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Generation of Intermediate Plasmids for the Investigation of Sequence-Dependent Ligase Activity due to Location of Sequences Relative to the Restriction Endonuclease Nick Site

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T4 DNA ligase has been shown to perform ligation reactions more efficiently with HindIII-digested λ DNA fragments than NdeI-digested λ fragment. The reasons for this observation are currently under investigation. One hypothesis is that ligation efficiency might be dependent on sequences adjacent to the restriction endonuclease nick site. To test this hypothesis, an experiment was designed to compare ligation efficiency between a control DNA fragment and a substrate with an insertion mutation of 3 nucleotides in length at the position five bases away from the Restriction endonuclease nick site. In order to generate these substrates, it was necessary to amplify a control and a mutagenized DNA fragments by polymerase chain reaction (PCR) and clone these inserts into pUC19 to produce plasmids pJW3 and pJW4. Several attempts had been made through a 1-step cloning procedure, but no clones were obtained. This cloning failure is potentially due to low restriction enzyme activity at the end of PCR-amplified DNA fragments. As a result, an alternative cloning strategy was employed to clone the inserts into intermediate products via single 3'-thymidine overhangs. This new strategy yielded 3 clones with the control insert and 2 clones containing the mutagenized insert, which were named pJW1 and pJW2 respectively. The successful generation of pJW1 and pJW2 will provide more convenient means to construct the pJW3 and the pJW4 plasmids. These plasmids will eventually allow the isolation of substrates needed for the ligation efficiency test.

DNA ligases play a pivotal role in DNA replication, DNA repair, and DNA recombination (10). They have also become essential tools for in vitro DNA manipulation in a wide range of applications in molecular biology, in the detection of specific nucleic acid sequences or protein analytes, and in DNA nanotechnology (4). One of the most common applications is to generate recombinant DNA molecules in which DNA fragments of interest are covalently combined into vector molecules (Invitrogen Corporation, T4 DNA ligase: Technical bulletin 15244-2, 2002).

The conservation of activities for these ATP-dependent DNA ligases is evident from their sequence and structure conservations. Although they vary in size, they contain highly conserved motifs in their catalytic domain. The prototype of these DNA ligases is the most commonly used T4 DNA ligase. The crystal structure of this 41kDa enzyme has been resolved and shows that the enzyme comprises of two domains with a deep cleft between them (3). It is also interesting that T4 ligase is able to catalyze the joining of blunt-ended dsDNA, which is not observed in other ligases (7).

Although it is known that T4 ligase have broad substrate specificity, substrates can also play a role in ligation efficiency (2). It has been observed that T4 ligase performs ligation reaction more efficiently with HindIII-digested dsDNA fragments than certain NdeI-digested fragment (2). Ligation of this specific NdeI-digested fragment is not improved by prolonged incubation and the reason for this low ligation efficiency is currently unknown. This observation might be explained if specific nucleotide sequences adjacent to the restriction endonuclease nick site have significant effects on the efficiency of ligation reactions.

One way to study this subject is to investigate whether ligation efficiency is affected by sequences adjacent the nick site. It is difficult to study this directly because these nucleotides are generally not conserved. The length and the location of the adjacent sequences affecting nucleotide-enzyme interactions are also unknown. However, inserting a conserved sequence five nucleotides away will twist the subsequent sequences so that specific interactions will be distorted. This specific location represents a distortion at the opposite end of the DNA helix, where specific nucleotide-enzyme interactions may be present. As a result, the insertion will change subsequent sequence interactions with any protein but retain the majority of the original sequence. Therefore, it was hypothesized that insertion of a short sequence 3 nucleotides (nts) in length at the position five bases away from the nick site will have an effect on the ligation efficiency. An experiment was designed to use a mutagenized HindIII-digested λ fragment as a

ligation substrate. This fragment has been demonstrated to be effectively ligated by T4 DNA ligase (2). As a result, any changes in ligation efficiency will indicate an effect caused by sequences away from the nick site. In this report, the attempts to generate this reaction substrate as well as a control substrate are described in detail.

