Journal of Experimental Microbiology and Immunology (JEMI) Vol. 2:68-80

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

JILLIAN CLARK, JACQUI HUDSON, ROBIN MAK, CHRISTA McPHERSON, AND CARMEN TSIN

Department of Microbiology and Immunology, UBC

There is little known about the methods by which bacterial cells take up foreign DNA. There are however, a number of theories as to how this occurs. The first suggests that plasmid DNA, if condensed into small clusters, can pass through calcium channels present in a bacterial membrane. This theory is potentially flawed in that the calcium channels are too small to allow the passage of condensed DNA. A second theory suggests that plasmid DNA may not require any form of channel. Instead of simply being allowed to approach a bacterial membrane, the plasmid can allow loops of DNA to pass through the lipid bilayer into the cytoplasm of the cell, this initial action is followed by subsequent loops entering the cell until the entire length of the plasmid has passed through the membrane. Lastly, it has been suggested that an open-circular form of plasmid DNA may be able to use the calcium channels to enter a bacterial cell. In knowing that, the trivalent cations spermine and sperimidine, when used at specific concentrations, were capable of condensing plasmid DNA into spherical clusters, it was decided to test this action alongside the standard Hanahan protocol for transformation. Later, spermine and sperimidine were tested without the calcium chloride called for in the Hanahan protocol to assess the relative effects of spermine and sperimidine on the ability of bacterial cells to take up plasmid DNA without the presence of the competence inducing calcium ions. It was found that condensing DNA into spherical clusters was not conducive to increasing transformation efficiencies, therefore it can be suggested that plasmid DNA is not taken up better by bacterial cells when in a condensed form. The remaining theories regarding DNA uptake by bacterial cells remain to be investigated.

The mechanism for DNA uptake during transformation is not completely understood, however Hanahan *et al*. described bacteria (*Esherichia coli*) as preferentially interacting with, and taking up double-stranded DNA (4). Hanahan also described the existence of a transitory state of competence for transformation, which is generally related both to conditions of growth and to the circumstances under which the cells and DNA are combined. It is also to be noted that divalent cations are thought to play an important, and sometimes essential role in the early stages of DNA uptake; hence the use of calcium ions in the afore mentioned protocol (4).

DNA transfer into *E. coli* was first demonstrated by Mandel and Higa, who reported that bacteriophage DNA could be transfected into cells with the consequent appearance of infectious centers of virus. Transfection occurred when the cells and DNA were combined in the presence of 50 mM Ca^{2+} at 0^oC, and subjected to a brief heat pulse at 37 to 42^oC. The consensus that arises from this observation and other investigations is that *E. coli* cells and DNA interact productively in an environment of calcium ions and low temperature, and that heat pulse is important, though not strictly required. Several other factors have been shown to stimulate the efficiency of DNA transfer: combination of Ca^{2+} and Mg^{2+}, substitution of other alkali earth metals for Ca^{2+}, and extended incubation of Ca^{+2} treated cells at 0^oC (4). Low molecular weight poly-3-hydroxybutyrate (PHB) has been observed to complex with Ca^{+2} channels in the bacterial membrane (5). These structures have been proposed to facilitate DNA import after Ca^{+2} treatment to render bacterial cells artificially competent for DNA uptake (transformation). It should be noted that genetic competence of *E. coli* by Ca^{+2} treatment correlates with the PHB content of the bacterial membrane (5).

Ion distribution profiles, ion binding properties, and competition effects in the association of different counter-ions to DNA have been studied. The distribution of the three charges on complex multivalent ions (i.e. $spermidine^{+3}$) have been shown to display drastically different behaviour from molecules with point charges (or spherical) trivalent

ions (i.e. calcium chloride). Later research showed that the linear, non-chiral polyamines such as spermine and spermidine bind and stabilize DNA duplexes and triplexes by electrostatic interactions (4). Likewise, an in vitro study of the kinetics of DNA condensation by the trivalent cation hexaammine cobalt (III) by Matulis et al., showed that DNA condensation occurs only above a critical [$Co(NH_3)(6)^{+3}$] for a given DNA and salt concentration (6). At the onset of condensation, the hexaammine cobalt (III) to DNA-phosphate concentration ratio is close to 90% charge neutralization, which is necessary for condensation (1).

The binding of the trivalent cations cobalt hexaammine and spermidine to plasmid DNA has been studied by isothermal titration calorimetry (6). Two stages were observed in the course of titration, the first attributed to cation binding and the second to DNA condensation. As predicted from their relative sizes, the binding constants of the cations were indistinguishable, but cobalt hexaammine had a much greater DNA condensing capacity because it is more compact a molecule than spermidine. It was shown that DNA condensation occurred when about 67% of the DNA phosphate charge was neutralized by cobalt hexaammine and 87% by spermidine. During condensation, the remaining DNA charge was neutralized (6).

It has been noted that the phosphates of the β-DNA backbone are the primary targets of both spermidine and spermine interactions. The modest dependence of polyamine binding on base composition in genomic DNA suggests that sequence context plays a secondary role in polyamine recognition (3). Importantly, each polyamine bound DNA retains its β conformation when highly condensed.

