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Cloning the Potential Insertion Site of a Promoterless Kanamycin Resistance Cassette Recombined by the Lambda-Red Recombinase System

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A previous study by Jaeger *et al.* attempted to disrupt the *lac*I gene by using the lambda red recombinase system to insert a promoterless kanamycin resistance (Kan^R) cassette into the gene. Instead of the expected deletion of the *lac*I gene, subsequent study found that the insert actually occurred outside *lac*I. Our study started to map the insertion site of the Kan^R cassette by constructing a genomic library of the mutated *E.coli* C29 cells using the pUC19 vector. To screen for relevant clones which contain the Kan^R cassette, a double digest using two restriction enzymes which cut within the Kan^R gene was performed. Clones that showed the expected 400 bp fragment were screened for kanamycin resistance. Our study have identified 5 clones which potentially have pUC19 carrying a Kan^R cassette flanked by the insertion site.

The lambda-red recombinase system has been touted as an efficient method for the one-step inactivation of specific chromosomal genes of up to 82 kb (2). This technology involves the activation of three Lambda-red recombinase genes which mediates the recombination of any two DNA sequences with sequence homology of 36-50 nucleotides (1). Therefore, if a linear DNA fragment carrying a selective marker flanked by sequences homologous to the gene of interest is present, the selective marker of this fragment will be inserted into the host DNA and the gene of interest will be knocked out.

A previous study (3) attempted to knock out the *lac*I gene in a C29 strain of *E. coli* with the kanamycin resistance (Kan^R) cassette using the lambda-red recombinase system (3). However, they found that their kanamycin resistant C29 cells did not show the expected phenotype (i.e. there was *lacZ* expression in the presence and absence of IPTG) (3). Jaegar et al. then discovered that they had erroneously designed the primers used to clone the Kan^R; therefore, the sequences flanking the Kan^R cassette targeted a region within the *lac*I gene rather than the start of the *lac*I coding sequence. (3) This observation led Jaegar et al. to offer several explanations based on assumption that the Kan^R cassette was inserted into the lacI gene. However, a subsequent study showed that the point of insertion of the Kan^R cassette was not actually in the *lacI* gene. (5)

Our study starts to locate the insertion site of the Kan^R cassette in the resistant *E. coli* C29 isolated by Jaeger et. Chromosomal DNA of these mutant cells was first digested and cloned into pUC19. The recombinant plasmids were transformed into *E. coli* DH5α and selected for in a red-white screen. To further screen clones for the Kan^R cassette, a double digest was performed on the isolated clones by using two restriction enzymes that each cut once within the Kan^R cassette. To further test the potential presence of the Kan^R gene, the clones were tested for kanamycin resistance. It is hoped that by mapping out the insertion site of the Kan^R cassette, this study will help explain the phenotype of the mutant cells obtained by Jaeger et al.(3), and assess the efficacy of the lambda-red recombinase system for gene inactivation in *E. coli*.

MATERIALS AND METHODS

Strains *E.coli* JSST1 is the kanamycin resistant *E.coli* C29 isolated by Jaeger *et.al* (3). *E.coli* DH5α was used for cloning (6).

Plasmids. Plasmid pUC19 (2686 bp) contains *ori*, *lacZ* and *bla* (confers ampicillin resistance). Extracted plasmids were provided by the MICB 421 laboratory.

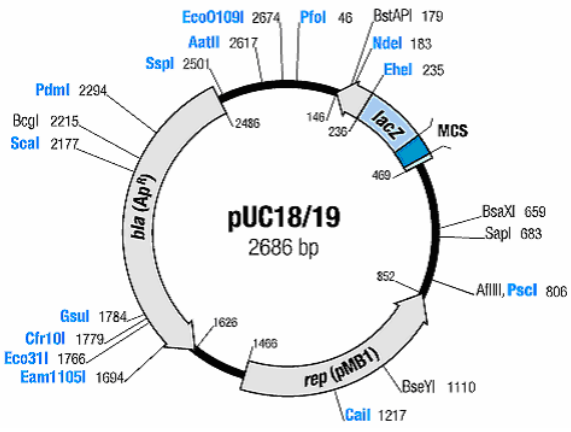

FIG 1. Restriction Digest map of plasmid pUC19

Media. Broth cultures of *E. coli* JSST1 cells (obtained from MICB 421 laboratory stock collection) were grown at 37 °C with shaking in Luria Broth (pH 6.9); (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 15% NaOH). The 2-YT media used for heat shock transformation composed of 16 g tryptone, 10 g yeast extract and 5 g NaCl in 1 L of H_2O. The MacConkey-Amp plate used to

