the DNA may have had difficulty penetrating the cell wall, thereby resulting in a lower than expected transformation frequency.

Originally, it was thought that treatment 3 and 4 could gain ampicillin resistance because both treatments were incubated in the presence of ampicillin during spheroplast formation. It was predicted that this could allow time for the cells to mutate and gain antibiotic resistance. However, the lack of ampicillin resistance colonies in the absence of pUC8 proved that incubation with ampicillin does not predispose the cell to gain antibiotic resistance.

It is also important to consider the growth characteristics of these cultures. Firstly, it appears that recovering cells grown overnight in sucrose (treatment 2; Table 3) had approximately a 10-fold decrease in cell density compared to cells grown overnight in LB only (treatment1; Table 3). One possible reason for this is that the high sucrose concentrations interfered with cellular sugar transport systems thereby making it more difficult for the cells to grow and divide (12). Furthermore, treatment 3 had the lowest cell density (Table 3) after growth in the overnight culture. One possible reason for this phenomenon is that spheroplasts cannot undergo cell division (8). Thus, the spheroplasts in treatment 3 first had to regain their rod morphology before division, and this requires time . As a result, the spheroplasts in treatment 3 grew slower in the overnight culture.

Another point to consider is that growing transformants overnight could skew the ratio of transformants to non-transformants. The reason is that maintaining a plasmid encoding an antibiotic resistance gene in the absence of the antibiotic can slow down the growth of the cell relative to non-transformed cells (10). Therefore over time, cells that did not receive the plasmid will be at a selective advantage (10). That is, the ratio of transformants to the total number of cells in the culture immediately after transformation may have been higher than the reported numbers in this study.

In conclusion, the use of ampicillin, in addition to a high concentration of sucrose, produced spheroplasts with the ability to transform at a frequency that was higher than the typical $CaCl_2$ treated cells. Although the viability of the spheroplasts was compromised, the fact remains that not only did the cells survive transformation with $CaCl_2$, but they also produced culturable transformants. The application of a more efficient transformation protocol involving the use of spheroplasts is important. In cases where DNA is limiting or the fragments are too large, spheroplasts may potentially be used to circumvent low transformation rates.

FUTURE EXPERIMENTS

Due to time constrictions, this study was only performed once and will need to be conducted again in order to confirm these findings. Some researchers have stated that the ability of spheroplasts to transform is highly inconsistent, with only 6 out of 10 trials producing transformants (5). Because the data for this study was obtained from only one trial, these experiments must be repeated in order to reduce the uncertainty in the data caused by human error. For example, about 20% of the plated samples in this study had minor errors, such as over-crowding of colonies, particularly at edges of the plate. Regardless, there is a 100-fold increase in transformation frequency for spheroplasts relative to wildtype *E. coli* cells, and this is unlikely due to experimental error.

Future experiments should also track the growth of the culture in addition to plate counts by taking optical density (OD_{660}) measurements during spheroplast formation to better characterize the changes that may occur in the samples. Moreover, one can test whether or not growth in the overnight culture skewed the ratio of transformants to non-transformants by plating the spheroplasts immediately after transformation using agar supplemented with sucrose (9). Furthermore one can test whether the cause of the low transformation frequency of the control (treatment 1), was to due to the use of a culture that was too old by utilizing an exponentially growing culture instead.

Another direction for future studies is to test whether or not spheroplasts themselves are naturally competent in the absence of $CaCl_2$ treatment. If $CaCl_2$ is not required for spheroplast transformation, this would indicate that the major role of calcium during transformation is to facilitate binding of DNA to the outer membrane. However if $CaCl_2$ is required, it suggests that calcium is also necessary to modify the cytoplasmic membrane permeability, likely by interactions with the PHB/PP_i complex in order to

allow the DNA to pass through. Conversely, it may be interesting to test whether or not electroporation, a method that is harsher than the $CaCl_2$ treatment (3), can work with spheroplasts. Although spheroplasts are more sensitive to osmotic pressure (8), the presence of sucrose in the medium may prevent cell lysis. If spheroplasts can survive electroporation, they may even have a transformation efficiency that is higher than wildtype cells because they have fewer barriers blocking DNA uptake.

Furthermore, spheroplasts can be used in order to determine whether the outer membrane hinders transformation of high molecular weight plasmids. If the outer membrane and the cell wall are the main physical barriers, then spheroplasts which are not subjected to $CaCl_2$ treatment should be able to transform more efficiently than competent wildtype cells. If however, the cytoplasmic membrane is the main barrier, then spheroplasts should not have an advantage in transformation when high molecular weight plasmids are used.

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