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Cloning of λ DNA fragments into pUC19 vector to study the ligation efficiency of *Nde*I-digested pUC19 and *HindIII*-digested pUC19 by T4 DNA ligase

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Ligation experiments were conducted to examine the ligation efficiencies of *Hind*III-digested pUC19 and *Nde*I digested pUC19 vectors by T4 DNA ligase. *Hind*III-digested pUC19 was shown to ligate more efficiently than *Nde*I-digested pUC19. Upon close examination, *Hind*III-digested pUC19 could also ligate intermolecularly and intramolecularly whereas *Nde*I-digested pUC19 can only ligate intermolecularly. Ligation of *Hind*III-digested pUC19 yielded monomeric and multi-meric, circular plasmid with varying degree of supercoiling. Ligation of *Nde*I-digested pUC19 only yielded multi-meric and possibly linear ligated fragments. Ligation temperature of 4, 16, and 22ºC showed that temperature did not significantly affect the ligation efficiency. In order to study the effect of plasmid size on ligation efficiency, cloning of pUC19 fragments of sizes ~3686, 4686, and 6686 kb was attempted. Specific regions from λ DNA were amplified by PCR and these 1000, 2000, and 4000 bp fragments were cloned into pUC19 vectors. However, clones could not be obtained due to time constraints and technical difficulties in the screening step. Improvements on the screening system may increase the likelihood of obtaining desirable clones.

T4 DNA ligase is a widely used enzyme in the field of molecular biology. It is commonly used in cloning to covalently join DNA insert of interest into the open ends of a cloning vector. In the presence of T4 DNA ligase and ATP, the 3' hydroxyl end and the 5' phosphate end of DNA fragment(s) are joined together by forming a phosphodiester bond (4). In ligation experiments, several parameters are optimized to increase the likelihood of obtaining the desirable clones. These parameters include DNA concentration, molecular ratio of the insert and vector, temperature of reaction, buffer concentration (4). The right parameters should maximize the yield of monomeric, circular ligation products, which can be efficiently transformed into competent cells for screening (4). The ligation efficiency of T4 DNA ligase may also be affected by the use of different restriction endonucleases prior to ligation (W. Ramey, personal communication).

*Nde*I and *Hind*III are two commonly used type II restriction endonucleases in cloning strategies (3). *Nde*I recognizes and cleaves the sequence 5'-CA↓TATG-3', and *Hind*III recognizes and cleaves the sequence 5'-A↓AGGCT-3' (NEB catalog, New England Biolabs Inc, Ipswich, MA). Both enzymes create overhangs in digested DNA fragments and these overhangs can be ligated with compatible ends of vectors or other DNA fragments. Previous studies have noted that *Nde*I-digested DNA fragments often had lower ligation efficiency than that of *Hind*III-digested DNA fragments (2). This observation was confirmed by Chang *et al* who demonstrated *Nde*I-digested λ fragments ligated less efficiently than *Hind*III-digested λ fragments did with the aid of T4 DNA ligase (2). However, the ligation patterns and efficiencies could not be further analyzed because the λ fragments were too large to be studied in detail by standard gel electrophoresis and therefore, the ligation patterns could not be studied.

This paper describes ligation efficiency of *Hind*III-digested pUC19 fragments and *Nde*I-digested pUC19 fragments by T4 DNA ligase. The small size of pUC19 vector (2868 bp) allowed more detailed analysis of ligation efficiency as well as ligation patterns. Furthermore, in preparation for studying the effect of plasmid size on ligation efficiencies, cloning experiments were performed to generate three new pUC19-based vectors. λ DNA fragments of various sizes were cloned into pUC19 to create plasmids with final sizes of 3868, 4868 and 6868 bp. Using the DNA fragments generated by these four plasmids, studies can be done to characterize T4 DNA ligase and study its ligation efficiency and pattern on various DNA fragments.

