Journal of Experimental Microbiology and Immunology (JEMI) Vol. 6:13-19

PCR Amplification, and Sequence Comparison of *lacI* gene in WT *E. coli* C29 cells and a presumptive *lacI* Knockout *E. coli* C29 cells to Determine the Difference in the Basal Expression Level of *lacZ* in Lac Operon

MEERA RAJ

Department of Microbiology and Immunology, UBC

***LacI* gene encodes for the repressor of Lac operon. An *Esherichia coli* (*E. coli*) strain with a *lacI* knockout would yield cells that can constitutively express ß-galactosidase. Use of these cells can considerably reduce the use on an inducer isopropyl-ß-D-1-thiogalactoside (IPTG) and reduce the induction time. Jaeger et al (2) prepared a presumptive *lacI* knockout in *E. coli* C29 strain using a λ Red recombination system. This knockout would, in theory, delete a large portion of gene that encodes 54- 348 amino acids from a total of 360 amino acids in a repressor. The presumptive deleted region of the *lacI* gene was replaced by a kanamycin resistant gene, which would confer the recombinant cells resistant to the antibiotic kanamycin. Interestingly, these cells were found to have low expression of ß-galactosidase, probably, due to partial repression of Lac operon. Also, it was found that when an inducer was added, these cells would show higher expression of ß-galactosidase. This study was carried out to determine why there was still repression in the expression of ß-galactosidase, whereas there should not be any repression at all. The multi-step approach used in this study was to PCR amplify the *lacI* gene from a WT *E. coli* C29 and the presumptive *lacI*$^-$ *E. coli* C29 cells, sequence the gene and confirm whether the deletional mutation took place in the presumptive *lacI*$^-$ *E. coli* C29 cells as specified. The sequencing results from this experiment showed that the deletional mutation did not take place as specified. The results suggest that the recombinant cells were kanamycin resistant either because recombination took place at some other location in the genome, or that the cells had acquired kanamycin resistance from some other source. They also indicate that the *lacI* gene may posses a point mutation that can cause the basal expression of *lacI* gene to vary between WT *E. coli* C29 cells and the kanamycin resistant *E. coli* C29 cells.**

Lac operon in *Esherichia coli* cells is an extensively studied gene model of regulation (2,3). It is comprised of three structural genes namely *lacZ, lacY,* and *lacA* (2,4). *LacZ* encodes for a ß-galactosidase, *lacY* encodes for a permease, and *lacA* encodes for a transacetylase (2,4). These three structural genes are coordinatively transcribed to metabolize lactose (2,4,7). *LacI* gene product, i.e. Lac repressor, in the absence of an inducer, binds tightly to the specific DNA sequence of an operator and prevents the transcription (2,4,7). In presence of an inducer, such as lactose or lactose analogues, the repressor is allosterically regulated and prevented from binding to a *lacO,* i.e an operator, to allow the transcription of these structural genes (2,4,7). However, two conditions are required for expression of Lac operon. One is the absence of glucose, and other is the presence of lactose (2,4,7).

The Lac repressor is a protein of 360 amino acids (4). To be functional, it forms a tetramer of 154,520 dalton molecular mass (4). Number of mutational studies carried out of *lacI* gene determines that the Lac repressor consists of several domains (3,4). Approximately 60 amino acids from amino terminal binds specifically to an operator; ~ 30 amino acids from a carboxyl terminal are responsible for tetramerization of a protein; and ~300 amino acids from a carboxyl terminal, which includes the tetramerization region, are responsible for binding to the inducer and its analogues, such as isopropyl-ß-D-1-thiogalactoside (IPTG). (3,4)

Since the Lac repressor acts as a regulatory molecular switch of a Lac operon, an *E. coli* strain with the *lacI* knockout could be made, which would constitutively express ß-galactosidase (2). Such a strain would not require an inducer, such as IPTG, for cell growth, and the steps for an incubation time necessary for sufficient production of ß-galactosidase would be eliminated. This would be beneficial for research purposes (2). An attempt to knockout the *lacI* gene in the *E. coli* C29 strain was made by Jaeger et al (2), which resulted in an *E. coli* C29 strain that partially repressed the Lac operon. The experiment involved a multi-step process that should have resulted in a linear DNA fragment, coding for the 10 N-terminal amino

acids (from position 43 to 53 N-terminal amino acids) of *lacI* gene, the antibiotic kanamycin resistance genes (kan^R), and 12 C-terminal amino acids (2). Thus, this sequence of a DNA should have contained a part of DNA binding domain, but lacked an inducer-binding domain for being allosterically regulated (2). The PCR product to induce the insertion was designed to have a flanking region homologous to the termini of the *lacI* gene that would facilitate recombination by the Red recombination system (2). The recombination should have resulted in deletion of nucleotides encoding for 54-347 amino acids from *lacI* gene replaced with kan^R (2, 3). Figure 1 shows the schematic diagram of the expected recombination event between WT $lacI^+$ *E .coli* C29 gene and the mutant $lacI^-$ *E. coli* C29 gene .