MATERIALS AND METHODS

PCR Amplification of λ Fragments. Control λ (ctrλ) and mutagenized λ (mutλ) fragments were amplified from λ genome (#25250-010, Invitrogen) by polymerase chain reaction (PCR) as previously described (9) using the final concentrations of 0.3mM dNTP, 1mM MgSO4, 0.3μM primers and 1.25U of Pfx DNA polymerase (#11708-013, Invitrogen) in 50μL reactions. The initial reactions were amplified using 200 ng of template at the annealing temperatures of 58oC, 55oC, 51oC for ctrλ and 60oC, 57oC, 54oC for mutλ. More optimized conditions include 50 ng of insert at the annealing temperatures of 52oC, 49oC for ctrλ and 54oC, 51oC for mutλ. All fragments were amplified for 35 cycles and 5μL of the reactions were then electrophoresed through a 1% agarose gel (8).

Sense primers JWCF (5'-TCTCGGATCCTATTTTCATAAAGCTTT-3') and JWMF (5'-TCTCGGATCCTATTTTCATAAAGCTTTTCGATTAATTTAAC-3') were used for ctrλ and the mutλ respectively. Antisense primer JWR (5'-TTGGACTCAAGAATGCTGCC-3') was used for both inserts. The introduced BamHI restriction site for the sense primers were underlined whereas the insertion mutation is indicated in bold. An EcoRI site is present approximately 45 nucleotides upstream of the antisense primer. These primer combinations allowed the amplification of a 1 kb fragment from the λ genome at positions 25140-26166.

Digestion and purification of pUC19 and λ Fragments. The 50μL PCR reactions were purified using a DNA cleanup kit (#28006, Qiagen). These reactions and 2 mg of pUC19 (UBC Teaching Lab, Frozen Stock, Dept. Microbiology & Immunology, University of British Columbia) were digested with 5 U of EcoRI (#15202-021, Invitrogen) and BamHI (#15201-031, Invitrogen) in 70μL reactions containing 1X React buffer and incubated at 37oC overnight. For pUC19, 2 μL of the reaction was removed and analyzed by gel electrophoresis. All reactions were then PCR purified. After this purification step, the linearized pUC19 was dephosphorylated by Calf Intestinal Alkaline Phosphatase (#M0290S, New England Biolabs) (8) and purified again by the DNA cleanup kit.

Ligations and Transformations of pUC19 and λ Fragments. Approximately 95 ng of the linearized pUC19 plasmid were ligated with 30 ng and 90 ng of inserts using T4 DNA ligase (#EL0014, Fermentas) with 2 μL of the supplied 10X ligase buffer in 20 μL reactions. Ligations were performed at 14oC overnight. The same ligation procedure was used in both trial 1 and trial 2.

Ligated samples in trial 1 were transformed by the standard chemical method and electroporation method (Strategene, Achieve the highest transformation efficiency, Strategies. 18:1-2., 2002). In both trials, a transformation control of circularized pUC19 was included. In trial 2, samples were transformed by electroporation and a mixture of linearized pUC19, ctrλ, mutλ, and circularized pUC19 was included as a control. Transformants were plated on ampicillin plates (50μg/mL) and incubated at 37oC overnight.

Ligations and Transformations of pCR2.1-TOPO and λ Fragments. In order to generate single 3' adenine overhangs required for TOPO cloning reactions, 10 μL of previously amplified PCR reactions from ctrλ and mutλ were amplified with 2.5 U of Taq polymerase (#10966-018, Invitrogen) for 5 cycles according to previously described method (6). These segments were then mixed with 10 ng of pCR2.1-TOPO linear vector (#K4500-01, Invitrogen) in 6μL reactions containing 200 mM NaCl and 10 mM MgCl2. This reaction does not require DNA ligases and the reactions were incubated for 5 min at room temperature. Freshly ligated samples were transformed using the chemical method and plated on ampicillin plates (50μg/mL). These plates were incubated overnight at 37oC.

