

Construction of a plasmid for studies on the efficiency of *Escherichia coli* transformation by ligating the chloramphenicol acetyltransferase gene from pFM005 into the multiple cloning site of the plasmid pUC19

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To test whether the greater efficiency of transformation of pUC19 relative to pBR322 into *Escherichia coli* was due to differences in size rather than sequence, we normalized the sizes of these two plasmids by inserting a 1.9kb cm^R gene into pUC19 to construct a 4.6kb plasmid (p3B) with the pUC19 replicon. By including a marker gene, different combinations of cell transformation events can be distinguished phenotypically. Phenotypic and genotypic data suggest the ligation of the 1.9kb cm^R gene and pUC19 was successful. This was shown phenotypically by growth of a white transformed DH5 α colony on ampicillin and Xgal plates; and further confirmed genotypically using restriction enzyme digestion with Xba1 and gel electrophoresis. Although a ligation reaction was obtained, the expression of chloramphenicol resistance by the chloramphenicol acetyltransferase gene could not be confirmed.

Previous studies carried out on the transformation efficiency of the pBR322 and pUC19 plasmids into DH5 α *E. coli* showed that the smaller pUC19 (2.7 kb) was more efficiently taken up than the larger pBR322 (4.4 kb) plasmid (1). We hypothesized that this variation in the efficiency of transformation was due to the size difference of the two plasmids. In order to observe the effects of sequence and thus conformation on the efficiency with which DNA is transformed into *E. coli*, it is important to be able to ensure that size is eliminated as a variable (3); our challenge was thus to eliminate this distinction between pUC19 and pBR322. In previous work, an attempt was made to normalize the size of the two plasmids by inserting a 1.7 kb lambda DNA fragment into pUC19 digested with NdeI using T4 DNA ligase and to test whether the difference in transformation efficiency persisted (1). The attempted ligation was not successful.

In this experiment, we ligated a 1.9 kb DNA cassette encoding the marker gene for chloramphenicol acetyltransferase (CAT) into the multiple cloning site of pUC19. The chloramphenicol resistance gene was obtained from an XbaI digestion of the previously prepared plasmid pFM5 (provided by the Fernandez lab, UBC). By using a chloramphenicol resistance marker rather than a non-distinguishable insert it should have been feasible to phenotypically distinguish all possible combinations of transformations from a mixed p3B (ligation product) and pBR322 plasmid reaction. Although the size-adjusted product desired from the ligation was obtained, there was no phenotypically observable expression of chloramphenicol resistance in the transformed cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Wild type DH5 α cells (6) were used for all transformations. These cells were rendered competent using the CaCl₂ protocol (6) and stored at -70°C. pUC19 (Appendix I) and pBR322 (Appendix I) were supplied in wild type DH5 α cells by Dr. Ramey at UBC. pFM5 (Fernandez lab, UBC) was provided at 120 μ g/ml. In the second round of experiments, 25 μ l of 140 μ g/ml pUC19 was provided by Jennifer Sibley at UBC. All DH5 α cells were grown in Luria-Bertani (LB) medium (6) and plates at 37°C. Ampicillin was added to the LB media and plates at 100 μ g/ml and chloramphenicol at 30 μ g/ml when necessary. The chloramphenicol used in the concentration gradient test for low chloramphenicol resistance (cm^R) expression were added in concentrations of 5, 10, and 20 μ g/ml. For mutant screening, 40 μ l of stock 200 mg/ml Xgal in conjunction with 4 μ l of 1M IPTG were added to the LB plates 30 minutes prior to adding the transformed cells.

Making competent cells. Five millilitres of over night culture were made from a single colony. One ml of this culture was diluted into 100 ml LB and grown at 37°C on a shaker until an OD₆₆₀ of 0.4 was reached. Cultures were transferred to 50 ml disposable sterile tubes and spun at 3500 RPM at 4°C for 10 minutes. Cells were air dried and resuspended in 10 ml ice cold 50 mM CaCl₂. Cells were left on ice for 10 minutes and spun down at 3500 RPM at 4°C for 10 minutes. Cells were resuspended in 4 ml of 50 mM CaCl₂ + 15% glycerol and placed in chilled microfuge tubes, allowed to air dry, then stored at -70°C.

Transformation. Transformation with all of the plasmids was performed using a standard CaCl₂ protocol (6). Cells were heat shocked at 42°C for 30 seconds and then incubated in LB for 1 hour before plating. Cells were then plated with the appropriate antibiotic to select for cells able to maintain the transformed plasmid.

Phenol/Chloroform purification. To purify the XbaI-digested pUC19 and pFM5 plasmids, a phenol/chloroform DNA purification was used (6). Ten ml of phenol was mixed with 10 ml of chloroform, then 300 μ l of the bottom layer was removed, added to each DNA sample and mixed by shaking. The mixtures were centrifuged at 12000 rpm in a Brinkman centrifuge 5415 for 3 minutes. The top aqueous layer was transferred into a fresh microfuge tube and was treated again with 300 μ l from the bottom layer of the phenol/chloroform mixture. After another round of centrifugation, the top aqueous layer was removed and washed in a 25x volume of 95%

salted ethanol (sodium acetate). Samples were incubated at -80°C for 30 minutes and again centrifuged at 4°C at a speed of 12000 rpm for 15 minutes. The pellets were washed with 70% ethanol and centrifuged again under the same conditions. These pellets were dried and resuspended in 30 μl of distilled water.

