Journal of Experimental Microbiology and Immunology (JEMI) Vol. 2:57-67

The Influence of the Size and Number of Digested pBR322 Fragments on the Inhibition of Transformation of *E. coli* HB101 Cells by the Plasmids pBR322 and p328.5

ANDREW FLYNN, DYLAN JONES, ERIC MAN, STEPHEN SHIPMAN, AND SHERMAN TUNG

Department of Microbiology and Immunology, UBC

Transformation reactions can be inhibited by a number of different contaminants. We were interested in investigating the inhibitory effect of linearized pBR322 on the transformation of *E. coli* HB101 cells with pBR322 and p328.5. In particular, we wanted to determine the relative influences of pBR322 fragment size and number on the level of transformation inhibition. To that end, we digested pBR322 with various restriction enzymes to produce digests with identical sequences, but differing fragment sizes and numbers. These digests were individually used to inhibit transformation reactions. Unfortunately, overall cell transformation levels were too low to permit any reasonable conclusions. Therefore, we cannot confidently state that either fragment size or number are crucial factors in influencing levels of transformation inhibition.

Plasmid transformation is the process by which extracellular plasmids pass through a bacterial cellular membrane and subsequently becomes part of the genome of the bacterium. Due to the low transformation frequency of plasmids in nature, competent cells are prepared to facilitate the transformation process and thereby increase the rate of transformation. Two common methods used to prepare competent cells are the Hanahan Protocol and Electroporation. The Hanahan Protocol uses an ice-cold $CaCl_2$ solution to facilitate DNA binding to the bacterial cell surface. The cells are then heat-shocked to induce the formation of pores (1) through which the plasmids are transported. Electroporation uses high voltage to cause the temporary formation of small pores in the cell membrane whereby the plasmids may enter (2). Contaminants such as phenol, protein, RNA, and DNA may all interfere with the process of transformation and thus, if present during the transformation procedure, may reduce the observed transformation frequency. Some possible mechanisms of inhibition may include the blockage of pores and the binding to plasmids or cellular surfaces.

Results from a previous study (3) demonstrated that the presence of chromosomal DNA decreased the transformation efficiency of p328.5 into *E. coli* HB101. However, the study did not test whether contaminating plasmid DNA would have a similar inhibitory effect on p328.5 transformation rates. Since plasmids are composed of DNA molecules, they should demonstrate a similar inhibitory effect as chromosomal DNA if the interference is independent of the DNA sequence. In addition, factors such as the length and the number of contaminating plasmid fragments may have direct effects on transformation inhibition. Longer DNA fragments may have greater affinity in binding to plasmids or cells and blockage of pores; while a greater number of fragments may demonstrate a greater ability to bind to a higher proportion of the cellular pores.

Therefore, an experiment was conducted to investigate the interference caused by plasmid pBR322 on the transformation rate of p328.5 into *E. coli* HB101. Various sizes and numbers of pBR322 fragments were used to inhibit the transformation in order to determine the role of these factors in transformation inhibition. To eliminate mass-dependent factors, transformations were performed using the concentration of p328.5 that produces the maximum transformation frequency. In addition, a constant mass of contaminant pBR322 was used. In doing so, the effects of concentration can be negated and the effects of the number and the length of contaminant DNA fragments on transformation efficiency were investigated.

MATERIALS AND METHODS

The materials and methods for this experiment are outlined in the Microbiology 421 laboratory manual (3), experiment B6, pages 1-6. Procedures from the manual were followed, with a number of modifications. Plasmid isolation was not confined to p328.5, but also included pBR322 and pUC19 (pUC19 was not used for any of the transformation procedures). As part of the experiment, the concentration of plasmid for optimal transformation was determined by using the Hanahan Protocol to transform competent cells with various amounts of plasmid DNA. The plasmid used for this part of the experiment was p328.5. After plating and overnight incubation at 37°C, colonies were counted to determine the plasmid concentration for optimal transformation. This concentration was then used for the second part of the experiment (i.e. p328.5

transformation inhibition). Due to the errors encountered in this part of the experiment, the optimal concentration for transformation was taken from the results of the previously completed experiment B6. This concentration was deemed to be 10 µg/ml.

