

Investigation of an Unknown Plasmid

By

Jyoti Sahi

Partner: Jonathan Sims

Group # 6

Biology 450

Dr. Claire Rinehart

May 6, 2004

INTRODUCTION

Plasmids are DNA molecules that can replicate and regulate their replication independent of the host genome. Plasmids generally have a closed, circular conformation and are 2-12 kb in size. One or more genes are usually carried by plasmids. These genes are important because they mark a significant trait displayed by the host bacterium. For example, the ability to survive in toxic amounts of ampicillin is due to a plasmid, which has the antibiotic resistance gene, in the bacterium.

Gene cloning allows identical copies of a gene of interest to be produced in a host, via a vector, in order to allow amplification for further studies (1).

Fifty ng of an unknown plasmid were made available at the onset of this study; the only information available about this plasmid was that it was capable of replicating in *E. coli*, carried the ampicillin resistance gene (*bla* gene) and had a single Eco RI site. The aim of this project was to determine as much possible about this plasmid using a variety of techniques, including standard procedures for DNA amplification, purification, quantification and restriction enzyme analysis. The cloning vehicle used in this project was the pGEM 7zf(+) vector, which has a size of about 3000 bp (1).

MATERIALS & METHODS

Transformation

The unknown plasmid (5 ng/μl) and pGEM vector (1 ng/μl) were transformed into previously prepared competent cells (Biotechnology Center, WKU) of *Escherichia coli* XLI-blue. Transformations followed the Promega protocol (2).

Small Scale Plasmid Preparation

Promega's Wizard® *Plus* Minipreps DNA Purification System, with a few changes, was followed for plasmid preparation (3). After overnight incubation, the bacterial culture had to be spun an additional fifteen minutes in order for pellet formation to occur; plasmid purification was done using a vacuum manifold. DNA was stored in water in -20°C until further use.

Quantification of DNA

The samples were subjected to absorbance analysis using a Shimadzu UV-1601 spectrophotometer. This presented values for determination of concentration and purity of DNA. Absorbance values were recorded at 260 nm and 280 nm. Ratio determination (A_{260}/A_{280}) aided in determining total amount of DNA present.

Gel Electrophoresis & Restriction Enzyme Digests

A 1% agarose gel (0.50 g agarose + 50 ul 1x TAE) was used to run samples. Digests were compared to a 1 kb ladder (Promega Part No. G571A). Gels were run at approximately 70 volts/cm. Gels were stained in 2.5 μl Ethidium Bromide for 15-25

minutes, as needed. Visualization of gels was via an ultraviolet light box; a Kodak digital camera captured the image.

The following restriction enzymes were used: BamH I, Cla I, EcoRI, Hind III, Sph I, Xba I, Xho I, Apa I, and Kpn I; digests followed manufacturer's recommended protocols (NEB). Restriction digests were stopped by adding 1/5 the volume of 0.4 M EDTA if digest could not be run on gel immediately after 37°C water bath incubation (4.). In some cases, digest mixes were so large in volume that the mix had to be split into two lanes on gel so as not to overfill well. DNA fragments from agarose gel were isolated for DNA recovery; purification was done using vacuum manifold. Promega's Wizard® SV Gel & PCR Clean-Up System was used for the protocol (5).

Large Scale Plasmid Purification

A larger scaled plasmid preparation technique was performed in order to obtain larger amount of the unknown plasmid DNA. Promega's Wizard® *Plus* Midipreps DNA Purification System was used with slight modifications (6). Forty ml cultures of LB with amp were grown overnight for the plasmid prep.

Kirby-Bauer Test

Antibiotic resistance tests were performed according to the Antibiotic Sensitivity Testing protocol (7). Liquid cultures of unknown plasmid were subjected to antimicrobial susceptibility tests overnight in 37°C for the following antibiotics: Streptomycin (S10), Penicillin (P2), Sulfonamides (G300), Gentamicin (GM10), Vancomycin (VA30), Carbenicillin (CB100), Cephalothin (CR30), Ampicillin (AM10), Clindamycin (CC2), Tetracycline (TE30), and Cefoxitin (FOX30).

RESULTS

Transformation of the unknown plasmid and pGEM vector into XLI Blue cells were successful. Growth was observed on both 50 and 200 µl plates for both plasmid and vector, indicating that DNA had been transformed into *E. coli* XLI-Blue competent cells; as expected, no growth was seen on the negative-no DNA-control.

Two sets of broth cultures (one for unknown plasmid and one for vector) were grown in five ml of LB with ampicillin (50 mg/ml). Promega's Wizard® *Plus* Minipreps DNA Purification System yielded a small amount of DNA. A plasmid DNA midiprep (6) was thus done in order to recover a larger quantity of DNA; the overall concentration of DNA was approximately the same for both the mini- and midipreps.

Restriction digests produced gels displaying the unknown plasmid being cut multiple times by enzymes such as BamH I and Hind III around 5000-6000 bp, as well as 600 bp. Other restriction enzymes used included Xba I, Xho I, Cla I, EcoRI, Sph I, Apa I and Kpn I. These restriction enzymes cut at least once. Kpn I and Xba I produced only one fragment around 5000-6000 bp. Apa I and Xho I fell produced a band around the 4000-5000 bp range. Cla I and Sph I produced a band similar to that of Kpn I and Xba I in the 5000-6000 range (8).