Construction of p3B. pUC19 plasmids were isolated from wildtype DH5 α cells using the Qiagen Miniprep Kit (#27104) according to manufacturer's protocol. This plasmid was digested using the XbaI restriction endonuclease for 1 hour to make a linear form of the plasmid. The 1.9 kb insert containing the chloramphenicol resistance cassette was obtained by digesting pFM5 (Appendix I) with XbaI. The insert and linear vector were purified using either the Qiagen MinElute gel extraction kit (#28604) according to the manufacturer's protocol, the Qiagen MinElute reaction cleanup kit (#28204) according to the manufacturer's protocol, or the phenol/chloroform purification as described above. Five μl of each purified sample was run on a 1.5% agarose gel. All band intensities were estimated using the Alphamager[®] Imaging System (Alpha Innotech Corporation, San Leandro, CA). Based on the intensities obtained, the 1.9kb insert and the 2.7kb pUC19 vector (from the gel extraction) required a relative ratio of 1:1 by volume for the ligation, according to Invitrogen's technical Brief on T4 DNA ligase (#15244-2). Several ligation reactions with relative molar ratios of 3:1, 5:4, 3:4, 36:5 (insert:vector) and λ DNA/HindIII digestion fragments (Invitrogen) were set up over night at 22°C . Five μl of each ligation reaction was run on a 1.5% agarose gel, 5 μl of the each reaction was used for the transformation reaction, and the rest was stored at -20°C . Ethidium bromide (EtBr) was either added to the gel prior to polymerization at 0.12 $\mu\text{g/ml}$ or the gel was soaked in a 0.2 $\mu\text{g/ml}$ ethidium bromide bath for 40 minutes (specified where relevant). All the low-melting point gels (#15517022, Invitrogen) were run at 50 volts for 60 minutes, whereas the conventional agarose gels (#15510027, Invitrogen) were run at 100 volts for 40 minutes.

RESULTS

The entire experiment was attempted twice with minimal variation. During the first round, pUC19 was isolated from wildtype DH5 α cells using the Qiagen Miniprep Kit. Following the digestion, the samples were run on a low melting point agarose gel (Fig. 1). The concentration of pUC19 was so low that the ligation reaction was not performed. pUC19 also appeared to have not properly been digested by XbaI as the enzyme was diluted in this trial. Therefore, we proceeded with the second round of the experiment in which pUC19 was provided to us in high concentration (140 $\mu\text{g/ml}$).

As in the first round, pUC19 and pFM5 were digested with XbaI, this time undiluted. Following digestion in this round, only half of the digested plasmids were run on a low melting point gel to be purified via gel extraction. The other half was purified using the phenol chloroform method as described above. Finally, 5 μl of each reaction was run on an agarose gel to observe the relative degrees of purification attained using each method (Fig. 2). Using the Alphamager Imaging System, the intensities of the bands obtained were measured. The normalized intensities of 1.9kb insert and 2.7kb pUC19 plasmid (from gel extraction) were 290 and 106, respectively. From this result, we concluded that the concentration of

insert was approximately three times higher than that of pUC19.

According to the Invitrogen Technical Brief (15244-2) concerning the use of T4 DNA ligase, the relative molar ratios of insert to vector in the ligation reaction should be 3:1. Therefore, insert and vector isolates were mixed in a 1:1 volume ratio as well as 5 μl :12 μl , 1 μl :4 μl and 12 μl :5 μl . All of the ligation reactions, along with λ DNA/HindIII digestion fragments (Invitrogen) used as a ligation control were run on a gel (Fig. 3).

In order to confirm the identity of the DNA products isolated from the p3B ligation reaction, the reaction products as well as the undigested pUC19 and the λ DNA/HindIII digestion fragments (Invitrogen) were run on two identical agarose gels. The first was prestained with 0.12 $\mu\text{g/ml}$ ethidium bromide by direct addition to the agarose/TAE buffer mixture prior to polymerization, and the second gel was post-stained by soaking for 40 minutes in a 0.2 $\mu\text{g/ml}$ ethidium bromide bath. As ethidium bromide is known to affect the molecular topologies and migration rates of DNA (7), this step was included to further characterize the conformational states of the ligation products as well as of the plasmid isolates from the DH5 α transformants later on.