Digestions of pBR322 were performed using 10 µg of plasmid DNA and 10 units of the appropriate enzyme (Gibco BRL). Plasmids were digested at 37°C overnight. Reactions were subsequently terminated by a 10-minute incubation at 65°C. Digests were verified by agarose gel electrophoresis on a 1.2% gel. The gel was run at 90 V for 2 hours. Predicted fragment sizes for the pBR322 digestions can be seen in Figure 1.

Two different sets of transformations containing contaminating DNA were performed. The DNA masses per transformation used in the first were 1 µg of pBR322 and 1 µg of contaminating DNA (pBR322 fragments). The DNA masses per transformation used in the second were 10 µg of p328.5 and 7.5 µg of contaminating DNA. The level of inhibition due to each contaminant was reported as the decrease in number of transformants seen on Luria-Bertani ampicillin plates (2). All plating procedures were performed in triplicate, but due to low plate counts, the replicates were combined to provide more statistically-relevant data.

RESULTS

Three attempts were performed to determine the concentration of plasmid DNA for optimal transformation. The first experimental trial ran into errors and no results were obtained. The second experimental trial provided countable plates only at the 0.5 µg level (data not shown). The plates with the remaining concentrations (1.0 µg - 20 µg) were deemed to be unusable as they all were too numerous to count. Therefore, the optimal plating concentration was initially determined to be 1 µg of plasmid DNA. In the third experimental trial (Table 1) the number of transformants did increase as the concentration of the plasmid DNA was increased, however, the optimal concentration was not found within the range tested, but was over 20 µg.

Table 1 – Plate counts for competent cells (*E. coli* HB101) transformed using various concentrations of plasmid DNA (p328.5) grown on Luria-Bertani plates with 50 µg/ml ampicillin.

[plasmid DNA] µg/ml	CFU count	Volume plated (µl)	Corrected CFU count to 160 µl
0.1	1	160	1
0.3	0	250	0
0.5	4	250	3
0.7	1	250	1
1.0	3	160	3
2.5	15	160	15
5.0	21	160	21
10.0	56	160	56
20.0	77	160	77

Table 2 – Plate counts for competent cells (*E. coli* HB101) transformed with a set concentration of plasmid DNA (1.0 µg/ml pBR322) in the presence of various contaminants(1.0 µg/ml).

Plate content	CFU count
Uncut pBR322 (no contaminant)	12
EcoRI cut pBR322 alone	1
Uncut pBR322 + EcoRI cut pBR322	10
Uncut pBR322 + EcoRI & HindIII cut pBR322	3
Uncut pBR322 + EcoRI & SalI cut pBR322	2
Uncut pBR322 + NdeI & EcoRI cut pBR322	11
Uncut pBR322 + DraI cut pBR322	8

The gel (Figure 2) was of poor quality and therefore difficult to analyze. In lanes 5 and 6 no bands were visible. The bands observed in lanes 3,4, and 7 were quite faint but of the expected size (i.e. ~ 4300 bp).

Two attempts were made at inhibiting the transformation with various contaminants. The first experimental trial utilized pBR322 as both the transforming plasmid and (in fragmented form) the contaminant. Importantly, the transformation using linear pBR322 alone (i.e. no uncut plasmid) produced only one colony (Table 2). This indicated that plasmid fragments were inefficient at intracellular re-circularization and therefore did not contribute to transformation efficiency. Unfortunately, no trend was observed between size and/or number of contaminating fragments and transformation efficiency (Table 2).