In theory, the *lacI* gene from $lacI^-$ *E.coli* C29 cells that were prepared would posses ~53 amino acids from a DNA binding domain, ~12 amino acids from tetramerization domain, and the remaining amino acids for kanamycin resistance (kan^R). The absence of an inducer-binding domain suggests that the $lacI^-$*E. coli* C29 cannot be induced if the residual DNA binding domain is active. However, ß-galactosidase assay of these $lacI^-$*E. coli* C29 cells showed an increased production of ß-galactosidase on addition of IPTG (2). Though we consider that there could be an experimental error that could explain the change in the basal level of ß-galactosidase activity, it would be difficult to explain the induction of ß-galactosidase by an inducer in the absence of an inducer-binding site. Interested as to whether a change in the *lacI* gene, which resulted in producing a functional repressor, this experiment was designed to test whether a deletional mutation had actually took place in presumptive $lacI^-$ *E. coli* C29 cells as theoretically suggested.

MATERIALS AND METHODS

DNA extraction. In this experiment, the total chromosomal DNA is isolated from an original $lacI^-$ *E. coli* C29 cells constructed by Jaeger et al (2) and were obtained from Microbiology and Immunology department of UBC. The wildtype *E. coli* C29 cells (*lac* I^+ *E. coli* C29) are also included in this experiment as a control. The *lacI* gene was then amplified by a PCR reaction. Two PCR primer sets were used to obtain two different PCR products. Primer set 1 was designed to obtain complete the $lacI^+$ gene from WT *E. coli* C29 cells and the $lacI^-$ gene from mutant *E.coli* C29 cells. Whereas, Primer set 2 that resembled the primers used to construct the original linear fragment of DNA containing a mutant *lacI* gene is used to amplify a *lacI* gene from a WT and a mutant *E. coli* C29 genome as well as to identify any possible non-specific amplification that may take place elsewhere in a genome with the homologous sequence of the *lacI* gene of duplicate *lacI* gene. Amplification of *lacI* region using primer set 1 and primer set 2 is schematically represented in figure 2. Thereafter, the PCR product obtained from primer set 2 is cloned into commercially available TOPO TA Cloning kit that facilitated sequencing.

The QIAGEN DNeasy™ Tissue Kit to extract DNA from both $lacI^+$ *E. coli* C29 cells and presumptive $lacI^-$ *E. coli* C29 cells was used to extract 0.5 µg of chromosomal DNA. For the protocol, please refer to the QIAGEN DNeasy™ Tissue Kit Protocol from a QIAGEN DNeasy™ Tissue Kit Handbook.

Primer designing. Two sets of primers were designed called primer set 1 and primer set 2. Each set comprised of a forward and a reverse primer. The sequence of the *lacI* gene was obtained from the bacterial genome sequence of an *E. coli* K12 strain (GenBank accession number NC_000913) from the NCBI genome database. Primers were designed using Primer designer software. For primer set 1, the sequence of the forward primer was :5'-AGAGAGTCAATTCAGGGTG-3', and the sequence of the reverse primer was: 5'-CCGCTCACAATTCCACACAAC-3'. The designed sequence was blasted against the *E. coli* K12 genome to confirm the specificity of primers.

Primer set 2 was designed using the original primers used in the paper by Jaeger et al (2). This team used primers that contained a part of *lacI* gene and a part of kan^R gene. The Kan^R region of those original primers were excluded to obtain the sequence of the forward primer (named, FWRM03): 5'-ATGGCGGACCTGAATTACATTC CCAACCGCGTGG CA-3', and the sequence of the reverse primer: (named, RVRM04) 5'-TCACTGCCCGCTTTCCAGTCGGGAAAC CTGTCGTGC-3'. The designed sequence was blasted against an *E. coli* K12 genome to confirm the specificity of primers. Two non-specific queries were observed against the reverse primer of primer set 2. However, both the queries resulted in no complementation/alignment of precisely 10 and 21 nucleotides that resulted in a hanging 3' end. This suggested that the amplification of the *lacI* gene would not occur through this non-specific binding due to the unavailability of the 3' –hydroxyl end for elongation. Also, the queries with 10 and 21 nucleotides overhangs were 250622 and 3959664 nucleotides upstream to the specific complementary primer sequence suggesting that PCR products would not form or interfere with the desired PCR reaction.

PCR amplification. The dried pellet of PCR primers were obtained from Nucleic Acid Protein Service Unit (NAPS Unit) (UBC, Canada) and were resuspended in the sterile distilled water. On a final volume of 50µl, the reaction mixture contained: 5 µl of 10x Buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of 1µM primers, 1 µl of 10 mM dNTPs, H_2O, 0.25 µl of 5U/ml Taq DNA polymerase (Invitrogen Life Technologies) and 0.5 µl (25- 80 ng of DNA) of template DNA from $lacI^-$ *E. coli* C29 cells and $lacI^+$ *E. coli* C29 cells. The reaction mixture was set-up as specified by the Invitrogen Life Technologies protocol. The PCR reaction conditions for primer set 1 were 180 sec at 95°C for the first cycle, followed by 40 cycles of 60 sec at 95°C, 60 sec at 55°C, and 60 sec at 72°C, followed by the a final extension cycle of 10 min at 72°C. The PCR reaction conditions for primer set 2 were 120 sec at 95°C for the first cycle, followed by 30 cycles of 45 sec at 95°C, 45 sec at 58.5°C, and 90 sec at 72°C, followed by a final extension cycle of 10 min at 72°C.

