The Effect of Increasing Plasmid Size on Transformation Efficiency in *Escherichia coli*

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Based on the observation that the transformation of *Escherichia coli* was more efficient with pUC19 than with the larger plasmid pBR322, we hypothesized that transformation frequency is somehow affected by size. To test this hypothesis, we attempted to insert a 1.7kb lambda *NdeI* fragment into pUC19 to generate a plasmid (pHEL) of the same size as pBR322. The two plasmids of equal size were then to be used to transform *E. coli* in order to compare transformation efficiencies. After two rounds of cloning, we were unable to generate pHEL. In lieu of using pHEL and pBR322, *E. coli* were transformed with previously prepared plasmids of varying sizes: pUC8 (2.6 kb), pUC8 0-690 (4.3 kb), and pUC8 0-690::pKT210 (16.1 kb). The results of these transformations indicate that increasing plasmid size correlates with a decrease in transformation efficiency.

Transformation is an important technique in molecular cloning for transferring genetic material to bacteria. It can be done by either heat shock or electroporation. The former involves the preparation of competent cells, incubation of the cells with DNA at 0°C and the completion of DNA uptake by heat pulse. Competent cells are capable of taking up DNA. They can be prepared by cold treatment with calcium chloride. The exact mechanism of the uptake of DNA is not known. However, it is thought that the presence of calcium ions allows the negatively charged DNA to come in close contact with the similarly charged cell membrane by acting as the cross bridge. Together with low temperature, the divalent cations affect the integrity and organization of the lipopolysaccharide layer of the cells and stabilize the binding of DNA to the cell membrane near channels through which the transport of DNA might occur (9). These channels are associated with zones of adhesion, where the outer membrane and the inner membrane are fused through holes in the cell wall, allowing transport of macromolecules (2). In addition, the chloride ions that are permeable through the cell membrane are taken up into the cell along with water molecules, resulting in swollen cells which are more vulnerable and able to take up DNA ([http://www.genome.ou.edu/protocol_book/protocol_adxF.html](http://www.genome.ou.edu/protocol_book/protocol_adxF.html)). The heat shock step acts on a later phase of transformation by transiently melting the membrane to complete the uptake process (2). The efficiency of transformation depends upon many different factors including the competent state of the cells and the properties of the DNA to be transformed.

In a previous experiment, the transformation efficiency of two plasmids, pUC19 and pBR322, were compared in *Escherichia coli*. It was observed that the number of transformants that resulted with pUC19 was significantly higher than with an equal mass of pBR322. Because the two plasmids are of different sizes, it was therefore postulated that the size of the plasmid might have affected the transformation efficiency: assuming it is more difficult for larger molecules to get through the channels in the cell membrane. To test this hypothesis, we tried to normalize the size of the two plasmids by constructing a plasmid with a 1.7 kb lambda fragment inserted into pUC19 and examine whether the transformation efficiency of this new plasmid is the same as pBR322. Unfortunately, both attempts to obtain a transformant clone with the new desired plasmid were unsuccessful. As a result, a different set of available plasmids were used instead to examine the effect of plasmid size on transformation efficiency: pUC8, pUC8 with a 1.7 kb insert (pUC8 0-069) and fusion of pUC8 0-690 with pKT210. In addition, to eliminate the effect of different numbers of plasmid molecules and total mass of DNA, equal molar concentrations and equal mass of the two plasmids were used.

**MATERIALS AND METHODS**

Construction of pUC19 plasmid with 1.7 kb insert (pHEL)

*E. coli* strain DH5α containing the pUC19 plasmid (Dr. Ramey, UBC) was used to isolate pUC19 DNA (Appendix II) using the Promega Wizard DNA purification system according to the manufacturer’s protocol. pBR322 (Appendix II) DNA was also isolated in the same manner from DH5α. *E. coli* containing that plasmid (Dr. Ramey, UBC). pUC19 DNA was digested with *NdeI* (Invitrogen) in standard conditions at 37°C overnight to linearize the plasmid. The 1.7 kb insert for ligation with the pUC19 plasmid was generated by digesting lambda DNA previously cut with *HindIII* (Dr. Ramey, UBC) with *NdeI* overnight. The resulting fragments were excised from a 0.8 % agarose gel, and extracted using a Bio-
Rad gel extraction kit according to the manufacturer’s directions. A ligation was set up with an insert to vector ratio of 3:1 using New England Biolabs Ligation Buffer and T4 DNA Ligase, and the reaction was incubated at 4°C overnight. Wild-type DH5α cells were rendered competent by calcium chloride treatment (6) and used for transformation (6) with the ligation reaction and plated on Luria Broth plates (Appendix I) with ampicillin at a concentration of 100 µg/ml for selection. An equivalent amount of unnanipulated pUC19 DNA was also used in a transformation reaction as a positive control. The resulting transformants were tested for correct plasmid size by Slot Lysis (7, Appendix I) using E. coli containing the pBR322 plasmid as well as the pUC19 positive control for comparison.