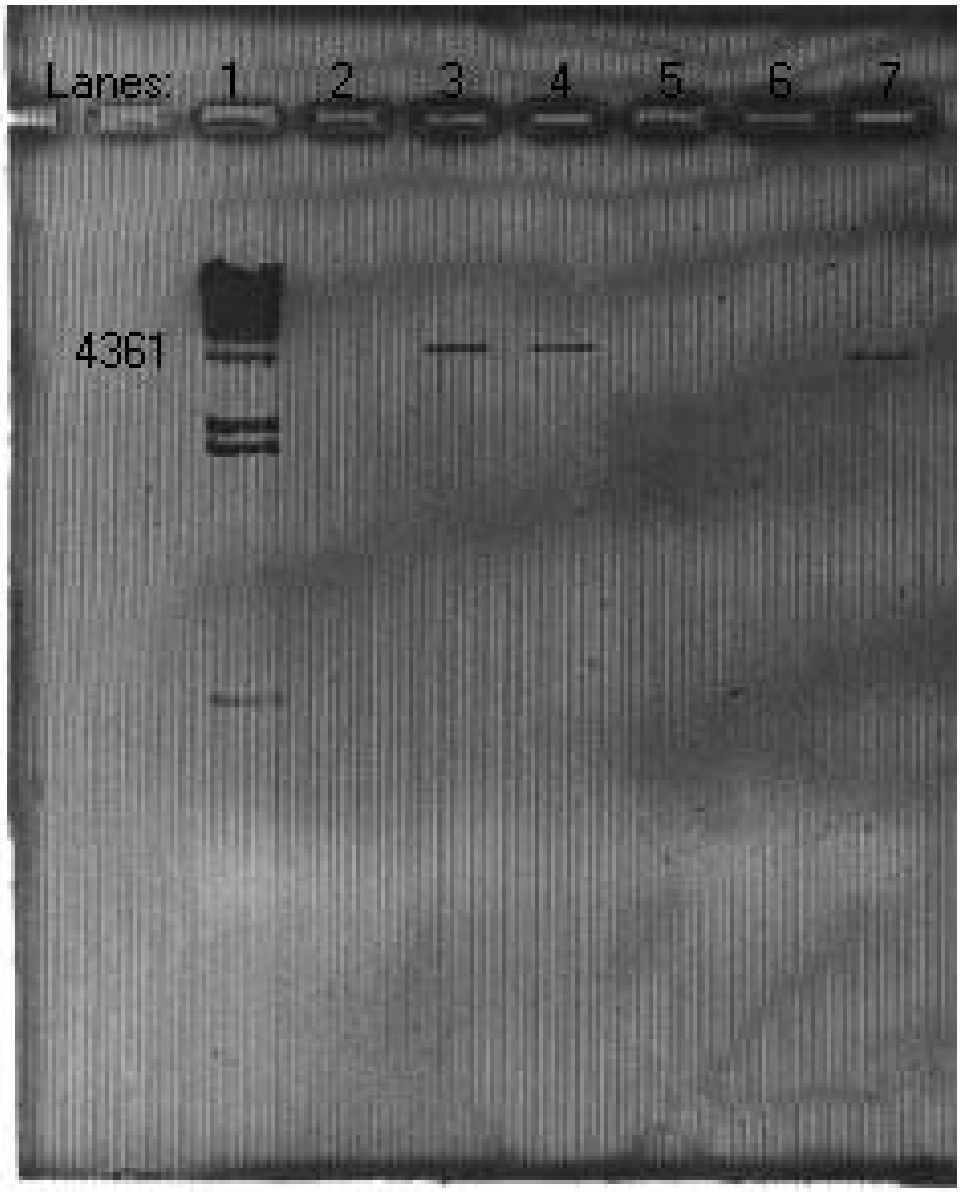

Figure 2 – Agarose gel displaying 1 µg pBR322 digested with various restriction enzymes. Lanes: 1- λ/*Hin*dIII ladder, 2- undigested pBR322, 3- pBR322+*Eco*RI, 4- pBR322+*Eco*RI+*Hin*dIII, 5- pBR322+*Eco*RI+*Sal*I, 6- pBR322+*Eco*RI+*Nde*I, 7- pBR322+*Dra*I.

Table 3 - Plate counts for competent cells (*E. coli* HB101) transformed with a set concentration of plasmid DNA (10µg/ml p328.5) in the presence of various contaminants(7.5 µg/ml).