Given the above conclusions drawn from the research involving DNA ion binding and DNA uptake by bacterial cells, it might be hypothesized that the calcium ions employed in the Hanahan protocol facilitate the formation of Ca^{+2} channels in the bacterial membrane that facilitate DNA import. Likewise, the use of trivalent cations that are known to bind to and conformationally compact DNA may be useful in enhancing DNA uptake by Ca^{+2} competent bacterial cells, by providing a form of DNA that is not only compact and therefore easy to transport through a channel, but also has a reduced negative charge (thereby allowing it to approach a bacterial membrane with less electrostatic repulsion). A discrepancy with this theory is that the diameter of the Ca^{+2} channels in the bacterial membranes are noted to be smaller than what is agreed to be the smallest size of condensed plasmid DNA (W. D. Ramey, personal communications). This condensed DNA would likely not pass through such channels. Nonetheless, there could be larger, as of yet uncharacterized, bacterial membrane channels that allow the passage of condensed DNA. If this is the case one might expect an increase in uptake efficiency for plasmid DNA treated with trivalent cations.

However, to further add complication to confusion, there is yet another theory as to bacterial DNA uptake. This theory hypothesizes that plasmid DNA in an open-circular state can upon approaching the bacterial membrane (facilitated by charge neutralization by cations, uni- or multi-valent); drop a short loop of DNA through the lipid bilayer. Intracellular DNA binding proteins then bind this small loop or bit of DNA, and they serve to anchor the loop inside the cell (Figure 1). Thermal kinetics and other forces then allow other sections of the plasmid to pass (or get pulled) into the cytoplasm. Given enough time and sufficient DNA binding proteins to anchor the plasmid, its entire length would eventually pass through the lipid bilayer (W. D. Ramey, personal communications). If this is the method of DNA uptake used by bacterial cells, pre-condensation of the plasmid DNA may not actually be of any benefit, as condensation would eliminate any free loops of plasmid that would be able to loosely pass through the lipid bilayer to become entrapped in DNA binding proteins. Thus said, any benefit observed by the addition of cations to transformation preparations may lie in the ion's ability to neutralize the negative charges present on both the DNA and bacterial surfaces. Further inference from this would suggest that point cations, or single charge cations would be most useful, as they are least likely to change the open conformation of the plasmid ring.

Lastly, another speculated theory of DNA uptake by bacterial cells could involve open-circular (non-condensed) DNA passing through the Ca^{+2}channels in a loop form (Figure 1). This could allow the DNA to enter into the cells despite the restricted diameter of the calcium channels. If this is the case, perhaps the Ca^{+2} ions employed in the Hanahan protocol assist not only in forming the calcium channels, but also in allowing the plasmid DNA to approach the bacterial membrane and enter a channel.

The protocols currently employed to transform bacterial cells with plasmid DNA have very low efficiency rates, approximately transforming just 1 in 10^4 cells (7). Such inefficacy makes for lengthy experimentation protocols for those wishing to transform bacterial cells with specific plasmid constructs. Of the few bacterial cells that actually take up and express the genes of a foreign plasmid, many such cells will contain a plasmid lacking the desired insert. This is perhaps unavoidable due to the nature of the protocols employed to create plasmid constructs, but if bacterial cells could be influenced to take up DNA more readily, one would be left with a greater pool of cells to further screen for plasmids of the desired construction.

Testing transformation efficiencies in *E. coli* in the presence of novel ionic molecules will perhaps help establish more efficient protocols with which to transform bacterial cells. If unable to better the transformation efficiency

protocol, one may at least be able to hypothesize further on the likely mechanism (channel or lipid bilayer passage) used by bacterial cells to take up plasmid DNA.