Since the previously detailed ligation was unsuccessful, modifications were made to increase the probability of success. A larger amount of lambda DNA was used in the digestion reaction, and a larger amount of pUC19 was isolated from cells by adding chloramphenicol at a concentration of 170 µg/ml when the culture had reached an OD600 of 0.8, and incubating the culture for a further 3 hours at 37°C in a shaking water bath. As well, the 5’ phosphate groups of digested pUC19 were removed to decrease re-circularization of the vector during the ligation reaction. pUC19 DNA was isolated in the same manner as described above, and digested with NdeI for 3 hours. The digestion was carried out in NEBuffer 4 (New England Biolabs) so that the resulting fragment could be dephosphorylated with Calf Intestinal Alkaline Phosphatase (New England Biolabs) without a change in buffers. Following digestion, the NdeI enzyme was heat inactivated at 65°C for 20 minutes. Five hundred units of CIAP (New England Biolabs’ instructions calls for 0.5 Unit/µg vector DNA. Less than 1 µg of DNA was present in the digestion reaction, which would require the addition of 0.5 Units of CIAP. However 500 Units was added due to the ambiguous labelling of the tube containing CIAP) was added to the digestion reaction and incubated according to the manufacturer’s instructions and the resulting dephosphorylated vector was isolated from an agarose gel as previously described. The 1.7 kb insert was generated by digesting uncut lambda DNA (Dr. Ramey, UBC) with NdeI and excising the 1.7 kb band from an agarose gel as described above. A ligation reaction was set up with an insert to vector ratio of 4:1 using reagents from New England Biolabs, and incubated at 4°C overnight. DH5α competent cells were generated and transformed with the ligation reaction, and unnmanipulated pUC19 DNA was again used as a positive control for the transformation. As no colonies were observed after incubation of plates containing the cells transformed with the ligation reaction, slot lysis was not performed for analysis.

Testing Plasmid Size and Equal Molar Amounts on Transformation Frequency

Plasmids were obtained (Smit Laboratory, UBC) that allowed for testing the effects of increasing plasmid size on transformation frequency, and that closely resemble the construct we had tried to create above. pUC8 is the parent plasmid of pUC19; pUC19 is identical in sequence to pUC8 except for the inversion of the multiple coding sequence and the addition of approximately 10 nucleotides to create additional restriction enzyme sites (Appendix II). pUC8 0-690 (Appendix II) was created by ligating a 1.6 kb insert into the BamHI and HindIII sites in pUC8 to create a 4.3 kb plasmid that is approximately the same size as the pHEL vector that was intended to be constructed above. pUC8 0-690:pKT210 was constructed by ligating EcoRI-digested pKT210 (Appendix II) into the EcoRI site in pUC8 0-690, and the resulting plasmid is approximately 16.1 kb in size. pKT210 is a high copy number plasmid with sequences that allow for broad host range, as well as a chloramphenicol resistance gene for selection (www.dsmz.de/lit/lit5183.htm).

DNA was isolated from the following strains using an Alkaline Lysis protocol modified from the Maniatis Manual by treating samples with phenol-chloroform before performing the ethanol precipitation (3): DH5α E. coli containing the pUC8 plasmid, JM101 E. coli containing the pUC8 0-690 construct, DH5α E. coli containing the pUC8 0-690:pKT210 vector, and DH5α E. coli containing the pBR322 plasmid. Samples were treated with RNase at 50 µg/ml, and incubated at room temperature for 1 hour to remove any RNA contamination. Transformations were performed as previously described with equal molar amounts of each of the plasmids to test increasing plasmid size on the efficiency of transformation: 0.95 µg of pUC8, 1.52 µg of pUC8 0-690, and 5.2 µg of pUC8 0-690:pKT210. To test the influence of unequal molar amounts of DNA, transformations using 1.52 µg and 0.95 µg of pUC8 0-690:pKT210 were performed as well for comparison. A positive control transformation was performed with 0.95 µg pUC19 DNA (Dr. Ramey, UBC). Transformants were plated on LB plates with ampicillin at 100 µg/ml, except for cells transformed with pUC8 0-690:pKT210 which also had chloramphenicol added at 20 µg/ml. The result was an absorbance reading at A260 and A280. The isolated pUC19 and pUC8 0-690::pKT210 vector, and DH5α containing the pBR322 plasmid is approximately 16.1 kb in size. pKT210 is a high copy number plasmid with sequences that allow for broad host range, as well as a chloramphenicol resistance gene for selection.