MATERIALS AND METHODS

Media. Luria-Bertani (LB) broth was composed of 10 g/L trypton (BD Biosciences, Cat. 211705), 10 g/L yeast extract (BD Biosciences, Cat. 212750) and 10 g/L NaCl (EMD Chemical, Cat. SX0420-3). LB agar was

Table 1. Sequences of primers designed to amplify regions of λ DNA. Along with a λ template, F6019 could be used in conjunction of R7030, R8021, and R10006 to yield PCR products of 1000, 2000, and 4000 bp, respectively.

Name	Sequence	%GC	T_m (°C)
F6019	5'-CGGGATCCTGCCAGCGACGAGACGAAAA-3'	60	78
R7030	5'-CGGGATCCTTAATGCCTTCGCGCTGTGC-3'	60	77
R8021	5'-CGGGATCCACGGCTGAGGTTTTCAACGG-3'	60	75
R10006	5'-CGGCAGCCTGAGAAATTGCCTCCGTGGG-3'	60	76

composed of LB broth and 15 g/L agar (Marine BioProducts, Cat. M12-500). Modified MacConkey agar was composed of 17 g/L trypton (Difco, Cat. 0123-02), 3 g/L tryptose (Difco, Cat. 0124-01), 5 g/L NaCl, 10 g/L lactose (Allied Chemical, Cat. 1816), 0.03 g/L neutral red (Sigma, Cat. N6634) and 13.5 g/L agar.

Plasmids. The 2686 bp pUC19 has one of each *Nde*I, *Hind*III, and *BamH*I restriction sites (Fermentas catalog, Fermentas International Inc, Burlington, Ontario). QIAfilter Plasmid Maxi Kit (QIAGEN, Cat. 12263) was used to extract a large amount of pUC19 from *E. coli* DH5α strain carrying pUC19 (UBC MICB 421 frozen stock). This strain of *E. coli* was grown in 100 ml LB broth containing 100 μg/ml ampicillin (Sigma, Cat. A9518) at 37ºC overnight with shaking. Plasmid extraction was performed by following the manufacturer's instructions. Commercial pUC19 (Fermentas, Cat. SD0061) was used for the second set of cloning experiment.

Primer Design. Four primers (Table 1) were designed using Primer Designer software (Scientific and Educational Software, USA) to amplify 1000, 2000, and 4000 bp fragments from specific regions of λ DNA and introduce *BamH*I restriction sites to both ends of the products through PCR. The primers were synthesized by Nucleic Acid Protein Service Unit (UBC, Vancouver) and dissolved in autoclaved, distilled water to 100 μM before use. Forward primer F6019 resembled the nucleotide sequence starting from position 6019 of λ DNA. Reverse primers R7030, R8021, and R10006 resembled the complementary nucleotide sequences ending at positions 7030, 8021, and 10006 of λ DNA, respectively (GenBank ID J02459). Through PCR, primer sets F6019/R7030, F6019/R8021, and F6019/R10006 can amplify ~1000, 2000, and 4000 bp λ DNA fragments, respectively. All four primers were 28 bp long, and they contained a 5'-CGGGATCC sequence which represents the *BamH*I restriction sites.

Polymerase Chain Reaction. PCR were incubated in the Biometra T gradient PCR machine (Whatman, Germany). An annealing temperature optimization for each primer set was performed using the temperature gradient setting. Each 50 μL PCR reaction contained: 0.3 μM forward and reverse primers, 200 ng λ DNA (Invitrogen, Cat. 25250-010), 0.3 mM dNTP mixture (Invitrogen), 1.25 U Platinum *Pfx* DNA polymerase (Invitrogen, Cat. 11708-013), 1X *Pfx* Amplification buffer, 1 mM $MgSO_4$, and autoclaved, distilled water. PCR settings are listed in Table 2.

Table 2. PCR conditions for amplification of λ DNA.

