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Novel Suicide Elements for Gene Disruption in Escherichia coli

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NOVEL SUICIDE ELEMENTS FOR GENE DISRUPTION IN ESCHERICHIA COLI

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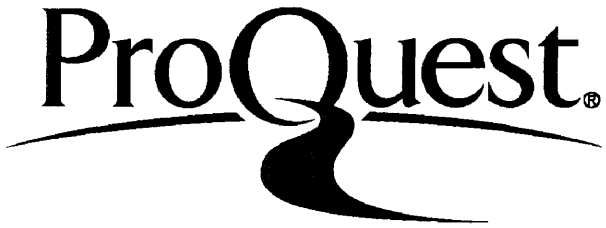

APPROVAL SHEET

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TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The purpose of this study was to construct and test novel suicide elements for their efficiency and practicability in making insertional mutations in the lac operon of Escherichia coli. Suicide elements are generally used to insert DNA into the host chromosome. They are DNA molecules that can not replicate in a target cell either because of the environmental conditions in which the cell is propagated or because of cell specific traits. Due to its lack of replicative ability in the target cell, the suicide element must integrate into the chromosome in order to pass on the selectable phenotype it carries. Suicide elements can be either plasmids or linear DNA.

The first element constructed was a suicide plasmid which contained an origin of replication that was flanked by unique restriction sites. The plasmid could be altered to remove the origin and then self-ligated. This process would produce an originless circular DNA molecule that would be incapable of replication in any bacterium. It would either integrate into the chromosome or be lost in subsequent generations. Unfortunately, no integrants were obtained with this suicide element.

The second element constructed was a suicide plasmid based on the origin of replication of the R6K plasmid. R6K can not replicate in cells which lack a copy of the pir gene. When introduced into a cell lacking pir, the plasmid integrates into the genome or is lost in subsequent generations due to lack of replicative function. Using this vector, it was possible to obtain large numbers of integrants with insertions in the lac operon.

The third suicide element constructed was a linear DNA suicide element that contained Chi sites. Linear DNA is normally degraded by intracellular nucleases. However, Chi sites have been shown to protect linear DNA from degradation and to promote recombinational activity in their vicinity. When our linear DNA suicide element containing Chi sites was introduced into E. coli, integrants at lac were obtained.

The results show that the suicide elements constructed would be useful for making insertional mutations in E. coli. It is believed by the investigator that these vectors could also be used for making point mutations and deletions in E. coli as well as other gram-negative bacteria.

NOVEL SUICIDE ELEMENTS FOR GENE DISRUPTION IN ESCHERICHIA COLI

INTRODUCTION

General Background

The gram negative bacterium *Escherichia coli* has been instrumental in the study of genetics. Its ease of propagation, small genome size, and rapid cell cycle make it ideal for this type of research; consequently, it is one of the most intensely studied and genetically characterized organisms in science. In fact, the first cloning experiments used *E. coli* (Cohen *et al.*, 1973), and most current dogma of operon structure, expression, and regulation is based on Jacob and Monod's work with the *lac* operon (Figure 1) of *E. coli* (Jacob, 1966; Monod, 1966). Because *E. coli* is very amenable to detailed genetic analysis, it is also a valuable system for understanding complex cellular processes. A common approach to such analysis is the use of reverse genetics. Reverse genetics involves mutating a gene of interest and studying the accompanying change in phenotype. This method was an advance over earlier methods which generally looked for the genetic basis of a particular phenotype rather than altering genes and looking for the resultant phenotype. Previous genetical work took the phenotype to gene approach because the technology to alter DNA directly was not yet available. However, with the advent of recombinant DNA technology, it is now possible to

target specific changes to the DNA complement of various cell types. The ability to alter chromosomal DNA is a very powerful tool for understanding the genetics of various organisms, particularly the prokaryotes. The types of changes that can currently be made to the chromosome can be grouped into three general categories: insertion of DNA, deletion of DNA, or conservative changes to the DNA sequence that do not result in addition or deletion of nucleotides. These alterations can be important if a change in the cell's phenotype results, because observations of cells with such alterations can provide valuable insight into the regulation or role of a particular gene, and insertion of DNA into the genome can give new and useful genotypes.

Types of Chromosomal Changes

The types of changes that can be made to the chromosome can be grouped into three categories: insertions, deletions, and point mutations.

1. DNA can be inserted into specific regions of the chromosome for many different purposes. Firstly, a gene of interest can be inserted into the chromosome. This change can produce new and useful genotypes that can be used for study or commercial purposes. Secondly, a marker gene such

as an antibiotic resistance gene can be inserted into the chromosome. The marker can then be used for genetic mapping or for specialized selection procedures. Thirdly, a specific gene can be made nonfunctional or inactive by the insertion of DNA at certain locations in the gene, creating a mutation referred to as a knockout mutation. A knockout is produced when extraneous DNA interrupts the coding region of a gene such that a non-functional protein product is produced or when the promoter of a gene is disrupted such that the gene is not transcribed. Finally, by inserting a new promoter in front of a gene, it is possible to change the regulation of that gene.

2. Recombination with the chromosome can also be used to delete regions of the genome. Entire genes or specific parts of a gene can be deleted to study the role of particular regions of the genome. When an entire gene is deleted, a copy of the gene can be provided extrachromosomally where it can be more easily manipulated by the investigator.

3. Recombination with the chromosome can be used to mutate specific sequences of a gene. In this process, no DNA is added to or deleted from the

chromosome. These changes are called point mutations when only one or a few nucleotides of the DNA sequence are changed. These point mutations can give insight into the function of a protein and its various domains, and may pinpoint particularly important amino acids for protein function.

The Use of Suicide Elements to Make Chromosomal Alterations

One way of integrating new genetic material into the chromosome is through the use of suicide elements. Suicide elements are DNA molecules that are introduced into cells in which they can not replicate. The elements can not replicate either because of incompatibility with the host cell or nonpermissive conditions in the host cell. Suicide elements can be either plasmids or linear DNA, and are introduced by either transformation, or in the case of mobilizable suicide plasmids, by mating via an Hfr cross (de Wind et al., 1985; Parker and Marinus, 1988). After introduction to a cell, suicide elements must recombine into the chromosome at a region of homology in order to impart their selectable phenotype to the cell. Otherwise, they will be lost in subsequent generations due to the lack of replicative ability. Thus, suicide elements do not leave unintegrated plasmids or DNA in the cell.

Specifics of the Project

The purpose of this project is to expand the number of suicide elements and methods of integrating into the chromosome by constructing novel suicide elements for *E. coli* that efficiently integrate DNA into the chromosome. Specifically, various ways of integrating into the chromosome were tested for their efficiency and practicability in creating knockout mutations in the *lacZ* gene. The knockouts were achieved by recombining a portion of *lacZ* that was interrupted by an antibiotic resistance determinant into the chromosomal *lacZ* gene using either a linear suicide element or a suicide plasmid. Presumably, a portion of the *lacZ* gene is still transcribed, but a functional protein product is not created due to the inserted extraneous DNA. Recombinants were selected on the appropriate antibiotic for the particular insert and were subsequently checked for the presence of functional β-galactosidase, the product of the *lacZ* gene. Our suicide elements were shown to efficiently knockout *lacZ* gene function in this system.

Suicide Plasmids

Two of the elements constructed and tested were suicide plasmids. Suicide plasmids are circular DNA molecules that can not replicate in particular cells. This lack of replicative ability is very important, because replicating

plasmids can not stably recombine into the chromosome at regions of homology. Therefore, they are unsuitable for chromosomal insertion or alteration. However, some suicide plasmids, such as temperature sensitive plasmids, must exist in the cell for a period of time as "non-suicide" plasmids before integrating into the chromosome. It is for this reason that some of the problems of "non-suicide" plasmids should be elaborated. These problems include the fact that if it is necessary to introduce another plasmid into the cell, then the antibiotic resistant markers on the two plasmids must be different. Furthermore, if the origins of the plasmids are similar, then one plasmid may compete with the other for replication, one eventually outcompeting the other. So, in most cases, a suicide plasmid is the preferable means to integrate into the chromosome.

