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Title: Understanding the impact of orientation on gene expression of *lux* operon in pKN800 transformation into *Escherichia coli* DH5α

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Purpose

Isolate and purify a pKN800 plasmid from *Escherichia coli.* Using restriction mapping determine the orientation of the Luciferase (*lux*) operon with respect to the plasmid backbone. Then transform the plasmid into *E. coli* strain DH5α to analyze the expression of the *lux* operon and the efficiency of transformation.

Methods

We began with an *E. coli* strain that contained an unknown pKN800 plasmid. We purified this plasmid and then processed it using a restriction digest technique. Tubes of uncut plasmid DNA and cut plasmid DNA were prepared. The "cut" plasmid DNA was cut with a *PstI* enzyme. Following incubation these tubes were loaded onto an agarose gel for gel electrophoresis. Plasmids were transformed into *E. coli* DH5α and were plated onto LB and LBamp50 plates to analyze for antibiotic resistance and luciferase activity.

Procedures followed for this experiment are on pp. 17-23 of section titled "Experiment 2 – Plasmid Purification and Restriction Mapping" with the following exceptions: Step A-7 on pp. 17, students were not required to spin down cells since cells had been spun down prior to the start of lab. Step B-7 on pp. 19, tubes were incubated for 45 minutes at 37◦C. Step D-13 on pp. 22, plates were incubated for four days prior to observation of colonies.

Results

We received an *E. coli* strain with an unknown pKN800 plasmid. Plasmid was purified and orientation of plasmid was analyzed using restriction mapping to determine if the orientation of the *lux* operon effects its expression in *E. coli.* We then transformed pKN800 plasmid DNA into *E. coli* DH5α and recorded the number of ampicillin-resistant and luminescent colonies to confirm the function of pKN800 plasmid in luciferase production.

To determine the orientation of the pKN800 plasmid, the plasmid was processed using a restriction digest with the enzyme *PstI.* Using gel electrophoresis we separated the uncut pKN800 plasmid and the *PstI* cut restriction fragments. Lane 1 of the gel contained the 1000-bp DNA ladder and following gel electrophoresis exhibited distinct banding (Fig. 1). Lane 8 contained my *PstI* enzyme cut plasmid. This lane exhibits 2 distinct bands; the top band is more intense than the bottom band. Lane 9 contains my sample of uncut pKN800 plasmid. This lane exhibits a single intense band, this band represents the uncut supercoiled plasmid DNA (Fig. 1).

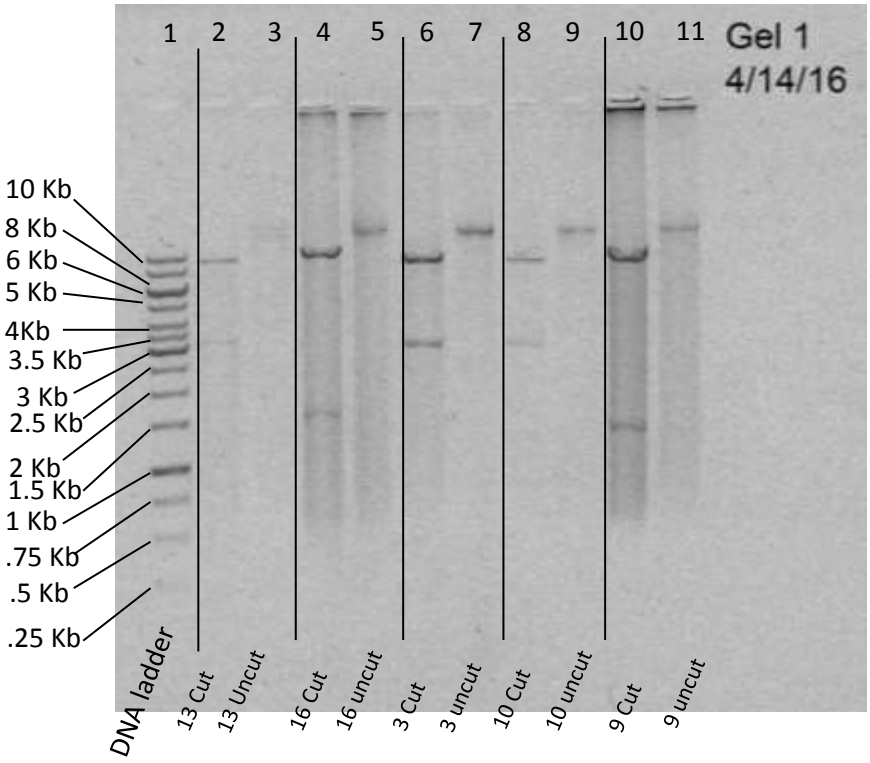

Figure 1. Agarose gel electrophoresis of *PstI* cut and uncut pKN800 plasmid. Lane 1 contains a 1000bp ladder, which provides reference for restriction fragment size in subsequent lanes. Lane 8 contains *PstI* cut pKN800 plasmid from my sample. Lane 9 contains uncut plasmid sample.