The transformed p3B cells were plated on 20 $\mu\text{g/ml}$ chloramphenicol/Xgal as well as (50 $\mu\text{g/ml}$) /ampicillin Xgal in order to determine whether a successful ligation reaction occurred as well as if plasmid expression was occurring. This resulted in 120 blue colonies and 1 white colony on the ampicillin /Xgal plate and no growth on chloramphenicol /Xgal plates. To determine whether the single white colony was a successful construct, two tests were performed. Firstly, plasmid DNA from an overnight culture of the white colony was isolated, digested with XbaI and electrophoresed on a 1.5% agarose gel. Secondly, cells were directly transferred from the single colony to both chloramphenicol and ampicillin plates. No growth was obtained on the chloramphenicol plate, but numerous isolated colonies grew on the ampicillin plate. As these colonies were known to have an inactivated *lacZ α* gene, twelve isolated colonies were plated on ampicillin/Xgal, resulting in the growth of eight light blue strains, two dark blue strains, and two white strains. Furthermore, to test the possibility that the chloramphenicol concentration used in the previous attempts of isolating a resistant strain were too high (20 $\mu\text{g/ml}$), cells were grown on a gradient of chloramphenicol concentrations. Plates containing 5, 10 and 20 $\mu\text{g/ml}$ of chloramphenicol yielded no growth of either of the 2 white strains or of the blue strain tested. As a control, the pFM5 *E.coli* cells used to isolate the CAT gene did grow on all concentrations of

chloramphenicol tested. All 4 strains also grew on an LB agar plate containing no chloramphenicol.

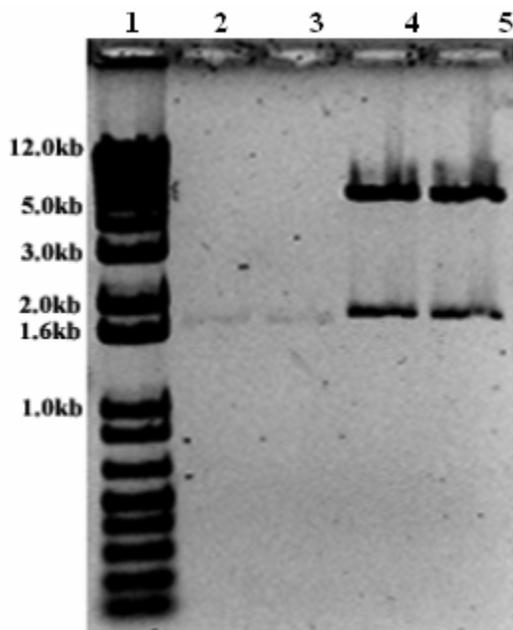


FIG. 1 XbaI-digested pFM5 and pUC19 on a 1.5% low melting point agarose gel used for DNA purification. The plasmids were isolated using the Quiagen Miniprep kit, digested for 1 hour, and run at 60 volts for 90 minutes. Lane 1- 1kb plus DNA ladder; Lane 2,3- XbaI-digested pUC19; Lane 4,5- XbaI digested pFM5.

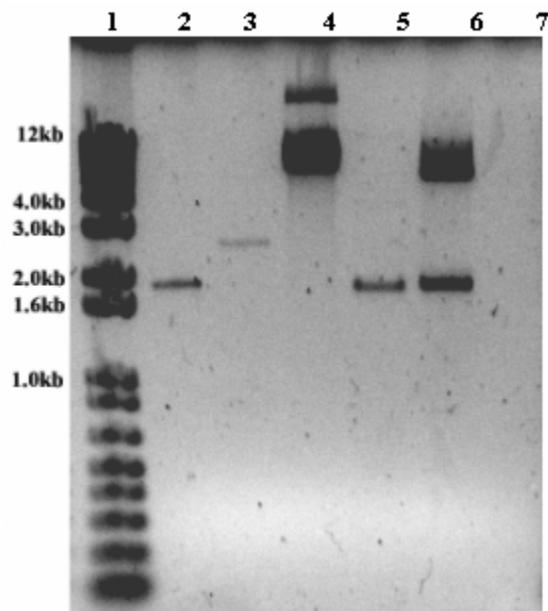


FIG. 2 Undigested pFM5 and pUC19 plasmids and their XbaI digested derivatives following gel extraction and phenol/chloroform purification. Ethidium Bromide was directly added to the agarose in this case. Lane 1- 1kb plus DNA ladder; Lane 2- XbaI digested and gel-extracted 1.9kb pFM5 fragment; Lane 3- XbaI digested and gel-

extracted pUC19; Lane 4- undigested pFM5; Lane 5- undigested pUC19; Lane 6- XbaI digested pFM5 purified with phenol/chloroform; Lane 7- XbaI digested pUC19 purified with phenol/chloroform.

DISCUSSION

The ligation of the 2.7 kb pUC19 plasmid with the 1.9 kb pFM5 fragment seems to have been successful in producing a 4.6 kb DNA molecule. Whether this molecule contains the complete CAT gene however, has not been established. Based on our findings, we believe that the gene is present, but that its expression is not occurring at a phenotypically detectable level.