There are many types of suicide plasmids, each using a different method to integrate into the chromosome by homologous recombination:

1. The first group of suicide plasmids are those that carry an origin that can not replicate in certain bacteria. This group is made up of the species specific plasmids and the polA-dependent plasmids. Both of these suicide plasmids are introduced into a cell in which they can not

replicate and therefore must integrate into the genome in order to pass on the selectable marker they carry.

Species specific plasmids are capable of replication only in certain microorganisms. They become suicide plasmids when introduced into a bacterial species in which they can not replicate. Under these conditions, the plasmid must integrate into the chromosome or be lost (Dunn, 1991). A disadvantage of this type of suicide plasmid is that two different species of bacteria must be maintained in the same laboratory.

The second member of this group are suicide plasmids based on the ColE1 origin. Plasmids containing these origins are incapable of replication in polA strains of bacteria and will be lost if they do not integrate into the chromosome (Gutterson and Koshland, 1983; Saarilathi and Palva, 1985). Again, a disadvantage of the ColE1 plasmids is that they must be transformed into a special strains of bacteria.

2. The second group of suicide plasmids are plasmids that can only replicate under certain conditions. This group consists of plasmids normally referred to as temperature sensitive

plasmids. They are often used as elements to integrate DNA into the bacterial genome (Hamilton et al., 1989; Chan, 1993; Maguin et al., 1992). Temperature sensitive plasmids contain a temperature sensitive replicon and are introduced into cells at the permissive temperature for the plasmid's replication. After introduction and selection for the antibiotic resistance gene carried on the plasmid, the temperature is changed to the nonpermissive temperature and the plasmid either integrates into the chromosome or is lost due to the lack of replicative function. Because the plasmid must exist in the cell for a limited period of time as a non-suicide plasmid, this method has the disadvantage of having to possibly deal with the plasmid compatibility problems mentioned earlier. Other disadvantages include the fact that the cells must be cycled between the permissive and nonpermissive temperatures to force integration of the plasmid into the chromosome and that the plasmids can not be used in temperature sensitive strains of E. coli.

It was the intention of this project to create suicide plasmids that avoided the aforementioned problems of suicide plasmids currently in use.

The first plasmid constructed utilized the unique properties of the origin of the R6K plasmid. R6K is a naturally occurring 38 kilobase conjugative plasmid with about 10-15 copies per cell that carries resistance to ampicillin and streptomycin (Kontomichalou et al., 1970). The R6K origin is unique in that in order to replicate, it requires a supply of the Pi protein from the R6K encoded pir gene (Inuzuka and Helinski, 1978). The Pi protein exhibits sequence specific binding to direct repeats of the R6K origin (Stalker et al., 1979) and initiates replication of the plasmid by host proteins (Germino and Bastia, 1983; Filutowicz et al., 1985a). Pi can be provided in either cis or trans (Kolter et al., 1978). Some of the host proteins necessary for replication of the plasmid include integration host factor, which compacts the R6K origin prior to replication (Dellis et al., 1992; Filutowicz and Appelt, 1988; Filutowicz and Inman, 1991) and DnaA (MacAllister et al., 1991; Wu et al., 1992). Pi autoregulates its own synthesis (Kelley and Bastia, 1985; Filutowicz, et al., 1985b) and also regulates the R6K plasmid's copy number. If Pi is overproduced, it couples the origins of several R6K plasmids and prevents their replication (McEachern et al., 1989).

The first suicide plasmid constructed in this study was pCD11LAC (Figure-7). It was designed to integrate into the chromosome by a single crossover as shown in Figure-11. pCD11 (Figure-6) was based on the plasmid pGP704 (Miller and

Mekalanos, 1988), and contained the origin of the R6K plasmid (lacking pir), an ampicillin cassette, and a multiple cloning site (MCS). The MCS was contained in a 'lacZ fragment capable of α-complementation, allowing color selection for recombinant plasmids. Both the multiple cloning site and the color selection for recombinant plasmids of pCD11 improved pGP704, and it was believed that this would be a very useful suicide element.

The second suicide plasmid tested contained an origin of replication that could be easily removed. The plasmid could then be religated to itself and introduced into a cell. Without an origin, the plasmid would either have to integrate into the chromosome or be lost. It was believed that this construct would also make a suicide element suitable for making insertion mutations. Both of these plasmids have the advantages of not having to be transformed into special strains of bacteria like the species specific or polA plasmids, and neither do they have to be cycled through different temperatures as is required for the temperature sensitive plasmids.

Linear DNA Suicide Elements

Suicide elements are not always plasmids. Linear DNA can also be used as means to alter chromosomal DNA. Linear DNA is normally transformed into special recBC sbcB or recD bacterial

strains (Jasin and Schimmel, 1984; Winans et al., 1985). Special strains must be used because linear DNA is degraded by wildtype E. coli after introduction (Smith et al., 1981) in part because of degradation by intracellular nucleases like RecBCD (Hoekstra et al., 1980). Degradation is avoided in recBC sbcB or recD strains because both strains are defective in the exonuclease activity of the RecBCD enzyme and do not degrade linear double stranded DNA. However, these rec mutants are still capable of recombination (for review see Smith, 1988) and therefore can be transformed with linear DNA without the newly introduced DNA being degraded before it can recombine into the chromosome. Again, one of the problems with this method is that linear DNA has to be introduced into special strains of bacteria.

Our third suicide element avoids this problem because it uses linear DNA containing Chi sites to recombine into the E. coli chromosome (see Figure-12). Linear DNA containing Chi can be transformed into non-rec strains because of the unique characteristics of Chi. A Chi site is a short stretch of DNA with the sequence 5'-GCTGGTGG-3' (Smith et al., 1981) which was first identified in recombinational mutants of bacteriophage lambda (Lam et al., 1974; Stahl et al., 1975). It has been noted as a RecA dependent recombinational hotspot (Lam et al., 1974; Smith et al., 1981; Smith, 1983) and its presence has been shown to protect single and double stranded linear DNA (containing Chi) from degradation (Dabert et al.,

1992). Both of these activities involve the RecBCD protein.

RecBCD is a trimeric protein composed of the nonidentical subunits RecB, C, and D (Smith 1990) that exhibits ATPase, helicase, and exonuclease activity (Palas and Kushner, 1990). The RecBCD protein interacts with Chi sites and is involved in recombination at that site (Roman et al., 1991). This interaction is site specific, with RecBCD recognizing the 8 base pair Chi site and making a nick in the DNA (Ponticelli et al., 1985). RecBCD must approach Chi from the 3' side for a nick to occur. If Chi is in the correct orientation, RecBCD cuts towards the 3' side of Chi and unwinds the DNA helix, yielding single stranded DNA. This single stranded DNA is a substrate for RecA and single-stranded binding proteins (Dixon and Kowalczykowski, 1991; Holbeck and Smith, 1992). These and other proteins catalyze recombination towards the 5' side of the cut (Taylor et al., 1985).

As mentioned previously, RecBCD degrades single and double stranded linear DNA by its ATP-dependent single stranded DNA exonuclease activity (Roman and Kowalczykowski, 1989; Roman and Eggleston, 1992). However, Dabert et al. (1992) showed that Chi sites, in the correct position and orientation, protect against RecBCD degradation of linear DNA in vivo. In later work by Dixon and Kowalczykowski (1993), this protection of linear DNA was shown to be due to an attenuation of the exonuclease activity of RecBCD. Because of the unique qualities of the Chi site, it was hypothesized that

linear DNA containing Chi sites could be introduced into cells and integrate into the chromosome with possible applications for gene disruption.

Summary

In summary, this project's goal was to create both plasmid and linear DNA suicide elements and to test their efficiency and utility in integrating into the E. coli chromosome at lac. Two suicide plasmids and one linear DNA suicide element were used to create knockout mutations in the lacZ gene. It was hoped that these elements would avoid some of the problems of suicide elements currently in use (eg. having to be transformed into special strains of bacteria) and be able to be used as a generalized method for transforming any competent strain of E. coli. Perhaps, in the future, these suicide elements could be exploited in other organisms as well. The ultimate goal of the project was to provide new tools for use in integrating into the chromosome.