RESULTS AND DISCUSSIONS

Preparation of pHEL (pUC19 + 1.7kb insert)

The isolation of pUC19 and pBR322 from E. coli DH5α strains containing these plasmids was achieved using the Promega Wizard Kit. Concentrations of 75.0 ng/µl of pUC19 and 76.5ng/µl of pBR322 were obtained according to absorbance readings at A260 and A280. The isolated pUC19 and HindIII-digested lambda were then digested with NdeI and gel extracted. These digested samples and the undigested pUC19 and pBR322 were run on an agarose gel to determine sample concentrations (Figure 1). The concentrations were determined to be: NdeI-digested pUC19 – 5 ng/µl, NdeI-digested lambda – 3.5 ng/µl, pUC19 undigested – 5 ng/µl, pBR322 undigested – 2.5 ng/µl. These concentrations are significantly lower than the concentrations determined by the absorbance readings. To ensure these absorbance readings were not due to contamination by RNA, chromosomal DNA, or protein, samples could have been run on an agarose gel with a concentration ladder prior to digestion and gel extraction. At the time the absorbance readings were taken, there was no reason to suspect the absorbance readings were too high due to contamination because the Promega Wizard Kit excludes RNA and chromosomal DNA, purifying for plasmid DNA. However, the discrepancies between the concentration values may be due to low absorbance readings outside the optimal range of the spectrophotometer or the presence of proteins or sugars both of which can affect absorbance readings. The decrease in the DNA concentration values likely were not due to the manipulations of the digestion or gel extraction procedure performed on pUC19 as these procedures were not performed on the pBR322 sample. The calculated DNA concentration values for both manipulated and unnmanipulated samples decreased by similar amounts (approximately 70 ng/µl).
Figure 1. Sizes and concentrations of Ndel-digested plasmid DNA samples and undigested plasmid DNA samples. Plasmids were isolated using Promega Wizard DNA Purification System and run on 0.8% agarose gel. Lane 1 – 1 kb plus DNA ladder; Lane 2 – Lambda HindIII ladder (23.1 kb, 9.4 kb, 6.6 kb, 4.4 kb, 2.3 kb, 2.0 kb); Lane 3 – Ndel digested lambda; Lane 4 – Ndel digested pUC19; Lane 5 – undigested pUC19; Lane 6 – undigested pBR322. DNA concentration was estimated by comparing the intensity of the band to the 2.0 kb fragment of the lambda DNA ladder which corresponds to 21 ng.

The 2.6 kb fragment of Ndel-digested pUC19 and a putative 1.7 kb fragment of the Ndel-digested lambda were gel extracted (gel not shown). The band containing the 1.7 kb lambda fragment was smeared and was not distinct from the band containing a 2 kb lambda fragment that was generated from the previous HindIII digestion. Therefore, both 1.7 kb and 2 kb lambda fragments may have been extracted. The presence of the 2 kb fragment was not detected as a separate band on the gel used to check concentration (see Figure 1). These bands are of similar size and therefore may not have resolved as separate bands. The 2 kb lambda fragment is cut by HindIII at both ends (Appendix II). Because the two restriction enzymes are incompatible, the 2.0 kb fragment cannot ligate to the Ndel-digested vector and it will remain linear. From the concentrations determined above, a ligation reaction with T4 ligase was set up with an insert to vector ratio of 3:1 (56 ng ~1.7 kb fragment from lambda: 29.6 ng pUC19). However, the presence of the 2.0 kb fragment in the sample implies that the ratio was in fact less than 3:1 and might have decreased the probability of successful ligation. Table 1 shows the transformation frequencies of the ligation reaction into competent cells.