	Temperature (°C)	Time (h:m:s)
1	94.0	0:2:0
2	94.0	0:0:15
3	Gradient 55-77°C	0:0:30
4	68.0	0:4:0
Steps 2-4 were repeated for 34 cycles		
5	68.0	0:4:0
6	4.0	Pause

The 1000 bp PCR products from F6019/R7030 were designated as ***A***; the 2000 bp PCR products from F6019/R8021 were designated as ***B***; and the 4000 bp PCR products from F6019/ R10006 were designated as ***C***. Five microlitre of PCR products were analyzed on a 0.8% agarose gel to determine the success of the reactions. The sample with most desirable PCR products and least impurities was used for cloning.

Restriction Digest. PCR products ***A***, ***B***, and ***C*** were digested with *BamH*I (Invitrogen, Cat. 15201-023). Each 50 μL digestion contained: ~45 μL DNA, 5 U *BamH*I (Invitrogen, Cat. 15201-023), 1X REact 3 Buffer (Invitrogen), and autoclaved, dH_2O. The samples were incubated at 37ºC for 1.5 hours and then deactivated at 65ºC for 20 minutes. pUC19 DNA was also digested in the same manner.

Vector Dephosphorylation. pUC19 was dephosphorylated to reduce the chances of self re-ligation. *BamH*I/pUC19 was incubated with 2.5 U Antarctic Phosphatase (NEB, Cat. M0289), NEB Buffer 3 and dH_2O at 37ºC for 1.5 hours and then deactivated at 65ºC for 20 minutes.

PCR Purification. The digested and/or dephosphorylated DNA samples were purified using QIAquick PCR Purification kit (QIAGEN, Cat. 28104) following the manufacture's instructions. DNA

samples were eluted in 30 µL EB Buffer and quantitated using a UV spectrophotometer.

Ligation Reaction. The digested, purified ***A***, ***B***, and ***C*** products were ligated to digested, dephosphorylated, and purified pUC19 vector. Each ligation reaction contained: 30 fmol pUC19, 90 fmol ***A, B*** or ***C***, 1 µL T4 DNA Ligase (Invitrogen, Cat. 15224-017), 1X Ligase Buffer, and dH_2O. Ligation reactions occurred at room temperature for 16 hours. A ligation control, which contained pUC19 without any PCR products, was also included.

Transformation. Ligation product was transformed into *E. coli* DH5α competent cells (UBC, MICB 421 frozen stock) using the heat shock method. Ligation products was mixed gently with thawed competent cells, and the mixture was incubated for 30 minutes on ice, 2 minutes at 42ºC, and 2 minutes on ice again. LB broth was added to a total volume of 1 ml and the culture was incubated at 37ºC for 1 hour with shaking before plating. A ligation control consisted of purified pUC19 was included. Transformants were initially plated on LB agar containing 100 µg/ml ampicillin, 800 µg X-gal, and 400 µg IPTG, and later transformants were plated on modified MacConkey agar containing 100 µg/ml ampilcillin. For both types of plates, white colonies were selected for screening.

Screening. Colonies of potential clones were grown overnight in 3 ml LB media containing 100 µg/ml ampicillin at 37ºC with agitation. Plasmids from 1 ml of overnight culture were isolated using the mini-prep in ten minutes protocol (6). The isolated plasmids were digested with *BamH*I as previous described in the Restriction Digest section. The isolated, digested plasmids were analyzed on 0.8% agarose gel to determine the success of cloning.

Ligation Efficiencies of T4 DNA Ligase. Ligation efficiency of *Nde*I digested pUC19 and *Hind*III digested pUC19 were compared. One batch of pUC19 was digested with *Nde*I (Invitrogen, Cat. 15426-018) in 1X REact 6 Buffer, and the other batch of pUC19 was digested with *Hind*III (Invitrogen, Cat. 15207-020) at 37ºC for 1.5 hours. Heat inactivation occurred at 65ºC for 20 minutes.