Plate content	CFU count
Uncut p328.5 (no contaminant)	22
EcorI & HindIII cut pBR322	0
Uncut p328.5 + EcorI cut pBR322	0
Uncut p328.5 + EcorI & HindIII cut pBR322	0
Uncut p328.5 + EcoRI & SalI cut pBR322	0
Uncut p328.5 + NdeI & EcorI cut pBR322	0
Uncut p328.5 + DraI cut pBR322	3

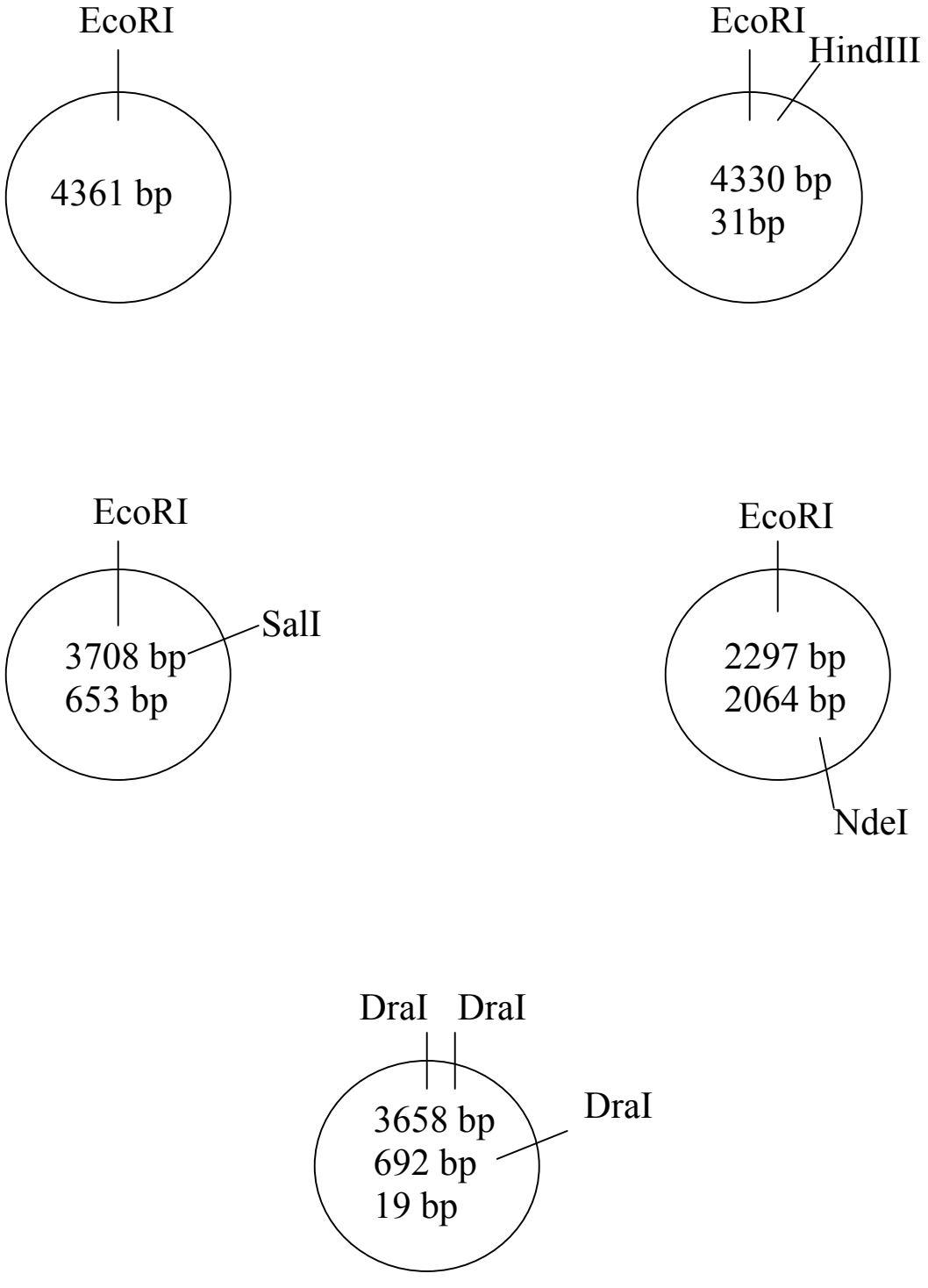

Figure 1 - Predicted fragment sizes for the pBR322 digestions (figures are not drawn to actual orientation in all cases)

The second experimental trial utilized p328.5 as the transforming plasmid and pBR322 fragments as the contaminants. Growth was observed in only the uncontaminated plate and the plate with the DraI digested contaminant DNA (Table 3).

