

Analysis of an Unknown Plasmid

by

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Introduction

A plasmid is a circular molecule of DNA that replicates independently in a bacterial cell. It is typical for a plasmid to carry one or more genes, which are responsible for the useful characteristics, such as antibiotic resistance, displayed by the bacterial host. Plasmids are able to multiply independently of the bacterial chromosome because they contain their own origin of replication. A few types of plasmids can also be stably inserted into the bacterial chromosome in order to replicate (1). Plasmids are extremely important because of their ability to carry genetic information between cells. For this reason, it is imperative to learn as much as possible about plasmids of unknown origins isolated from the environment. With an increased knowledge of plasmid DNA it will be possible to better understand the characteristic functions expressed by a bacteria, and could lead to new possibilities for the manipulation of the genetic information carrier.

This experiment began with the isolation of an unknown plasmid. The knowledge originally ascertained about this piece of DNA established that it carries an ampicillin resistance gene, the *bla* gene, has a single Eco RI site, and has the ability to replicate in *E. coli*. With this basic knowledge, a series of proven procedures were used to help in the further identification of this plasmid.

Materials and Methods

Transformation of the plasmid into competent cells. The unknown plasmid DNA was transformed into XL1 Blue competent cells in order to facilitate further analysis. This method was used to yield a high number of functional bacterial colonies that contain the plasmid of interest. 10 ng of plasmid DNA was added to each competent cell aliquot. This was mixed, heat-shocked, incubated, and then spread on agar plates that had been supplemented with ampicillin. The procedures listed here follow the Promega protocol (2).

Preparation of broth culture for purification purposes. Common culturing techniques for acquiring a large yield of bacterial growth were used in this instance. Single colonies were selected from the plates prepared after transformation and inoculated in LB. This was then incubated, with shaking, overnight at 37°C for maximum growth.

Purification Processes. Promega Wizard® Plus kits were used exclusively for the purification of the plasmid DNA. All protocol for proper use of the Promega Midiprep kit can be found in the Promega Wizard® Plus Midiprep DNA Purification System manual (3).

Determination of DNA quantity by spectrophotometric equipment. The absorbances were determined using a 220-350 nm wave scan. The absorbance values at 260, 280, and 320 nm were used to calculate the relative DNA amount present in each sample since one absorbance unit equals 50 g/ml.

Restriction enzyme digests. Single digests were performed on the purified DNA samples using nine common restriction enzymes. The procedures performed here are taken from Promega protocol (2).

Gel Electrophoresis. All digested samples were run on a 1% agarose gel at 70 volts. A 1 Kb size ladder, purchased from Promega, was used for the determination of the cut DNA sizes. 2.5 ul of Ethidium Bromide was supplemented in all gels for the purpose of viewing with an ultraviolet light box. Pictures of all gels were captured using a Kodak Digital Science DC-40 camera.

Testing of antibiotic resistances using the Kirby-Bauer Method. A single colony was taken from the original transformation plate and emulsified in 3 ml of prepared saline solution. Using a cotton swab, this was spread across a plate of Mueller-Hinton II agar, which was obtained from Mark Clauson, according to his instruction. The Kirby-Bauer method tests the sensitivity of an organism to a number of different chemotherapeutic agents (4). Twelve different antibiotic disks were placed on the surface of the streaked plate and the plate was incubated overnight at 37°C. Some of the disks caused zones of clearing on the plate of microorganisms, most likely because of the disruption of protein production; while others yielded no effect on sample. The zones were measured and the effectiveness of each antibiotic was noted (4) (Table 1).

Results

The unknown plasmid grew well on the LB/ampicillin (50 ug/ml) plate after transformation into XL1 Blue cells. The knowledge of the ability of the plasmid to grow in the presence of ampicillin was also tested using the Kirby-Bauer method. The plasmid was resistant to 5 of the 12 antibiotics used for testing. Growth of the bacteria was not inhibited around the penicillin, carbenicillin, clindamycin, tetracycline, and ampicillin disks (Table 1).