The digested pUC19 samples were purified using a phenol extraction of DNA sample and ethanol precipitation of DNA (1). pUC19 was then dissolved in dH_2O and stored at -20ºC. Two hundred and fifty microgram of digested, purified pUC19 DNA was ligated in a 20 µL reaction mix of 1 µL T4 DNA ligase, 1X Ligation buffer, and dH_2O for 16 hours 4, 16, and 22ºC. Subsequent ligation experiments occurred at 22ºC. Ligation products were visualized on 0.8% agarose gel. Same amount of *Hind*III/λ DNA fragments (Invitrogen, Cat 15612-013) were also ligated in parallel as a control.

RESULTS

DNA Extraction. Two QIAGEN maxi-prep plasmid extractions were performed and the expected yield was up to 500 µg. The first extraction using an old kit yielded 9 µg of pUC19 plasmid, and the second extraction using a brand new kit yielded 46.08 µg of pUC19 plasmid. The yields from both extractions were much lower than the expected.

PCR Optimization. The four primers successfully amplified the expected regions from λ DNA. Both primer sets F6019/R7030 and F6019/R10006 performed well within the annealing temperature range of 60-75ºC. PCR products B1-B5 and C1-C5 contained large amount of amplified λ fragments of 2000 and 4000 bp, respectively (data not shown). However, there were some impurities observed in gel electrophoresis analysis. Primer sets F6029/R7030 performed optimally at an annealing temperature of 56.2ºC and a distinct band of 1000 bp was observed in all products (A5-A10) (data not shown).

Screening of Potential Clones. The first set of cloning experiments involved cloning A7 into pUC19, B3 into pUC19, and C3 into pUC19. Large numbers of white and blue transformant colonies were observed on X-gal agar plates. White colonies sometimes turned blue under prolonged incubation at 37ºC or prolonged storage at 4ºC. A total of 30 white colonies from each A7/pUC19 and B3/pUC19 ligation, and 25 colonies from C3/pUC19 ligation were selected for screening. Among these colonies, only nine A7/pUC19, four B3/pUC19, and two C3/pUC19 were able to grow overnight in LB broth with ampicillin. Visualization on 0.8% agarose gels of plasmid DNA extracted from these colonies reveal that these colonies contained only the pUC19 vector and no inserts of desirable sizes (Figure 1). Undigested plasmids (Figure 1, Lanes b) were ~2000 bp in size due to their supercoiled nature, and digested plasmids (Figure 1, Lane a) had a size of ~2600 bp, which corresponded the size of pUC19 vector. No inserts of 1000 bp, 2000 bp or 4000 bp were observed in these samples.

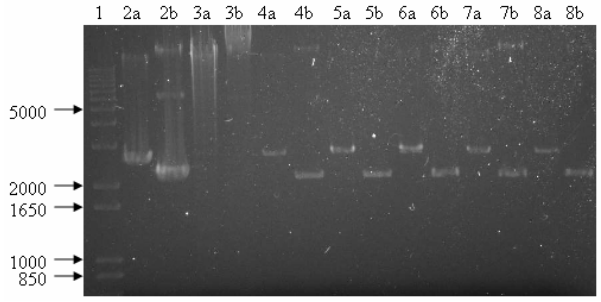

FIG 1. Screening of potential transformants of A7/pUC19, B3/pUC19, and C3/pUC19 clones. Lanes (b) were isolated plasmids from overnight transformant cultures and Lanes (a) were *BamH*I-

digested plasmids. Lane 1 was 1 µg of 1 kb plus DNA ladder (Invitrogen, Cat. 12308-011), Lane 2-5 were potential A7/pUC19 clones, Lane 6-7 were potential B3/pUC19 clones, and Lane 8 was potential C3/pUC19 clones.

The second set of cloning experiments included an additional step of vector dephosphorylation to minimize background pUC19 vector due to self re-ligation in cloning. The number of transformants on modified McConkey agar plates was significantly less than that from the first set of cloning experiments. Most transformants appeared to have a red tint in the centre of the colonies. When these colonies were observed under the microscope, a chimera of red and white bacteria were seen to make up a colony. A total of three white colonies of potential A9/pUC clones were screened by plasmid extraction and agarose gel (Figure 2). One potential clone (Figure 2, Lane 3a and 3b) contained plasmids that had an unexpected digestion pattern because the bands do not resemble pUC19 or any of the inserts.