MATERIALS AND METHODS

Homologous Recombination with the Origin Deficient Suicide Plasmid pALTERSSLAC

Construction of Strain BN102. CAG5851 (tet^R) and MC1061 ($strp^R$) were mated for 15 minutes at 37°C and then plated on LB plates supplemented with tetracycline (20 μg/ml), streptomycin (0.00033%), 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and Isopropyl-β-D-thiogalactopyranoside (IPTG). This new strain was named BN102.

Construction of pALTERSSLAC. A 524 bp HpaI fragment containing a truncated 'lacZ' was isolated from pMC1871 (Figure-4) and ligated into the SmaI site of the pALTERSS (Figure-2) polylinker. Escherichia coli strain NM522 was transformed with this ligation mixture (pALTERSSLAC) using $CaCl_2$/MOPS transformation (Sambrook et al., 1989) and plated on LB plates supplemented with tetracycline (20 μg/ml), 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and Isopropyl-β-D-thiogalactopyranoside (IPTG). The plates were incubated overnight @ 37°C. A blue control colony, and five white colonies were picked from this plate and restreaked on an LB plate supplemented with tetracycline, X-Gal, and IPTG.

This plate was incubated overnight @ 37°C. A rapid screen for recombinant plasmids was performed on the control colony and the five white colonies from this plate (Sambrook et al., 1989) to look for plasmids containing an insert. Based on the rapid screen, 100 ml of LB supplemented with tetracycline (20 μg/ml) was inoculated with a white colony that appeared to contain a recombinant plasmid. The presumptive plasmid, pALTERSSLAC, was isolated from this culture using an alkali plasmid preparation (Sambrook et al., 1989) and checked by digestion with the restriction enzyme ClaI (BRL). The insert has the final orientation shown in Figure-3.

Transformation of BN102 with ori-deficient pALTERSSLAC. pALTERSSLAC was digested with ApaII. The larger 5.0 kb fragment was eluted from an agarose gel and religated to itself (Sambrook et al., 1989). This origin deficient circular DNA molecule was used to electroporate BN102 using a BTX Electro Cell Manipulator 600 as per the directions of the manufacturer.

Homologous Recombination with the R6K Suicide Plasmid pCD11LAC

Construction of Strain BN101. The BamHI fragment of the recA gene was ligated into the unique BamHI site of pBR322 (Figure-5). Escherichia coli strain NM522 was transformed with this ligation mixture (pBR322RECA) using $CaCl_2$/MOPS transformation (Sambrook et al., 1989) and plated on LB plates supplemented

with ampicillin (125 μg/ml), X-Gal, and IPTG. The plates were incubated overnight @ 37°C. A blue control colony, and five white colonies were picked from this plate and restreaked on an LB plate supplemented with tetracycline, X-Gal, and IPTG. This plate was incubated overnight @ 37°C. A rapid screen for recombinant plasmids was performed on the control colony and the five white colonies from this plate (Sambrook et al., 1989). Based on the rapid screen, 5 ml of LB supplemented with ampicillin (125 μg/ml) was inoculated with a white colony that appeared to contain a recombinant plasmid. The presumptive plasmid, pBR322RECA, was isolated from this culture using an alkali plasmid preparation (Sambrook et al., 1989) and checked by digestion with the restriction enzyme ClaI (BRL). This plasmid was used to transform Escherichia coli strain S17-λ pir. A bacteriophage-λ lysate was made from these transformants and was used to infect JM109 (recA$^-$) (Sambrook et al., 1989). Transductants were restreaked on an LB plate. This plate was incubated overnight @ 37°C. Colonies from this plate were transformed ($CaCl_2$/MOPS transformation) (Sambrook et al., 1989) with pCD11 (Phillips and Dresser, unpublished; Figure-6) and plated on LB plates supplemented with ampicillin. Cells that could be transformed with pCD11 were named BN101. BN101 is a λ-Pir lysogen strain capable of α-complementation.

Construction of pCD11LAC. pMC1871 (Figure-4) was digested with the blunt end cutting restriction endonucleases HpaI and

SmaI. A 6.4 kb fragment was isolated from this digest and was self-ligated. This 6.4 kb plasmid, pMC1871ΔHpaI-SmaI, was digested with EcoRI and the 2.0 kb lacZΔHpaI-SmaI fragment was eluted from an agarose gel. This fragment was cloned into the polylinker of the plasmid pCD11 (Figure-6) that had been digested with EcoRI. E. coli strain BN101 was transformed with this new construct (pCD11LAC) using $CaCl_2$/MOPS transformation (Sambrook et al., 1989) and plated on LB plates supplemented ampicillin (100 μg/ml) , X-Gal, and IPTG. The plates were incubated overnight @ 37°C. A blue control colony and seventeen white colonies were picked from the plate and restreaked on an LB plate supplemented with ampicillin (100 μg/ml), X-Gal, and IPTG. This plate was incubated overnight @ 37°C. A rapid screen for recombinant plasmids was performed on the control colony and the five white colonies from this plate (Sambrook et al., 1989). Based on the rapid screen, 5 ml test tubes of LB supplemented with ampicillin (100 μg/ml) were inoculated with white colonies that appeared to contain recombinant plasmids (based on the rapid screen). The presumptive plasmid, pCD11LAC, was isolated from these cultures using an alkali plasmid preparation (Sambrook et al., 1989) and checked by digestion with the restriction enzyme EcoRV (BRL). A white colony was chosen based on the digestions to be inoculated into 100 ml of LB supplemented with ampicillin (100 μg/ml). The plasmid pCD11LAC was isolated from this culture using a CsCl plasmid preparation

(Sambrook et al., 1989) and checked by digestion with the restriction enzyme EcoRV (BRL). The insert has the final orientation shown in Figure-7.

Transformation of BN102 with pCD11LAC. pCD11LAC was used to electroporate BN102 using a BTX Electro Cell Manipulator 600 as per the directions of the manufacturer. Cells were plated on LB plates supplemented with ampicillin (100 μg/ml), X-Gal, and IPTG. Blue and white colonies were enumerated and restreaked to LB plates supplemented with ampicillin (100 μg/ml), X-Gal, and IPTG to check resistance to ampicillin. A rapid screen for recombinant plasmids was performed on the control colony and the five white colonies from this plate (Sambrook et al., 1989). Colonies were also restreaked to LB plates supplemented with tetracycline and streptomycin.

Transformation and Recombination of Linear DNA Containing Chi Sites into Bacterial Chromosome.

pKSLACZKAN. The plasmid pKSLACZKAN was provided by Dr. Gregory Phillips and is shown in Figure-8.

Electroporation of BN102 with Linear DNA Suicide Element. pKSLACZKAN was digested with the restriction endonucleases SstI(SacI) and KpnI. The 4.6 kb fragment was isolated from this digest by elution from an agarose gel. Both the fragment and the entire digest were used to electroporate BN102 using a BTX Electro Cell Manipulator 600 as per the directions of

the manufacturer. The electroporated cells were plated on LB plates supplemented with Kanamycin (50 μg/ml), X-Gal, and IPTG. Blue and white colonies were enumerated. One blue colony and five white colonies were restreaked to LB plates supplemented with 20 μg/ml Kanamycin, X-Gal, and IPTG, and then to LB plates supplemented with ampicillin (100 μg/ml), X-Gal, and IPTG to check resistance to ampicillin. Restreaked colonies on the Kan plate were tested for the presence of plasmid using a rapid screen for recombinant plasmids (Sambrook et al., 1989).