For each insert, 24 clones were selected and grown overnight at 37oC. Plasmid DNA from these clones was isolated by TENS miniprep (11) and resuspended in 50 μL of Tris-EDTA (TE) (10mM Tris, pH8.0, 0.1mM EDTA). To check for the presence of inserts in these clones, 5 μL of plasmids from all clones were analyzed by gel electrophoresis after digestion with EcoR1 restriction endonuclease at 37oC in a final volume of 20μL.

RESULTS

PCR Amplification of Control and Mutagenized λ Fragments. The initial PCR amplification intended for introducing the insertion mutaion generally yielded 2 intense bands of 6.0 and 1.0 kb (Figure 1). Strong smearing was observed in all samples but ctrλ had more intense bands than mutλ for the expected 1.0 kb fragment. As the annealing temperature decreased, this 1.0 kb fragment became more intense. However, the non-specific 6.0 kb product and the smearing were still present at the lower annealing temperature. By lowering the amount of template to 50 ng, the specificity was improved even though faint bands of higher molecular weights were still present (Figure 2). Further decreases in annealing temperature had no effect on ctrλ. The higher temperature of 54oC for mutλ also resulted in higher intensity for the 1.0 kb fragment even though some faint band persisted.

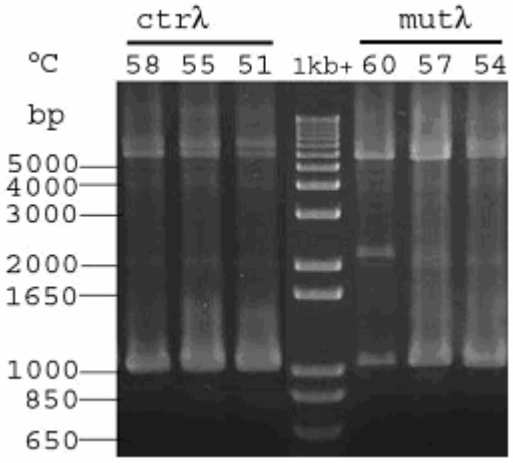

FIG.1 PCR amplification with 200 ng of λ genome as template. Various annealing temperatures that were used are indicated above the lanes. Samples were amplified for 35 cycles and 5 μL of the 50μL reactions were loaded on a 1% agarose gel. Both the ctrλ (lanes 1-3) and mutλ (lanes 5-7) were expected to have a single 1.0 kb fragment. The fourth lane (indicated by 1kb+) contains 1 μg of the 1kb plus ladder and sizes of the DNA fragments of the ladder are indicated on the left margin.

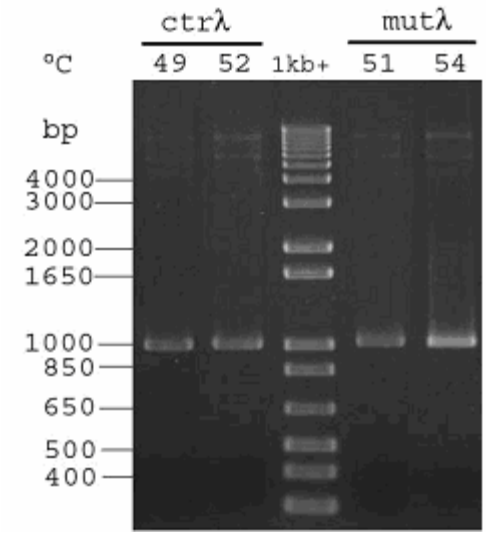

FIG. 2 PCR amplification with 50 ng of λ genome as template. Various annealing temperatures that were used are indicated above the lanes. Samples were amplified for 35 cycles and 5 μL of the 50μL reactions were loaded on a 1% agarose gel. Both the ctrλ (lanes 1-2) and mutλ (lanes 4-5) were expected to have a single 1.0 kb fragment. The third lane (indicated by 1kb+) contains 1 μg of the 1kb plus ladder and sizes of the DNA fragments of the ladder are indicated on the left margin.