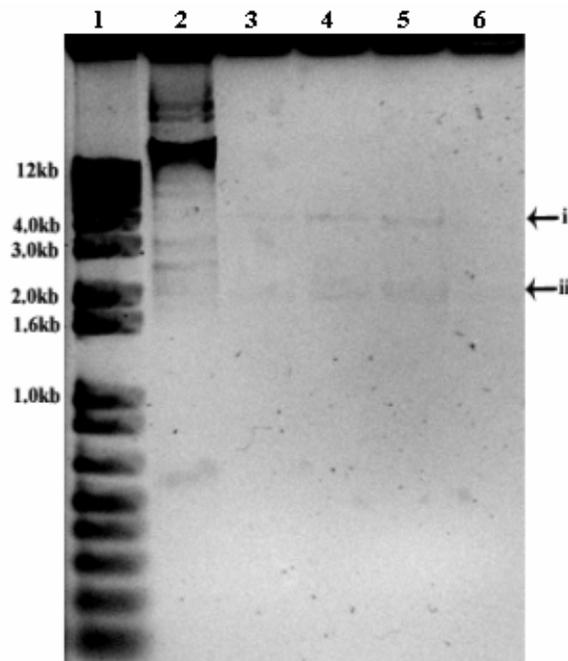


FIG. 3 1.5% Agarose gel showing the products of the four ligation reactions with pUC19 and the 1.9kb pFM5 fragment. A λ DNA/HindIII fragment digest was used as a ligation control. Lane 1- 1kb plus DNA ladder; Lane 2- λ DNA/HindIII fragment ligation control; Lane 3,4,5,6- ligation products from reactions of various insert:pUC19 ratios: (7 μ l:7 μ l, 5 μ l:12 μ l, 3 μ l:12 μ l and 12 μ l:5 μ l respectively)

We approached this problem by first ensuring that the 2.7 kb pUC19 (at 2.9 kb position, Fig. 2) and the 1.9 kb chloramphenicol resistance gene (at the 2.0 kb position) were properly purified by gel extraction. On the same figure (Fig.2), we can see that the phenol/chloroform purification of the same DNA samples yielded higher concentrations of both products, however the limited amounts of pUC19 plasmid forced the use of the purer insert product. The obvious purity distinction lies in that the pFM5 DNA extracted by the

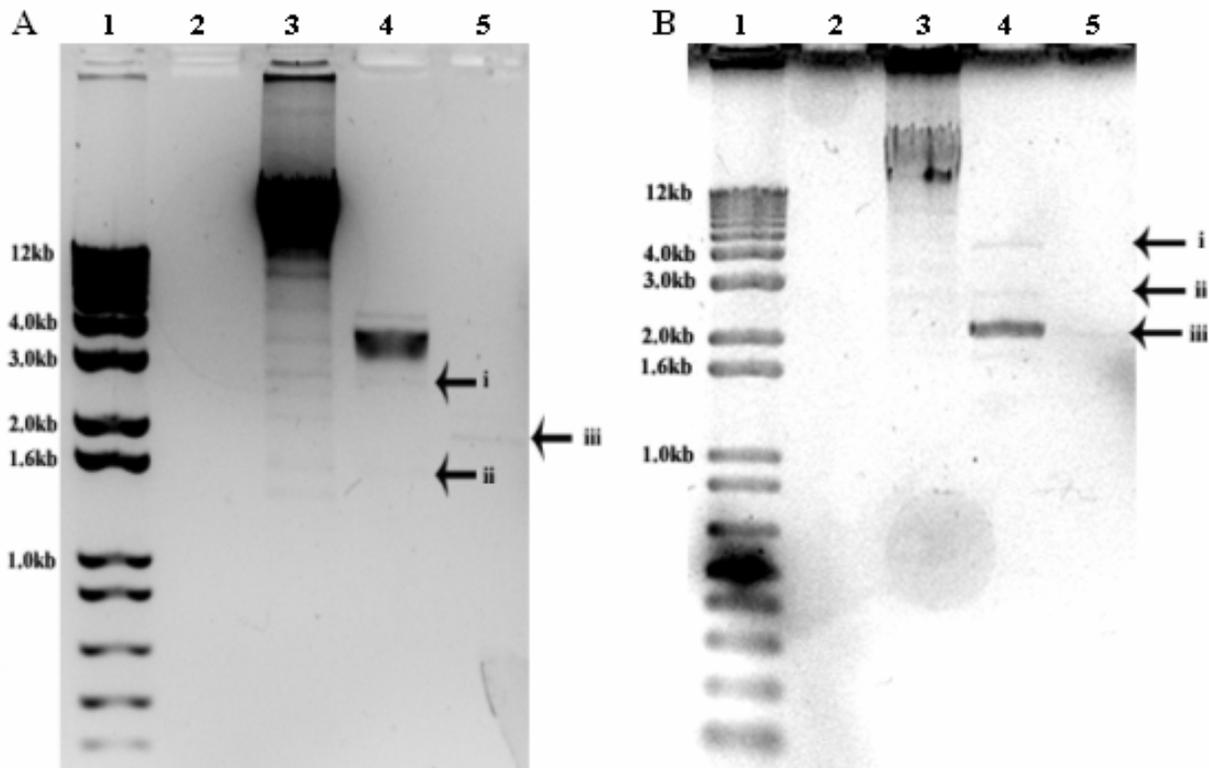


FIG. 4 The effect of Ethidium Bromide on the migration of pUC19 and the p3B ligation reaction on a 1.5% agarose gel. The gel-purified XbaI digestion products were ligated overnight at a 3:1 molar ratio. A λ DNA/HindIII digest was used as a ligation control. (a) Ethidium Bromide was added to the 1.5% agarose at a concentration of 0.12 $\mu\text{g/ml}$ prior to polymerization. (b) The 1.5% agarose gel was soaked in a 0.2 $\mu\text{g/ml}$ Ethidium Bromide bath for 30 min following electrophoresis. Lane 1- 1kb plus DNA ladder; Lane 2- empty; Lane 3- λ DNA/HindIII fragment ligation control; Lane 4- undigested pUC19; Lane 5- products of the p3B ligation reaction.