Mapping of the antibiotic markers of the inserts @ lacZ

A generalized transducing phage, P1, was used to infect lac$^-$ transformants of BN102 (lacZ::kan/amp) that had either been transformed with pCD11LAC or the linear DNA suicide element (Sambrook et al., 1989). Lysates were made from these cultures and were used to infect CAG18439, a lacZ$^+$, tet^S, strep^S, kan/amp^S E. Coli strain. Transductants were plated on LB plates supplemented with kanamycin or ampicillin, X-Gal and IPTG. These plates were incubated overnight @ 37°C. Transductants were restreaked to a Kan/Amp LB plate and then to a Tet or Strep LB plate. These plates were incubated overnight @ 37°C. The number of Kan^R/Kan^S, Strep^R/Strep^S, and Tet^R/Tet^S colonies were enumerated.

Southern Analysis of pCD11LAC and pKSLACZKAN transformants

BN102, along with the pCD11LAC and pKSLACZKAN transformants, were each inoculated into 5 ml of LB supplemented with the appropriate antibiotic. Genomic DNA was isolated from each of these cultures by the methods of Chen and Kuo (1993). The genomic DNA from this preparation was then digested with BstEII, run on an agarose gel, and transferred to a Genescreen membrane. This membrane was probed with a P^{32} labeled EcoRI fragment isolated from the pCD11LAC plasmid (Sambrook et al., 1989). Several exposures were taken of the membrane at various time points and were later analyzed.

TABLE 1

CONCENTRATIONS OF VARIOUS CHEMICALS AND ANTIBIOTICS USED IN LB PLATES

Chemical/Antibiotic (Source)	**Concentration**
Low Concentration Ampicillin (Sigma)	40 μg/ml
High Concentration Ampicillin (Sigma)	125 μg/ml
Tetracycline	20 μg/ml
Streptomycin	0.001%
Kanamycin	50 μg/ml
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma)	10 mM
Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma)	5 mM

TABLE 2

CHARACTERISTICS OF BACTERIAL STRAINS

E. coli Strain	**Antibiotic Resistance**	**Relevant Genotype**	**Reference**
NM522	None		
BN102	Tet/Strep	*lacZ*$^+$	
BN101	None		
JM109	None		
CSH142	None	*lacZ*$^+$	
MC4100	Strep		
CAG5851	Tet	*lac*$^+$, *lacI*^{42}::Tn10, *lacZ* U118	

RESULTS

Electroporation and Transformation Efficiencies

Origin Deficient Suicide Element pALTERSSLAC. No transformants were obtained using this suicide element either with electroporation or transformation.

R6K based Suicide Plasmid pCD11LAC. pCD11LAC was tested for its efficiency in integrating into the chromosome at lac. Both electroporation and $CaCl_2$/MOPS transformation were used to introduce the plasmid to BN102 (lac$^+$).

Electroporation Efficiency. The electroporation efficiency of transformation with pCD11LAC is shown in Table 3. White colonies are transformants with an integration at lac.

Transformation Efficiency. pCD11LAC was able to transform BN102 using standard $CaCl_2$/MOPS transformation techniques, but was very inefficient.

Characterization of pCD11LAC Transformants. Transformants grew very poorly or not at all on LB plates containing a high concentration of ampicillin (120 μg/ml), but grew well on LB plates containing a low concentration of ampicillin (40 μg/ml). This is consistent with a single chromosomal insertion of bla. Rapid screens for the presence of plasmid DNA were performed on blue and white transformant colonies, see Figure-9. It was found that blue colonies contained the

pCD11LAC plasmid while the white colonies did not appear to have plasmid DNA. Blue and white transformant colonies would also grow on LB plates supplemented with tetracycline and streptomycin.

Linear DNA Suicide Element based on pKSLACZKAN. Our linear DNA suicide element was also tested for its efficiency in integrating into the chromosome at lac. Only with electroporation did we obtain transformants of BN102. The efficiency of this method is shown in Table 4. White colonies are transformants with an integration at lac.

Characterization of the Linear DNA Sucide Element Transformants. Rapid screens to test for the presence of plasmid DNA were performed on blue and white transformant colonies (Figure-9) and it was found that blue colonies contained the pKSLACZKAN plasmid while the white colonies did not appear to have plasmid DNA. Blue and white transformant colonies would also grow on LB plates supplemented with tetracycline and streptomycin, confirming that these transformants were of the original strain BN102 and not a contaminant. White colonies restreaked to LB plates supplemented with ampicillin would not grow.

TABLE 3

ELECTROPORATION EFFICIENCIES OF THE R6K BASED SUICIDE PLASMID pCD11LAC

# of White Colonies	# of Blue Colonies	Amt of DNA	# of Cells in Cuvette		# of White Colonies /ug DNA
4800	533	1.2 ug	$2.07*10^9$		4000
4700	490	1.2 ug	$2.07*10^9$		3917
5000	503	1.2 ug	$2.07*10^9$		4166
				mean	4028
				std. dev.	126.78

TABLE 4

ELECTROPORATION EFFICIENCIES OF THE LINEAR DNA SUICIDE ELEMENT

# of White Colonies	# of Blue Colonies	Amt of DNA	# of Cells in Cuvette		# of White Colonies \ug DNA
45	200	0.83 ug	$2.07*10^9$		54.2
39	215	0.83 ug	$2.07*10^9$		47.0
36	325	0.83 ug	$2.07*10^9$		43.4
37	179	0.83 ug	$2.07*10^9$		44.6
35	237	0.83 ug	$2.07*10^9$		42.2
32	204	0.83 ug	$2.07*10^9$		38.6
33	297	0.83 ug	$2.07*10^9$		39.8
48	154	0.83 ug	$2.07*10^9$		57.8
35	245	0.83 ug	$2.07*10^9$		42.2
				mean	45.5
				std. dev.	6.48

Linkage Mapping of Inserts by P1 Transduction

A linkage map of our inserts was constructed to genetically confirm that our suicide elements had integrated into the chromosome at lac.

Mapping of R6K based Suicide Plasmid pCD11LAC. A bacteriophage P1 lysate was made from transformants and was used to infect CAG18439 (lac$^+$, lacI^{42}::Tn10, lacZ U118). All ampicillin resistant transductants were lac$^-$, so lac and Amp were 100% linked.

Mapping of Linear DNA Suicide Vector based on pKSLACZKAN. A lysate was made from transformants and was used to infect CAG18439 (lac$^+$, lacI^{42}::Tn10, lacZ U118). All kanamycin resistant transductants were lac$^-$, so lac and Kan were 100% linked.

Physical Characterization of lacZ Recombinants

A southern blot of genomic DNA from our suicide vector recombinants was probed with a P^{32} labeled truncated lacZ to physically show that our suicide elements had integrated into the chromosome at lac. The probe hybridized with a band of the predicted size (6.3 kb) in the control lane of the Southern which contained chromosomal DNA from BN102 without an insert. As predicted, transformants of both pCD11LAC and the linear DNA suicide element had larger bands than the 6.3 kb control band. The size of the bands was correspondingly

larger than the control by the size of the respective insert.

Southern Blot of R6K based Suicide Plasmid pCD11LAC Transformants. Our lacZ probe hybridized with a band of the predicted size (10.9 kb), indicating the presence of an insertion at lac. The insert's size corresponds to the size of our putative plasmid insert (see Figure-10).

Southern Blot of Linear DNA Suicide Element Transformants. Our lacZ probe hybridized with a band of the predicted size (7.6 kb), indicating the presence of an insertion at lac. The insert's size corresponds to the size of our putative suicide element insert (see Figure-10).

DISCUSSION

The rationale behind using the origin deficient plasmid pALTERSSLAC was that by deleting the origin and recircularizing the molecule *in vitro*, the plasmid would not be degraded once in the cell. This alteration would increase the likelihood that a recombinational event would occur between the plasmid and the chromosomal copy of *lac*. A scheme using site-specific recombination of originless circular DNA molecules was successful in inserting DNA at the *attB* site in *E. coli* (Diederich *et al.*, 1992). However, Diederich's vector is not generally as useful as the vector we had envisioned. Our vector recombines by homologous recombination and therefore allows change to be affected anywhere in the *E. coli* genome. On the other hand, Diederich's vector recombines by site specific recombination and can only insert DNA at *attB*. Furthermore, their system required the use of a temperature sensitive plasmid containing the *int* gene of bacteriophage lambda while the pALTERSSLAC suicide vector did not require the use of a secondary or helper plasmid. These two differences make a vector like the one envisioned in this project much more useful. Our system used an origin deficient plasmid, as did Diederich's, but it would not require a helper

plasmid or be limited to the part of the chromosome which could be altered.