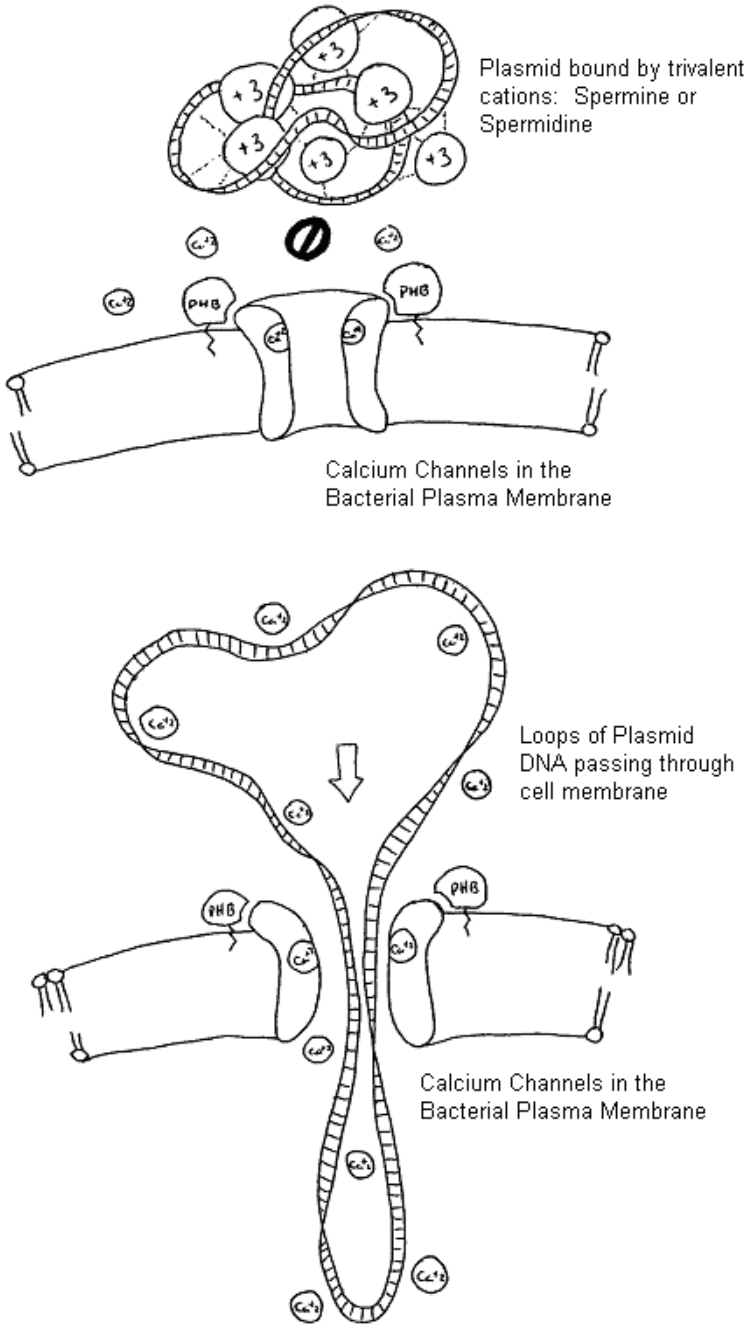

Figure 1: Plasmid Entering Cell via Two Hypothesized Methods

Such information may assist, and in turn, provide models for simple eukaryotic systems, which could then be applied to more complex eukaryotic systems. Potentially such findings may aid in the development of efficient DNA vaccines, whose underlying mechanism relies on having mammalian cells take up plasmid DNA encoding the vaccine, followed by expression of this encoded protein, to which the body's immune system elicits an immune response against the epitopes.

METHODS AND MATERIALS

Strains: *Escherichia coli* DH5α (previously transformed with pUC19) was used in plasmid isolation of pUC19. *Escherichia coli* DH5α with no plasmid (tested on ampicillin to ensure sensitivity) was used in transformation with pUC19.

Plasmid Isolation: *Escherichia coli* DH5α culture was grown to mid-exponential growth in Luria broth containing 50 μg/ml ampicillin, then treated with 100 μg/ml chloramphenicol to inhibit growth and amplify the autonomous resident plasmid (pUC19). Fifty-six ml of this culture was used to isolate pUC19, by modifying previously described methods (8). Dialysis was omitted and alternatively, ribonuclease treatment lasted for 2 hours, followed by the addition of 1.5 ml acidified salt buffer, 3 ml ethanol and centrifugation at 8500 rpm with the #819 rotor for 10 minutes. The supernatant containing ribonucleotides was removed and the drained pellet was re-suspended in 0.25 ml of TE buffer. A 1-in-40 dilution of the isolated plasmid was made in a final volume of 1 ml saline. The absorbance of this dilution was measured at 260, 270 and 280 nm using a double beam spectrophotometer to determine DNA purity and concentration. Quality of the isolated plasmid was assessed by electrophoresis (digested with restriction endonuclease HindIII), visualized with UV light, and photographed (data not shown). This isolated pUC19 was used in the first round of transformations. 1 ml of 70 μg/ml pUC19 generously donated by Dr. W. D. Ramey was used in the second round of transformations to circumvent the problematic nature of the original isolated plasmid (see Discussion section). Prior to use, this plasmid was subjected to electrophoresis (as mentioned above) alongside the original isolated plasmid to assess purity (Figure 2).

Transformation I: The transformation was performed as previously described (8), modified by the incorporation of spermine or spermidine into the preparation of certain DNA samples. One ml of 0.3 M stock solutions of either spermine tetrahydrochloride and spermidine trihydrochloride were prepared, filtered into microfuge tubes, then stored in the freezer for later use. A 10^{-6} dilution of each stock was prepared just prior to use during the DNA sample preparation. Five DNA samples were prepared as follows: 1 microfuge tube serving as a control contained 3 μg plasmid. The remaining four tubes each contained 3 μg of plasmid, in addition to either 10 μl of diluted spermine tetrahydrochloride or spermidine trihydrochloride, or 20 μl of either trivalent cation. As a viability control, plasmid DNA was omitted from three tubes, which contained either 20 μl of spermine tetrahydrochloride or spermidine trihydrochloride, or 50 μl Tris buffer (10 mM Tris [pH 7.4]). Finally, sufficient Tris buffer was added to all other tubes to achieve a final volume of 50 μl then chilled on ice for 5 minutes (allowing the plasmid to interact with either spermine or spermidine). Fifty μl of competent cells were then added to each tube. The tubes were subsequently chilled, heat shocked and incubated for 1 hour as per protocol (8). Samples were plated onto Luria plates and/or Luria plates with ampicillin (50 μg ampicillin/ml) as follows:

For all tubes containing plasmid, two ampicillin plates for each sample were prepared by plating 50 μl of undiluted culture or 5 μl undiluted culture plus 45 μl saline. As well, Luria plates to a final plated dilution of 10^{-5} and 10^{-6} were prepared from the samples containing 20 μl spermine or spermidine, and that which contained plasmid DNA in isolation. Luria plates were prepared in a similar manner for all samples without plasmid, as well as one additional ampicillin plate with 50 μl undiluted culture from the sample lacking plasmid or trivalent cation. Plates were incubated overnight at 37°C and colonies counts were recorded the next day.