PCR products were resolved at 120 V by gel electrophoresis on a 1.5% agarose gel. Gels were then stained with ethidium bromide (EtBr) solution (1 mg/ml) for 15 min, and visualized under uv.

Cloning of *lacI* PCR products into a sequencing vector pCR2.1-TOPO®. The cloning used the Invitrogen Life Technology TOPO TA Cloning® (TOPO cloning) Kit containing pCR®2.1-TOPO® (pcR2.1) using chemically competent TOP10 cells. The TOP10 *E. coli* strain of cells facilitated blue/white screening. Two reaction mixtures containing 2 L and 4 L of PCR products were set up for each $lacI^+$ *E. coli* C29 genome and presumptive $lacI^-$ *E. coli* C29 genome using the Setting Up the TOPO® Cloning Reaction Protocol described in the kit (Invitrogen Life Technology).

The TOP10 competent cells were transformed using the Transforming One Shot® DH5™-$T1^R$, Top10, and Top10F' Competent cells protocol outlined in the TOPO TA Cloning® manual. To confirm the presence of the right insert in the cloning vector and to reduce the chances of false positive readings, restriction digestions were carried out using 1.5-2.0 µg of plasmids isolated from the white isolated colonies. *Eco*RI restriction endonuclease digestion of the plasmid constructs would yield ~1221 bp insert and 3913 bp of vector. However, in an attempt to distinguish the $lacI^+$ from the

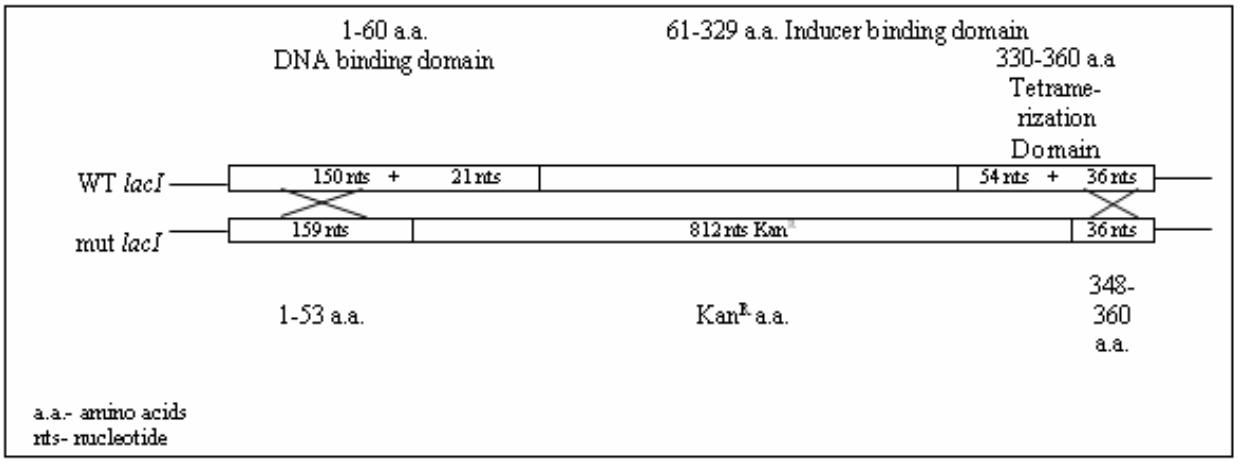

FIG. 1 A schematic diagram of recombination between the WT *lac I* gene and the mutant *lac I* gene.