phenol/chloroform purification was a mixture of the linear plasmid and the desired fragment. Though a ligation could have been performed with this isolated insert sample, the emergence of a ligated 4.6 kb fragment from the ligation of the gel-extraction products was sufficient to continue on with the experiment. As the ligation reaction was performed using 4 different insert:plasmid ratios (based on AlphaImager pixel counts of Fig. 2), a variety of 4.6 kb product concentrations were obtained and can be seen in figure 3. The highest quantity of 4.6 kb ligation product seems to have been obtained from molar ratios of approximately 5:4 and 3:4 insert to plasmid, respectively. As the linearized pUC19 plasmid and the pFM5 fragment in lanes 3, 4 and 5 of this same figure have largely disappeared, there is compelling evidence that some degree of ligation did occur. The control ligation of the λ DNA/HindIII fragments supports this possibility (lane 2, Fig.3). Furthermore, the appearance of the most prominent band at a position of 4.6kb further accentuates this proposition and suggests the p3B ligation product was obtained. As we used a 1 kb

Plus linear DNA ladder (Invitrogen), this suggests the ligation product was largely linear. The last lane (Fig. 3) also shows similar results as lanes 3-5, but in much lower concentration. This DNA was only discernible by computer visualization, and could not be adequately photographed. Perhaps less obvious in figure 3 is the presence of a band at the 2.0 kb position in lanes 3-6. This band likely represents a combination of 1.9 kb cm^R fragment, pUC19 in its re-ligated circular form (compare to lane 5, Fig.2), and perhaps even fully ligated and supercoiled p3B plasmid. As we have not yet observed purified and established p3B plasmid on a gel however, we are unsure at this stage of its exact migrational behavior.

It has been shown that the intercalation of ethidium bromide by a DNA molecule reduces the average rotation angle of its double helix (3,7). This concept becomes significant when trying to identify nucleic acid bands in electrophoresis gels; especially when the products are of an unknown structural conformation. This was the case following the ligational production of p3B, and thus by characterizing the effects of ethidium

on the migration of other products, much was learned about the state of the plasmid used in the transformation. It was further demonstrated that ethidium does not have any significant effect on the migration of linear DNA molecules. This differential 'selection' by ethidium allows for the discrimination between linear, circular, and negatively supercoiled DNA. Oppenheim et al. (4) have demonstrated that ethidium causes the relaxation of negatively supercoiled DNA, slowing down its migration, but has an opposite effect on closed circular DNA molecules that are either relaxed or slightly positively supercoiled. Molecules that are in these two forms positively 'tighten' when exposed to ethidium and thus migrate at an increased rate (4).

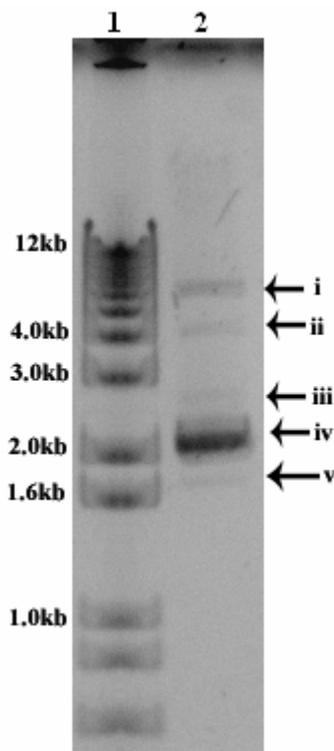


FIG. 5 XbaI-digested plasmid DNA from the single white colony of p3B transformants grown on ampicillin and X-gal. The gel was post-stained in an ethidium bromide bath. Lane 1- 1 kb Plus DNA ladder; Lane 2- XbaI-digested plasmid DNA from the *E.coli* strain not expressing β -Galactosidase.

DNA which was loaded on agarose gels pre-stained with ethidium bromide was much more visible than when the gel was post-stained in a bath following the final ligation of p3B. This observation suggests the results presented in figure 4b may not be fully representative of all conformations of DNA present following this ligation. In lane 4 of figure 4a (undigested pUC19), the most prominent band is located at about the 3kb position relative to the ladder.