Transformation II: Based on observations from Transformation I, further modifications to the original protocol were carried out. These included the use of the supplied plasmid rather than isolated plasmid, treatment with spermine or spermidine in the absence of $CaCl_2$, and finally decreasing the manipulated dilutions of these trivalent cations 100-fold (10^{-4}). Seven DNA samples were prepared for use during the transformations. Again, one microfuge tube served as the control, containing only 3 μg plasmid. Of the remaining six tubes, four contained 3 μg of plasmid, in addition to either 10 μl of diluted spermine tetrahydrochloride or spermidine trihydrochloride, or 20 μl of either trivalent cation. These four tubes received cells treated in ice-cold Tris (10 mM Tris [pH 7.4]), rather than ice-cold $CaCl_2$ as dictated by the standard protocol (8). The remaining two samples also contained 3 μg of plasmid, 20 μl of spermine tetrahydrochloride or spermidine trihydrochloride, however received 50 μl of cells having undergone the standard $CaCl_2$ treatment. As a viability control, plasmid DNA was omitted from five tubes, three of which contained either 20 μl of spermine tetrahydrochloride or spermidine trihydrochloride, or only Tris buffer, and received 50 μl of $CaCl_2$ treated cells. The remaining two tubes contained either 20 μl of spermine tetrahydrochloride or spermidine trihydrochloride however received those cells which had been exposed to ice-cold Tris. Finally, sufficient Tris buffer (10 mM Tris [pH 7.4]) was added to appropriate tubes to achieve a final volume of 63 μl, chilled on ice for 10 minutes, heat shocked and incubated for 1 hour. Samples were plated onto Luria plates and/or Luria plates with ampicillin (50 μg ampicillin/ml) as follows:

For all tubes containing plasmid, two ampicillin plates for each sample were prepared by plating 50 μl of undiluted culture (done in duplicate) or 5 μl undiluted culture plus 45 μl saline. As well, Luria plates to a final plated dilution of 10^{-6} and 10^{-7} were prepared from the samples containing 20 μl spermine or spermidine, and those which contained plasmid DNA in isolation regardless of type of cells received. Luria plates were prepared in a similar manner (although done in duplicate) for all samples without plasmid, and one additional ampicillin plate (50 μl undiluted culture) was made up for each of these samples. Plates were incubated overnight at 37°C and colonies counts were recorded the next day.

RESULTS

Figure 2 shows undigested and digested forms of the experimentally isolated plasmid and supplied plasmid. Lanes 1 and 5 show the undigested isolated and supplied plasmids, respectively. The digested isolated and supplied plasmids appear in lanes 2 and 4. Lane 3 contains a 1-kB DNA ladder. Several distinct bands are visualized in all lanes corresponding to different topological isomers of pUC19.

With respect to the first round of experimentation, Figure 3 shows that the addition of the trivalent cations spermine and spermidine to the transformation reactions without the addition of the pUC19 plasmid, enhanced cell viability, resulting in higher colony forming unit (CFU) counts. When the pUC19 plasmid was added to the transformation reaction with spermine and spermidine, cell viability decreased, resulting in lower CFU counts. The calcium chloride control subjected to transformation conditions in the absence of plasmid showed lower cell viability counts than the calcium chloride control in the presence of pUC19.

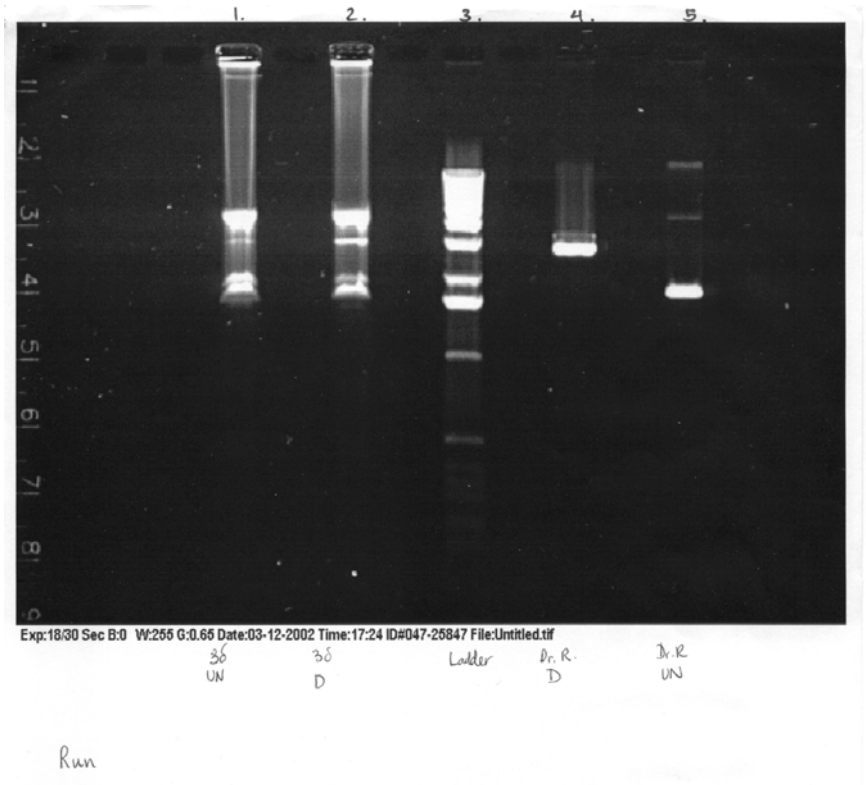

Figure 2: Photograph of the electrophoresis gel with both our isolated plasmid sample and pUC19 plasmid sample. Lanes: 1 – undigested sample, 2 – digested sample, 3 – DNA 1Kb ladder, 4 – digested pUC19, 5 – undigested pUC19.