Unfortunately, our vector did not yield transformants with insertions at lac. In fact, we got no transformants at all. There are many possible reasons as to why transformants were not obtained using this method. The first is that the plasmid may need to be supercoiled in order to efficiently recombine into the chromosome. Diederich received transformants without supercoiling his plasmid. This is most likely because the lambda system uses site specific recombination, which is more efficient than homologous recombination. Therfore, less DNA was required to integrate at attB by site-specific recombination than would be required to integrate at lac by homologous recombination. In the future, one might wish to use topoisomerase to supercoil an origin deficient plasmid and determine if this supercoiled circular DNA could integrate into the genome. The second reason that this method may not have worked is that there may not have been a sufficient amount of recircularized DNA to electroporate adequately. This could be due to the fact that after digesting the DNA, eluting the fragment, and then self-ligating the DNA, the yield of intact circular DNA may have been low. However, every effort was made to increase the amount of DNA used to electroporate the cells. The amount of DNA was checked on an agarose gel prior to electroporation and quite a substantial amount of DNA was used in each experiment.

If these problems could be overcome and transformants obtained, I believe that this would be a very useful suicide vector.

pCD11LAC integrated very efficiently at *lac* in the *E. coli* chromosome (Table 2) and is a good suicide vector. However, before this method could see widespread application, the method of propagation of this suicide plasmid would need to be altered. In our system, the plasmid was propagated in a strain of *E. coli* that was a λ-*pir* lysogen. When pCD11LAC was electroporated into BN102, a background of blue colonies appeared on the plate with the white integrant colonies. It was thought that some of the bacteriophage lambda from BN101 became lytic and copurified with the plasmid. These phage could then infect BN102 and support the replication of pCD11LAC. This would explain the background of blue colonies that appeared with the white integrant colonies. A rapid screen confirmed that the blue colonies contained the pCD11LAC plasmid, and the only way that they could replicate the plasmid is if they had a copy of *pir* provided by bacteriophage lambda-*pir*. This explanation for the presence of blue colonies is consistent with the observation that the blue colonies contained distinct white spots in them. These colonies would be cells that had undergone a recombination between pCD11LAC and the chromosomal *lacZ* gene to give *lac*$^-$ cells. A way to avoid the problem with the phage would be to engineer a strain to propagate pCD11LAC that had *pir* inserted

on the chromosome instead of using a strain that was a lambda-pir lysogen. This has recently been done by Metcalf et al. (1994).

Many successful suicide vectors have been constructed that exploit the unique qualities of the R6K origin. An R6K based mobilizable suicide plasmid has been shown to function well as a vector for gene replacement in Rhodobacter sphaeroides (Penfold and Pemberton, 1992) and Skrzypek et al. (1993) transformed Yersiniae pestis with a suicide vector similar to pCD11 and were able to replace genes on the chromosome. However, our work is the first to show that an R6K based suicide plasmid is suitable for making changes to the E. coli genome. Our work also supports the view of Skrzypek et al. that an R6K based suicide plasmid would work in many different types of gram negative bacteria.

Our linear DNA suicide vector did give stable integrants, but it was not very efficient compared to pCD11LAC (which was 100 times more efficient at integrating into the genome). As with our origin deficient plasmid, this reduction could have been due to a low yield of DNA after cutting with restriction enzymes. However, every effort was taken to increase the amount of DNA used to electroporate the cells.

These data represent the first successful use of linear DNA to transform a non-rec mutant. However, it is inconclusive as to whether Chi sites were responsible for this success. In any case, if Chi is responsible for the success

of these vectors due its attenuation of the exonuclease activity of RecBCD, then the transformation efficiencies of linear DNA containing Chi would be expected to be close to that received from transforming linear DNA into a RecBCD mutant. Russell et al. (1989) linearized plasmids and transformed them into recD strains of E. coli. He obtained approximately 10^3 times more transformants using 10^3 times less DNA than was used in our linear DNA experiments. As mentioned earlier, if Chi is really attenuating the exonuclease activity of RecBCD and increasing recombination at Chi, then we would expect to obtain at least as many recombinants with our Chi vector as Russell obtained using recD mutants. More work would is required in order to determine why such discrepancies occurred, but differences may be due to the fact that Russell was not using the same strain of E. coli as was used in our experiments and that he had a larger region of homology between his linearized plasmid and the chromosome. Furthermore, he was not making changes in the same operon or region of the E. coli chromosome. All of these factors could be responsible for the discrepancies seen between the two experiments. Only experiments using similar strains and insertional targets will be able to give further insight.

From the results of these experiments, it can be concluded that of the methods tested, pCD11LAC was the most efficient in integrating into the chromosome. The high number of transformants can likely be attributed to the fact that

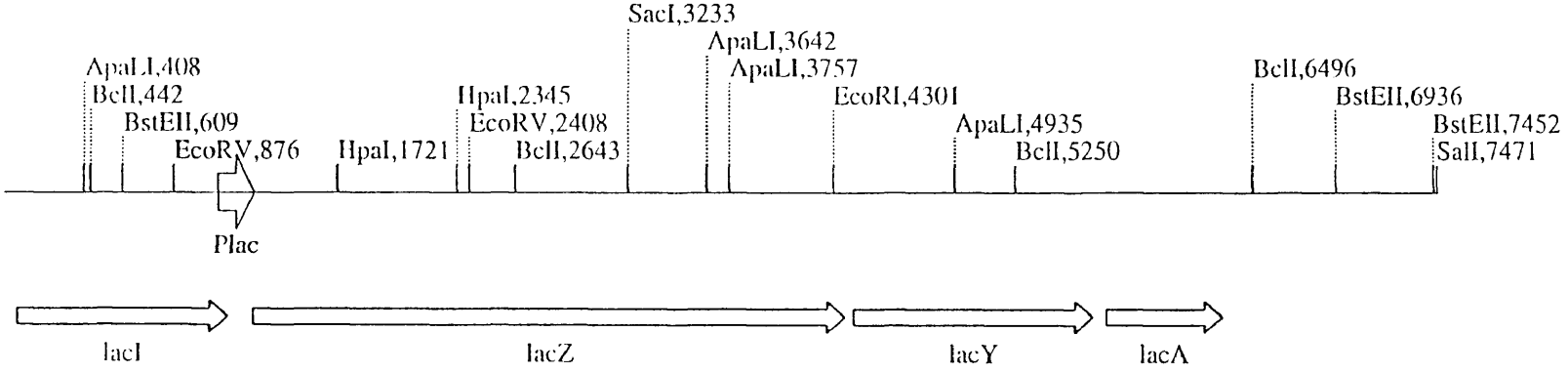
pCD11LAC is an intact supercoiled plasmid. Also, with the pCD11LAC plasmid, the amount of DNA that one uses for transformation is not limited by the amount of DNA one can ligate, cut, or supercoil. Our linear DNA suicide element and pALTERSSLAC were affected by these limitations.

Linear DNA sucide elements have qualities that make them valuable to use for insertional mutations, especially when transforming with suicide plasmids has been unsuccessful. First, a linear DNA suicide element can be more useful than suicide plasmids when one would like to select only for double crossovers between the suicide element and the chromosome. This advantage is due to the fact that linear DNA can only recombine into the chromosome with a double crossover while suicide plasmids can recombine with a double or single crossover. Plasmids that recombine by a single crossover insert the entire sequence of the plasmid into the chromosome and in some cases, this result is undesirable. Furthermore, the scheme to select for double crossovers between suicide plasmids and the chromosome may be complicated. Therefore, if one wishes to have only double crossovers, using a linear DNA suicide element is often preferable to using a suicide plasmid.

A goal of future experiments would be to determine why such low numbers of transformants were obtained using the linear DNA suicide vector and whether the transformants obtained were definitely due to the presence of Chi sites.