The ctrλ and mutλ fragments were prepared in order to generate plasmids as test substrates for ligation efficiency test. Although the initial reaction conditions yielded large amounts of non-specific products, the specificity was much improved by lowering the template amount. The optimized annealing temperature for mutλ appears to be 54oC whereas both 49oC and 52oC have similar results for ctrλ. Attempts have also been made using digested λ genome as templates but the specificity was not improved (data not shown). Since the majority of the amplified products were the desired 1.0kb fragment, the PCR reactions were purified using a DNA cleanup kit and used for further ligation reactions. They were not gel purified to minimize the loss of DNA from purifications.

Ligations and Transformations of pUC19 and λ Fragments. The pUC19 vector was digested by EcoRI and BamHI. This reaction was confirmed to go to completion to produce a single 2.7kb fragment (Figure 3). It was also tested that either EcoRI or BamHI alone was able to linearize the pUC19 plasmid (data not shown). As a result, this linearized pUC19 vector should have contained the compatible ends needed for ligations with the PCR amplified λ fragments.

The digested pUC19 and λ fragments were mixed together and ligated with T4 DNA ligase and transformed into DH5α competent cells. However, no clones were obtained after 2 trials of ligations and three transformation attempts (Table1). In all cases, the transformation control of circularized pUC19 had over 500 colonies. Both chemical and electroporation methods were attempted but yielded similar results. In order to determine whether the inserts and the vector had any toxic effect on the competent cells, a mixture of all the inserts, the linearized pUC19 vector, and circularized pUC19 was transformed in trial 2. This mixture also had over 500 colonies. These results suggest that the limited number of transformants might be resulted from failures at the ligation step.

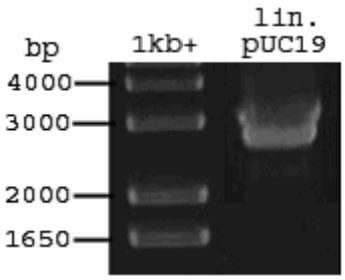

FIG. 3 EcoRI- and BamHI-digested pUC19 vector. Lane 1 (indicated by 1kb+) contains 1μg of the 1kb plus ladder and the sizes of the DNA fragments of the ladder are indicated on the left margin. Lane 2 is linearized pUC19. The plasmid pUC19 was simultaneously digested with EcoR1 and BamH1 overnight. A portion of the 70μL digestion reaction was removed to ensure complete digestion. A dominant 2.7kb fragment was observed.

Several attempts were made with to ligate the PCR amplified inserts and pUC19. However, no clones are obtained in all cases, even though the chemical transformation method is replaced by the more efficient electroporation method (Strategene, Achieve the highest transformation efficiency, Strategies. 18:1-2., 2002). In all these attempts, the transformation controls had more than 500 colonies and this result indicated that the transformation reactions are successful. The pUC19 vector was also shown to be linearized and it was demonstrated that either enzymes is able to linearize the vector (data not shown). These independent tests show that the enzymes were active. However, it was difficult to show the appropriate double cuts on the vector and the insert because the fragment arising from the double digest was too small to resolve in the gel and they were too small to affect the mobility of the larger fragment. However, by method of elimination, we suspect that the cause of the failed ligation attempts was in the digestion step for the inserts. It is known that restriction enzymes generally do not perform as well towards the ends of DNA fragments, although it has been shown that the BamHI enzyme can cleave >90% of substrates with 2bp overhangs in 2hours (Fermentas International Inc., Molecular biology: Catalog & product application guide, 2005) and the BamHI sites in the amplified PCR fragments were designed to be located 4-nts away from the end of the fragments to avoid this problem. This

enzyme property is believed to be the cause of the failed ligations.