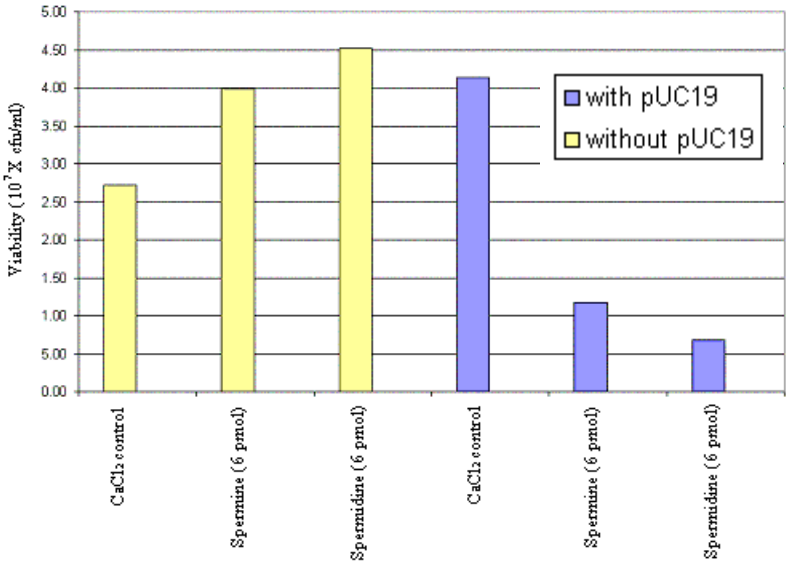

Figure 3: Viable Cell Count of E. coli DH5α Grown in Luria Plates with Different Concentrations of Spermine or Spermidine and Calcium Chloride.

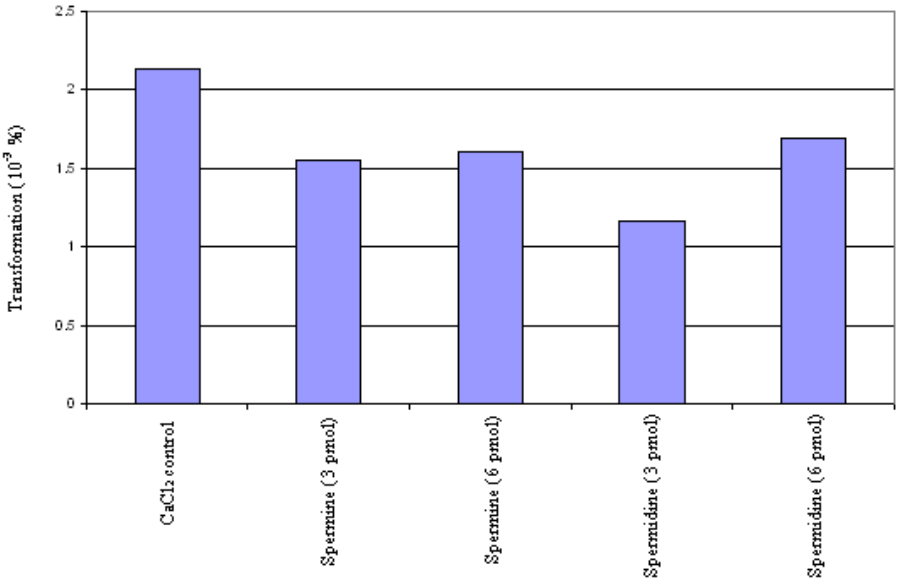

Figure 4: Transformation Frequencies of E. coli DH5α with pUC19 Plasmid in the Presence of Different Concentrations of Spermine and Spermidine Compared to the Calcium Chloride Control

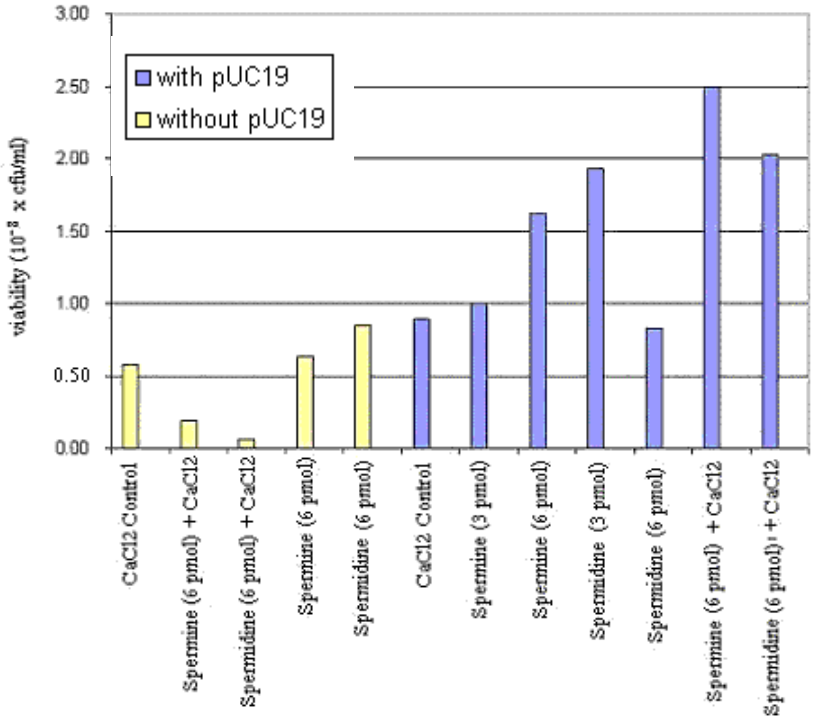

Figure 5: Viable Cell Count of E. coli DH5α Grown on Luria Broth Plates with Different Concentrations of Spermine or Spermidine with and without Calcium Chloride