DISCUSSION

Initially our intent was to determine the inhibitory effect of varying sizes of homologous *E. coli* HB101 chromosomal DNA fragments on transformation efficiency. Unfortunately several factors prevented us from continuing to explore this hypothesis. Our attempts to use the Marmur Method to isolate chromosomal DNA were unsuccessful. Due to our inability to isolate a sufficient quantity of this DNA, we obtained 700ug of *E. coli* HB101 chromosomal DNA from Dr. William Ramey. However, upon digestion with RsaI we were unable to observe the expected DNA smear on an agarose gel (data not shown). Therefore, we were unable to excise the desired fragment sizes from the gel. As a result we modified our hypothesis to consider the inhibitory effects of plasmid rather than chromosomal DNA.

Hanahan determined that there is no sequence specificity in plasmid transformation of *E. coli* (1). We wished to extend this analysis to the relative influence of fragment length and size on transformation inhibition. Therefore, we digested pBR322 with various restriction enzymes (Figure 1) to act as contaminants. If transformation inhibition is independent of fragment size and number we would expect all digest products to display an equal inhibitory effect. However, if transformation inhibition is solely dependent upon fragment number, then we would expect to see equivalent transformation inhibition by the DNA digests which produce two fragments (i.e. EcoRI+HindIII, EcoRI+SalI, and EcoRI+NdeI) but different levels of inhibition for digests producing three (i.e. DraI) or one (i.e. EcoRI) fragments. Conversely, if transformation inhibition is solely dependent upon fragment size, then we would expect to see equivalent transformation inhibitions by EcoRI and EcoRI+HindIII and also by EcoRI+SalI and DraI. Finally, if transformation inhibition is dependent upon both fragment size and number then we would expect to observe differing transformation inhibitions by all digestion products.

Unfortunately, the numbers of transformants obtained in both experimental trials (Table 2 and 3) were too low to permit the establishment of a correlation between fragment size/number and transformation inhibition. The low overall numbers of transformants could be due to such factors as insufficient bacterial competence, impure DNA samples, or low transforming plasmid concentrations, but is consistent with the low frequencies of transformation seen at low plasmid concentrations (Table 1). In addition, the absence (or presence in very low numbers) of transformants in the second trial (Table 3) may be due to toxic properties (e.g. other contaminants) of the inhibitory preparations. Furthermore, we encountered difficulty in obtaining reproducible cell viability counts, which interfered with our ability to express our results as transformants per viable cell.

Finally, the banding patterns observed in our gel (Figure 2) were discouraging. No bands were present in lanes 5 and 6, and a ~700 bp fragment was missing in lane 7. It is therefore unclear as to whether the digestion products were the desired fragment sizes.

In conclusion, we were unable determine whether the inhibition of transformation by contaminating pBR322 DNA is dependent on fragment size or number.

ACKNOWLEDGEMENTS

We would like to thank Andre Comeau, Karen Smith, and W.D. Ramey for their valuable help and input on the design, set up, and analysis of our experiment.