The colony morphology of the cells on the negative control was similar to that of the colonies on the positive control and transformation plates implying that this growth likely was not due to random contamination. However, cells from the positive control may have contaminated the negative control plate if the spreader was not submerged in enough ethanol to kill all bacteria on spreader and/or not sufficiently flamed. Colonies on the negative control indicate that the competent cells without the plasmid were able to grow on the selective plates. This suggests a problem with the selection process. The transformed cells were selected for using ampicillin as pUC19 and pHEL contain an ampicillin resistant marker. A possible explanation is that the ampicillin was not effective. The ampicillin provided in the lab was first opened in 1983. Ampicillin is subject to degradation in the presence of moisture. During the 19 years that the antibiotic has been in use it is possible that enough hydration has occurred to
render the ampicillin completely or partially ineffective. In this scenario, selection would be weakened or eliminated as both transformed and non-transformed cells would form colonies.

Table 1: Frequencies for the transformation of E. coli DH5α with pHEL

<table>
<thead>
<tr>
<th>Plate containing DH5α +</th>
<th>Volume plated (µl)</th>
<th>CFUa</th>
<th>CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DNA in transformation</td>
<td>1000</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>pUC19 (undigested)</td>
<td>1000</td>
<td>TNTC</td>
<td>TNTC</td>
</tr>
<tr>
<td>pUC19 + 1.7 kb</td>
<td>100</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>pUC19 + 1.7 kb</td>
<td>900</td>
<td>87</td>
<td>97</td>
</tr>
</tbody>
</table>

a Colony forming units
b Too numerous to count

Unmanipulated pUC19 was transformed into competent plasmid free DH5α cells. There was a large amount of growth on this positive control indicating that the transformation procedure was successful. This also indicates that some selection was occurring on the ampicillin plates as the negative control had far fewer CFU/ml than the positive control plate. Another control that could have been performed would be to transform pUC19 digested with NdeI and religated with T4 ligase without the lambda 1.7 kb insert. This would have been beneficial in determining whether the problems were with the digestion or the ligation reactions.

When the products of the pUC19 + 1.7 kb lambda fragment ligation reaction were transformed into plasmid-free DH5α cells, approximately 100 CFU/ml were obtained. These colonies represent candidates for transformed DH5α cells containing pUC19 + 1.7 kb insert. A slot lysis was performed to identify a transformant with the correct size plasmid (4.3 kb). Figure 2 shows representative picture of the slot lysis gels. Despite growth on the negative control plate of the transformations, we decided to proceed with the slot lysis. Considering the volumes plated, the number of colonies on the negative control was significantly lower than the number of colonies on the positive control and the pUC19 + lambda transformation plates. Significantly lower numbers of colonies on the negative control plates indicates that some selection was occurring. This was likely due to partial activity of the ampicillin. Therefore, it was assumed that the majority of the colonies on the non-control transformation plates contained plasmids with the ampicillin resistance marker, and that some of these transformants may contain plasmids of the desired size.

Lane 1 of Figure 2 contains DNA from a colony picked from the pUC19 positive control plate, however no band representing plasmid DNA can be seen. This is likely due to the very small size of the colony used in preparing the sample for loading on the gel. Lane 16 shows the DNA from another colony from the pUC19 positive control plate. Lane 2 contains pBR322, which represents the target size and was used to compare the bands in lanes 3 – 15 and 18 – 30. These bands represent plasmid DNA isolated from colonies selected for on ampicillin plates. These colonies should represent transformants; however, as discussed above, the selection process may not have been completely effective. None of the plasmids in Lanes 3 – 15 or 18 – 30 ran at the same size as supercoiled pBR322 (4.3 kb), therefore a clone containing the desired plasmid (pHEL) was not detected. Several of the lanes contained bands running at lower molecular weights, possibly representing religated pUC19. This is plausible because of the small amount of lambda insert available for ligation into pUC19. With very little lambda DNA (~56 ng) available as insert in the ligation reaction, a 3:1 insert to vector ratio meant that only a small amount of the vector DNA (29.6 ng) was used, resulting in low total amount of DNA. In addition, a final volume of 30 µl in the ligation reaction may have been too high for the total amount of DNA used in the ligation reaction, prohibiting sufficient interaction of insert DNA with vector DNA. This dilute reaction would promote the religation of pUC19 with itself instead of ligating the insert into the vector. Though this positive control gives a comparative reference which suggests that the plasmids present in the transformed colonies are religated pUC19, there was no supercoiled molecular weight...
ladder run for size comparison to definitively conclude whether the bands seen represent a supercoiled molecule of 2.6 kb. Considering the results in Figure 2, the cloning of pHEL was unsuccessful, presumably due to the ligation step.

