BENG0004 Biochemistry and Molecular Biology

DNA Practical Lab Report

Identification and Expression of Tinsel Purple in *E. coli*

Group 10

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This experiment used various molecular biology techniques to identify two unknown plasmid samples. The samples were restriction digested by EcoRI and KpnI to cleave the plasmids before being gel electrophoresed. The UV-fluorescent bands were used to identify antimicrobial Tinsel Purple from toxic Dreidel Teal by cross-comparing the experimental and theoretically expected results. Using heat shock, Tinsel Purple plasmids were then transformed into competent TOP10 E. coli cells. It was finally determined that agar plates with both kanamycin and IPTG resulted in the most expression of Tinsel Purple.

1 Introduction

This experiment aims to first distinguish a plasmid encoding for a purple antimicrobial protein – Tinsel Purple [1], from another plasmid encoding for a blue toxic protein – Dreidel Teal, given two unknown plasmid samples, A and B, and then transform the former into *E. coli*, using molecular biology techniques.

Plasmids A and B were first digested with restriction enzymes. Restriction digestion is the process of cleaving DNA strands into fragments using restriction enzymes, which is often performed during DNA sequence identification [2]. Restriction enzymes are a particular class of protein enzymes that can recognise a specific DNA sequence and cleave the DNA strands [3]. The enzymes used in this experiment (*Eco*RI and KpnI) are type II restriction enzymes which cleave both DNA strands at a specific location within the recognition site [4]. This procedure splits the circular plasmid into linear DNA fragments depending on the nucleotide sequences.

The digested DNA strands were then electrophoresed in an agarose gel. Agarose gel electrophoresis is a technique used to separate charged particles by size under an electric field [5]. Since the DNA backbone is negatively charged [6], it is attracted by the positive electrode. The agarose gel provides a resistive medium to aggregate the DNA by size. Their sizes can be estimated if a DNA ladder of known sizes is electrophoresed alongside the DNA samples. This technique is most effective for DNA between 100bp and 25kbp long [7]. The DNA samples are stained using a staining dye that forms UV-fluorescent complexes with DNA strands for visualisation [8]. The unknown plasmids can be identified by comparing the experimentally observed fragment size against expected DNA fragment sizes from Tinsel Purple and Dreidel Teal.

Tinsel Purple was then transformed into competent *E. coli* cells. Transformation is the introduction of plasmid DNA into cells to facilitate DNA cloning or expression [8]. The cells were pre-treated with $CaCl_2$ to chemically induce competence [9], which allows them to uptake foreign DNA. The cells were then heat-shocked to increase cell membrane permeability so that they could more readily uptake the plasmids [10].

The cells were finally plated and incubated with antibiotics and an inducer to isolate and induce the expression of the desired Tinsel Purple protein. The plasmids contain an antibiotic resistance gene, which allows successfully transformed *E. coli* to survive the antibiotics on the agar plate. The chromoprotein gene in the Tinsel Purple plasmid is preceded by a T5 promoter and a *lac* operator [1]. In usual conditions, the *lac* repressor binds to the *lac* operator; it suppresses the expression of its succeeding gene (Tinsel Purple) by inhibiting transcription from the T5 promoter as a form of gene regulation [11]. The introduction of IPTG dissociates the *lac* repressor from the operator and thus re-enables the expression of the gene [12], resulting in an induced purple colour in *E. coli* cells.

2 Materials & Methods

2.1 Restriction Enzyme Digestion

Three tubes of sample mixtures, each with 10µL of plasmid, were prepared for each of plasmids A and B. One of which was the control, to which 5µL of loading buffer (0.1M Na_2EDTA, 40% sucrose, and 0.15mg/mL bromophenol blue) was added, while 1.5µL of restriction buffer (50mM of TrisHCl pH7.5 and 5mM $MgCl_2$) was added to the other two tubes. Among the two testing tubes, 1.5µL of EcoRI was added to both, and 1.5µL of KpnI was added to only one. The six tubes were vortexed to mix and placed in a water bath at 37°C for 45 minutes.

As can be seen from the plasmid maps in Figure 1, Tinsel Purple contains one recognition site for KpnI and two for EcoRI, and Dreidel Teal contains two for the former and one for the latter. Thus, Tinsel Purple is expected to be cleaved into two fragments in the test tube without KpnI and three fragments in the tube with KpnI, while Dreidel Teal is expected to be cleaved into one linear fragment without KpnI and three fragments with KpnI. This difference can be contrasted in the next step for the discrimination of Tinsel Purple.