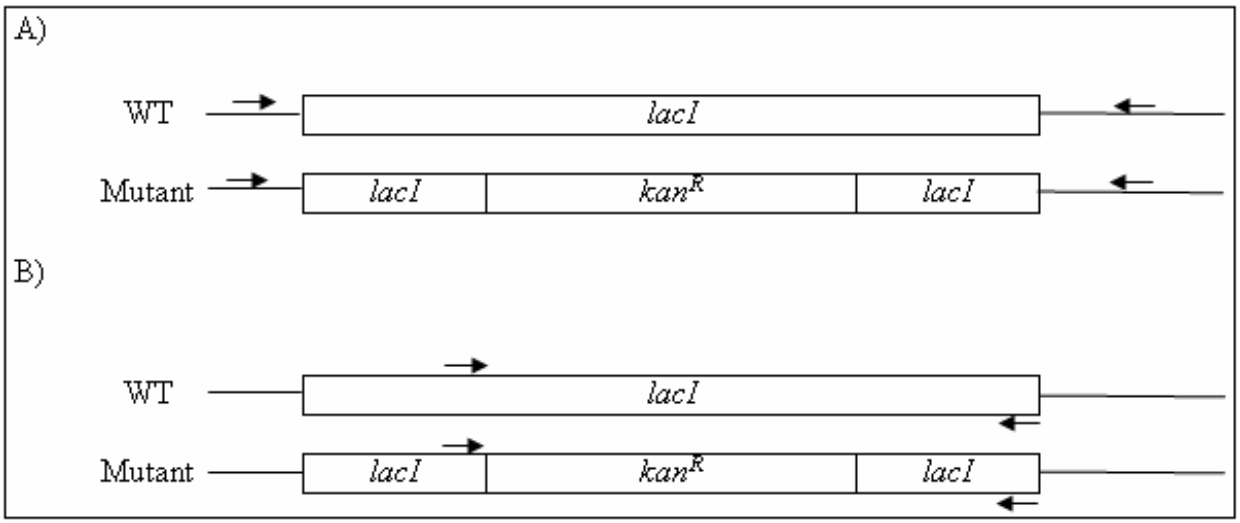

FIG. 2 Schematic representation of the PCR primers and the corresponding PCR amplification region. A) PCR amplification region using PCR primer set 1. B) PCR amplification region using PCR primer set 2.

presumptive *lacI*$^-$ gene, these constructs were also digested with *Hind*III and *Sma*I restriction enzyme that would cleave *kan*^{R} gene.

Mini preparation of a plasmid for the purpose of confirming successful cloning into the TOPO cloning vector. The QIAGEN Plasmid Mini Kit was used to obtain up to 9-20 µg of plasmid from 6 discrete white colonies of the *lacI*$^+$ containing pCR®2.1-TOPO® vector (*lacI*$^+$:pCR2.1) and the presumptive *lacI*$^-$ containing pCR®2.1-TOPO® vector (*lacI*$^-$:pCR2.1) using the Mini kit protocol. (Invitrogen Life Technology)

Medium preparation of an extra-purified plasmid for the purpose of sequencing. QIAGEN Plasmid Maxi Kit was used to obtain up to 500 µg, i.e a midipreparation, of the plasmid using the Maxi Protocol. (Invitrogen Life Technology)

Sequencing. The *lacI*$^+$:pCR2.1 vector and the *lacI*$^-$:pCR2.1 vectors that were isolated using the QIAGEN Plasmid Maxi Kit were sequenced by NAPS Unit with an Applied Biosystems PRISM 377 automated sequencer and Applied Biosystems BigDye™ v3.1. The forward primer M13R and the reverse primer T7 were used for the purpose of sequencing by terminator chemistry. Both the *lacI*$^+$ and the presumptive *lacI*$^-$ gene sequence were analyzed using MegaAlign software. (DNASTAR, Inc.)

RESULTS

DNA extraction and PCR amplification. A total of 5.67 – 8.09 µg of DNA from *lacI*$^+$ *E. coli* C29 and presumptive *lacI*$^-$ *E. coli* C29 cells was successfully isolated following PCR amplification of *lacI* gene primed with the primer set 1. Figure 3 shows the successful amplification of a ~1204 bp band corresponding to *lacI* gene with primer set 1. However, each lane shows the appearance of an approximately >2000 bp faint band corresponding to a non-specifically amplified PCR product.

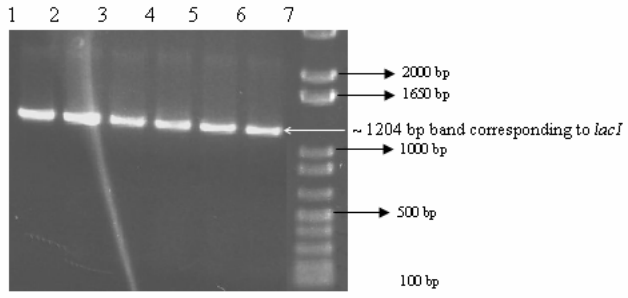

FIG. 3 Agarose gel electrophoresis (1.5% agarose, 0.2 µg/mL EtBr) of the amplified *lacI* genes. Bacterial genome was isolated from the presumptive *lacI*$^-$ *E. coli* C29 and the *lacI*$^+$ *E .coli* cells. The *lacI* genes were amplified using primer set 1 to obtain a PCR fragment of ~1204 bp band. Lane 1-3. Loaded 15 µl of PCR product from *lacI*$^+$ *E .coli* C29, Lane 4-6. Loaded 15 µl of PCR product from presumptive *lacI*$^-$ *E. coli* C29, and Lane 7. loaded 1.5 µg of ladder plus + 1x gel loading buffer.

Figure 4 shows the successful amplification of ~960 bp bands corresponding to *lacI* gene with primer set 2. Again, a faint band of approximately >2000 bp and <100 bp bands corresponding to a non-specifically amplified PCR product was observed.