select for transformed *E. coli* DH5α contained 17g of peptone, 3g of protease peptone, l0.0 g lactose, 1.5g Bile salt No. 3, 5.0 g NaCl, 13.0 g agar, 0.3 g neutral red, 0.001 g Crystal violet, and 100 ug/mL ampicillin in 1 L of H_2O.

Growth. *E. coli* JSST1 cells were grown in LB containing 50 ug/mL kanamycin. $CaCl_2$ chemically competent DH5α cells (obtained from Micb 421 laboratory stock collection) transformed with the pUC19:insert were grown in 2-YT and plated onto MacConkey plates containing 100 ug/mL ampicillin. All broth cultures were incubated overnight at 37° C with aeration.

Genomic DNA extraction. Genomic DNA E. Coli JSST1 was extracted using QIAmp DNA Mini Kit (Qiagen. Catalog No. 51304) according to the manufacturer's instructions with the following modifications. When the QIAmp DNA Mini Kit with bacterial DNA extraction was used, broth cultures of the cells were centrifuged at 14, 000 rpm for 2 minutes. The pellet was resuspended in 200 uL of buffer AL. Then 180 µl of buffer ATL was added and then incubated at 56°C for 10 minutes with occasional vortexing until the pellet was completely lysed. The mixture was then combined with 200 µl of absolute ethanol and mixed by pulse-vortexing for 15 s. The mixture was applied to the QIAamp spin column, which holds a silica gel membrane, and spun for 1 min at 8000 rpm. The spin column was washed with 500 uL buffer AW1 and buffer AW2 sequentially by centrifugation at 8000 rpm for 1 min and at 14000 rpm for 3 min, respectively. The DNA was eluted by centrifugation with 50 µl of buffer AE after a 5 min incubation at room temperature. The extracted DNA was assessed for purity and concentration based on the A_{260nm}/A_{280nm} readings and stored at −20°C until further use.

Plasmid DNA extraction Plasmid DNA was isolated by standard alkaline lysis method (6). TENS (0.1N NaOH, 0.5% SDS, 10mM Tris-HCl pH 8.0, 1 mM) was added, followed by sodium acetate (pH 5.2). After collecting the supernatant, ethanol precipitation was done at -20°C for 1 hour.

Restriction Digest. Digestions were performed according to the manufacturer's instructions. Both the extracted genomic DNA and pUC19 plasmids were digested using the restriction endonuclease BamH1 (Invitrogen. Catalog No. 15201). The BamH1 restriction digest mixture consisted of 0.1 to 0.4 ug of DNA, 5 uL of 10X React 3 buffer, 1 unit of BamH1 per ug of DNA and autoclaved H_2O up to 50 uL. Overnight digests were performed at 37°C. Plasmid DNA from white colonies was digested with the restriction endonucleases DdeI and XhoI (Invitrogen. Catalog No. 15238 and 15231). The restriction digest mixture consisted of 7-9 ug of DNA, 1ul of 10x React 2 Buffer, 1 unit each of XhoI and DdeI and autoclaved H_2O up to 10ul. Successful digestions were visualized on a 1.2% agarose gel that was electrophoresed at 100V for 40 minutes. Digestions were visualized using a transilluminator after staining the gel in an ethidium bromide bath (0.2ug/ml) for 15 minutes.

Phenol/Ethanol Purification. Purification was performed based on the protocols from Sambrook (6). An equal volume of phenol/chloroform was added to the digested DNA and centrifuged at 14,000 rpm for 15 seconds. The top aqueous layer was collected and the extraction was repeated. After 1/10 volume of sodium acetate (pH 5.2) was added, ethanol precipitation was performed for 15 minutes in dry ice by adding 2.5 volume of cold 100% ethanol. Following centrifugation at 14,000 rpm for 5 minutes, pellet was washed with 70% ethanol and resuspended in 10 ul of TE (pH 8).