Table 1 displays single digests performed with Xba I, Xho I, Cla I, EcoRI, Sph I, Apa I, Kpn I, BamH I and Hind III:

Table 1: Plasmid Restriction Digest Approximate Fragment Size

Xba I	Xho I	Cla I	Sph I	Apa I	Kpn I	BamH I	HindIII
5000-6000	4000-5000	5000-6000	5000-6000	4000-5000	5000-6000	5000-6000	5000-6000
						600	600

Gels were also run for pGEM, which showed linear fragments for BamH I and Hind III digests at 3000 bp.

Restriction digests of recombinants were done using restriction enzymes that would cut at the same site used for cloning indicating that an insert was ligated into the vector. Transformations were plated onto LB/Amp plates with IPTG and XGAL for blue/white screening. Blue colonies outnumbered white colonies, but the presence of the white colonies indicated that a few recombinant molecules had been created. A restriction enzyme digest was performed after the plasmid miniprep was done post ligation using BamH I and Hind III; the digest showed beautiful bands at approximately 3000-4000 bp. This indicated that the recombinants had been successfully introduced into the pGEM vector.

The Kirby-Bauer test antibiotic sensitivity was used to determine which antibiotics the plasmid was resistant or sensitive to. The results of the tests are shown in Figure 2.

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

Figure 2: Antimicrobial Susceptibility: Kirby-Bauer Test

*Zone of clearing: edge of disk to edge of clearing

**S = sensitive, I = intermediate, R = resistant

DISCUSSION

The unknown plasmid is known to have the capability of replicating in *E. coli*, carries the ampicillin resistance gene and has a single Eco RI site. However the actual identity of the plasmid was not determined from the tests that were run.

Some of the restriction enzyme digestions, those with single cuts-BamH I and Hind III- indicate that the overall size of the unknown plasmid falls approximately 5000- 6000 bp. Xba I, Xho I, Cla I, EcoRI, Sph I, Apa I, and Kpn I produced one band each, indicating that the unknown plasmid did have these cut sites present.

The initial intent of the Kirby-Bauer test was to compare the sensitivities of the unknown plasmid to that of a proposed identity for it; this would thereby provide confirmation when trying to support a choice for the identity of the unknown plasmid.

According to the antimicrobial susceptibility test, the plasmid was resistant to penicillin, carbenicillin, clindamycin, and tetracycline. It was already known that the plasmid was ampicillin resistant. Upon closer observation, one finds that ampicillin and carbenicillin fall under a larger class of the penicillin family. All three of these antibiotics contain β -lactam rings. Thus, the plasmid β -lactamase gives it the capability to cleave, the beta-lactam rings present in these antibiotics, thereby making the plasmid resistant. Clindamycin falls under a larger family known as microlides. Tetracycline falls under its own class of antibiotics.

The competent cells, XL1-blue, used for growing the plasmid has tetracycline resistance. Therefore, further tests would have to be done to propose that this is present in the unknown plasmid.

In order to suggest an identity for the unknown plasmid, additional analysis would have to be done. For example, obtaining a workable sequence could be used to run a BLAST search, thereby enabling one to find commercially available plasmids that match to the unknown.

CONCLUSION

The aim of this project was to determine as much possible about an unknown plasmid presented at the beginning of class using a plethora of techniques. It can be concluded that this unknown plasmid, approximately 5000-6000 bp in size, contains beta-lactamase; the unknown plasmid was cut multiple times by only two restriction enzymes – BamH I and Hind III. The plasmid has an ampicillin resistant site; therefore it is also resistant to carbenicillin and penicillin. However, it does not antibiotic resistant genes for streptomycin, sulfanomides, gentamicin, and ceftiofur. A final conclusion as to what the identity of the unknown plasmid is, however, cannot be proposed at this time.

LITERATURE CITED

- (1) Brown TA. (2001) "Gene cloning and DNA analysis," Blackwell Science, Osney Mead, Oxford, p. 14.
- (2) Doyle K. (1996). "Protocols and applications guide," 3rd Ed., Promega Catalog # P1610, p. 46.
- (3) Promega's Wizard[®] *Plus* Minipreps DNA Purification System: Part # TB117, revised 9/02.
- (4) Doyle K. (1996). "Protocols and applications guide," 3rd Ed., Promega Catalog # P1610, p. 8-10.
- (5) Promega's Wizard[®] SV Gel & PCR Clean-Up System: Part # TB 308, revised 10/02.
- (6) Promega's Wizard[®] *Plus* Midipreps DNA Purification System: Part # TB173, revised 9/02.
- (7) Benson Harold J. "Antibiotic Sensitivity Testing." Microbial Applications: Lab Manual in General Microbiology. 8th Ed. McGraw Hill, 2002, p. 118.
- (8) Sambrook J, Fritsch EF, and Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