![Figure 2](image)

**Figure 2. Slot Lysis of isolated pBR322 or DH5α transformants of pUC19, pUC19 with 1.7 kb insert.** Single colonies were resuspended in protoplasting buffer, lysed in individual wells and run on a 0.8% agarose gel. Lane 1, 16 - undigested pUC19 transformants; Lane 2 – isolated pBR322; Lanes 3 – 15, 18-30 – ligation reaction transformants; Lane 17 – empty.

**Preparation of pHEL – Trial 2**

Based on the explanation for the negative results in Trial 1, the cloning procedure was repeated with modifications designed to increase the possibility of creating the desired clone. A chloramphenicol amplification was performed to increase plasmid yield as chloramphenicol stops cell division while replication of plasmid DNA continues, resulting in a higher plasmid to chromosome ratio. The concentration of DNA after isolation with the Promega Wizard Kit was not determined until after the NdeI digestion and gel extraction of pUC19 and lambda DNA. pUC19 was then treated with a phosphatase enzyme to eliminate the possibility that the vector would religate with itself. However, we have since learned that T4 ligase is dependent on the presence of a 5' phosphate (4). T4 ligase uses the 5' phosphate to seal the breaks between the 5' phosphate and the 3' hydroxyl group on each strand of the DNA (4). NdeI cuts leave a 5' overhang and therefore a 5'phosphate group (http://www.nick.med.usf.edu/GCGdoc/Data_Files/enzyme_data_files.html). This phosphate would have been cleaved from the phosphatase-treated, NdeI-digested pUC19, but not from the NdeI-digested lambda. This would leave two 5' phosphates available for ligation where four are required, resulting in unstable ligation products. Incomplete ligation could have decreased the transformation frequency below a level detectable with the dilutions and methods of this experiment.
The concentrations of the DNA at this time were 7.5 ng/µl for pUC19 and 6.5 ng/µl for the lambda fragment (Figure 3). In Figure 3, dephosphorylated and NdeI-digested pUC19 (Lane 4) ran at a molecular weight of ~3 kb. This is unexpected as the known size of pUC19 is 2.6 kb. To confirm the plasmid was in fact pUC19, the E. coli cells from which the plasmid was isolated were streaked onto a LB plate with X-gal. Blue colonies were observed after a 2 day-incubation. Since pUC19 is the only plasmid available in the lab that can α-complement the partial lacZ gene in DH5α, it was concluded to be the correct strain and that the plasmid should be pUC19. One possible explanation for the unusual migration is that residual binding matrix from the gel extraction procedure may have remained bound to the DNA, impairing its movement through the gel. The ligation reaction was performed with a 4:1 insert to vector ratio (117 ng 1.7 kb lambda fragment: 45 ng pUC19) to increase the possibility of the insertion of the 1.7 kb fragment into the digested pUC19. The total amount of DNA (162 ng) used in this ligation reaction was greater than in the Trial 1 ligation reaction. In addition, there was only a 5 µl difference in total volume between the Trial 1 (30 µl) and Trial 2 (35 µl) ligation reactions. The total amount of DNA and final volume of the Trial 2 ligation reaction provided more favorable conditions for ligation of the insert into the vector. Therefore, low transformation frequencies likely were not due to these factors. The results of the transformations using the ligation reaction (pUC19 + insert) showed no growth; no transformants were detected. The lack of transformants was likely due to the use of the phosphatase. In this case, both vector and insert DNA may have remained linear, and would be degraded when taken up by competent cells. An alternative possibility for the lack of transformants was the very small amount of DNA used in the transformations. The products of the ligation reaction were used at several dilutions in the transformation reaction – 9.2 ng DNA/10 µl; 46 ng DNA/20 µl; 23 ng DNA/10 µl and 69 ng DNA/15 µl. This range of DNA concentrations was used in the transformations to ensure that enough DNA would be present for the frequency of transformations to be detected while diluting the components of the ligation reaction so that they would not inhibit transformation. Unfortunately, the DNA concentrations were not high enough which rendered the frequency of transformations below a detectable level. Our second attempt at producing pHEL was unsuccessful probably due to the use of phosphatase on NdeI-digested pUC19 and the small amounts of DNA available for the each transformation reaction.