Ligation. Ligation of digested genomic fragments and pUC19 was performed using T4 DNA ligase (Invitrogen. Catalog No. 15224) according to manufacturer's instructions. Ligation ratios (pUC19 vector:genomic DNA) of 1:2, 1:3 and 1:6 were tested. A ligation reaction consisted of 4 uL of 5X ligase buffer, 45 fmol pUC19 vector, 90-270 fmol genomic DNA, 0.1 units of T4 DNA ligase and autoclaved H_2O up to 20 uL. Two controls were included. One control consisted of digested pUC19 vector only. The other control consisted of Hind III digested lambda DNA fragments (Invitrogen. Catalog No. 15612). Overnight ligations were performed at 22°C in a thermocycler. Successful ligations were visualized on a 1.2% agarose gel that was electrophoresed at 100V for 40 minutes. Ligations were visualized using a transilluminator after staining the gel in an ethidium bromide bath (0.2ug/ml) for 15 minutes.

Heat shock transformation. Fifty microlitres of chemically-induced *E.coli* DH5α was transformed with 3 ul of ligated pUC19 through heat shock transformation (6). The mixture of DNA and DH5α was heat shocked in a 42°C waterbath for 90 seconds, followed by incubation at 37°C with aeration for 1 hour. Transformed cells were plated and selected on MacConkey plate with 100 ug/mL ampicillin.

Pooling. White colonies were transferred to both a master MacConkey agar plate and a LB broth, each containing 100 ug/mL ampicillin. These plates were incubated at 37°C overnight and white colonies were selected on the next day. To expedite the screening of white colonies, 5 recombinant colonies were pooled and grown together in LB-Amp broth. The alkaline lysis method, as described above, was then used to extract the plasmids from these mixed cultures.

RESULTS

Plasmid pUC19 was singly digested with BamH1. This digestion yielded a single band of size 2.7 kb, which is the expected size of the linearized plasmid.

Chromosome of the kanamycin resistant *E. coli* JSST1 from Jaegar et al.'s study was digested with BamH1, a six-cutter enzyme which would give fragments with an average size of approximately 4.1 kb. These fragments were then ligated into the BamH1-digested pUC19 for either 2 hours or overnight. Following heat shock transformation into *E. coli* DH5a competent cells, transformed cells were plated on MacConkey-Amp plates. These plates contain lactose and neutral red, which turns red in acidic conditions (i.e. lactose fermentation) (4). Only successful transformants that contained pUC19 with an insert would appear white on these plates because they do not have a functional *lacZ* due to fragment insertion into the BamH1 multiple cloning site within the *lacZ* gene. Adequate growth was observed on all plates. As expected, *E. coli* DH5a cells transformed with either the undigested or re-circularized pUC19 yielded only pink colonies. The overnight ligation yielded double the number of successful ligation transformants compared to the ligation performed for 2 hours. In addition, ligation ratios (pUC19 vector:

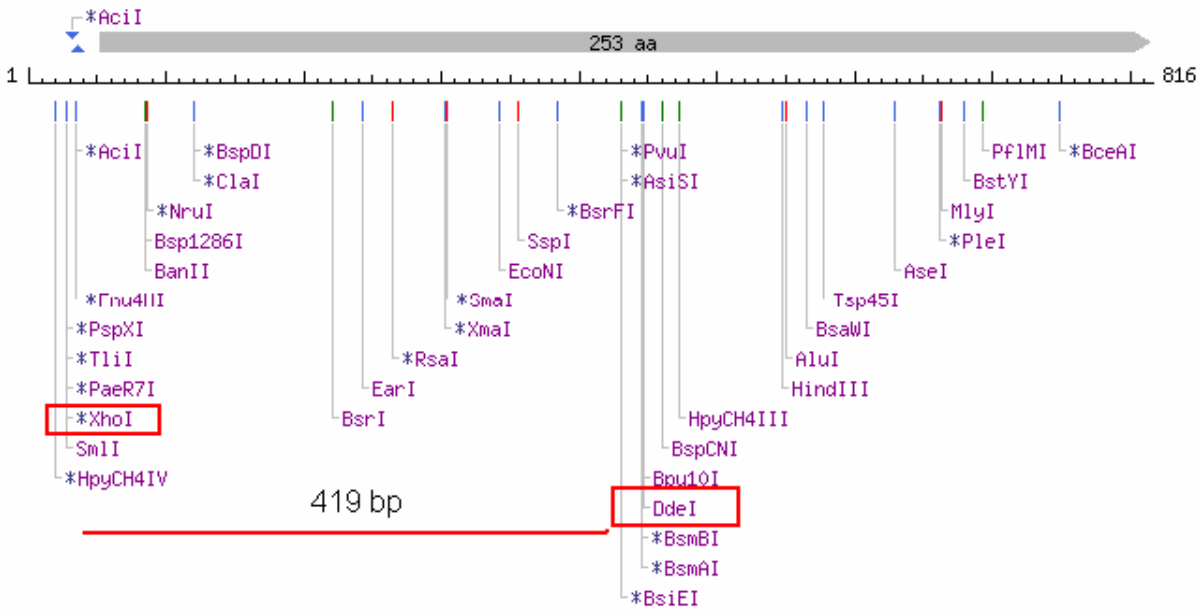

FIG. 2 Diagram showing the expected size of the digestion fragment when the kanamycin resistance cassette is cut with XhoI and DdeI. Figure generated by NEB cutter program at www.neb.com

genomic DNA fragments) of 1:3 and 1:6 yielded double the number of Amp^R white colonies compared to the l:2 ligation ratio.

Each individual Amp^R white colony was streaked on MacConkey-Amp master plates to help keep track of the identity of each colony. These same colonies were also pooled and grown in LB-Amp broth overnight for mini-prep plasmid isolation the next day. Interestingly, not all the colonies on the master plate were white, as initially seen in the transformation plates (Table 1). This was most likely due to cross-contamination from neighbouring red colonies when transferring the white colonies on the master plate.

Restriction digest was used for further screening of the Amp^R white colonies. The plasmid DNA from pooled samples was digested with XhoI and DdeI, both which cut within the Kan^R cassette. This should yield a fragment of approximately 400 bp (Fig.2), leaving a fragment greater than 2.7 kb which corresponds to the pUC19 plasmid and residual genomic DNA from the insert. White colonies that contained the 400 bp band in their digest were then subjected to a final screen on LB-Kan plates. Colonies grown on these plates should contain the Kan^R cassette because the untransformed the *E.coli* DH5a cells are kanamycin sensitive.

Gel electrophoresis of the digest showed that 11 pooled samples yielded the expected 400 bp band; moreover, 10 of these samples also had a 500 bp band (Fig.3). Of these 10 pooled samples that showed both bands, only 6 contained a fragment greater than 2.7kb, the expected band size of linear pUC19 (Table1). Out of the 67 clones that were streaked, 23 grew on LB-Kan plates (Table 1). Five clones met all the selection criteria, as described in the discussion below.

DISCUSSION

Five out of 204 colonies (clones X1, X6, X44, X46, X50 based on Table 1) were selected as potential clones that contained a plasmid with the genomic fragment containing the Kan^R cassette. Selection was based on satisfaction of all of the following criteria (i) contained the pUC19 with an insert because they appeared white on the MacConkey-Amp plates; (ii) contained a fragment of about 400 bp, the expected size of the kanamycin fragment digested by both Xho1 and Dde1; (iii) contained a fragment of size greater than 2.7 kb, the expected size of the linearized pUC19; (iv) grew on LB-Kan plates.