To determine the size of the restriction fragments of the pKN800 plasmid a standard curve for a 1000-bp DNA molecular weight standard was performed, found in lane 1 (Fig. 1). Using this information we could estimate the size of the fragments (Fig. 2). The distance traveled by the two *PstI* cut bands in lane 8 were 6.0 cm and 3.6 cm (Fig. 1). When compared to the 1000-bp molecular weight standard these distances correspond with a fragment size of about 10,000bp and 3,000bp respectively. The uncut sample traveled 3.0 cm which indicates that it is larger than 10,000bp.

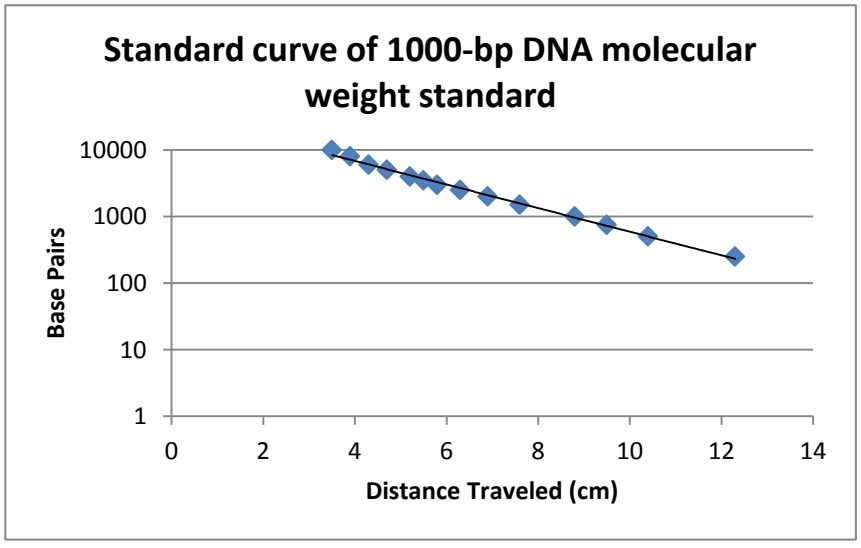

Figure 2. Standard curve of 1000-bp DNA molecular weight standard

Transformation of pKN800 plasmid into *E. coli* DH5α was performed to confirm luciferase activity. Reporter gene expression was also observed. For this experiment ampicillin resistance was the reporter gene used (Table 1).

Table 1. Data from plated transformed cells showing the number of ampicillin resistant and luminescent transformant cells.

Type of Plasmid DNA	Type of Plate	Incubation Temp	Dilution	Count	Average ampicillin resistant & luminescent colonies
PstI Cut DNA	LBamp50 agar	30◦C in dark room	$1x10^{0}$	0	0
				0	0
Uncut DNA	LBamp50 agar	30◦C in dark room	$1x10^{0}$	1	2[a]
			$1x10^{0}$	2	
			$1x10^{-1}$	0	0
			$1x10^{-1}$	0	0
No DNA	LBamp50 agar	30◦C in dark room	$1x10^{0}$	0	0
			$1x10^{0}$	0	0
	LB agar		$1x10^{-5}$	21	0
			$1x10^{-5}$	17	0
			$1x10^{-6}$	2	0
			$1x10^{-6}$	2	0

[a] average was 1.5.

To calculate the efficiency of transformation the following formula was used.

$$\frac{number\ of\ ampicillan-resistant\ and\ luminescent\ transformants}{amount\ of\ DNA\ used\ for\ transformation}$$

Counts for all plates were insignificant; therefore the efficiency of transformation could not be determined with confidence. However, we still estimated the amount of uncut pKN800 DNA by comparing the intensity of the uncut plasmid DNA band, lane 9 (Fig. 1), with the band intensity on the on the 1000-bp DNA molecular base pair standard (Fig. 3).