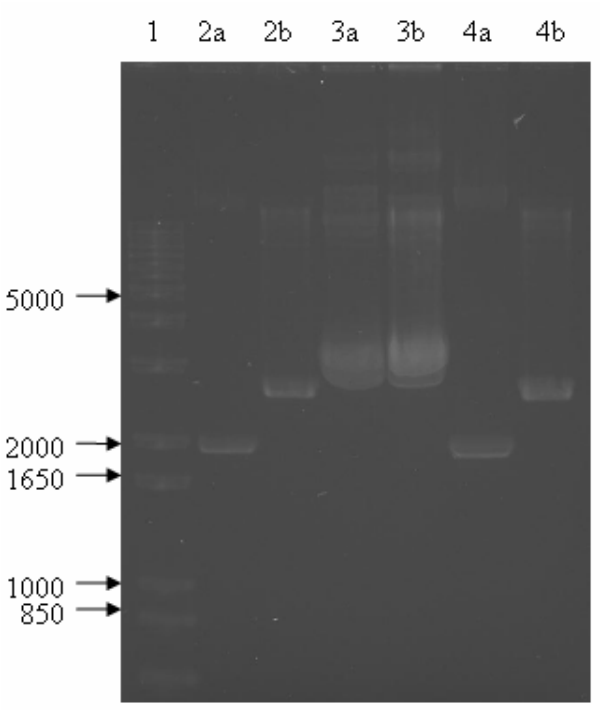

FIG 2. Screening of potential transformants of A7/pUC19 clones. Lanes (b) were isolated plasmids and Lanes (a) were *BamH*I-digested plasmids. Lane 1 was 1 µg of 1 kb plus DNA ladder, Lane 2-4 were potential A7/pUC19 clones.

Ligation Efficiency of *Hind*III/pUC19 and *Nde*I/pUC19 by T4 DNA Ligase. Ligation experiments (Figure 3) showed that the ligation efficiency of commercial *Hind*III/λ DNA fragments (control) was very high. Lane 4 shows that the five bands disappeared and ligated into bands with sizes of more than 23 kbp. Lane 3 shows that *Hind*III/pUC19 fragments underwent complete ligation to generate multiple, new bands with less intensities. A high molecular weight band of ~23 kbp was also observed. *Nde*I/pUC19 fragments were ligated poorly because only half of the original fragments were ligated into bands of ~5000 bp and higher (Figure 3, Lane 2a and 2b).

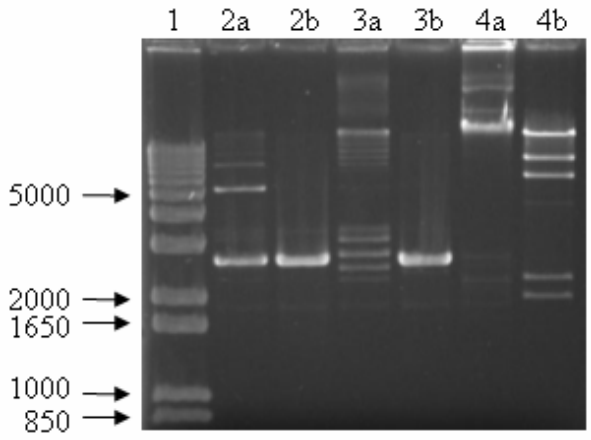

FIG 3. Ligation efficiency of *Hind*III/pUC19 and *Nde*I/pUC19 fragments by T4 DNA ligase. Lane 1 was 1 µg of 1 kb plus DNA ladder, Lane 2 was loaded with 0.250 µg of *Nde*I/pUC19, Lane 3 was loaded with 0.250 µg *Hind*III/pUC19, and Lane 4 was loaded with 0.250 µg *Hind*III/λ as a control. Lanes (a) were samples after ligation, and Lanes (b) were samples before ligation.