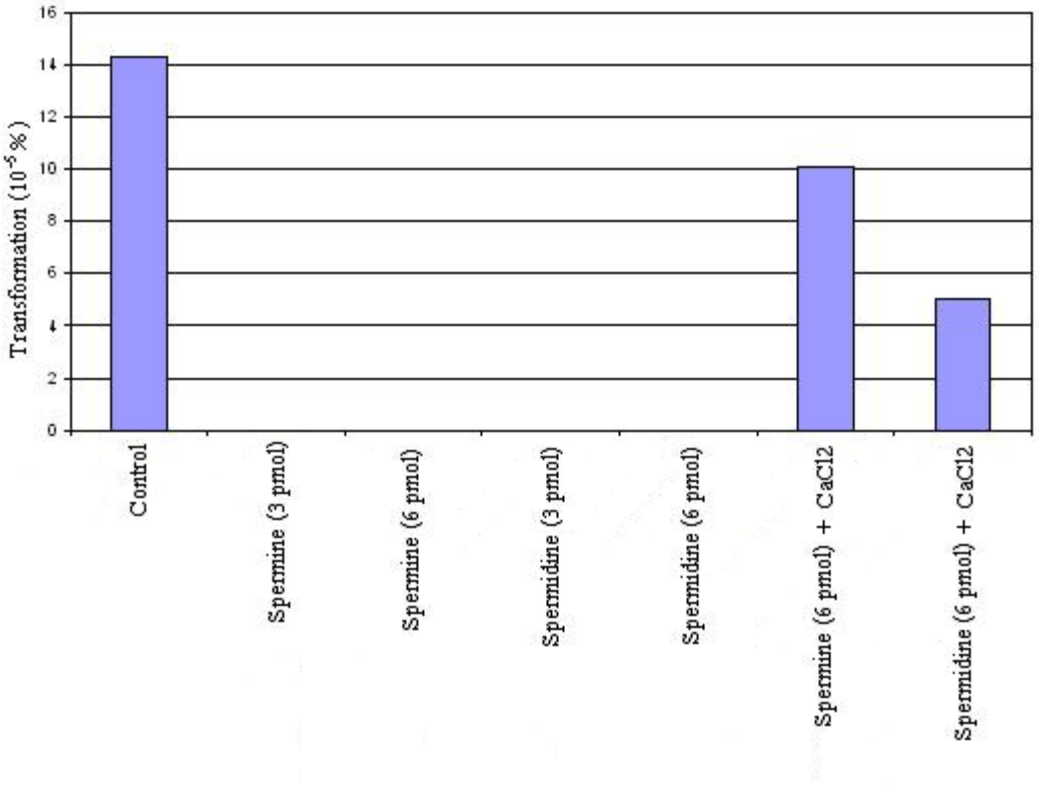

Figure 6: Transformation Frequencies of E. coli DH5α with pUC19 Plasmid in the Presence of Different Concentrations of Spermine and Spermidine Compared to the Calcium Chloride Control

Figure 5 shows the cell viability counts for all conditions of round 2 of experimentation. The observed results demonstrate great variation in cell viabilities following the transformation protocol. It is unclear whether the variability seen in the cell viability was due to the conditions of the experiment (presence or absence of calcium, spermine and/or sperimidine) or simply sampling discrepancy. Without having done experimental replication for all conditions, trends are difficult to assess. Thus it was decided to generate transformation frequencies using the calcium control cell viability counts as a standard reference. Future work may be able to determine the relative effects of the presence or absence of various agents on cell viability, but not wishing to draw hasty conclusions we have not. The addition of trivalent cations and/or plasmid should not impede cell survival. Due to these variances in cell viabilities, transformation frequencies were generated using the calcium control cell viability counts (Figures 4 and 6).

Figure 4 shows that the transformation frequencies for all conditions (excluding the calcium chloride control) decreased. Although, the observed decrease was slight, it was potentially still significant given the consistency of the trend for all experimental conditions involving both calcium and trivalent cations. Figure 6 shows transformation frequencies for the second round of experimentation where (accidentally) the concentrations of spermine and spermidine were one hundred times greater than in the first round of experimentation. The original intent was to investigate transformation efficiencies for cells treated without calcium chloride and only with spermine and spermidine. However, direct comparison of results obtained from round 2 and round 1 (excluding the calcium chloride control results), was not possible due to the (accidental) change in concentrations of the trivalent cations used. Figure 6 still manages to depict the effects spermine and spermidine on transformation efficiency of *E. coli* cells. Namely, these two trivalent cations are shown to have a deleterious ability to enhance DNA uptake by bacterial cells. Even with the addition of calcium chloride, spermine and spermidine decreases the transformation efficiency, which is consistent with the observed results from round 1 of experimentation (Figure 4). Although a trend is observed in both rounds of experimentation, direct comparison of data is difficult due to the different concentrations of spermine and spermidine used. However, estimated comparisons of the data seen in Figures 4 and

6 show a higher overall percentage of successful transformations in round 1 experimentation (approximately two-fold) than in round 2 experimentation. This may be related to the spermine and sperimidine concentrations used or other unaccounted experimental deviations.