Table 1:

Antibody	Status	Zone of Clearing
Streptomycin	S	10 mm
Penicillin	R	-----
Sulfonamides	S	18 mm
Gentamicin	S	12 mm
Vancomycin	I	2 mm
Carbenicillin	R	-----
Cephalothin	I	1.5 mm
Ampicillin	R	-----
Clindamycin	R	-----
Tetracycline	R	-----
Cefoxitin	S	9 mm

Kirby-Bauer method results

Zone of clearing: measured edge of disk to edge of clearing

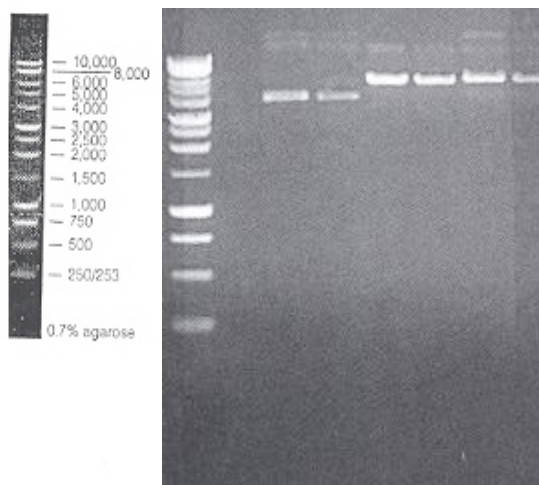
S = sensitive, I = intermediate, R = resistant

Only low quantities of DNA were purified using the Promega Wizard® Plus kits. Spectrophotometric techniques helped to verify this. In the beginning stages of this experiment the Promega Miniprep kit, designed for small-scale samples, was used. It

became apparent that a large amount of the plasmid sample was needed, so the Promega Midiprep kit was used to effectively separate the plasmid DNA from the bacterial chromosomal DNA. A DNA concentration of approximately 0.1 ug/ul, in 40 ul total, was obtained using the Promega Miniprep system. A DNA concentration of 0.065 ug/ul, in 300 ul total, was obtained using the Promega Midiprep system. These values were used to determine the amount of DNA needed for the restriction digests.

The pictures taken after the gel electrophoresis showed that every restriction enzyme made at least one cut of the plasmid sample. The restriction enzymes used were Bam HI, Eco RI, Xba I, Xho I, Cla I, Sph I, Hind III, Apa I, and Kpn I. The majority of these digests had a distinguishable bright band at around 5000-6000 bp (Figure 1), with others, Bam HI and Hind III, showing varying other bands around the 600 bp mark on the ladder.

Figure 1:



Gel picture displaying size of unknown plasmid

Gel Picture displaying cuts made by restriction enzyme digests.

Promega 1 kb ladder used (Part # 9PIG571)

Discussion

Most of the restriction enzyme digests yielded a large fragment approximately 6000 bp in size, giving a good indication that the size of the unknown plasmid is somewhere near this. The overwhelming majority of bands of this size confirm this with reasonable certainty. It is beneficial to determine which restriction enzymes will cut the DNA sample, and specifically, how many times each will cut. Some of the bands viewed on the agarose gels were small enough to be introduced into a known vector and sequenced. Bam HI and Hind III both had bands approximately around the 600 bp mark on the ladder. With more time and study these bands of DNA could be purified and sequenced to yield a portion of the plasmid sequence.

It was known that the plasmid carried an ampicillin resistance gene, so in an effort to verify other resistance genes that might be present in the plasmid the Kirby-Bauer method was used. The five antibiotics that the plasmid was resistant to were penicillin, carbenicillin, clindamycin, tetracycline, and ampicillin. Clindamycin is classified under the group known as the Microlides. Tetracyclines are classified under the broad class of tetracyclines. Penicillin, carbenicillin, and ampicillin are in the same category known as the β -Lactams. These last three all have a distinctive β -Lactam ring present in their structure. The unknown plasmid was known to have a resistance gene to ampicillin, so it is reasonable that the plasmid also had the ability to grow in the presence penicillin and carbenicillin. The ampicillin resistance gene, β -lactamase, which is present in the plasmid, has the ability to chop up the β -Lactam ring in the antibiotic's structure. Therefore, it most likely can chop up β -Lactam rings in the structure of other antibiotics.

The XL1 Blue cells used for the purpose of growing up the plasmid had a tetracycline resistance gene present in them, so further study would have to be done to say with certainty that this gene is also present in the unknown plasmid.

Conclusion

There is insufficient data present to successfully identify the unknown plasmid. The size of the plasmid is approximately 5000-6000 bp. A few genes of this plasmid have been successfully identified, and with further analysis through sequencing these could be verified.

References

1. Brown, T.A. Gene Cloning and DNA Analysis. 4th Edition. Blackwell Science, 2001. p. 14-16.
2. Doyle, K. ed. "Promega Protocols and Applications Guide." 3rd Edition. Promega Corporation, 1996.
3. "Wizard Plus Midiprep DNA Purification System." Promega Corporation, Revised Sept. 2002. Part # TP173.
4. Benson, Harold J. "Antibiotic Sensitivity Testing." Microbial Applications: Laboratory Manual in General Microbiology. 8th Edition. McGraw Hill, 2002. p.118.