Some white colonies that contained a 400 bp fragment did not show a fragment size of equal or greater than the 2.7 kb pUC19 fragment. Instead, digests of these colonies contained fragments of size smaller than 2.7 kb. This observation suggests the presence of nucleases in the plasmid sample due to insufficient purification during the alkaline lysis plasmid isolation. Another possible explanation is that DdeI and/or XhoI might exhibit star activity, leading to cleavage of similar but not identical restriction sites. Star activity is most evident if there is an excess of restriction enzyme or suboptimal digestion conditions. There is also the remote possibility that the pUC19

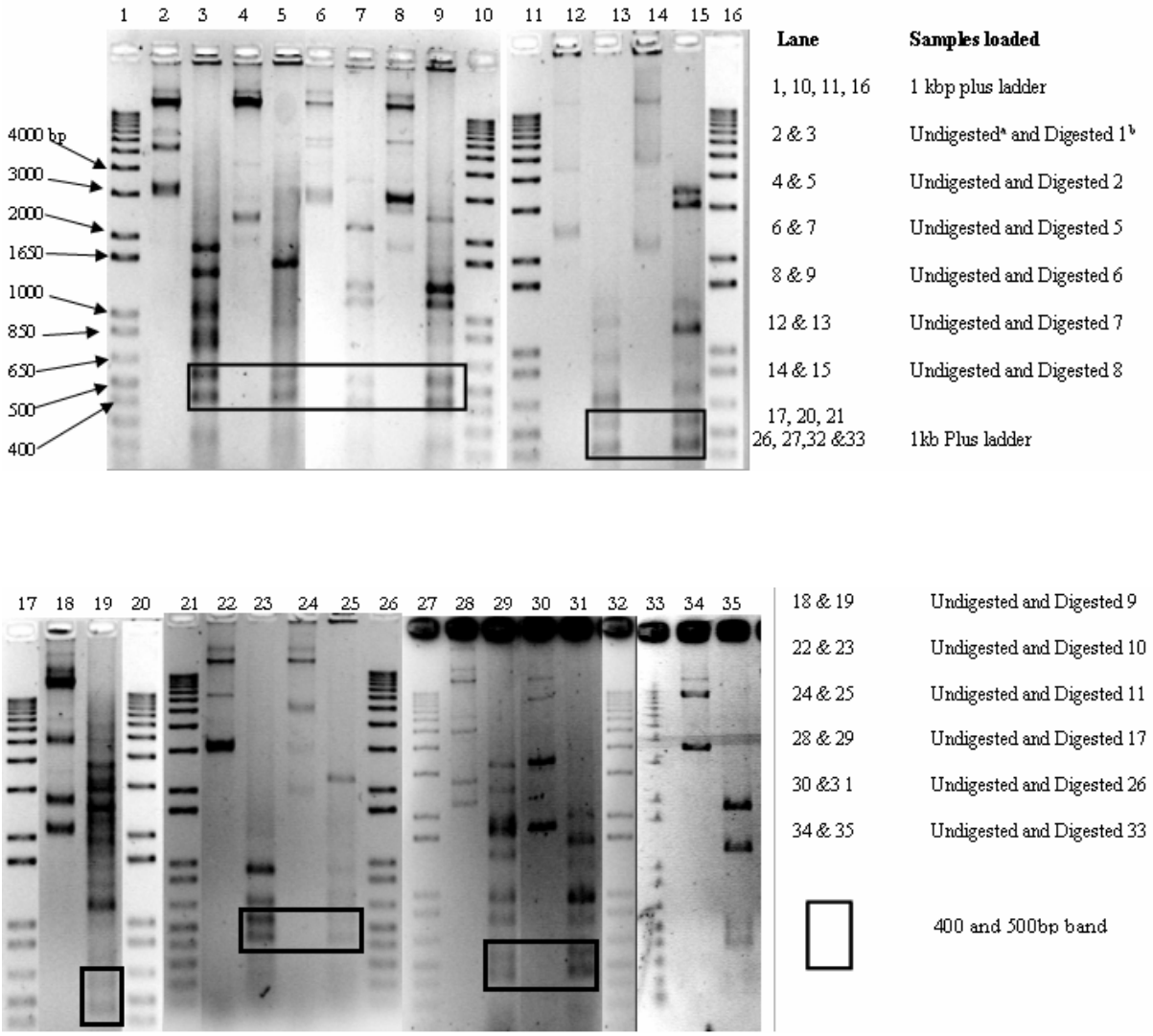

FIG. 3 Plasmid DNA from pooled white colonies digested with XhoI and DdeI, electrophoresed on 1.2% agarose gel. [a] The term undigested refers to the plasmid DNA isolated from white colonies, which were not digested with XhoI and DdeI. [b] The number for each digest corresponds to the pooled sample number in Table 1.

used in these experiments may actually have mutations that created DdeI and/or XhoI cut sites.