DISCUSSION

Prior to performing transformations, it was necessary to assess the quality and purity of the plasmids. This is done via gel electrophoresis, and the resulting bands are displayed in Figure 2. The supplied plasmid appears pure and intact as seen by the very distinct bands in lane 4, as well as the one distinct band in lane 5. The fastest migrating band seen in lane 5 represents pUC19 in its supercoiled conformation, followed by a faint open-circular band, and finally a slow migrating band of knotted plasmid. After digestion, a pure band of approximately 2.96-kB results from complete linearization. Plasmid DNA isolated from DH5α shows multiple bands in the gel in lanes 1 and 2. Both lanes show bands migrating at the molecular weight expected for linear pUC19. This was expected in the digested sample (lane 2), while the lane 1 band at the representative molecular weight may have arisen from DNA breakage during its handling in isolation. However, the observed band intensity differs between the lanes, increasing in lane 2 as a result of digestion. Lane 2 shows evidence of incomplete digestion as two faster running bands also appear. These bands are also evident in lane 1. The faster running DNA fragment of the two corresponds to supercoiled plasmid (as seen in lane 5) and the slightly slower running band of DNA likely represents the presence of a catenene. Following digestion, this band appears less intense as a direct result of cleavage separating the replicated plasmids. These two lanes both contain a large diffuse band of higher molecular weight, which as described for lane 5, represent the plasmid in its open-circular form. Overloading of the gel in lanes 1 and 2 accounts for the appearance of streaking throughout these lanes. Similarly, ethidium bromide labeled smears appearing in the loading wells indicate the presence of contaminating chromosomal DNA. Because of the apparent chromosomal contamination in the self-isolated plasmid sample, the supplied plasmid was substituted for round 2 of experimentation.

As seen in Figure 3, cell viability was substantially reduced in the presence of the trivalent cations and the isolated pUC19 plasmid. One explanation could be that the trivalent cations provided membrane stability to the cells during the transformation process; however, the addition of plasmid may have interacted with the trivalent cations, decreasing the number of available cations providing protection and stability. The possibility of plasmid contamination decreasing cell viability is being ruled out because the calcium chloride control with the pUC19 plasmid showed comparable if not higher cell viability as the calcium chloride control cells treated without the plasmid. Without replica plates, it is difficult to determine if the observed results were due to randomness in procedures and samples or the actual effects of the trivalent cations on the plasmid.

The decrease in transformation frequencies observed for all spermine and spermidine concentrations (as seen in Figure 4) may be due to the action of trivalent cations condensing the plasmid DNA, yielding spherical clusters too large to fit through calcium channels. The objective was to achieve condensation using a trivalent cation concentration that would bind 87% of the DNA phosphates (6). For spermine and spermidine to condense the DNA rather than simply neutralize the charge, each molecule of trivalent cation must bind three consecutive DNA phosphates. A ratio of one-third trivalent cation molecules to the number of phosphates present in the DNA backbone of the plasmid sample was utilized to accomplish this. Due to the presence of calcium chloride competing for DNA binding, the above ratio was increased to two-thirds for certain samples. No significant differences in transformation frequencies were observed between the two different concentrations.

Had more replicates been done, subtle differences may have come to light to better predict the effect of the DNA conformational state on transformation frequency. Presumably by increasing the concentration of trivalent cation such that each binds only one phosphate, no condensation would occur and an open-circular, neutralized DNA strand similar to that achieved in the presence of calcium chloride would result (see Figure 1). If under these circumstances, the transformation frequency is similar with the calcium chloride control, then it could be argued that the mechanism underlying DNA uptake by bacterial cells requires an open-circular form of plasmid DNA. Perhaps an open-circular molecule of DNA can "thread" its way into a cell via the Ca^{+2} channel (Figure 2). It could then be argued that the presence of cations may be required only to assist plasmid DNA in approaching the bacterial membrane via charge neutralization.

The possibility of plasmid DNA looping through a membrane channel, calcium-derived or otherwise, is only one hypothesis explaining bacterial DNA uptake. A second idea, as illustrated in Figure 1, suggests that DNA may be able to enter into cells without the assistance of any channels. Potentially, plasmid DNA in an extended circular form, assisted by charge neutralization could approach and 'sit down' upon a bacterial membrane. Via the dynamics of plasma membrane kinetics, loops of DNA could be allowed to pass through the lipid bilayer. Once a section of

DNA has entered the cytoplasm it would likely become bound by DNA binding proteins, which could serve to anchor the loop of DNA inside the cell. Subsequent loops of DNA 'falling' through the plasma membrane would eventually allow the passage of an entire plasmid into the bacterial cell. If this is the actual method bacterial cells take up DNA, cations (trivalent or otherwise) would serve no purpose other than to neutralize the negative charges present on both the DNA and bacterial membranes, allowing closer association of the two.

In light of the two theories discussed in the introduction regarding DNA entry into bacterial cells, it appears from our results that condensation of the DNA into spheres does not facilitate DNA uptake by competent bacterial cells.

Figure 6 from round 2 of experimentation revealed that in the absence of $CaCl_2$, spermine or spermidine were unable to allow transformation to occur. Therefore it appears that calcium chloride is necessary in the reaction for transformation to occur. Accidentally a 100-fold increase in concentration of both spermine and sperimidine were used in round 2, and the initial fear was that perhaps no transformants were the result of the DNA having been precipitated out of solution. However, this is known to occur at a concentration of 0.1 mM of spermine or spermidine (2), which far surpasses the concentrations used in round 2. Therefore, the most likely explanation for no observable transformants is lack of competency of the bacterial cells. It appears the spermine and spermidine interact only with the DNA, having no affect on achieving cell competency, suggesting a key role for calcium chloride in achieving this. This trend was confirmed in round 1 of experimentation, where the presence of spermine and spermidine decreased transformation frequency even in the presence of calcium chloride (Figure 4).