REFERENCES

1. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**: 557-80.
2. **Hinze E. and Dill B.** 2000. Microbiology 323/421: Microbiological Techniques II Laboratory Manual. Laboratory 2: 22-23. Department of Microbiology & Immunology. University of British Columbia
3. **Ramey W.D.** 2002. Microbiology 421: Manual of Experimental Microbiology. Experiment B6: 1-8. Department of Microbiology & Immunology. University of British Columbia

Appendix

Calculations

1. The following formula was used to calculate the concentration of plasmids.

[Plasmid] = (A_{260}) * (50µg/ml) * (Dilution factor)

Note:

[plasmid] is expressed as µg/ml

A_{260} = Absorbance of plasmid at wavelength 260nm

Dilution factor = [total assay volume (TE buffer + plasmid)] / plasmid volume

This formula is only applicable if the plasmid is relatively pure where the ratio of A_{260}:A_{280} is greater than 2 and A_{270} is less than A_{260} (2). All plasmids used in this experiment were found to be relatively pure.

Sample Calculation:

20µl of pBr328.5 was diluted to 600µl. The absorbance reading at 260nm is 0.580.

[pBr328.5] = (0.580) * (50µg/ml) * (600µl / 20µl) = 870µg/ml

2. The following formula was used to calculate the concentration of viable competent cells:

[Viable cells] = (number of colonies) * (1ml / volume plated) * (dilution factor)

Sample calculation:

Two plates were each plated with 100µl of a 10^-4 diluted solution of competent cells. A total of 36 colonies were found.

[Viable cells] = (36) * (1ml / 0.2ml) * (1 / 10^-4) = 1.80 * 10^6 cells/ml

3. The following formula was used to calculate the percentage of transformants per viable cell:

% transformants per viable cell

= (number of transformants) * (1ml / volume plated) * (1 / [viable cells]) * (100%)

Sample calculation:

A total of 160µl of competent cells transformed with 20µg of pBR328.5 were plated onto Luria broth with ampicillin plates. A total of 77 colonies were found.

% transformants per viable cell

= (77) * (1ml / 0.160ml) * (1 / 1.8 *10^6 cells/ml) * (100%) = 0.0267 %

Experimental Errors

Why didn't the Marmur method work?

In our experiment, we were initially interested in using various sizes of homologous *E. coli* HB101 chromosomal DNA as contaminants in transformations. We attempted to isolate the *E. coli* HB101 chromosomal DNA using the Marmur Method as follows:

1. Grow *E. coli* HB101 in L-broth at 37°C to mid-to-late log phase (150 GreenKlett units)
2. Centrifuge out 90ml of solution and resuspend with 2 ml of 1.5M Tris, pH 8.2 with 0.4 M sucrose, then add:
   - 0.5ml of lysoszyme (200ug/ml)
   - 0.5ml of EDTA (40mM)
3. Incubate 15 to 30 minutes at 37°C
4. Dilute with 60 ml of 150mM Saline in 10mM EDTA with 1% SDS
5. Add equal volume of Phenol saturated with saline-EDTA
6. Collect aqueous phase
7. Precipitate with 2 volumes of cold 95%-to 100% Ethanol and wind out of solution with a "hooked" Pasteur Pipette
8. Resuspend with 0.5 ml TE buffer (10mM Tris, pH 7.4, 0.1 mM EDTA) in a sterilized

tube and add 100ul of chlororform)

9. Store at 37°C

We preformed this experiment 3 times and unfortunately we failed all 3 times. There were many other groups that also used this protocol and were also unsuccessful in isolating chromosomal DNA. The first attempt was unsuccessful and we suspected that our solutions were not made properly. The second attempt did not work as we were unable to extract the DNA from our sample; however, we knew that there was a small amount of chromosomal DNA in our sample. We used the spectrometer to determine the amount of DNA present and we had a reading of A260 doubling the reading of A280, which implies the DNA is relatively pure. From this, we suspected that the salt concentrations were not at the correct levels. We attempted the experiment for the third time. In addition to the MICB 421 Marmur method, we also used the MICB 201 Marmur method (provided by Karen Smith from the MICB 201 Lab Manual). The protocol was as follows:

1. Grow *E. coli* HB101 in L-broth at 37°C to mid-to-late log phase (150 GreenKlett units)
2. Add 0.05 ml of 10% SDS and 0.05mls ethyl EDTA to the broth culture. Gently mix tube.
3. Put the tube into a 65°C water bath for 10 minutes
4. Cool in the ice bucket provided for 5 minutes
5. Add 0.5 ml sodium acetate pH 5.0 aseptically
6. Gently layer approximately 2.5ml ethanol over the liquid using a 5.0 ml pipette
7. Return the tube to ice bucket for a further 5 minutes and wind out of solution with a “hooked” Pasteur Pipette