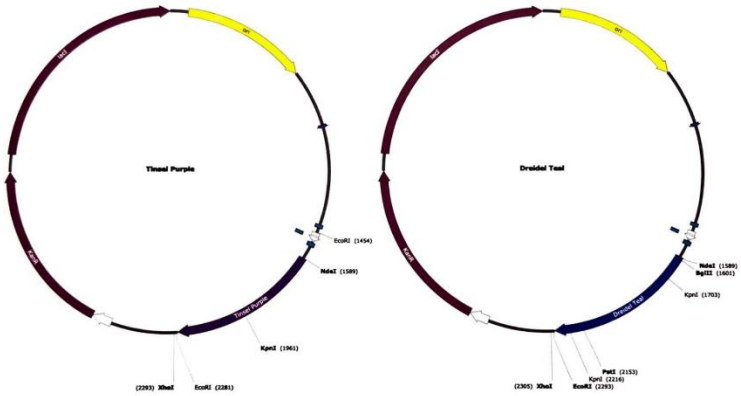

Figure 1 - Plasmid maps of the Tinsel Purple and Dreidel Teal plasmids.

2.2 Gel Electrophoresis

An electrophoresis trough with tris borate electrophoresis buffer (90nM tris base, 90mM boric acid, and 1nM Na_2EDTA) was prepared. 2.5µL of DNA Stain (ThermoFisher Scientific) [13] was added to each of the sample mixtures before loading 5µL of each into the agarose gel along with 5µL of DNA Ladder (ThermoFisher Scientific) [14] for reference. After gel electrophoresis was completed, the gel was imaged under UV light, and Tinsel Purple was identified.

2.3 Transformation

5µL of Tinsel Purple plasmid was added to 50µL of competent TOP10 *E. coli* cells. The tube was gently mixed by flicking before being allowed to sit on ice for 30 minutes. The cells were then heat-shocked at 42°C for 30 seconds to promote the uptake of plasmids. Finally, the cells were transferred to 1mL of LB broth and incubated at 37°C to allow for recovery.

2.4 Plating Bacteria

Four nutrient agar plates: one control, one with 20µg/mL kanamycin, one with 20µg/mL IPTG, and one with 20µg/mL of both were prepared, on which 50µL of the incubated cells were added and plated using a plate spreader. The plates were incubated for 24 hours before observing the resultant colour changes.

3 Results & Discussion

3.1 Gel Electrophoresis

Figure 1 shows that Tinsel Purple and Dreidel Teal should produce DNA fragments of the following sizes, as depicted in Table 1 below. Major products correspond to the assumption that each restriction site is cleaved, whereas minor products correspond to that where only some or none of the sites are cleaved.

Table 1 - Expected major and minor fragment sizes of Tinsel Purple and Dreidel Teal

Plasmid & Restriction enzyme	Major band sizes (bp)	Minor band sizes (bp)
Tinsel Purple 1	~2800 smear	
Tinsel Purple 2 (EcoRI)	820, 2000	~2800 smear
Tinsel Purple 3 (EcoRI & KpnI)	320, 507, 2000	827, ~2800 smear
Dreidel Teal 1	~2800 smear	
Dreidel Teal 2 (EcoRI)	2800	~2800 smear
Dreidel Teal 3 (EcoRI & KpnI)	77, 513, 2200	590, ~2700, ~2800 smear