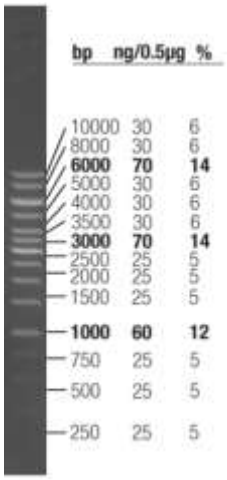

Figure 3. The 1000-bp molecular weight standard containing the intensity and size of each band. Bold bands indicate bands that have at least twice as much DNA as the other bands. Middle column indicates amount of DNA.

The amount of pKN800 plasmid DNA was 0.025µg. The efficiency to transformation was as follows:

$$\frac{2}{0.025} = 80\,{}^{transformants}/_{g\ of\ plasmid\ DNA}$$

**Note that this number is not significant

Discussion

In this experiment we investigated the affect that orientation of the *lux* operon has on gene expression in *E. coli* cells that have undergone transformation. To investigate this idea we purified an unknown pKN800 plasmid and treated it with a restriction enzyme, *PstI,* to determine the orientation of the *lux* operon in the plasmid. Plasmids were then transformed into *E. coli* cells to determine if the *lux* operon was expressed in its particular orientation within the plasmid.

It is possible that the pKN800 plasmid contains the *lux* operon. However, since plate counts were far out of what is considered to be significant it is impossible to say, with confidence, whether the operon was inserted into the plasmid with reasonable efficiency. Insertion of *lux* operon into cloning vector pBR322 should result in luminescent cells with a disruption in the cells tetracycline resistance. However, ampicillin resistance should remain intact.

There are two possible orientations of the pKN800 plasmid. The pKN800-A orientation occurs when the *lux* operon is inserted into the plasmid in the same direction as the tetracycline resistance gene. The pKN800-B orientation occurs when the *lux* operon gene is inserted in the opposite direction of the tetracycline resistance gene (1). Analysis was done to determine if the orientation of the *lux* operon affected its expression. To do this the plasmid was purified, orientation was determined, and pKN800 plasmid was transformed into *E. coli* DH5α. The transformants were then screened for luminescence and ampicillin resistance.

To determine the orientation of the *lux* operon the plasmid was cut using a *PstI* restriction enzyme. This enzyme cut the plasmid into two distinct sized fragments. These fragments underwent gel electrophoresis and were compared to a 1000-bp molecular weight standard to determine their

size. The sizes of the two fragments were determined to be 10 Kb and 3 Kb. These fragment sizes indicate that pKN800 plasmid is in the B orientation. In the B orientation we would expect to see fragments that are 9.98 Kb and 3.38Kb, according to Figure 2 on page 11 of the lab manual (1). These fragment sizes are very similar to the fragment sizes determined in our sample through gel electrophoresis. Further, our fragment sizes are much more similar to those of a pKN800-B oriented plasmid than a pKN800-A oriented plasmid, in an A oriented plasmid we would expect to see fragments that are 11.54Kb and 1.82Kb (1).

From analysis of the agarose gel used for the gel electrophoresis it can be determined that the *PstI* enzyme digested all pKN800 plasmids completely. This can be said because there were no extra bands on the gel. There were the two expected restriction fragment bands in the "cut DNA" lane, representing the 2 plasmid fragments that were created when the *PstI* restriction enzyme cut the DNA, and there was only a single band in the "uncut DNA" lane, representing the uncut supercoiled plasmid DNA.

The transformation of the pKN800 plasmid to *E. coli* DH5α was used to determine that the pKN800 plasmid encoded for functional luciferase proteins. The transformation of the pKN800 plasmid into *E. coli* DH5α is not considered to be successful. Although we were able to observe successful transformation in several colonies, these results are not considered to be significant, and therefore no definitive statements can be made regarding these results. Further, the efficiency of transformation for this experiment was incredibly low (80 transformants/g plasmid DNA), acceptable transformation efficiency for *E. coli* with plasmids is determined to be $1x10^9$ transformants/μg of plasmid DNA (2). This further indicates that the transformation of this pKN800 plasmid was unsuccessful.