Isolation of plasmid DNA from cultures of E. coli transformants

In an effort to obtain better yields of plasmid DNA from E. coli cultures than those that had been achieved using the Promega Wizards plasmid DNA purification system, the alkaline lysis protocol (3) was used to isolate plasmid DNA. Figure 4 shows the relative amounts of DNA that were acquired from 50 ml of overnight cultures of each of the E. coli cell lines containing the plasmids of interest. The total amounts of each plasmid that were isolated are as follows: pUC8 – 1.71 µg, pUC8 0-690 – 1.52 µg, pBR322 – 525ng, and pUC8 0-690::pKT210 – 9 µg. For the pUC8, pUC8 0-690, and pBR322 samples, several bands migrating at different speeds can be seen (this is especially evident for the pUC8 0-690 sample where at least four darker bands are present). These bands represent the various topoisomers of the plasmid DNA – supercoiled (perhaps various degrees of coiling are represented here by bands migrating at different rates), open circular (nicked), and linear DNA. Interestingly, fewer bands were seen when the plasmid DNA was purified using the Promega Wizards plasmid DNA purification system and run on a gel. However, the alkaline lysis protocol that was followed in this case requires several manipulations as compared to the miniprep kit protocol. Thus, overhandling could have caused a substantial amount of the DNA in each preparation to become nicked or fully linearized. In contrast, the appearance of only one band in lane 4 suggests that the pUC8 0-690::pK210 DNA appears to be wholly migrating at the same rate. However, it is unlikely that all of this plasmid is present in the same form. Rather, each of the bands is migrating to roughly the same location near the top of the lane due to the large size of the plasmid (approximately 16 kb). Running the gel for a longer period of time would likely result in resolution of these bands. Although the yields of pUC8 and pUC8 0-690 are roughly equivalent, the amount of pBR322 that was isolated is much lower. This is presumably due to the fact that the phenol extraction was performed twice in the isolation of pBR322 and a considerable portion of the DNA was lost in each step (since the protein layer was rather diffuse, only about 2/3 of the aqueous layer containing the DNA was removed in each step).
Figure 3. Sizes and concentrations of NdeI-digested and dephosphorylated pUC19, and NdeI-digested lambda fragment. pUC19 and lambda fragment were gel extracted and their concentrations were determined by comparing the band intensity to lambda ladder. Lane 1 – Lambda HindIII ladder; Lane 2 – 1kb Plus DNA Ladder; Lane 3 – NdeI-digested lambda fragment (~1.7kb); Lane 4 – NdeI-digested, dephosphorylated pUC19.

Figure 4. Agarose Gel Electrophoresis of pUC8, pUC8 0-690, pUC8 0-690::pKT210, and pBR322. Plasmids were isolated via the alkaline lysis method and 10µl of each preparation was run on a 0.8% agarose gel to determine the concentration (compared to a lambda ladder). Lanes 1, 6 – Lambda HindIII ladder; Lane 2 – pUC8; Lane 3 - pUC8 0-690; Lane 4 – pUC8 0-690::pKT210; Lane 5 – pBR322.
Transformation of *E. coli* DH5α with pUC8, pUC8 0-690, and pUC8 0-690::pKT210

The results from the transformations of *E. coli* with the various plasmids that were isolated are outlined in Table II. Because of the small yield of pBR322 DNA, we were not able to include it in this experiment. Concentrations of DNA were adjusted to normalize for both: a) molarity of the DNA (i.e. 0.95 µg of pUC8 vs. 1.52 µg pUC8 0-690) and b) total weight of DNA (pUC8 0-690::pKT210 transformations were done with 5.2 µg, 1.52 µg, and 0.95 µg of DNA). This was done in order to separate out any effects due to the concentration and/or weight of the DNA from those that are due to plasmid size. The growth of transformants on the pUC19 positive control plate confirms that the *E. coli* cells that were treated with CaCl₂ were in fact competent and transformable at an appreciable rate. It is therefore unlikely that any negative transformation results are due to the improper and/or unsuccessful preparation of competent cells. Comparing the transformation frequency of the pUC8 transformation (358 transformants/ml) with that of the pUC19 positive control (a nearly confluent lawn of cells was obtained from plating 1ml of the transformation reaction) reveals that the pUC8 transformation reaction was less efficient. This is unexpected since pUC8 and pUC19 are nearly identical in sequence and size and the same amount of DNA was used in each of the transformation reactions. This suggests that a component of the pUC8 DNA preparation may have inhibited transformation. It has been shown that linear DNA molecules in cell + DNA mixtures can inhibit the transformation of *E. coli* (5). The pUC8 and pUC8 0-690 DNA preparations contained linear DNA (as shown in Figure 4), and this may have contributed to a decrease in transformation efficiency. The pUC19 DNA that was used for the positive control was isolated on a Quiagen column in 2000 and stored as a frozen stock. The freezing may have caused the DNA to become nicked, however since the DNA was not run on a gel prior to its use we cannot be certain if this preparation contained a significant amount of linearized plasmid DNA.