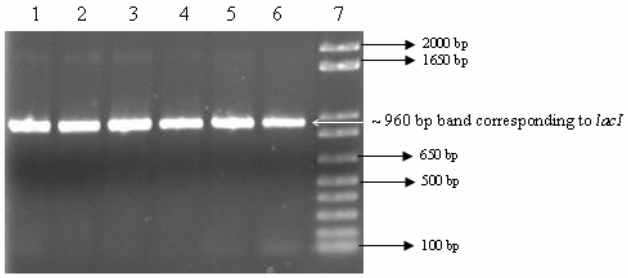

FIG. 4 Agarose gel electrophoresis (1.5% agarose, 0.2 µg/ml EtBr) of amplified *lacI* gene. Bacterial genome was isolated from *lacI* $^+$ *E. coli* C29 and *lacI*$^-$ *E. coli* C29 cells using QIAGEN DNeasy™ Tissue Kit using primer set 2. The *lacI* genes were amplified using primer set 2 to obtain a PCR fragment of ~ 960 bp band. Lane 1-3. Loaded 10 µl of PCR products from *lacI* $^+$ *E. coli* C29, Lane 4-6. Loaded 10 µl of PCR products from *lacI*$^-$ *E. coli* C29, and Lane 7. 1.5 µg of ladder plus + 1x gel loading buffer.

Cloning of *lacI* PCR products into a sequencing vector pCR2.1-TOPO® (pCR2.1). A total of 22 white colonies were successfully obtained that corresponded to bacteria containing *lacI* $^+$: pCR2.1. The *lacI* $^+$: pCR2.1 clones were than isolated from 6 discrete white colonies.

Similarly, a total of 9 white colonies were successfully obtained that corresponded to bacteria containing the presumptive *lacI*$^-$:pCR2.1. Presumptive *lacI*$^-$: pCR2.1 clones were then isolated from 6 discrete white colonies.

Mini preparation of a plasmid for the purpose of confirming successful cloning into the TOPO cloning. Successfully isolated a total of 2.96 – 7.83 µg of plasmid from minipreparations of a TOPO cloned vector. Approximately 1.5-2.0 µg of the minipreparations were double digested with *Eco*R1 +*Hind*III and *Eco*RI + *Sma*I. Figure 5 shows band corresponding to the expected 1221bp (insert), 3931bp (vector) and 5152bp (undigested vector) bands observed in lane 3, 6, 7, 12, 13 , 26 and 27 corresponding to partially digested *lacI*$^+$:pCR2.1 and *lacI*$^-$:pCR2.1. Lanes 5, 8, 9, 15, 20, 23, 24, 28, and 30 corresponds to the 3931bp pCR2.1 vector without an insert, suggestive of an unsuccessful cloning in those preparations. Lanes 11, 18, 19, 20, 21, 22, 24, 25, and 28 corresponds to undigested products comprising of concatemer, relaxed coiled, and supercoiled plasmid band.

Medium preparation of an extra-purified plasmid for the purpose of sequencing. Clones shown in figure 5, Lane 2 and Lane 29 corresponding to *lacI*$^+$:pCR2.1 and the presumptive *lacI*$^-$:pCR2.1 constructs respectively were grown and extracted to obtain a total of 2.47 µg and 37.35 µg of extra pure plasmids respectively. Lane 1 in figure 6 shows 3 bands, i.e. top band that corresponds to the relaxed plasmid; middle band that corresponds to the super coiled plasmid; and bottom band that corresponds to vector without an insert. Lane 2 in figure 6 also shows 3 bands, i.e. top band corresponds to concatemer; middle band corresponds to relaxed plasmid; and the bottom band corresponds to supercoiled plasmid.

Sequencing. The *lacI* sequence from *lacI*$^+$ *E. coli* C29 cells showed a sequence of 1030 nucleotides using a reverse primer T7, and showed a sequence of 125 nucleotides from the plasmid vector pCR2.1 without an insert using a forward primer M13R. The *lacI* sequencing from the presumptive *lacI*$^-$ *E. coli* C29 showed a sequence of 1036 nucleotides using the primers T7, and yielded 1030 nucleotides using the primer M13R.

A total of 1030 nucleotide sequence of the *lacI* gene from the *lacI*$^+$ *E. coli* C29 using the sequencing primer T7, a precise sequence of 699 nucleotides was found to be identical to the K12 *E. coli lacI* gene sequence, whereas the rest of the sequence flanking this region corresponded to PCR primers and regions from the *lacI* gene that could not be resolved due to the limitation of sequencing. A total of 1036 nucleotide sequence of the *lacI* gene from the presumptive *lacI*$^-$ *E. coli* C29 using the T7 primer yielded a 703 nucleotides long sequence that was identical to the K12 *E. coli lacI* gene sequence, whereas the rest of the sequence flanking this region corresponded to primers and regions from the *lacI* genome that could not be resolved due to the