Comparison between Figures 4 and 6 reveals a higher transformation frequency for round 1 than round 2. This contradicts an expected increase in transformation frequency, resulting from a more open-circular form of DNA due to the increased concentration of trivalent cations. The state of the cells could explain this lower transformation frequency. The cells from experiment 1 were grown up to 0.2 O.D. from a diluted overnight culture, while the cells in experiment 2 were not grown to the required 0.2 O.D. but rather diluted from the overnight culture and then used directly. These cells would likely have been in stationary phase rather than in exponential phase, therefore the overall second transformation procedure was carried out with fewer viable cells, or fewer rapidly growing and dividing cells. Additionally, transformations were performed in larger volumes for round 2 than round 1 to maintain the plasmid quantity of 3 μg, since the supplied plasmid was significantly more dilute than the isolated plasmid. The larger volume would be detrimental to the kinetics of the interactions involved during transformation. Furthermore, the isolated plasmid (used in round 1) contained a high salt concentration as a result of the isolation process, which may have assisted with creating competent cells or allowing the DNA to interact more closely with the bacterial membrane. Lastly, perhaps bacterial cells are triggered to take up DNA more efficiently when exposed to a variety of DNA lengths or types, as representative of DNA uptake in the natural environment. The isolated plasmid contained salt, chromosomal DNA, as well as all plasmid topologies. The presence of these combined elements may have served to trigger a greater uptake response in the cells. If this uptake response was significant enough to trigger bacterial cells to increase their ability to take up DNA, this may show increased transformation efficiencies.

On a final note, the relative transformation frequencies seen during this experiment, as compared to those previously documented, show that our calcium chloride control in round 1 had a relative transformation rate of 1 transformant per 4.7×10^4 viable cells. Compared to the industry standard of 1 transformant per 10^4 cells, a slight decrease in transformant frequency was observed (7).

The experimental results discussed discredit the theory regarding DNA uptake by bacterial cells via channels large enough to allow the passage of condensed spheres of DNA. The two remaining ideas concerning DNA uptake still require investigation to determine whether the plasmid is able to pass through the lipid bilayer, or requires the presence of a channel (calcium or not) to enter into a cell. Potentially these ideas could be investigated by creating a model system in which the calcium channels in the bacterial membrane are blocked. There would, however, remain the possibility of other unclassified or unknown channels that allow the passage of DNA into bacterial cells. An *in vitro* model consisting of an artificial lipid bilayer membrane with attached DNA binding proteins could aid in the investigation of DNA passage.

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APPENDIX I

Table 1: Transformation plate counts for round one of experimentation
Transformation Result

	Luria plate		Luria + Amp plate	
	Dilution		Volume (µl)	
	10*-5	10*-6	50	5
no pUC	195	35	0	
Spermine	237	56	0	
Spermidine	225	68	N/A	
CaCl2 control	216	61	44	2
Spermine 10µl*	N/A		32	0
Spermine 20µl+	6	23	33	3
Spermidine 10µl	N/A		24	4
Spermidine 20µl	15	12	35	8

*Spermine 10µl represents $3x10^{-12}$ moles, or roughly $1.8x10^{12}$ molecules of spermine, which would be equal to $5.4x10^{12}$ positive charges (three charges per spermine molecule)
This charge density was estimated to be able to neutralize roughly one third of the negatively charged phosphates present on the pUC19 plasmid.

+Spermine 20µl represents $6x10^{-12}$ moles, or roughly $3.6x10^{12}$ molecules of spermine, which would be equal to $1.1x10^{13}$ positive charges.
This charge density was estimated to be able to neutralize roughly one third of the negatively charged phosphates present on the pUC19 plasmid.

As both spermine and spermidine are both trivalent cations, the charge and molecular counts hold for both molecules.

Absorbance Readings for the 1-in-40 dilution of Isolated Plasmid

$A_{260} = 0.388$
$A_{270} = 0.331$
$A_{280} = 0.235$

Concentration of Isolated Plasmid

$[(63 \times 0.388) - (36 \times 0.235)] \times 40 =$ **639.36 µg/ml**

Table 2. Transformation plate counts for round two of experimentation
Transformation Result (3/21/02)

		Luria plate		Luria + Amp plate	
		Dilution		Volume (µl)	
		10*-6	10*-7	50	10
Control	1) no pUC + CaCl2	13	1	0	N/A
		58	0		
(no pUC)	2) Spermine 20µl + CaCl2	21	1	0	N/A
		16	5		
	3) Spermidine 20µl + CaCl2	7	0	0	N/A
		6	0		
	4) Spermine 20µl	86	2	0	N/A
		40	5		
	5)Spermidine 20µl	116	18	0	N/A
		53	6		
With pUC	6) CaCl2 control	89	10	9	4
			8	1	
	7) Spermine 10µl	N/A		0	0
				0	
	8) Spermine 20µl	162	17	0	0
				0	
	9) Spermidine 10µl	N/A		0	0
				0	
	10) Spermidine 20µl	83	16	0	0
				0	
	11) Spermine 20µl + CaCl2	250	6	7	0
				2	
	12) Spermidine 20µl + CaCl2	95	31	2	2
				1	

Transformation ingredients:

		pUC19 (µl)	Spermi (µl)*	Spermid (µl)*	Tris(µl)
Control	1) no pUC + CaCl2	\	\	\	63
(no pUC)	2) Spermine 2/3 + CaCl2	\	20	\	43
	3) Spermidine 2/3 + CaCl2	\	\	20	43
	4) Spermine 2/3	\	20	\	43
	5)Spermidine 2/3	\	\	20	43
With pUC	6) CaCl2 control	43	\	\	20
	7) Spermine 1/3	43	10	\	10

8) Spermine 2/3	43	20	\	\
9) Spermidine 1/3	43	\	10	10
10) Spermidine 2/3	43	\	20	\
11) Spermine 2/3 + CaCl2	43	20	\	\
12) Spermidine 2/3 + CaCl2	43	\	20	\

*1/1x10^4 dilution of 0.3M of Spermine and Spermidine were used

Note: 50μl of cells used for each transformation