### Table 2 – Transformation efficiency of *E. coli* DH5α with plasmids of varying sizes:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Total size (kb)</th>
<th>Amount DNA used (µg)</th>
<th>CFU per plated volume:</th>
<th>Efficiency (transformants/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>50µl</td>
<td>100µl</td>
</tr>
<tr>
<td>pUC8</td>
<td>2.7</td>
<td>0.95</td>
<td>59,29,0</td>
<td>1,72,0</td>
</tr>
<tr>
<td>pUC8 0-690</td>
<td>4.3</td>
<td>1.52</td>
<td>0,0,0,0</td>
<td>10,8</td>
</tr>
<tr>
<td>pUC8 0-690 + pKT210</td>
<td>16.1</td>
<td>5.20</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.52</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.95</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>-ve control (amp)</td>
<td>n/a</td>
<td>none</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>-ve control (amp + chloramphenicol)</td>
<td>n/a</td>
<td>none</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>pUC19 (+ve control)</td>
<td>2.7</td>
<td>0.95</td>
<td>TNTCb</td>
<td>n/a</td>
</tr>
</tbody>
</table>

a 3 plates were prepared with each volume at each of the DNA concentrations for pUC8 0-690 + pKT210  
b Only 1 plate was prepared each of the controls on which all of the cells in the transformation mixture were plated (1.07ml total volume)  
c Values from all of the plates were added and divided by the total volume plated  
d The standard deviation is calculated by taking the square root of the total number of colonies then dividing by the total volume plated.
As can be seen in Table II, the colony counts at each of the plated volumes are somewhat inconsistent - for example, plating 50 µl of the pUC8 transformation reaction yielded CFU’s of 59, 29, and 0. However, we believe that it is likely that the plates yielding surprisingly low colony counts (i.e. 0 or 1 colony as opposed to 72) are an experimental error. The randomization of colony counts may have occurred in one of two ways. The first is uneven spreading of ampicillin on the plates. As ampicillin was not included in the media when the plates were initially poured, the antibiotic was spread onto the surface of the agar before the transformed cells were plated. It is therefore possible that the plates yielding higher numbers of colonies had not received sufficient antibiotic for selection. However, the presence of small clumps of dense colony growth around the edges of these plates (where the ampicillin might not have been spread thus allowing growth of non-transformed E. coli) that are not present on the rest of the surface of the plate indicates that they were in fact treated with adequate ampicillin. The second and more likely explanation for the apparent randomization of colony counts is the use of a spreader that was not adequately cooled before plating the cells. If this were the case the hot spreader would have killed the cells, accounting for the plates without any growth.

Although the colony counts on the plates appear to be somewhat randomized, the calculated transformants/ml values (see Table II) nonetheless reveal quite a marked trend: the transformation efficiency decreases as plasmid size increases. The pUC8 transformation yielded 358 transformants/ml with 0.95 µg DNA. In contrast, the pUC8 0-690 transformation was much less efficient, yielding only 45 transformants/ml even though the amount of DNA was adjusted to normalize for molarity (i.e. 1.52 µg was used rather than the 0.95 µg used for the pUC8 transformation). This effect is even more apparent in the pUC8 0-690::pKT210 transformations, where none of the 18 plates that were prepared showed any growth. It is important to note in this case that this may not be a true representation of the results for this transformation. It was observed that the E. coli cells harboring the pUC8 0-690::pKT210 plasmid grew substantially slower than the E. coli cells harboring either of the smaller plasmids – the “overnight” culture that was used to isolate the plasmid was actually grown over a period of 2 days before it reached a sufficient turbidity. Since the transformation plates were only examined after 24 hours of growth, we cannot rule out the possibility that E. coli cells transformed with the pUC8 0-690::pKT210 plasmid would have grown had the plates been examined over a longer period of time. Nevertheless, the trend that is shown in the data suggests that we would expect to see few, if any, transformants with such a large plasmid.