In figure 4b however, the same major band traveled a longer distance to the 2.1 kb position. In the absence of ethidium, 90% of pUC19 is thought to exist in its supercoiled form (form I), and the remaining 10% as either relaxed (form II) or linear (form III) molecules. By looking at figure 4a, we suggest much of this naturally occurring supercoiling is of a negative configuration as ethidium seems to slow down its migration. As mentioned earlier, ethidium bromide is known to positively supercoil DNA, and thus we suggest it takes a negatively supercoiled pUC19 plasmid and relaxes it by coiling it in a positive direction. Figure 4b further supports this belief as in the absence of ethidium the most prominent form migrated at a faster rate, likely representing a supercoiled topology. Furthermore, the linearized pUC19 plasmid can be seen at the 2.7 kb position relative to the ladder as hypothesized in both gels; having migrational impartiality to ethidium bromide (bands (i) and (ii) in figures 4a and 4b, respectively). Therefore, we suggest from these results that the naturally occurring conformation of pUC19 is highly negatively supercoiled, and that ethidium bromide relaxes this structure thus slowing down its migration. The 1.5 kb band (ii) in figure 4a therefore likely represents positively supercoiled DNA, whereas the darkest band in lane 4 of figure 4b likely represents negatively supercoiled plasmid. The most relaxed conformation of pUC19 observed is found at the 5.0 kb position relative to the ladder.

Lane 5 in figures 4a and 4b contains the DNA products from the p3B ligation reaction. In both gels there is only one band visible at the 2.0 kb position (denoted as bands (iii) in both figures). This band is thought to be the linear form of the 1.9 kb pFM5 fragment containing the cm^R gene. As the ligation was performed using a 3:1 molar ratio of this insert to pUC19 plasmid, there was an expected excess of insert, which is what we see on both gels. As we later confirmed the presence of an insertion into the pUC19 *lacZa* gene (growth of a white DH5 α colony on Xgal and amp), we speculate that multiple ligation products are present in these gel lanes, however the wide variety of products prevents sufficient resolution for visualization.

The appearance of a white colony when transformed DH5 α cells were plated on ampicillin and Xgal further supports our hypothesis that the ligation reaction was successful. The lack of growth by the same transformants on 20 μ g/ml chloramphenicol and Xgal plates however, indicates the enzyme conferring resistance is not being expressed at a high enough level to acetylate a adequate number of chloramphenicol molecules in the cell. As growth of the p3B transformants plated on the gradient of chloramphenicol concentrations was also inhibited, we

conclude that though the cm^R gene may be present it is not being expressed to a level that is phenotypically detectable.

The results obtained from gel electrophoresis of the white colony's XbaI-digested plasmid DNA indicate that the ligation of pUC19 and cm^R gene insert was successful. The three bands in lane two (i, ii, and iv) of figure 5 can be explained by the presence of the three forms of p3B, open circular, linear and supercoiled respectively. However, the possibility that the 6.0 kb band (i) may also represent the linear ligation product of multiple pUC19 plasmids cannot be dismissed. The intensity of the smeared band (iv) can be explained by the possibility that this band may be a mixture of supercoiled pUC19 and pB3. The growth of blue colonies from single white colony further demonstrates this hypothesis. It is likely that cells containing only the pUC19 plasmid were also transferred into the overnight culture which was used to prepare the electrophoresis sample in figure 5. This would explain why there is such a high concentration of DNA at this location which corresponds to supercoiled pUC19 (Compare to Fig. 4b).

The 4.6 kb band (ii) which corresponds to the size of linear p3B plasmid, further supports the conclusion that a successful ligation was obtained. The 2.7 kb band (iii) corresponds to linear pUC19, and the faint 1.9 kb band (v) likely represents the linear cm^R fragment.

Finally, there are several possible explanations for the lack of expression of the cm^R marker gene when a successful ligation has been obtained. As only one cell strain was isolated containing a ligated p3B plasmid, there are several low probability events which may have caused a lack of expression of the insert. Such cells would likely have gone unnoticed if a larger number of successful ligation products had been obtained. First, it is possible that the inserted cm^R gene may have been damaged in an essential region resulting in a dysfunctional CAT enzyme product or no product at all. Lastly, if the insert was ligated into pUC19 backwards it would face the *lacZ* promoter which is constitutively expressed in the presence of IPTG. If both the *lacZ* promoter and the CAT promoter simultaneously transcribe in opposite directions, it is possible that the RNA polymerases are colliding on the plasmid; increasing the number of transcriptional initiations necessary to produce normal amounts of transcripts. This may also explain the appearance of light blue transformants on Xgal/amp plates. If this is in fact true, there must be some level of CAT present, however levels may be too small to detect at a phenotypic level.

FUTURE EXPERIMENTS

In order to be able to utilize the constructed p3B plasmid for transformation studies, it is important to understand whether or not the CAT gene is being expressed at all. If there are not signs of its expression at any level, it would be critical to determine why the expression is not taking place. Based on our hypothesis that RNA polymerase collision could still allow a low level of transcription to occur, some mRNA is expected to be present in the cells if expression is taking place. To test whether this is occurring at a very low level, a northern blot could be performed to identify mRNA transcripts of the gene of interest. A western blot could also be performed to test for the presence of minute amounts of CAT using enzyme-specific antibodies. Furthermore, as we encountered some level of difficulty in differentiating the products of the p3B digestion in electrophoresis gels, we would suggest digesting the plasmid with different restriction enzymes than XbaI for gel runs.

ACKNOWLEDGEMENTS

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APPENDIX I

The maps of the plasmids used and constructed in this project are shown below.