Ligations and Transformations of pCR2.1-TOPO and λ. Fragments. Due to the failure to obtain transformants from the ligase-treated mixtures of pUC19 and λ. fragments, an alternative strategy was employed. This strategy involved the cloning of the amplified λ. fragments into the pCR2.1-TOPO vector via single 3'-thymidine overhangs and Taq polymerase-amplified PCR fragments. This ligation and transformation reaction resulted in 35 clones for the ctrλ. and 69 clones for the mutλ. Out of these clones, 24 clones were selected and analyzed by restriction digestion with EcoRI. EcoRI was expected to release the insert from the vector and the presence of a 1.0kb and a 4.0kb bands would indicate a successful clone. For ctrλ, 3 of the 24 clones (named JW051 – JW053) were found to contain the insert (Figure 4) while 2 of the colonies (named JW054 & JW055) had mutλ cloned into pCR2.1-TOPO (Figure 5).

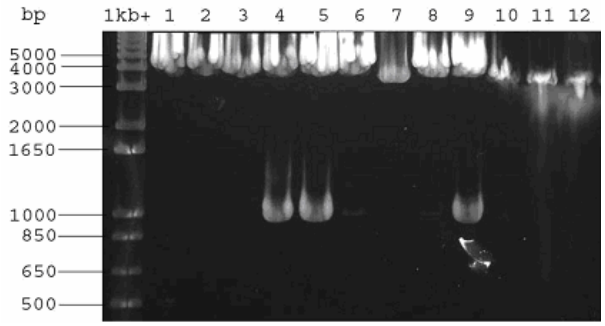

FIG 4. EcoRI digestion of pCR2.1-TOPO and ctrλ. clones. Plasmid DNA from 12 clones were isolated and digested with EcoRI. Lane 1 (indicated by 1kb+) contains 1μg of the 1kb plus ladder. Clone numbers are indicated above the other lanes. Clones #4, 5, and 9 showed the expected 1.0 and 4.0 kb pattern. Other clones contain only the 4.0 kb fragment.

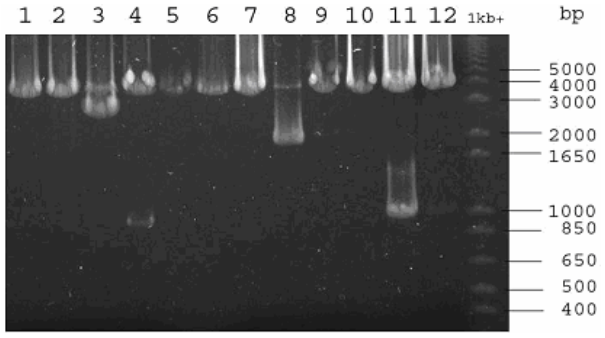

FIG. 5 EcoRI digestion of pCR2.1-TOPO and mutλ. clones. Plasmid DNA from 12 clones were isolated and digested with EcoRI. Lane 13 (indicated by 1kb+) contains 1μg of the 1kb plus ladder. Clone numbers are indicated above the other lanes. Only clones #4 and #11 showed the expected 1.0 and 4.0 kb pattern.

DISCUSSION

The original cloning strategy was designed to directly clone the control and mutagenized λ fragments into pUC19, and to use them to generate substrates to analyze ligase efficiency. The inserts are expected to insert into the vector via the EcoRI site at the 5' end and the BamHI site at the 3' end in the multiple cloning sites (MCS) to produce pJW3 with the ctrλ insert, and pJW4 with the mutλ insert (Figure 6). To generate substrates for the efficiency test, the plasmids can be linearized with BamHI, followed by dephosphorylation by CIP and then digested with HindIII. The resulting fragment will only have one end, the HindIII overhang, available for ligation. With this substrate, ligation efficiency can be analyzed by the rate of concatamer formations in time-point experiments and viewed by gel electrophoresis. Any significant differences observed between the control and the mutagenized substrates will indicate changes in ligation efficiency caused by the insertion mutation 5-nts downstream of the restriction site.