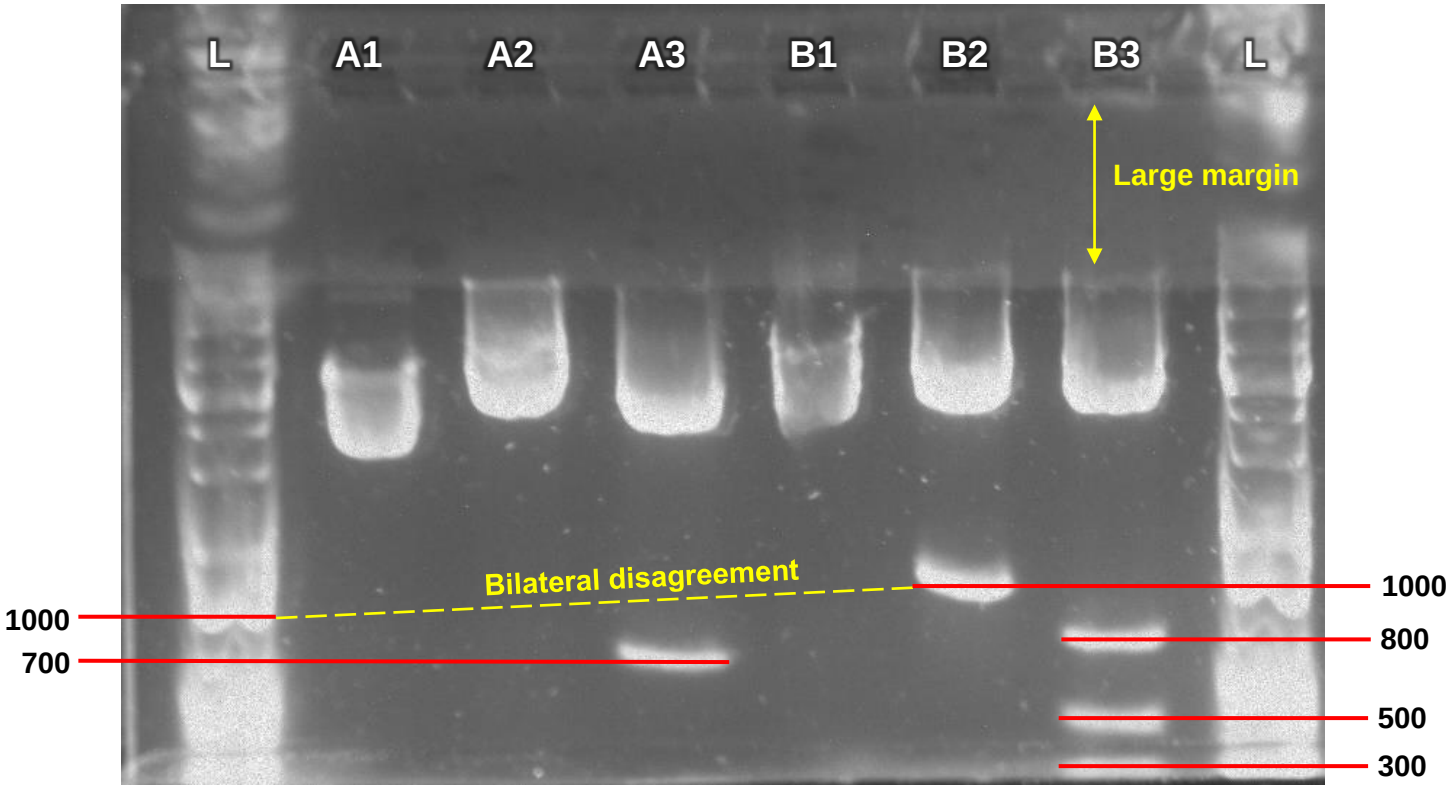

Figure 2 - Electrophoresed gel showing the restriction digestion products of plasmids A and B.

Lanes:
***L** – 5µL ThermoFisher GeneRuler 1kb Plus DNA Ladder,*
***A1** – 5µL Plasmid A, **A2** – 5µL Plasmid A restricted with EcoRI, **A3** – 5µL Plasmid A restricted with EcoRI and KpnI,*
***B1** – 5µL Plasmid B, **B2** – 5µL Plasmid B restricted with EcoRI, **B3** – 5µL Plasmid B restricted with EcoRI and KpnI.*

Highlighted in yellow, the bilateral disagreement between the ladders and the large top margin.

As seen in Figure 2, the gel electrophoresis was not of an ideal quality, as indicated by the intense smearing of the DNA ladder. The gel electrophoresis likely ran over time since the DNA ladder of the gel above this one can be seen to have escaped its gel's confines and overlapped the ladder of this gel. The largest bands can be observed to have migrated a significant distance from the wells, which is also indicative of an overtime gel electrophoresis. The DNA ladders can only be observed partially on the gel, and there was a non-negligible bilateral disagreement between the ladders, resulting in a tilted horizontal axis. Due to the discussed reasons, in combination with the intense smearing, it is very challenging to pinpoint the band sizes. Nevertheless, we still managed to deduce and discriminate the plasmids.

For lane B3, three small bands can be clearly observed, which highly correlated with the expected results of Tinsel Purple 3, namely the major 320bp and 507bp bands and the minor 827bp band. For lane A3, we can safely assume that the expected 77bp band had overran the gel boundaries and can be ignored. The only small band observed near 700bp correlated not insignificantly with the expected 513bp and 580bp bands, taking the tilted horizontal axis into account. Although the band observed in lane B2 correlated weakly with any expected band sizes, most of the evidence corroborated plasmid B as the desired Tinsel Purple and A as Dreidel Teal.

