Building an Improved Suicide Vector for Creating Mutations in Specific Genes

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ABSTRACT

Bacterial geneticists that wish to create mutations in specific genes currently use a suicide vector pSUP202, developed by Simon, Priefer, and Puhler in 1983 (3), as a carrier molecule. This vector is difficult to use because it has cloning sites scattered throughout the vector. In addition, this vector lacks color screening capabilities, which makes identification of cells containing cloned genes difficult and time-consuming. The purpose of this project is to construct a suicide vector based on the plasmid pSUP202. This new plasmid will allow use of a wider variety of restriction enzymes in future cloning strategies (5). This plasmid will also be engineered to contain the *lacZ'* gene which encodes the α-peptide of β-galactosidase. This feature will allow direct blue/white screening of recombinant clones by inactivation of α-complementation. In addition, the new vector will confer multiple antibiotic resistance to host bacteria. Towards these goals, a detailed cloning strategy has been developed using computer data base sequence manipulations and the Genetics Computer Group Sequence Analysis Software Package. A DNA fragment, derived from the plasmid pUC19 cut with *Hae* II, was separated by agarose gel electrophoresis. This DNA fragment, containing a multiple cloning site (MCS) and the *lacZ'* gene, was purified from the gel matrix using Gene-Clean™ and blunted with the enzyme T4 DNA polymerase. In separate experiments, vector DNA (pSUP202) was digested with the restriction endonuclease *Eco*R I and the resulting ends were also converted to blunt ends with T4 DNA polymerase. The vector and insert DNA were ligated with T4 DNA ligase and ligation products were transformed into E. *coli* DH5α. Once identified by histochemical screening, the recombinant plasmid will be purified and further tested.

INTRODUCTION

Genetics describes the heredity and variation of living organisms and the mechanisms by which this variation is brought about. The function of genes can be addressed in two ways: Traditional genetics examines methods by which mutation occurs randomly. Rare mutants that cannot carry out a particular metabolic function, i.e., cells that have the desired genotype, are recognized. The mutated genes are subsequently identified and characterized. Using inverse genetics, the specific gene of interest is first cloned, and a defined mutation is created in that gene. The changes in the cells' metabolic functions caused by the defined mutation are then investigated. This approach relies on recombinant DNA technology, genetic engineering, or gene cloning.

One way to generate defined mutations in specific genes is by marker exchange (4).

The following steps are usually used (see Figure 2).

Step 1. DNA from an organism of interest (for example *Rhodobacter sphaeroides*) is digested with a restriction endonuclease.

Step 2. A specific gene(s) is/are cloned into a plasmid vector (carrier).

Step 3. A selectable marker, for example antibiotic resistance, is inserted into the gene.

Step 4. The plasmid is transformed into competent cells (typically *E. coli*).

Step 5. The plasmid is then moved from the organism used to do the cloning, (*E. coli* cells), into a wild-type cell from which the specific gene was cloned (*Rhodobacter sphaeroides*). Note that the plasmid must fail to replicate in this host (a suicide vector).

Step 6. Inside the recipient cells, gene exchange occurs between the good copy and the copy of the same gene which contains the antibiotic resistance gene.

Step 7. The host cells are then grown in the presence of the antibiotic encoded by the modified gene.

The clones that have had the disrupted version of the gene (marker) exchanged for the normal copy in the chromosome are identified by resistance to the antibiotic. The effects of the mutation on the metabolism of the cell are then ascertained.

MATERIALS AND METHODS

Phase I. Preparation of a cloning strategy to build a better suicide vector.
The complete DNA sequence of pUC19 vector containing a MCS and the *lacZ*α gene was obtained. This sequence was downloaded from a database using the World Wide Web. A restriction endonuclease (*Hae* II) that has cleavage sites that will cut out the MCS and *lacZ*α gene as one fragment was identified. These sites were located using Genetics Computer Group Sequence Analysis Software Package (formerly available at UW-L). A map of the existing suicide vector, pSUP202, was obtained (4) and the availability of sites to insert the MCS and *lacZ*α were determined. A strategy to insert MCS and *lacZ*α gene into the existing suicide vector pSUP202 was designed based on enzyme compatibility.

Phase II. Vector construction, (see Figure 3).
Table 1 below shows the summary of the restriction digestion of pUC19 and pSUP202.

Table 1. Summary of restriction digestion of pUC19 and pSUP202. Incubation was done in a 37°C water bath for 30 minutes.