The effect of ligation temperature was investigated (Figure 4). The T4 DNA ligase efficiency on these samples did not vary significantly when different temperatures were used. The ligation patterns were very similar to those in Figure 3. The ligated pUC19 samples of *Hind*III/pUC19 did not have the same degree of supercoiling as the uncut pUC19 control.

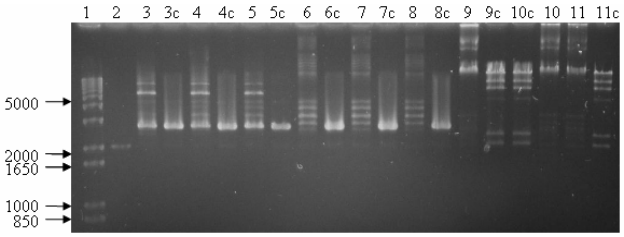

FIG 4. Ligation efficiency of *Hind*III/pUC19, *Nde*I/pUC19, and *Hind*III/λ fragments by T4 DNA ligase at 4ºC, 16ºC and 22ºC. Lane 1 was 1 kb plus DNA ladder, Lane 2 was untreated pUC19, Lane 3, 4, 5 were *Nde*I/pUC19 ligated at 4, 16, and 22ºC, respectively, Lane 6, 7, 8 were *Hind*III/pUC19 ligated at 4, 16, and 22ºC, respectively, and Lane 9, 10, 11 were *Hind*III/λ ligated at 4, 16, and 22ºC, respectively. Lanes (c) were ligation controls without T4 DNA ligase.

DISCUSSION

The synthesis of three new pUC19-based plasmids of ~3686, 4686 and 5686 bp in size began with PCR amplification of λ DNA. Successful PCR products were obtained but small amount of impurities were also observed due to random primer binding on the large λ DNA template (data not shown).

In the first set of cloning experiments, gel electrophoresis showed that no desirable A/pUC19, B/pUC19, and C/pUC19 clones were found among the selected transformant colonies (Figure 1). No 1000, 2000, or 4000 bp inserts were found in the BamH1-digested plasmids from the potential A7/pUC19, B3/pUC19, or C3/pUC19 clones during screening (Figure 1, Lane 2-8). In the second set of cloning experiments, the addition of vector dephosphorylation step significantly reduced the number of transformant colonies. This suggested that most transformants found in the first set of cloning experiments contain mostly pUC19 re-ligation plasmids. Dephosphorylation successfully eliminated the vector background as expected. Due to time constraints, only three A7/pUC19 transformants were screened. Out of these potential A7/pUC19 transformant colonies screened, two contained pUC19 vectors without any 1000 bp insert (Figure 2, Lane 2, 4), and one had an unexpected pattern, (Figure 2, Lane 3). This suspicious plasmid had a size of 3100 bp (Figure 2, Lane 3a) and after digestion by *BamH*I, two fragments of 3400 and 3000 bp were observed (Figure 2, Lane 3b). This unexpected clone could have been the ligation products of PCR impurities and pUC19 vector.

Some difficulties were encountered during selection and screening of transformants. The blue/white selection of colonies on X-gal plates was not accurate. The accuracy depended on how evenly X-gal was spread on the plate and the size and number of the transformant colonies on the plate. When colonies were under-incubated, the blue color was not obvious and this gives a false-positive error. This could explain why the colonies screened in the first set of cloning experiments did not contain any inserts. When modified McConkey agar plates were used, the colors of the colonies could be better distinguished because the red/white contrast was higher. Under close examination under the microscope, some single colonies were a chimera of red and white bacteria. It was possible that these colonies originated from a single transformant colony that had two types of vectors: pUC19 and pUC19 with an insert. As this single transformant grew and divided, some progeny inherited only the pUC19 vectors that gave a red color and some progeny inherited only the pUC19 plus insert that gave a white color (W. Ramey, personal communication). Another problem was that some of the selected colonies did not grow in LB broth. One possibility was that the selected colonies were satellite colonies that showed up after prolonged incubation, and they could not grow under ampicillin selection in LB broth.