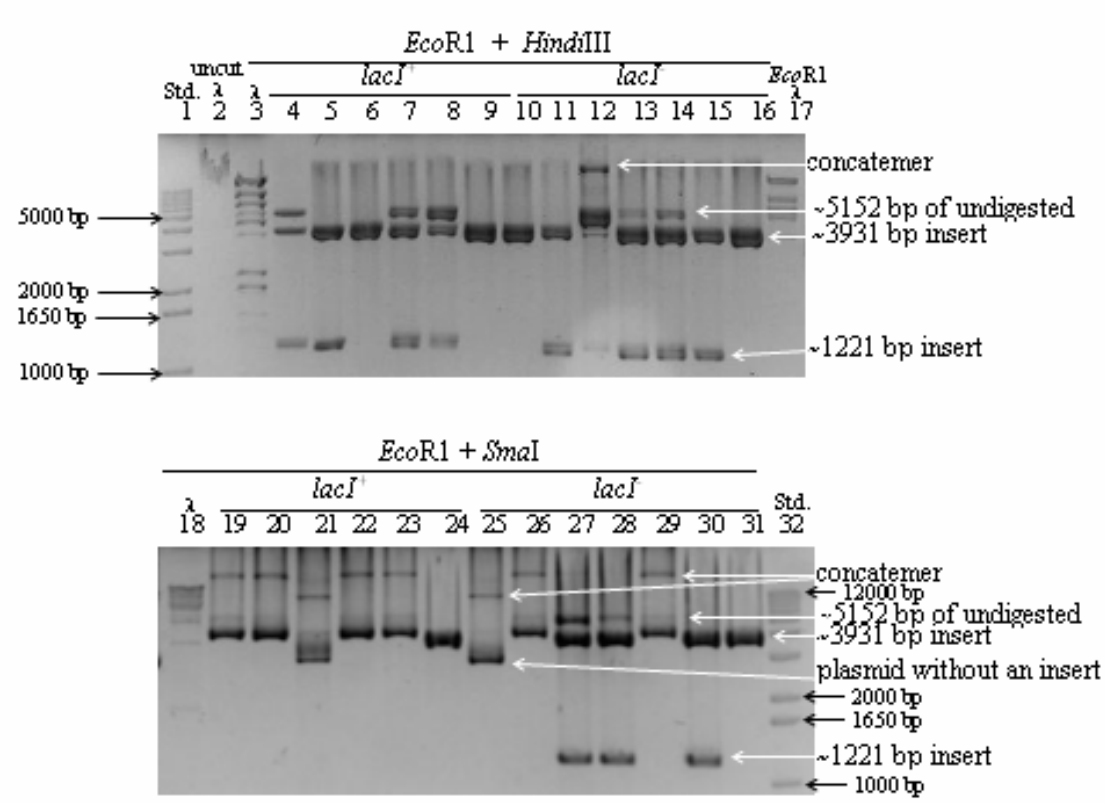

FIG. 5 Agarose gel electrophoresis of restriction enzyme digested *lacI*$^+$:pCR2.1 and presumptive *lacI*$^-$:pCR2.1. A total of 1.5-2.0 µg minipreparations of the plasmid construct were isolated and double digested with *Eco*R1+ *Hind*III and *Eco*R1 + *Sma*I. The resulting products were than resolved on 1.5% agarose, and the gel was stained with 0.2 µg/ml EtBr solution. Lane 2. Uncut lambda DNA, Lane 3. *Hind*III digested lambda DNA, Lane 4-9. *Eco*RI + *Hind*III digested *lacI*$^+$:pCR2.1 isolated from six topo cloned colonies obtained from PCR products, Lane 10-16. *Eco*RI +*Hind*III digested *lacI*$^-$:pCR2.1isolated from seven topo cloned colonies obtained from PCR products, Lane 17. *Eco*RI digested lambda DNA, Lane 18. *Sma*I digested Lane 19-24. *Eco*RI + *Sma*I digested *lacI*$^+$:pCR2.1 isolated from six topo cloned colonies obtained from PCR products, and Lane 25-31. *Eco*RI +*Hind*III cut presumptive *lacI*$^-$:pCR2.1 isolated from seven topo cloned colonies obtained from PCR products.

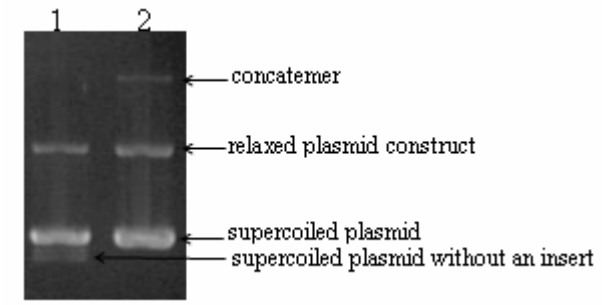

FIG. 6 Agarose gel electrophoresis of midipreparation. The midipreparation of the selected minipreparation using QIAGEN Plasmid Maxi Kit was carried out and, 1 µl of construct were resolved on 1.0% agarose gel and the gel was stained with 0.2 µg/ml EtBr solution. Lane 1. plasmid preparation from the *lacI*$^+$ *E. coli* C29 cells, and Lane 2. plasmid preparation from presumptive *lacI*$^-$ *E. coli* C29 cells.

sequencing limitations. Similarly, the *lacI* sequence from the presumptive *lacI*$^-$ *E. coli* C29 using the T7 primer yielded a 633 nucleotides long sequence that was identical to the K12 *E. coli lacI* gene sequence, except for position 470 that showed a point mutation to nucleotide C as appose to nucleotide A at position 470, whereas the rest of the sequence flanking this region corresponded to PCR primers and regions from the *lacI* gene that could not be resolved due to the sequencing limitation. The two sequences when combined yielded a complete the *lacI* gene of 1083 nucleotides.