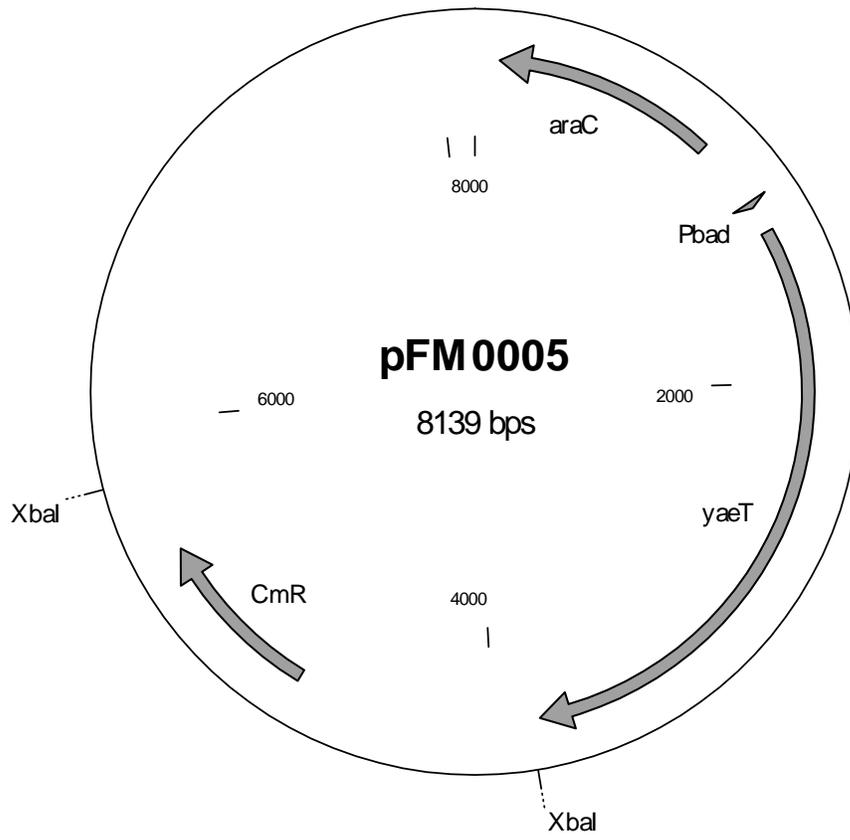


Fig. A1. Restriction Enzyme Map of pFM0005

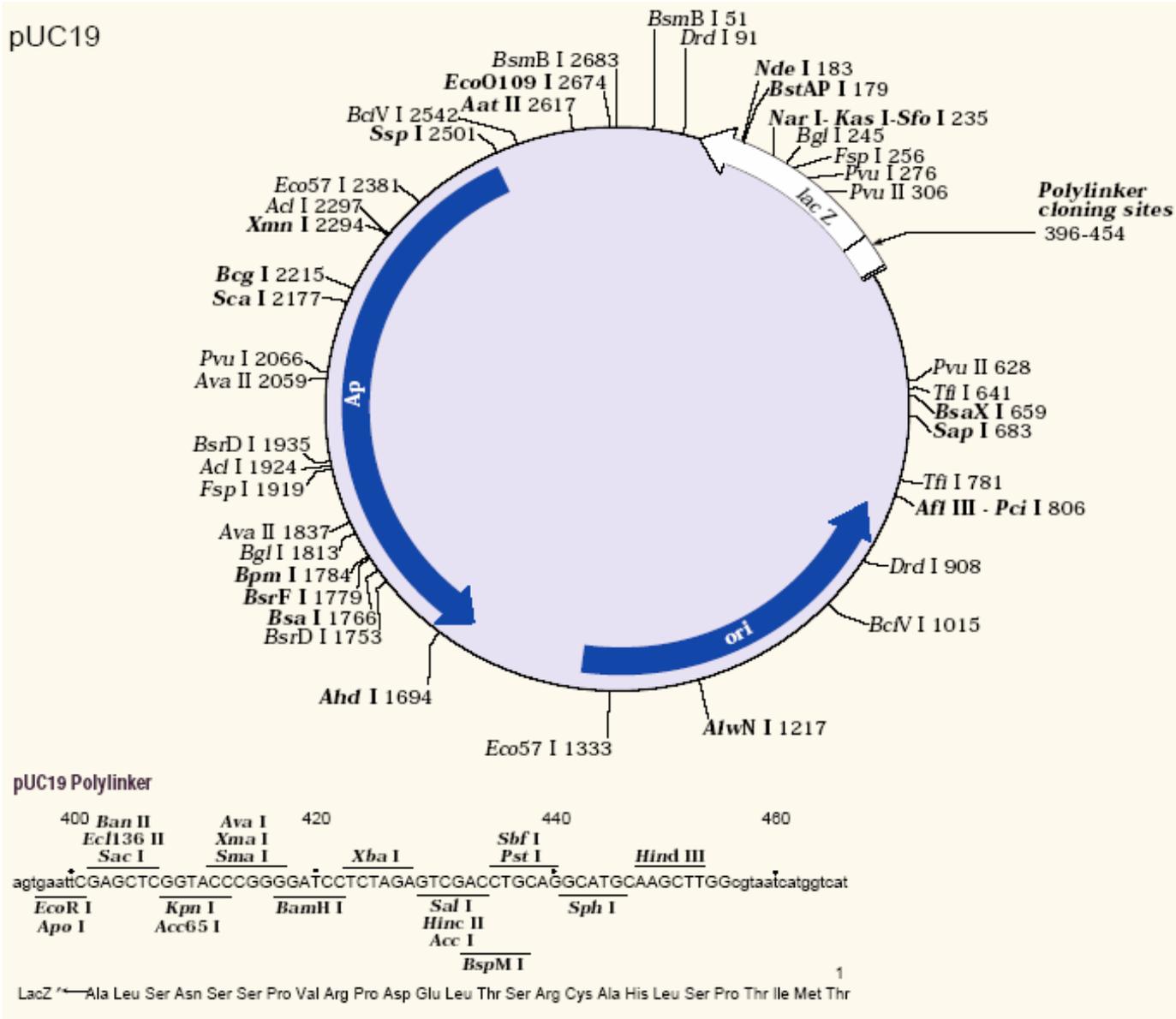