Experiment #	pUC19	pSUP202	10X buffer	H_2O	100X BSA	*Hae* II	*Eco*R I
1	47 µl (7 µg)	—	10 µl	41.5 µl	1 µl	0.5 µl	—
Negative control	1 µl	—	2 µl	17 µl	—	—	—
2	—	2 µl	2 µl	14 µl	—	—	2 µl
Negative control	—	1 µl	2 µl	17 µl	—	—	—

The plasmid pUC19 was cut with *Hae* II to generate a fragment containing the MCS and *lacZ*α gene. The desired fragment was separated using a 1.2% agarose gel electrophoresed overnight at 50V. A large 12" x 10" electrophoresis chamber was used to more effectively separate the two smaller fragments (Figure 3.A). The desired fragment (~445 bp) was purified from the agarose using Gene-Clean™. The suicide vector pSUP202 was digested with the restriction endonuclease *Eco*R I. The digested pSUP202 was visualized in an 0.8% agarose gel electrophoresed at 100V for approximately 1 hour (Figure 3.B). A standard size electrophoresis chamber was used for this purpose. Based on kinetic analysis of ligation reactions, a ratio of 5 nM insert for 7.5 kb vector is recommended. Following this protocol, the digested DNA fragments were converted to compatible blunt ends using 1 µl of T4 DNA polymerase, 0.33 mM solution of all four dNTPs, 0.5 µl 10X T4 DNA polymerase buffer and 3.5 µl DI water. Incubation was done at room temperature for 3 min. (2). After incubation, the enzyme was heat inactivated at 75°C for 10 min. The sample was then alcohol precipitated, resuspended in 1X ligase buffer and spiked with 1 µl T4 DNA ligase (1). Incubation was done at 16°C for 16 hours. The ligation mixture was transformed into competent *E. coli* DH5α cells (6). Plasmid pGEM was transformed into *E. coli* DH5α as positive control and cells without a plasmid were used as negative control. The transformation mixes were plated onto LB agarose plates containing X-gal (40µg/ml) and 50 µg/ml of ampicillin. All plates were incubated for 20 hours at 37°C. Identification of the transformants containing the new plasmid was done by histochemical screening.

RESULTS

According to the Genetics Computer Group Sequence Analysis Software Package, the plasmid pUC19 can be cut with *Hae* II to generate three fragments of three sizes, 1871 bp, 445 bp, and 370 bp. The 445 bp fragment contains the MCS and *lacZ*α gene is shown in highlight.

1. Complete genetic sequence of pUC19 was obtianed (Figure 1).

2. A crude map of pSUP202 was obtained (Figure 3). There is no available sequence for pSUP202. There are five enzymes known to cut pSUP202: *Eco*R I, *Pst* I, *Hind* III, *Bam*H I, and *Sal* I.

3. Digestion of pUC19 and pSUP202. Restriction digestion of pUC19 with *Hae* II resulted in 3' overhang while digestion of pSUP202 with *Eco*R I resulted in 5' overhang. T4 DNA polymerase was used to blunt both the insert and vector DNA fragments creating compatible ends for ligation. "This enzyme is derived from the *E. coli* bacteriophage T4. It is a DNA-dependent DNA polymerase that has 5' —> 3' polymerase activity and a 3' —> 5' exonuclease activity (6).

4. Transformation (see Table 2).

Table 2. Summary of transformation results.

	Volume plated(μl)	# of blue colonies	# of white colonies
Positive control	50	972	0
	200	Too many to count	0
Negative control	200	0	0
Transformants from ligation mixture	50	0	10
	100	0	31
	200	0	76

There were two types of white colonies, large and small. Both types of colonies showed morphological instability. After re-streaking a sample from a large colony, small colonies appeared with the large ones. The same results were observed when a sample was re-streaked from a small colony.

DISCUSSION

There are several possible reasons why no blue colonies were obtained:

1. Due to the unavailability of the complete sequence of pSUP202, the ideal site for inserting the MCS and *lac*Zα gene was difficult to determine. The *Eco*R I site chosen based on the crude map of pSUP202 might have resulted in the loss of ß-galactosidase's native configuration. Therefore, it's function may have also been lost.
2. Blunting and ligation of the fragments failed. If the enzyme T4 DNA polymerase failed to blunt either one or both of the fragments, ligation would have been impossible. The vector would have simply re-ligated on itself without the MCS and *lac*Zα gene. This would lead to white transformants.
3. The competent cells were simply not competent enough. The possibility of acquiring a recombinant plasmid is rare. If the competency of the cells used (*E. coli* DH5α) was low, the likelihood that a recombinant plasmid would have been successfully transformed would have been lowered.

TROUBLE-SHOOTING STRATEGY

It is unlikely that the complete DNA sequence of pSUP202 will become available. Therefore, trouble-shooting must be focused on the blunting and ligation of both insert and vector fragments. First, more insert and vector must be purified in large quantities. Second, each fragment must be blunted using the same procedures as before. In addition, each fragment must also be blunted separately and test whether blunting was successful. This can be done by re-ligating each blunted fragment with a known blunted fragment and then screen for transformants containing the recombinant plasmid. Note that good competent cells must be used to transform all recombinant plasmids. After ligation, the recombinant plasmid must be tested to determine whether it actually contains the desired insert. This can be done by digesting the recombinant plasmid with any of the restriction enzymes known to cut in the MCS of the insert (see Figure 3).