DISCUSSION

The *lacI* sequences amplified using the PCR amplification technique with the primer set 1 are expected to yield a band size of precisely 1204 bp (25 nucleotides upstream of the *lacI* gene including the forward primer + 1083 nucleotides of the *lacI* gene + 96 nucleotides downstream of the *lacI* gene including the reverse primer) from *lacI*$^+$ *E. coli* C29 cells, and band sizes of precisely 1092 bp (25 nucleotides upstream of the *lacI* gene including the forward primer +159 nucleotides coding for 53 amino acids from NH_2-terminal of the Lac repressor + 812 nucleotides of kanamycin resistant gene+ 96 nucleotides downstream

of the *lacI* gene including the reverse primer) from presumptive *lacI*$^-$ *E. coli* C29 cells. A difference of 112 bp should possibly exist in the PCR products obtained from the *lacI*$^+$ *E. coli* C29 and presumptive *lacI*$^-$ *E. coli* C29 cells. However, this difference is not observed in figure 3, which shows ~ 1204 bp band in all the lanes. This suggests that either the difference cannot be distinguished by resolving the products on traditional 1.5% agarose gel, or that the suggested deletion of nucleotides encoding for the amino acids at position 54- 347 did not occur. Additionally, the presence of faint bands of approximately >2000 bp corresponding to a non-specifically amplified PCR product shows that the primers are binding to a different region of the bacterial genome. This suggests that the recombination could occur at some other locations in the genome. Also, the possibility of having a second copy of the *lacI* gene was not revealed on this gel. However, none of the six clones showed a pattern consistent with a kanamycin insertion when they were digested with restriction endonucleases. So, an insertion into a duplicated *lacI* gene is unlikely.

Similarly, the *lacI* sequences amplified using PCR amplification techniques with the primer set 2 were carried out to observe if these primers, which were used for homologous pairing during recombination event within C29 cells, would bind in a non-specific manner to amplify any homologous gene elsewhere in the genome suggesting that the recombination event could take place elsewhere in the genome. The expected band size of the *lacI* gene using the primer set 2 is 906 bp (1083 bp of *lacI* gene including the forward and the reverse primer -123 bp encoding for of first 41 amino acids excluding the forward primer) from *lacI*$^+$ *E. coli* C29 cells, and 884 bp (812 bp of kanamycin gene + 36 bp forward primer + 36 bp reverse primer) from presumptive *lacI*$^-$ *E. coli* C29 cells. Again, a difference of 96 bp should possibly exist in the PCR products obtained from *lacI*$^+$ *E. coli* C29 and presumptive *lacI*$^-$ *E. coli* C29 cells. The prominent bands of ~906 bp were observed in each lane, and any changes in the band sizes were not visually observed on this gel suggesting that either there is no difference in the length of the *lacI* gene between *lacI*$^+$ *E. coli* C29 cells and presumptive *lacI*$^-$ *E. coli* C29 cells implying that deletion mutation did not take place or that recombination could have occurred at some other location in the genome. This is supported by the presence of faint bands of approximately >2000 bp in each lane due to the non-specific amplification by PCR reaction. Though, the possibility of having a second copy of *lacI* gene was not evident on this gel, the absence of another copy cannot be concluded, since the WT and mutant *lacI* gene are indistinguishable without digestion with specific restriction endonucleases.

In an attempt to distinguish the PCR products of *lacI*$^+$ *E. coli* C29 and the presumptive *lacI*$^-$ *E. coli* C29, the PCR products were cloned into TOPO cloning vector, and were double digested using restriction enzymes *Eco*RI and *Hind*III. The expected size of the *lacI* gene in *lacI*$^-$ *E. coli* C29 is 1109 bp (i.e. 1092 bp of the *lacI*$^-$ *E. coli* C29 PCR product + 10 and 7 bp of nucleotides flanking the PCR product), which would further be digested by *Hind*III at position 551 in the kanamycin region of the deleted *lacI* gene to yield a DNA fragment of ~ 742-745 bp and ~364-367 bp. The variability in the two fragment sizes can be accounted for by the direction in which the PCR products get inserted into the vector. Similarly, *Sma*I would digest at position 307 of the kanamycin region present in deleted *lacI* gene to yield a DNA fragment of ~ 552- 555 and ~554-557 bp. Since, the PCR products from *lacI*$^+$ *E. coli* C29 cells would not carry the sequence for kanamycin resistant in its *lacI* gene, the *Eco*RI digestion of the *lacI*$^+$:pCR2.1 construct would yield the *lacI* gene of 1221 bp (i.e.1204 bp PCR product from the *lacI*$^+$ *E. coli* C29 genome + 10 and 7 bp of nucleotides flanking the PCR product). Also, the presence of the kanamycin gene in the vector itself would further digest the 3931 bp pCR2.1 into three fragments of sizes 49 bp, 1995 bp and 1870 bp using *Hind*III, and two fragments of sizes 1995 and 1919 bp using *Sma*I. The presence of the band size of 1221 bp in lanes of the both the *lacI*$^+$ *E. coli* C29 and the presumptive *lacI*$^-$ *E. coli* C29 in figure. 5 suggested the *Eco*RI restriction digestion was achieved, which shows that the cloning was successful. However, further digestion with *Hind*III and *Sma*I was not observed suggesting that the Automated Restrictionmapper site used to determine restriction sites on kanamycin region was inaccurate. The partially digested lanes in figure 5 can be explained by the poor activity of the restriction enzymes, but lanes 2, 16 and 17 suggests that all the three enzymes were functional. Again, the *lacI* gene from the *lacI*$^+$ *E. coli* C29 and the presumptive *lacI*$^-$ *E. coli* C29 was indistinguishable using restriction enzymes; therefore, the sequencing was carried out.