Fig. A2. Restriction Enzyme Map of pUC19

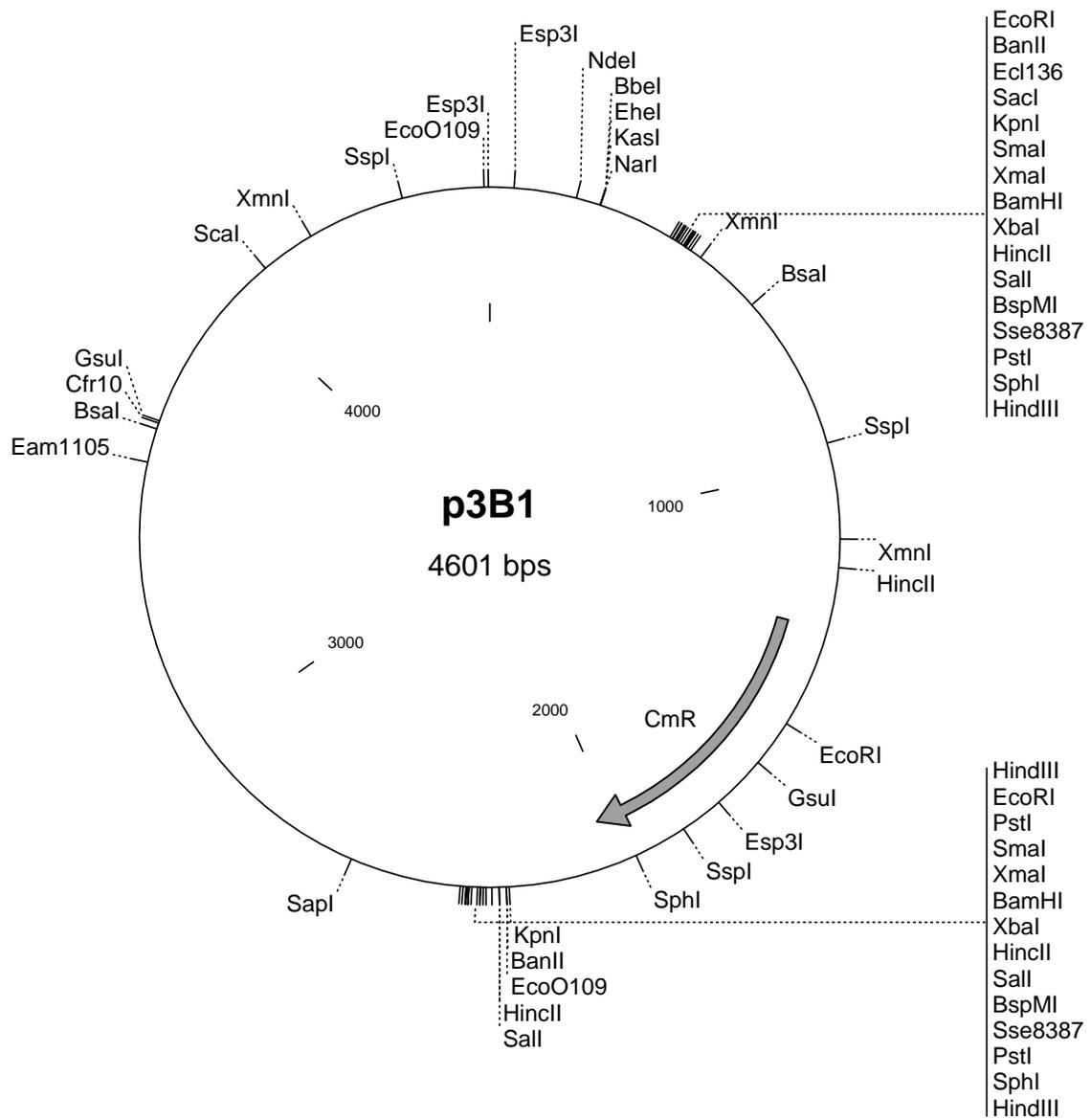


Fig. A3. Restriction Enzyme Map of p3B1