When considering what is occurring in the transformation reaction at the cellular level, it does not seem surprising that the transformation efficiency would decrease as plasmid size is increased. According to recent theory, incubation of E. coli at 0°C with CaCl₂ induces cell membrane restructuring and it is believed that this change forms and/or stabilizes holes in the outer membrane LPS network. When the DNA is added to the cells, it is then able to associate with these holes and can gain access through which it can enter passively into the cell. However, this access usually results in only a partial transfer of DNA into the cell, thus the necessity for the heat shock step. It is believed that the heat shock results in a “transient melting” of the crystalline membrane, changing its conformation such that DNA uptake can be completed. This step appears to be crucial to obtaining appreciable transformation rates (2). Since this membrane “melting” is a transient phenomenon, it seems logical that larger DNA molecules would have a lesser chance of completely entering the cell in this limited time frame than would smaller ones and would thus have lower transformation frequencies. In fact, a decrease in transformation efficiency with increasing plasmid size has previously been observed and documented in several studies on the transformation of E. coli (1, 2, 8).

Our results indicate that increasing plasmid size results in a decrease in transformation frequency. They do not, however, rule out the possibility that the decreased transformation frequency is due to the sequences that were inserted into the original plasmid. To test this possibility, a further experiment could be performed where sequences from pUC8 are ligated into the pUC8 plasmid to create a larger plasmid that contains sequences that are identical to the original plasmid. Two or more molecules of pUC8 could be ligated together to create a range of plasmid sizes to test this hypothesis. However, tandem sequence molecules are often unstable due to the presence of multiple origins of replication. To minimize this instability, the origins of replication could be removed by restriction enzyme digest, then religated so that only one origin of replication was present on the new plasmid. Experiments to directly test the mechanism whereby plasmid size affects transformation frequency is beyond the scope of current technology.

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Appendix I
Slot Lysis Protocol and Solutions:
i. Pour a 0.8% agarose gel in TBE with 0.5 µg/ml ethidium bromide and allow to solidify.
ii. Aliquot 10 µl of protoplasting buffer into 1.7 ml Eppendorf tubes.
iii. Pick a colony with a toothpick and touch the toothpick to a LB plate to obtain a patch colony of the transformant, then put the toothpick in an Eppendorf tube and vortex.
iv. Preload the gel slots with 4 µl of lysis buffer.
v. Load the protoplast suspension under the lysis buffer. Make sure not to leave the cells in the protoplasting buffer longer than 30 to 40 minutes before loading into gel.
vi. Electrophorese in TBE at 40V for 15 minutes to allow the cells to lyse completely, then increase the voltage and run until the blue dye migrates to the bottom of the gel.
vii. Visualize the bands under UV light.

Protoplasting buffer:
30 mM Tris-HCl, pH 8.0
5 mM EDTA
50 mM NaCl
20% Sucrose
50 µg/ml Rnase
50 µg/ml lysozyme

Lysis buffer:
89 mM Tris-HCl, pH 8.0
89 mM boric acid
2.5 mM EDTA
2% SDS
5% Sucrose
0.04% bromphenol blue
4.75 ml distilled water

Both solutions may be kept in aliquots at -20°C.

Luria Broth:
Tryptone 10 g
Yeast Extract 5 g
NaCl 5 g
Glucose 2 g
Distilled water to 1 L

Adjust pH to 7.0 with 0.1 M NaOH. Autoclave.
For LB Agar plates, add 15 g agar to 1 L broth.
Appendix II
Plasmid Maps
Sources:
Lambda (Figure A5), pBR322 (Figure A6) and pUC19 (Figure A7) maps are taken from New England Biolabs catalog 1996-1997
pUC8 (Figure A8), pUC8 0-690 (Figure A9) and pKT210 (Figure A10) maps are provided by Dr. J. Nomellini, UBC

Figure A5. Restriction Enzyme Map of Lambda DNA.
Figure A6. Restriction Enzyme Map of pBR322.
Figure A7. Restriction Enzyme Map of pUC19.
Figure A8. Restriction Enzyme Map of pUC8.
Figure A9. Restriction Enzyme Map of pUC8 0-690. The fragment from EcoRI 2232 to HindIII 3857 represents the RsaA protein, which is the subunit of Caulobacter S-layer.
Figure A10. Restriction Enzyme Map of pKT210.