The nucleotide sequences from the WT *lacI*$^+$ *E. coli* C29 and the presumptive the *lacI*$^-$ *E. coli* C29 were identical to the nucleotide sequence of *E.coli* K 12 strain. The results confirm that the expected deletion mutation of nucleotides encoding for the amino acids at location 54- 347 amino acids did not take place as suggested. However, it can be suggested that the difference in the basal level activity of the □-gal due to the inefficient repression and expression of *lacI* gene followed by induction might be accounted for by three possible explanations.

First, the C29 cells may have randomly acquired kanamycin resistance due to either intergenic or

intragenic suppression; or due to acquisition of Kan^R by some other mechanism. For example, change in cell wall properties of the cells that prevent the uptake of antibiotic kanamycin; or the acquisition of kan^R gene from some other source.

Second, if the change of nucleotides from C to A at location 470 observed in *lacI*$^-$ *E. coli* C29 sequence is not a sequencing error, than the gene in the presumptive *lacI*$^-$ strain would translate into a variant repressor protein with N159T mutation. This change from charged asparagines to uncharged threonine at the 159th location, i.e. within the inducer binding domain, may affect the structure of the protein resulting in the inefficient binding of the repressor to the operator of the Lac operon. However, the effect may be neutralized by binding of the inducer to this region and allowing normal induction *lacZ* gene.

Third, recombination may have taken place at different location within the genome. The λ Red recombination system used to prepare *lacI*$^-$ *E. coli* C29 cells has been used for PCR mediated deletion of exonuclease in *E. coli* and *Salmonella* (5,6). The system encodes for three major proteins namely λ protein, ß protein, and γ protein (5,6). The λ protein encodes for the enzyme exonuclease that digests the 5' ends of the dsDNA in the host's chromosome (5,6). The ß protein binds to the bacterial RecBCD and prevents the degradation of the linear piece of DNA introduced into the host cell (5,6). The γ protein binds to the ssDNA facilitating annealing during the recombination event (5,6). Additionally, efficient recombination requires optimization of the system for the particular strain and for the specific gene targeted (5,6). It has been shown that the λ Red recombination system used to delete five O157-specific islands of *E. coli* strain EHEC and EPEC occurred easily with high efficiency, however deletion mutation in *lacZ* gene within *E. coli* K12 occurred poorly (5,6). Non-specific and poor efficiency of recombination that takes place within the cells can be accounted for by possibly three main reasons: First is the non-specific pairing of the homologous region of dsDNA at growth temperature, which is different from the PCR annealing temperature of ssDNA, thus allowing non-specific recombination which may not be detected by the PCR; Second is the intact and coiled bacterial genome, that might render the target gene inaccessible to the recombination system; third is the excess linear DNA introduced into the cell that might get circularized and get inserted next to the WT gene through a single recombination event via one of the homologous region as a single recombination event is more likely to occur than a double recombination event. Such recombination may result in expression of both WT and variant *lacI* protein. The expression of variant protein may also interfere with the dimerization or tetramerization of Lac repressor, which may affect the DNA binding properties of the repressor.

FUTURE EXPERIMENTS

Absence of the deletion mutation in *lacI* gene can be accounted for by an inefficient recombination system. To obtain *lacI* knockout of *E. coli* C29 cells using λ Red recombination system, optimization of this system to suite *E. coli* C29 strain to target *lacI* gene is particularly required. It might be helpful to induce the lac operon in the C29 cells prior to the mutagenesis so that the gene sequence is likely exposed. Alternatively, the location of the kanamycin resistance in the cells could be mapped to determine if it is near the *lacI* gene. Also, an alternative approach to recombination may be used to obtain *lacI* knockout of *E. coli* C29 cells.

ACKNOWLEDGMENTS

This research project was supported by Microbiology and Immunology department at UBC to bring out research ability and skills, and to expand research background through MICB 447 course. Special thanks to William Ramey and Jen Sibley for their valuable comments, advice, and directional guidance in this project.

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