Although several ligation attempts have been made with this cloning strategy, no transformants were obtained. Since the enzymes were active and the transformants were obtained from the controls, the lack of colonies might be attributed to the ligation step. The failed ligation attempts lead to a new cloning strategy that will eventually generate the same pJW3 and pJW4 plasmids. This strategy involved intermediate plasmids pJW1 and pJW2 (Figure 7). The amplified inserts were first cloned into pCR2.1-TOPO via single 3' thymidine overhangs (1). Using these intermediate plasmids, the ctrλ and mutλ inserts could be excised out while avoiding digestion reactions at the end of DNA fragments. These inserts would also allow the desired fragments to be cut from a large sequence of DNA (the plasmid), so that there should not be any problem having restriction enzymes performing catalytic reactions near the end of small DNA fragments. These plasmids would also provide a simple way to analyze the plasmids by gel electrophoresis at different steps to ensure the presence of compatible ends because digestion by EcoRI and BamHI would generate large enough DNA fragments that could be differentiated and observed on an agarose gel. The inserts could then be ligated with BamHI- and EcoRI-digested pUC19 vector to produce pJW3 and pJW4.

This new strategy resulted in successful isolation of clones from both inserts. Cloning with mutλ and pCR2.1-TOPO yielded almost twice as many clones as those from ctrλ. The reasons for this discrepancy between the two inserts are unknown but it is potentially caused by unknown differences in the PCR and ligation steps. The screening of these clones

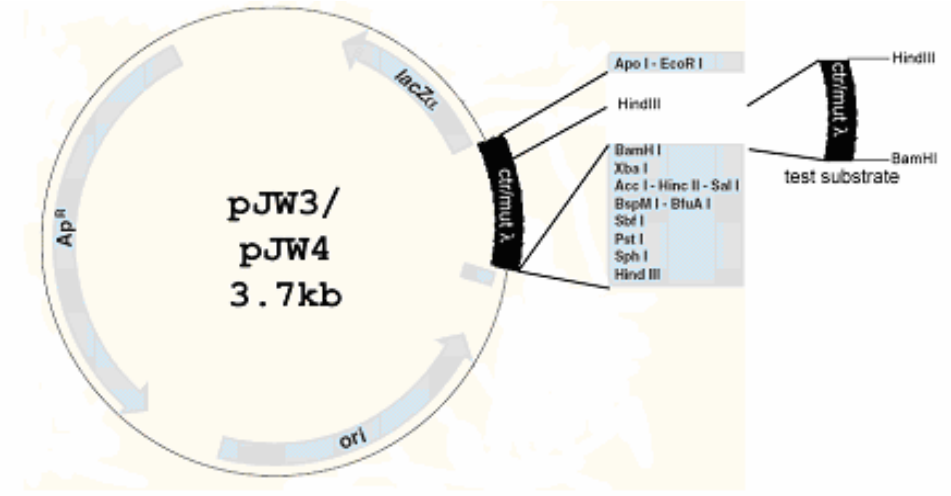

FIG. 6 The pJW3 and pJW4 plasmids. These two plasmids would be constructed by the insertion of ctrλ (pJW3) and mutλ (pJW4) inserts into pUC19 plasmid via the EcoRI and BamHI sites. The resulting plasmid would have a disrupted lacZ gene. The test substrate for ligation efficiency test could be excised out by restriction digest with HindIII and BamHI as indicated in the diagram.