Initial investigation of T4 DNA ligase ligation efficiency on *Nde*I/pUC19, *Hind*III/pUC19, and *Hind*III/λ was performed. T4 DNA ligase exhibited the highest ligation efficiency on control *Hind*III/λ DNA fragments because most *Hind*III/λ fragments disappeared and ligated to bands higher than 23000 bp in size (Figure 3, Lane 4). Because the *Hind*III/λ DNA fragments were commercially prepared, it potentially had less impurities to affect the ligation efficiencies of T4 ligase. Also, the nature and property of viral DNA may promote higher ligation efficiency than bacterial DNA. In addition *Hind*III/λ DNA fragments did not seem to ligate intramolecularly because small bands were not apparent. Rather, these fragments preferred to ligate intermolecularly to form larger fragments. The ligation efficiency of *Hind*III/pUC19 fragments was higher than that of *Nde*I/pUC19 fragments (Figure 3). This confirmed the finding of Chang *et al* that ligation of *Hind*III/λ fragments is more efficient than *Nde*I/λ fragments (2).

However, the ligation pattern of digested λ fragments seen in Chang *et al* study and this study was different from that of digested pUC19 fragments in this study. *Hind*III/pUC19 fragments ligated both intermolecularly and intramolecularly, whereas *Nde*I/pUC19 ligated inter-molecularly (Figure 3). When *Hind*III/pUC19 ligated intra-molecularly, five new bands of varying sizes appeared around the original band of cut pUC19 before ligation (Figure 3, Lane 3a). These bands were probably ligated, monomeric and circular pUC19 with varying degree of supercoiling but it must be confirmed. The same pattern was seen when *Hind*III/pUC19 ligated inter-molecularly to form higher sizes of DNA fragments. This suggested the higher molecular weight ligation products may also be circular. However, this trend was not observed in *Nde*I/pUC19 fragments. *Nde*I/pUC19 seemed to only ligate inter-molecularly and did not circularize to form bands of varying degree of supercoiling (Figure 3, Lane 2a). The differences in the structures of these cut pUC19 and how each molecule interactions interact with each other before ligation and how T4 DNA ligase acts on these cut pUC19 during ligation may explain the reason behind the differences in ligation patterns.

Initial ligation experiments using *Hind*III-digested pUC19 and *Nde*I-digested pUC19 provided new information about the ligation efficiency and ligation pattern by T4 DNA ligase. *Hind*III-digested pUC19 ligated more efficiently than *Nde*I-digested pUC19, and *Hind*III/pUC19 also ligated both intermolecularly and

intramolecularly and circularized to form plasmids of varying degrees of supercoiling. Cloning of pUC19-based plasmids with inserts of 1000, 2000, 4000 kb was attempted but clones could not be obtained due to time constraints and technical difficulties. With these plasmids of different sizes, investigation of plasmid sizes on ligation efficiency by T4 DNA ligase could be done.

FUTURE EXPERIMENTS

More screening of the potential transformants from the second set of experiments should be attempted to isolate pUC19 vectors of ~3686, 4686 and 6686 bp for the investigation of the effect of vector sizes on T4 DNA ligase ligation efficiency. The accuracy of color selection of the transformant colonies must be improved to increase the probability of finding the right clones. The ligation pattern of these two pUC19 patterns should be further investigated. Ethidium bromide could be use to confirm whether *Hind*III/pUC19 really formed circular pUC19 with varying degree because high concentration of ethidium bromide could drive close-ended, circular pUC19 into the same degree of supercoiling and therefore a single band would be observed (1).

Using these four different pUC19 vectors, the effect of fragment size on T4 DNA ligase ligation efficiency and the ligation patterns of *Hind*III/pUC19 and *Nde*I/pUC19 fragments could be investigated in detail in future experiments.

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