Smears can also be observed in addition to the relatively sharp and defined bands used above to discriminate the plasmids. DNA, including plasmids, naturally exists in a supercoiled state [15]. The degree of coiling of plasmids can vary to produce varying sizes, areas, and other properties [16]. This allows plasmids with the same number of base pairs to experience a spectrum of resistance when travelling through an agarose gel during gel electrophoresis, resulting in a smear pattern. Their high standard deviation in travelling speed, in addition to having a circular surface area instead of a linear one, makes it unsuitable for estimation using a standard linear DNA ladder.

3.2 Plating Bacteria

As shown in Figure 3, only the agar plates with kanamycin contain purple E. coli and the existence of IPTG results in a more vibrant purple. Agar plates without kanamycin exhibit lawn growth of *E. coli*.

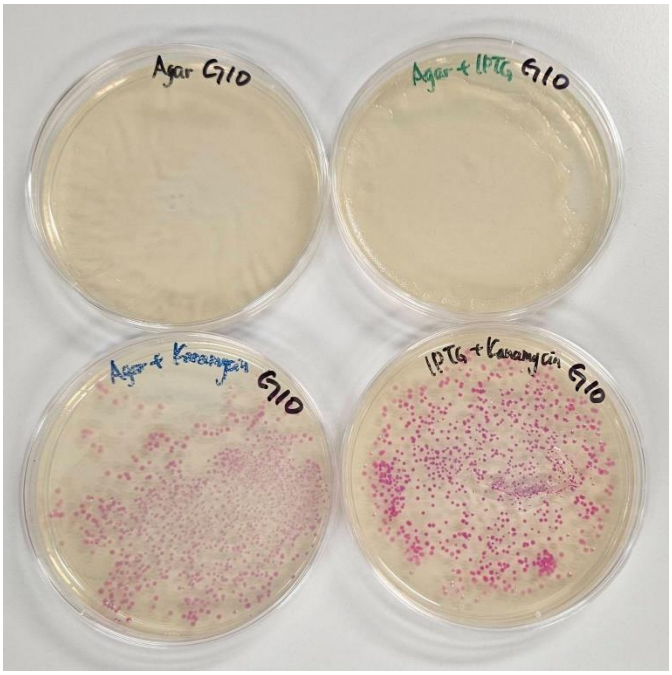

Figure 3 – Agar plates with incubated transformed E. coli.

Agar plates:
***Top left** – Transformed E. coli on agar,*
***Top right** – Transformed E. coli on agar with IPTG,*
***Bottom left** – Transformed E. coli on agar with kanamycin,*
***Bottom right** – Transformed E. coli on agar with kanamycin and IPTG*

Since the chromoprotein gene is packaged with the antibiotic-resistant gene, a retention of one necessitates that of the other. Therefore, the existence of purple colour is indicative of the existence of antibiotic resistance. As discussed in the Introduction above, kanamycin acts as a selection mechanism by providing selective pressure. We can observe this in the bottom agar plates where only coloured (transformed) colonies survived.

However, it might be intriguing that no purple colour can be observed at all in the top plates despite no selective pressure specifically against antibiotic resistance. While plasmids can provide utility to the bacteria, they can also be detrimental to possess if they are not actively providing said utility since plasmids are very costly to replicate and express [17]. This duality energetically disfavours bacteria hoarding dormant plasmids and promotes plasmid loss if there is no immediate utility [18]. Hence, transformed bacteria cells either lost their plasmids or got outcompeted in the non-selective environment of the top plates. This phenomenon also explains the lack of colour expression despite the IPTG inducer in the top right plate. The lack of pressure and abundance of nutrients resulted in an uncontrolled lawn growth of *E. coli* in the top plates.

At the beginning of the experiment, it was hypothesised that only the agar plate with both kanamycin and IPTG would display a purple colour. However, this was evidently disproven, seeing that the plasmid had seemingly been expressed without the need for inducers. As discussed in the Introduction above, the expression of the chromoprotein gene is mediated by the *lac* operator and repressor, with the latter requiring lactose or a lactose-mimicking molecule (IPTG) to dissociate with the operator and, therefore, allow transcription [11]. However, the *lac* operon is *leaky*, meaning it does not fully inhibit transcription and allows

basal gene expression [19]. With the addition of IPTG, this inhibitory mechanism is fully disabled, as can be observed by the vibrant purple in the bottom right plate.

3.3 Conclusion

From the restriction enzymes used to digest the plasmids, we cross-compared the experimental gel electrophoretic behaviour with the expected behaviour to determine that plasmid B was the desired antimicrobial Tinsel Purple, whereas plasmid A was the toxic Dreidel Teal. It was also discovered that transformed *E. coli* incubated on agar plates with both kanamycin and IPTG expressed the Tinsel Purple gene the most.

4 References

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