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LOCUS ARPUC19 2686 BP
DEFINITION Cloning vector pUC19 (high copy number plasmid)
ACCESSION X02514
NID g58136
SOURCE Cloning vector pUC19
BASE COUNT 666 a 675 c 686 g 659 t

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Figure 1. Complete genetic sequence of pUC19 (**Bold indicates the *Hae* II enzyme** cuts fragment purified in this experiment.)

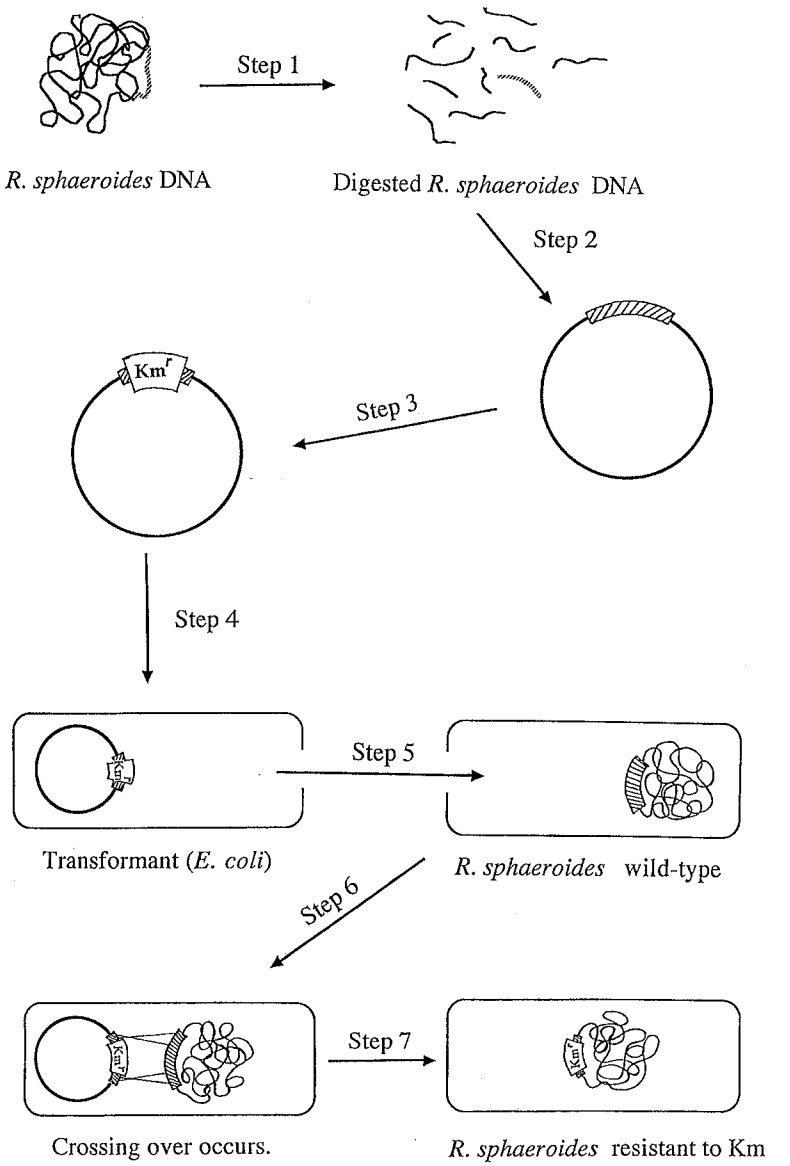

Figure 2. creating defined mutations in specific genes.