It is possible that the low efficiency of transformation is due to experimental techniques performed that inhibit high efficiency transformation. Research has been done to determine the optimal conditions for transformation in *E. coli* using the pBR322 cloning vector. This research suggests that heat shock of the cells to be transformed lowers the efficiency of transformation to half that of optimal efficiency. Further, transformation is greatly inhibited when the cell/DNA mixture contains linear DNA fragments (3). It is probable that any of these factors affected the efficiency of transformation in this experiment. However, the likelihood that linear DNA affected the efficiency of transformation is low since the results for gel electrophoresis show only a single band in the uncut DNA lane (Fig. 1). This indicates that only the supercoiled DNA was present, and that linear DNA fragments were not present. The more likely reason for our low efficiency results is that we used the chemical method to induce competency in our cells. This method utilizes heat shock which greatly reduces transformation efficiency.

The plated control, *PstI* cut DNA transformants, indicated that the *PstI* enzyme correctly and completely cleaved the plasmid DNA. This can be stated because no growth was present on the agar plates. This indicates that the ampicillin resistance genes of the plasmid were disrupted. However, when we look at the no DNA control plates we see that colonies without the plasmid DNA were able to grow on the LB agar plates, but were unable to grow on the LBamp50 plates. This indicates that the *E. coli* DH5α cells are not resistant to ampicillin when they do not contain the pKN800 plasmid.

This further indicates that all cells that were able to grow on the LBamp50 plates contained a pKN800 plasmid with an undisrupted ampicillin resistance gene.

From these results, it can be determined that the *lux* operon was inserted into the pKN800 plasmid in the B orientation, the orientation that it is in the opposite direction of the tetracycline resistance gene. However, since the transformation of this plasmid into the *E. coli* DH5α cells was unsuccessful, the impact of the orientation of the *lux* operon within the plasmid on gene expression cannot be determined by this data alone. We can also make no statements about the impact that the B orientation has on the gene expression of the *lux* operon in the pKN800 plasmid since no significant data was collected from the transformation.

Conclusion

The *lux* operon was inserted into the pKN800 plasmid in the B orientation, opposite direction of the tetracycline resistance gene. No conclusions can be made about the effects of orientation on gene expression for this operon from the data collect in this experiment due to insignificance.

References

1. **Mueller, R., Ream, W., Geller, B., Trempy, J., & Field, K.** 2016. *Molecular microbiology laboratory manual: Experiment 2- plasmid purification and transformation.* Online edition. Oregon State University Department of Microbiology. Pp. 1-17.
2. **Inoue, H., Nojima, H., Okayama, H.** 1990. *High efficiency transformation of Escherichia coli with plasmids.* Gene. **96:**1. 23-28.
3. **Norgard, M., Keem, K., Monahan, J.** July 1978. *Factors affecting the transformation of Escherichia coli strain x 1776 by pBR322 plasmid DNA.* Gene. **3:**4. 279-292.

Questions

1. No, the uncut plasmid did not form more than one band during gel electrophoresis. This single band represents the supercoiled uncut plasmid DNA.
2. The *lux* operon is in the B orientation. I know this because the 2 restriction fragment sizes measured in the gel electrophoresis were 10Kb and 3Kb. These sizes are most closely related to the expected restriction fragment sizes for the B orientation (9.98 Kb and 3.38Kb).
3. Yes, my *PstI* digestion went to completion. I know this because when I plated my cut plasmid DNA on the LBamp50 agar no colonies were able to grow, this indicates that my enzyme digestion was complete since it interrupted the ampicillin resistance genes on the plasmid.
4. No, the restricted plasmid DNA did not produce ampicillin-resistant transformants. I know this because no colonies were present on the LBamp50 plates where my “cut” DNA was plated.
5. Yes, although I did not see this in my results. There is a small probability that the *lux* operon was not inserted into the plasmid correctly and therefore would not be able to function. Whereas the ampicillin resistance gene may still be able to function. It is also possible that

the orientation of the *lux* operon affects its expression, which could also in turn yield a result where the ampicillin resistance is expressed but the *lux* operon is not.

6. The uncut DNA band was analyzed for several reasons. The first reason is that it provides a comparison for determining if the *PstI* cut DNA was cut correctly. Second, analysis of the intensity of the uncut DNA band allows for an estimation of the amount of DNA used to transform *E. coli.* This value can then be used to determine transformation efficiency.
7. We plated bacteria that did not contain any plasmid DNA as a negative control. These plates showed that *E. coli* DH5α are unable to grow in the presence of ampicillin without the plasmid DNA. Further it shows that the bacterial cells are viable when antibiotic selection does not occur.
8. To determine if the plate was at fault I would streak one of the ampicillin plates with a known ampicillin resistant strain. This way I would know that if there is no growth the plate is at fault. However, if this known ampicillin resistant bacteria grows on my plates I will know that I had extremely poor transformation.