8. Resuspend with 0.5 ml TE buffer (10mM Tris, pH 7.4, 0.1 mM EDTA) in a sterilized tube and add 100ul of chlororform)
9. Store at 37°C

After the third attempt with the MICB 421 method, we did not isolate any chromosomal DNA. However, with the MICB 201 method, we were able to isolate 77ug/ml of DNA. However, our experiment required 720ug/ml DNA.

In conclusion, the MICB 421 method did not work and we did not manage to isolate any chromosomal DNA. We know the problems were not related to the reagents as we followed each procedure very carefully for all three attempts. If we were to target which step failed based on our observations, we expect that there could have been a problem with the salt concentration. We may look into using a different salt concentration to isolate the chromosomal DNA because different strains may have different salt concentration requirements. There were no problems with lysing the cells as the spectrometer indicated that DNA was present in the solution. However, this method was used for isolating DNA from Bacillus strains so this procedure may not apply to *E. coli* HB101. In the future, I would recommend using the MICB 201 method to isolate small amounts of chromosomal DNA.

Why our optimal transformation did not work the first time?

Our first transformations were performed in order to determine the plasmid concentration required to produce optimal transformation efficiency. However our results were unexpected. The results showed confluent lawns in all our Luria-Bertani ampicillin plates. We suspected that there was something wrong with the ampicillin and/or the *E coli* HB101 strains. After narrowing down the problem to these two factors, a mini project was carried out to determine whether or

not these two factors were responsible for the observed confluent lawns of bacteria on ampicillin plates. Our mini project involved the following setup and results:

The following table displays various strains of *E. coli* HB101 grown on plates containing ampicillin from various sources.

	Original *E. coli* HB101 cells	Fresh Sample #1 of *E. coli* HB101 cells	Fresh Sample #2 of *E. coli* HB101 cells	Fresh Sample #3 of *E. coli* HB101 cells	Positive control *E. coli* HB101 cells with pBR322
Old Ampicillin 50ug/ml (1st trial)	Growth	Growth	Growth	Growth	Growth
Old Ampicillin 50ug/ml (2nd trial)	Growth	Growth	Growth	Growth	Growth
New Ampicillin from Dr. Hancock's Lab 100ug/ml	Growth	No Growth	No Growth	No growth	Growth

From these results, we were able to conclude that there was a problem with the ampicillin originally used in creating the plates. This can be determined because the *E. coli* HB101 cells from Sample #1 to Sample #3 were all able to grow in the old ampicillin but were unable to grow in the new ampicillin provided by Dr. Hancock's Lab. The possibility of human error involved in the production of the old ampicillin plates was low because the plates were made on two separate occasions. In addition other classmates reported the same problem with confluent lawns of bacteria for their transformation results. However, there was one group that did obtain results. This may have been due to the fact that this group may have used the ampicillin first and therefore deactivated the ampicillin for the other group members in the class. Unfortunately, the

new ampicillin that was used to compare with the old ampicillin was not equivalent and this may also explain why the *E. coli* HB101 cells grew in the 50ug/ml instead of the 100ug/ml. However, most literature states that 50ug/ml is sufficient to prevent non-transformed cells from growing. Our latter experimental results also showed that the fresh samples of *E. coli* HB101 were not able to grow in a new 50ug/ml ampicillin plate.

Another interesting finding of these results was that the original *E. coli* HB101 cells grew on the new ampicillin plate of 100ug/ml. From this observation, we could conclude that the experiment was faulty to begin with because the ampicillin was inactivated and *E. coli* HB101 cells that were provided already contained plasmids conferring ampicillin resistance.

In conclusion, from our mini project, we discovered that both the *E. coli* HB101 cells were contaminated and the Ampicillin from the fridge was deactivated. These factors lead to our confluent lawns in our first transformation.