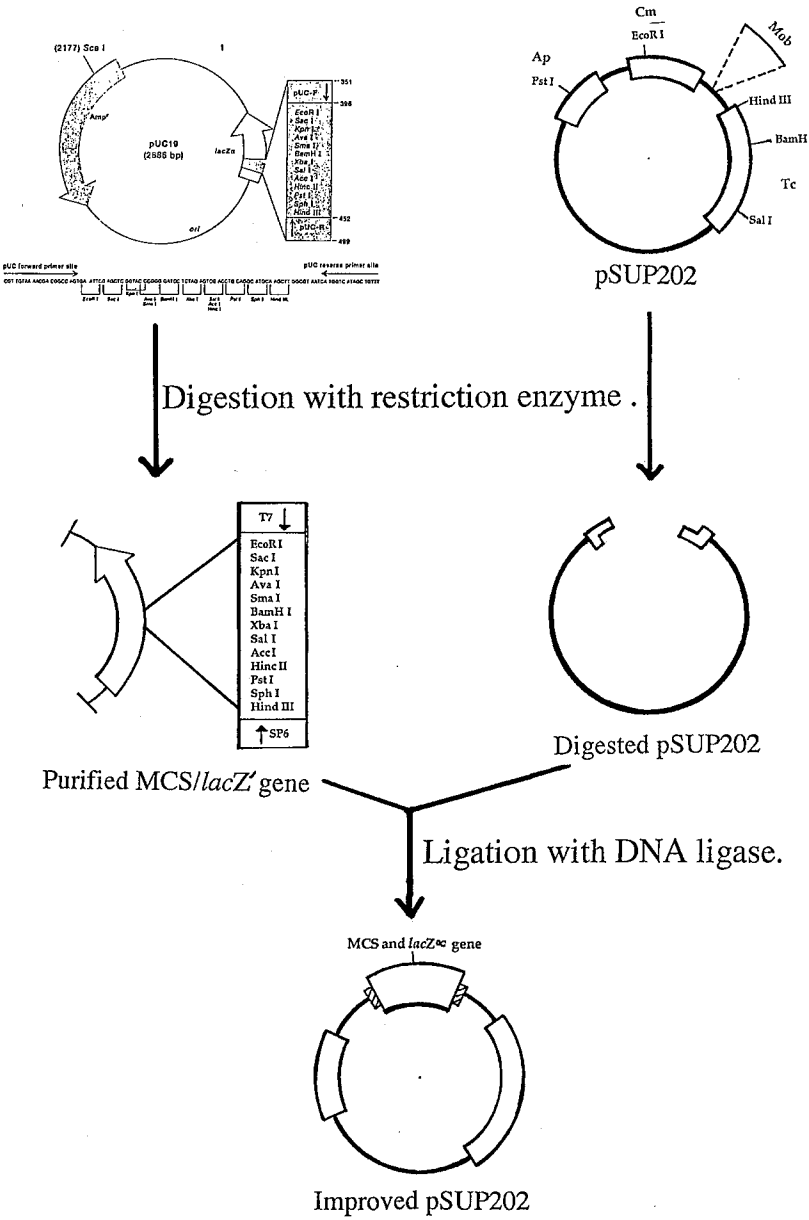

Figure 3. Overview of vector construction strategy

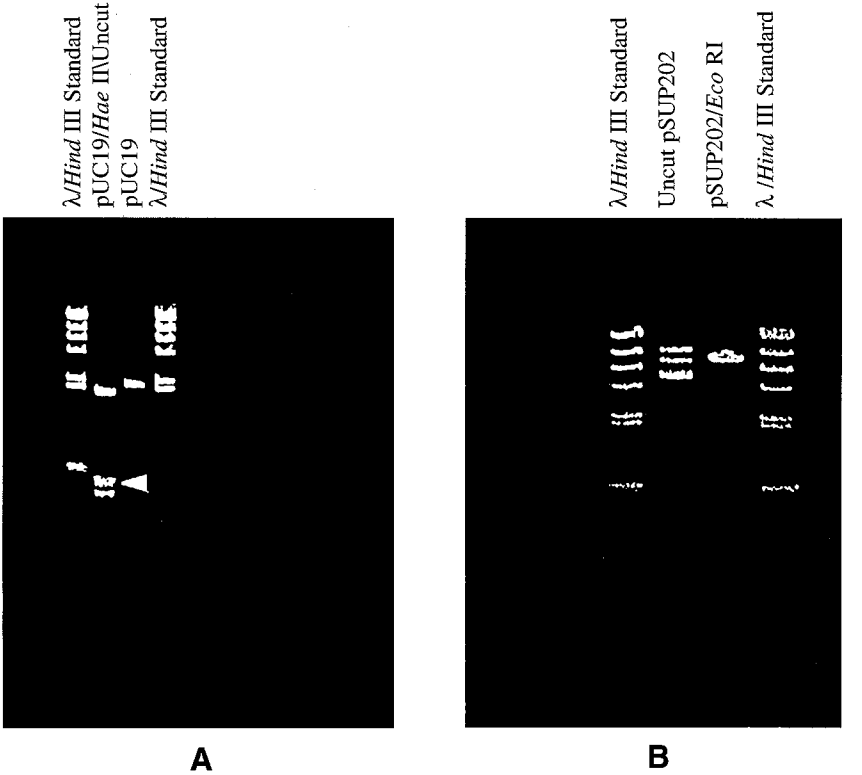

Figure 4. Agarose gel electtophoresis pattern showing the fragments produced by digesting A) pUC19 with *Hae* II, B) pSUP202 with *Eco*RI.