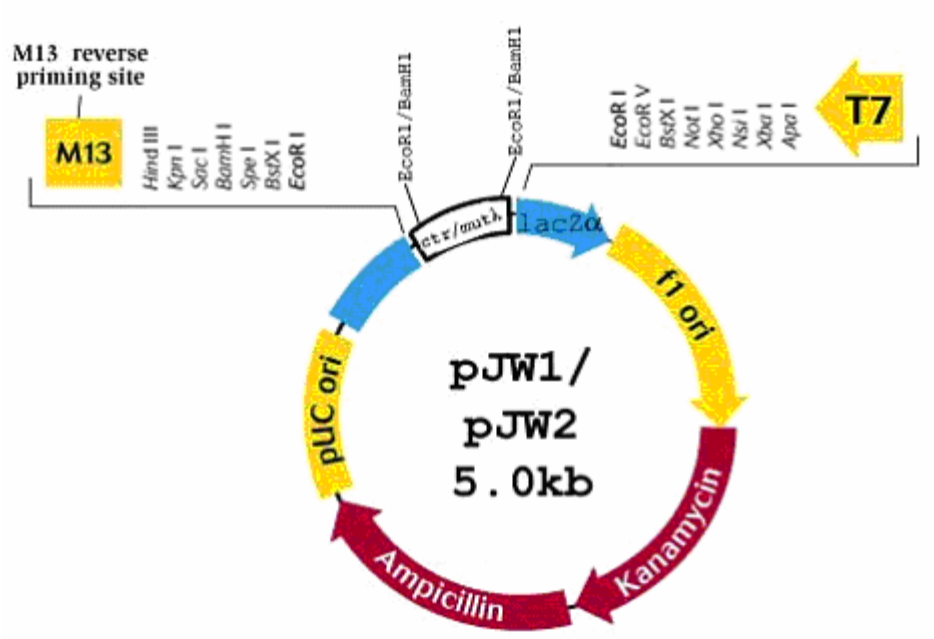

FIG. 7 The intermediate plasmids pJW1 and pJW2. These two plasmids have ctrλ (pJW1) and mutλ (pJW2) inserts cloned into pCR2.1-TOPO. The orientations of the inserts are currently unknown and the inserts contain an EcoRI site at one end and a BamHI site at the other end. All the restriction sites from the pCR2.1-TOPO plasmid are still intact and can be used for other cloning purposes.

surprisingly shows that only 3 clones contain ctrλ whereas mutλ is only present in 2 of the 24 clones (Figure 4 and 5). Vector re-circularization is potentially the main reason for this low number of clones with inserts. The identification of clones may be improved with α-complementation by growing the transformed cells in X-gal and IPTG containing medium as suggested by the manufacturer11. This will allow

observable colour differences to select clones with an insert. By increasing the chance of an insert in the clones that are checked, there is a higher chance of finding the right clone. The addition of a pCR2.1-TOPO vector control during the ligation step will also provide an estimation of the expected background level of clones. However, these additional analyses were not employed due to time constraints.

The successful generation of the pJW1 and the pJW2 plasmids will provide more convenient means to construct the pJW3 and the pJW4 plasmids. It should be noted that the orientation of the inserts in the pJW1 and pJW2 plasmids are currently unknown. However, the cloning strategies will not be affected by the orientations of the inserts. The inserts can also potentially contain mutations due to errors from PCR amplification. These unknowns should be addressed before continuing to the next cloning steps to generate pJW4 and pJW3. Production of pJW3 and pJW4 will eventually allow the isolation of substrates for the investigation of the relationships between ligation efficiency and downstream sequences away from the nick site.

FUTURE EXPERIMENTS

With the intermediate products of pJW1 and pJW2, it is necessary to ensure that the identities and the orientation of the inserts by different restriction digests and sequencing. Once the inserts are identified, the next step is to isolate them by sequentially digesting them with BamHI followed by EcoRI. This approach will allow analysis to ensure that the inserts are digested with both enzymes to produce the appropriate compatible ends. The inserts can then be ligated with BamHI- and EcoRI-digested pUC19 plasmid to generate pJW3 and pJW4, which will be used for further analysis.

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