Most colonies from the pooled samples that showed bands of 400 bp and 500 bp also grew on the LB-Kan plates. Conversely, those that did not contain both fragments did not grow on the LB-Kan plates. This correlation suggests that the 500 bp fragment is required for kanamycin resistance. An explanation for this observation is that this 500 bp fragment is a segment of the genomic DNA adjacent to the Kan^R cassette. This fragment may contain an endogenous promoter that drives the expression of kanamycin resistance. Hence this 500 bp fragment might possibly be the site where the Kan^R gene originally inserted into during mutation using the lambda red recombinase system in the study by Jaeger et al. (3)

Surprisingly, 23 out of 204 colonies (11%) screened were kanamycin resistant. This relatively high frequency is unexpected because genomic digest of the *E. coli* JSST1 with BamHI would potentially yield approximately 1500 different fragments; only one of which would contain the Kan^R cassette. Hence statistically, 1500 clones would need to be screened in order to yield the clone of interest (i.e. containing the Kan^R cassette). A possible explanation for this high frequency could be that the original untransformed wildtype *E. coli* C29 strain used by Jaeger et al (3) contained an unknown plasmid. If this plasmid contained sequences homologous to the Kan^R gene cassette used in the lambda red recombinase system, the gene cassette could have recombined in-frame into the plasmid and be expressed using an upstream promoter in this plasmid. If this plasmid has a high copy number and was extracted together with the

TABLE 1. Summary of characteristics of transformants from pooled samples that showed the expected 400 bp Dde1-Xho1 restriction fragment.

Pooled sample[a]	White on MacConkey-Amp Master Plates	Band(s) greater than 2.7 kb[b]	Kanamycin Resistant Colonies
1 (D1-5)	D1, D2, D5	No	D3
2 (D6-10)	D7, D8	No	None
5 (D21-25)	D21, D22, D24	Yes	D25
6 (D26-30)	None	Yes	D26
7 (D31-35)	D32, D35	No	D34
8 (X1-5)	X1-5	Yes	X1
9 (X6-10)	X6-10	Yes	X6
10 (X11-15)	X11-15	No	X12-X15
11 (X16-20)	X16, X17, X18, X19	No	X17
13 (X26-30)	X26	No	X30
16 (X41-45)	X44, X45	Yes	X41,X44
17 (X46-50)	X46-X50	Yes	None
26 (Y42-45)	None	No	Y44
33 (A41)	A41	No	None

[a] The number in the bracket represents the numerical designations of individual white colonies that were included in the pooled samples of five colonies.

[b] A 2.7 kb band corresponds to the band size expected for a linearized pUC 19.

genomic DNA; genomic digestion would yield a high number of fragments containing the Kan^R cassette. This would explain the high frequency of kanamycin resistant colonies observed, and the absence of the expected pUC19 fragments in some transformants

Although our study was unable to determine the insertion site sequence of the Kan^R cassette, it provides future researchers with 5 potential clones that contain genomic fragments of interest. Once the insertion site has been sequenced, the efficacy of the lambda red recombinase can be verified. The apparent correlation between the appearance of the 500 bp and 400 bp bands and kanamycin resistance suggests that this 500 bp band may be important in determining the insertion site of the Kan^R gene.

FUTURE EXPERIMENTS

Prior to any experiments, the five potential clones identified from this study should be streaked out on MacConkey-Amp-Kan plates to obtain pure cultures. Future studies could focus on further characterization of the plasmids from the five potential clones that fulfilled all four criteria mentioned. The pUC19 plasmid itself should also be digested with the restriction endonucleases XhoI and DdeI to confirm that these enzymes do not cut within the plasmid. Furthermore, to disprove the possibility that the constructed *E. coli* JSST1 contained a Kan^R cassette in a plasmid instead of the genome, miniprep should be performed. In addition, to determine the site of insertion of the Kan^R cassette, the fragment on the plasmids from the five clones could be sequenced.

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