Linear DNA that had one or both Chi sites removed, but left the region of homology with the chromosome intact would be good controls for this experiment. Also, mutated Chi sites would function as an excellent control. If these controls yielded fewer transformants than an intact vector with two functional Chi sites, then this would support the view that Chi sites are responsible for integration of linear DNA into the chromosome of non-rec bacteria. It would also be of interest to transform a recD E. coli strain with the various linear DNA constructs to compare the number of transformants received with the number received from transforming BN102. As mentioned earlier, pCD11 could be improved by changing the strain in which it was propagated and pALTERSSLAC could be tested again after supercoiling with topoisomerase. These few experiments would give insight into the mechanisms of linear DNA transformation and would help to improve the two suicide plasmids.

APPENDIX



(7476 bps)

FIGURE 1

THE LAC OPERON

FIGURE 2

pALTERSS

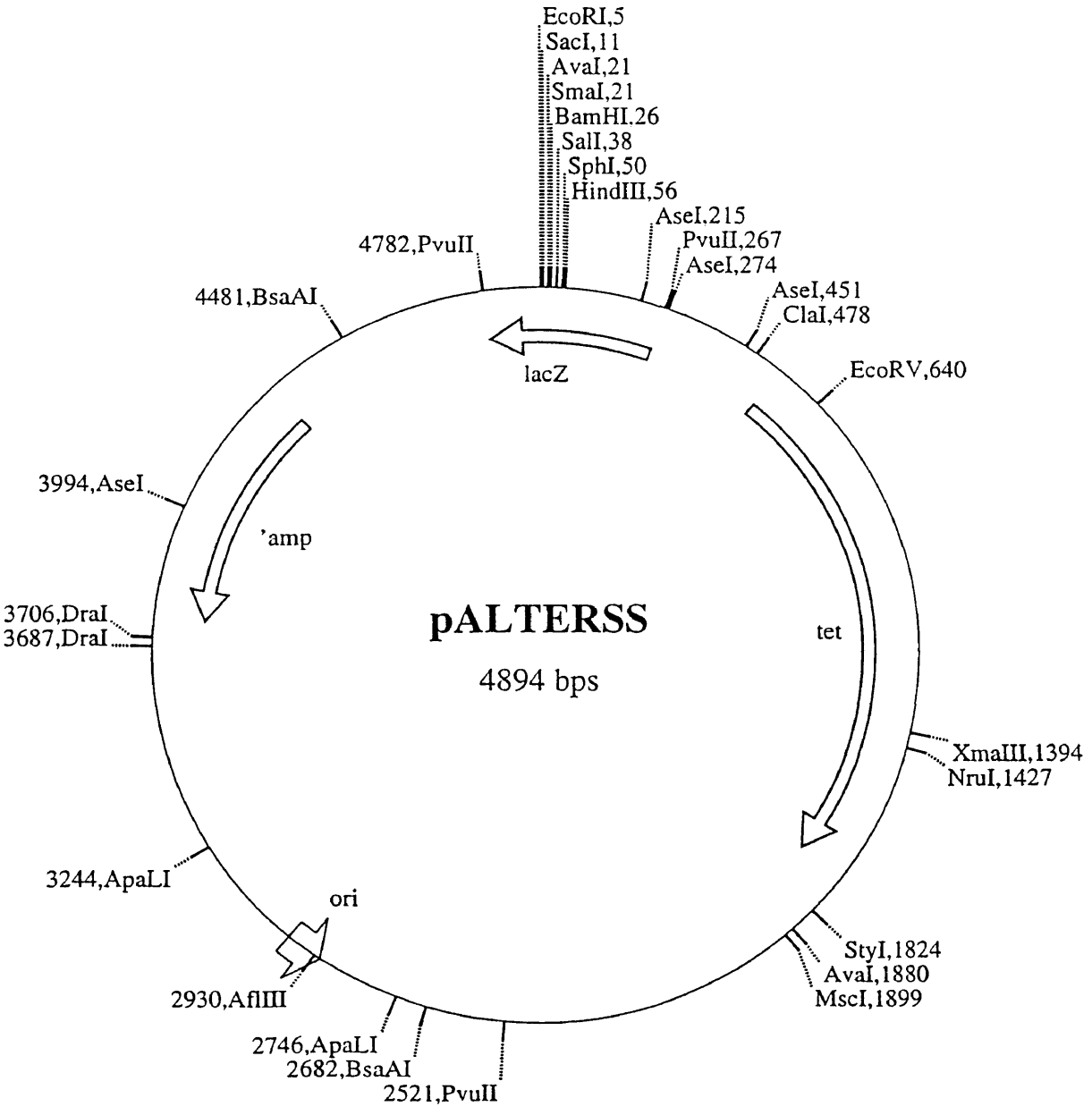


FIGURE 3

pALTERSSLAC

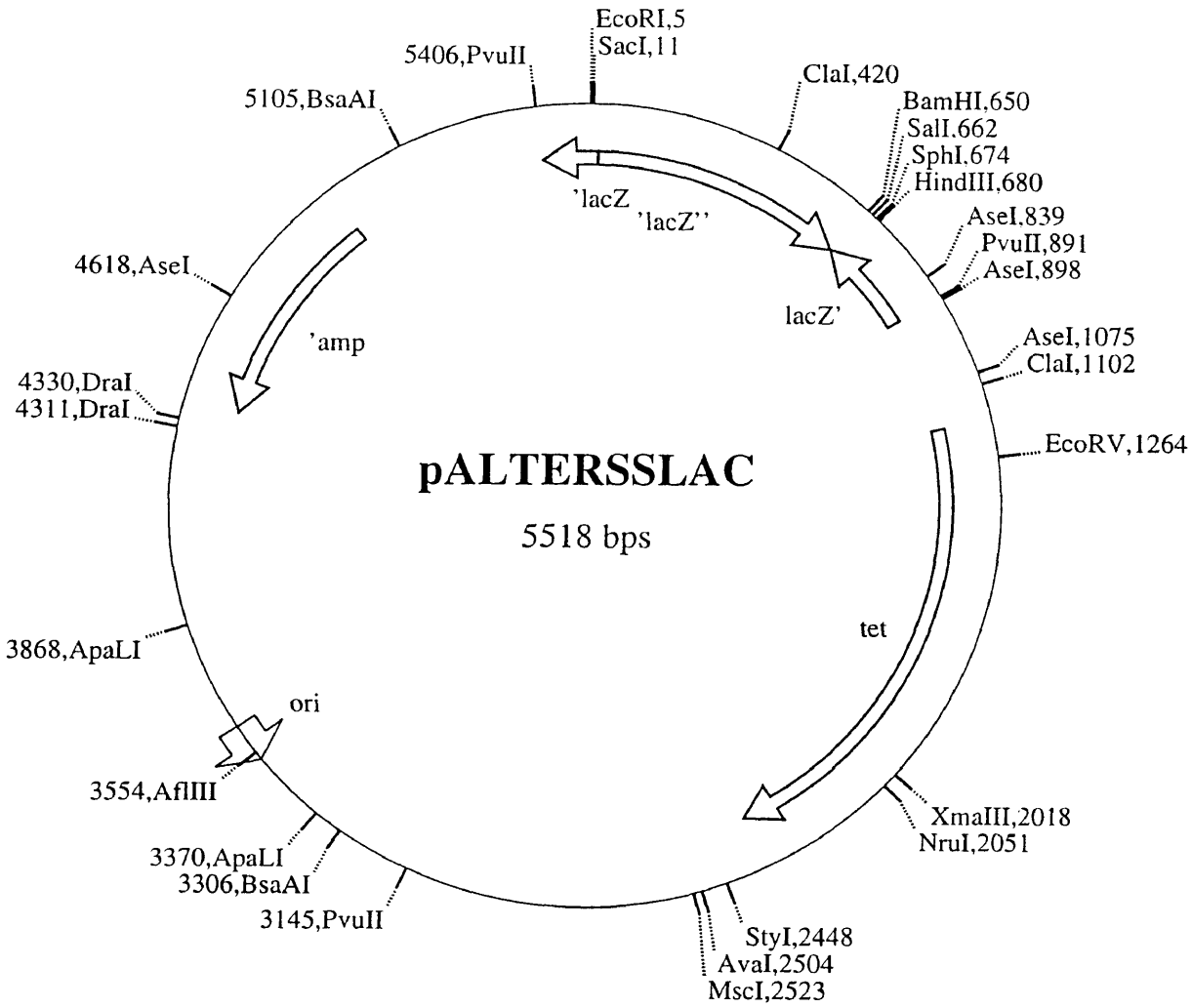


FIGURE 4

pMC1871

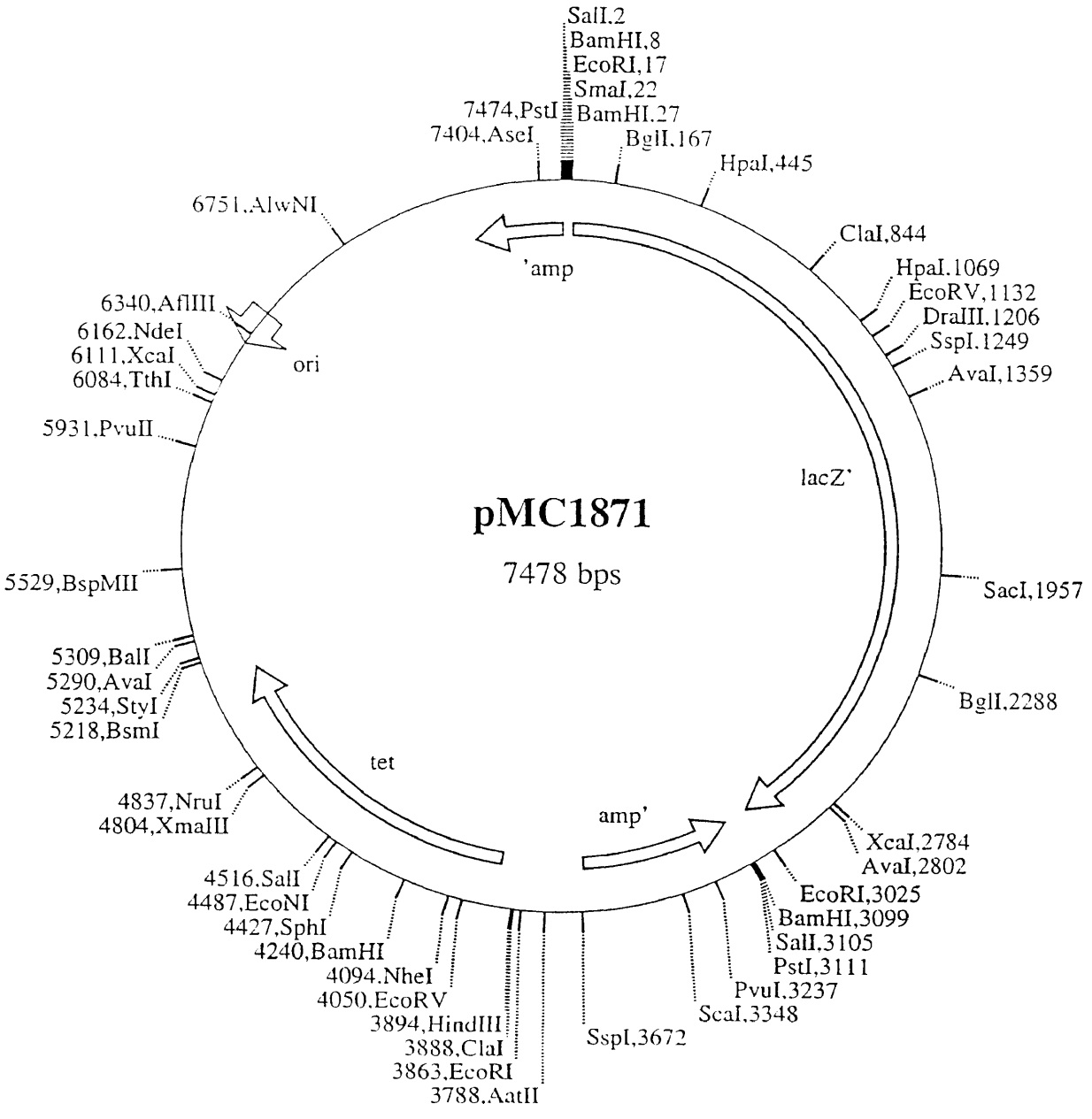


FIGURE 5

pBR322

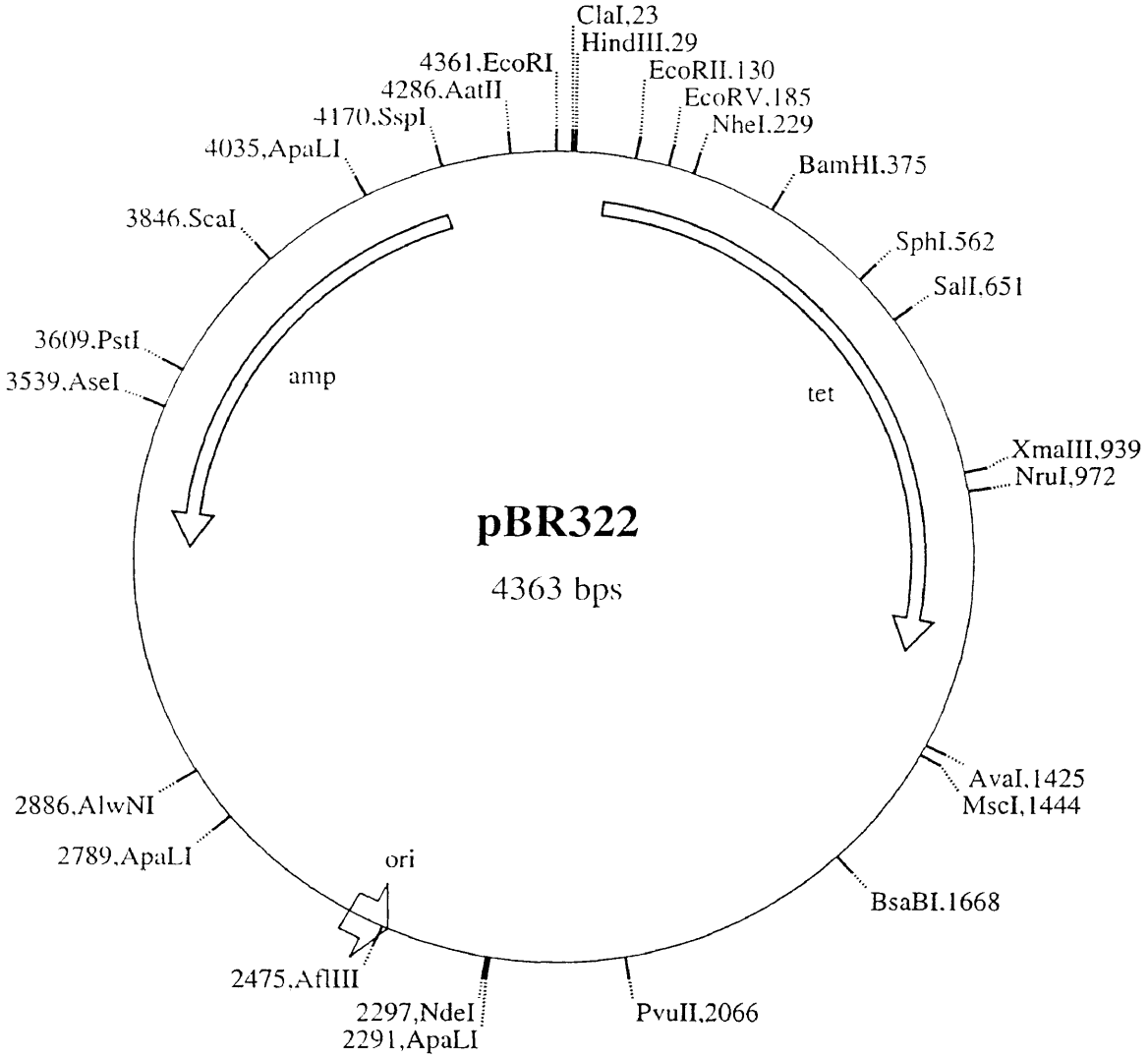


FIGURE 6

pCD11

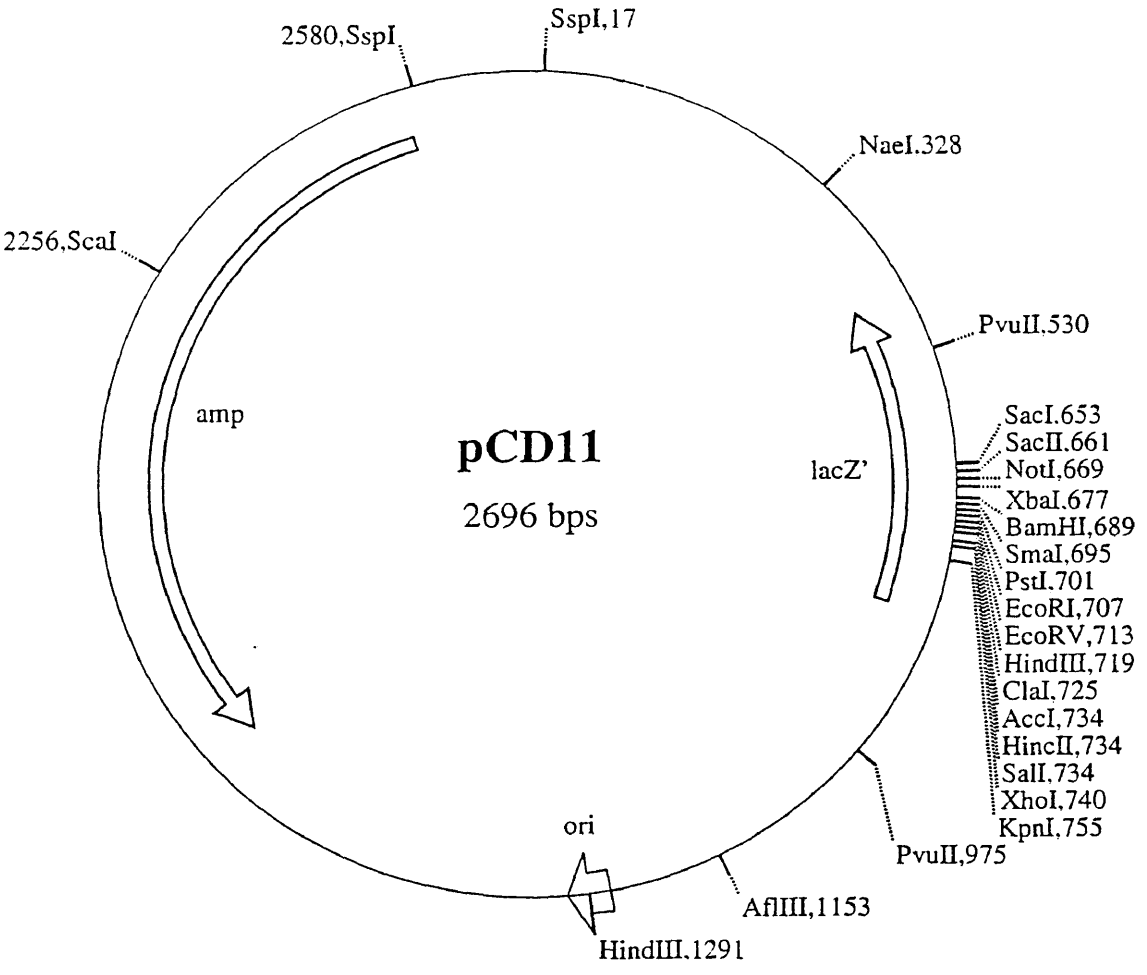


FIGURE 7

pCD11LAC

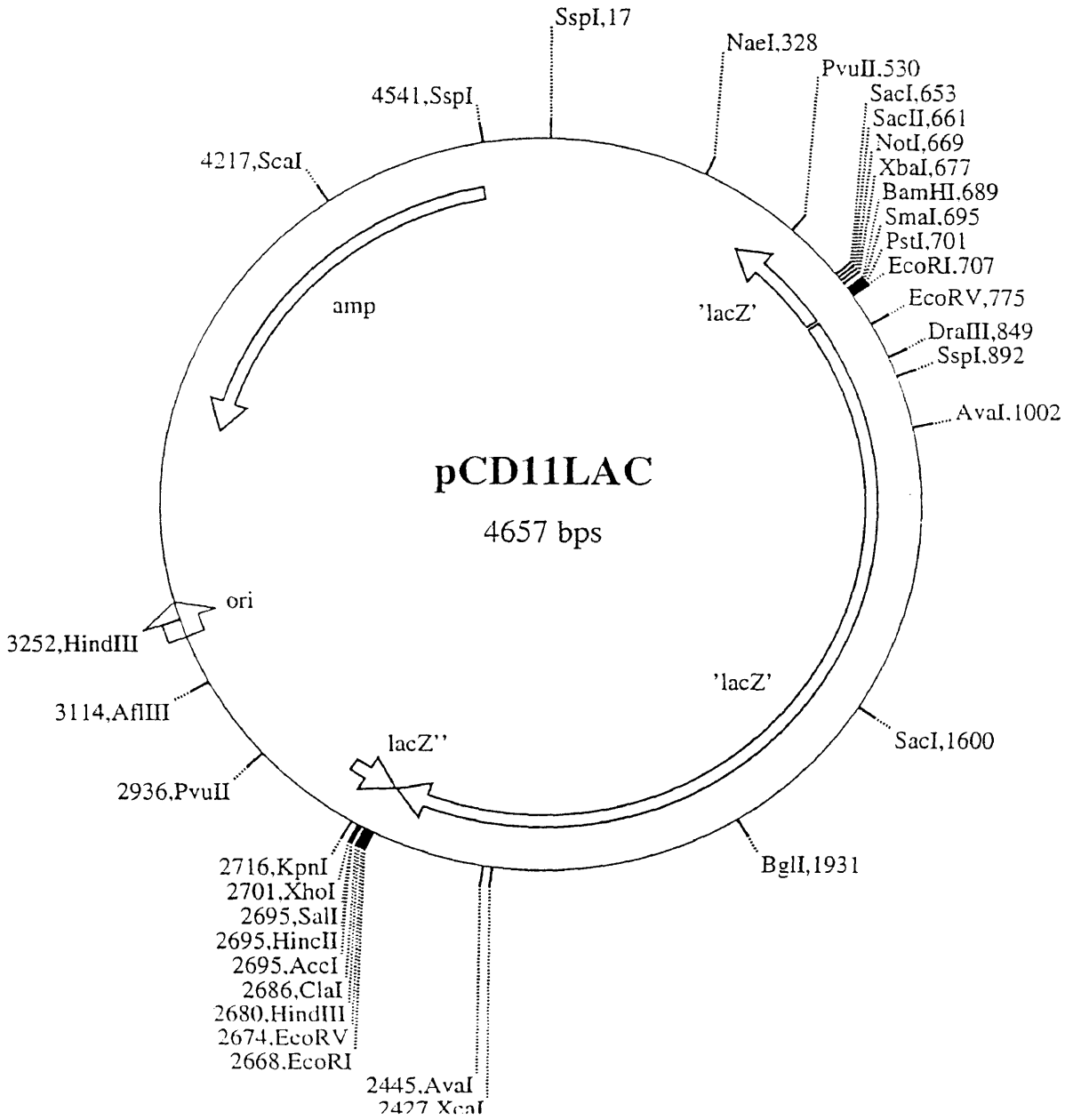


FIGURE 8

pKSLACZKAN

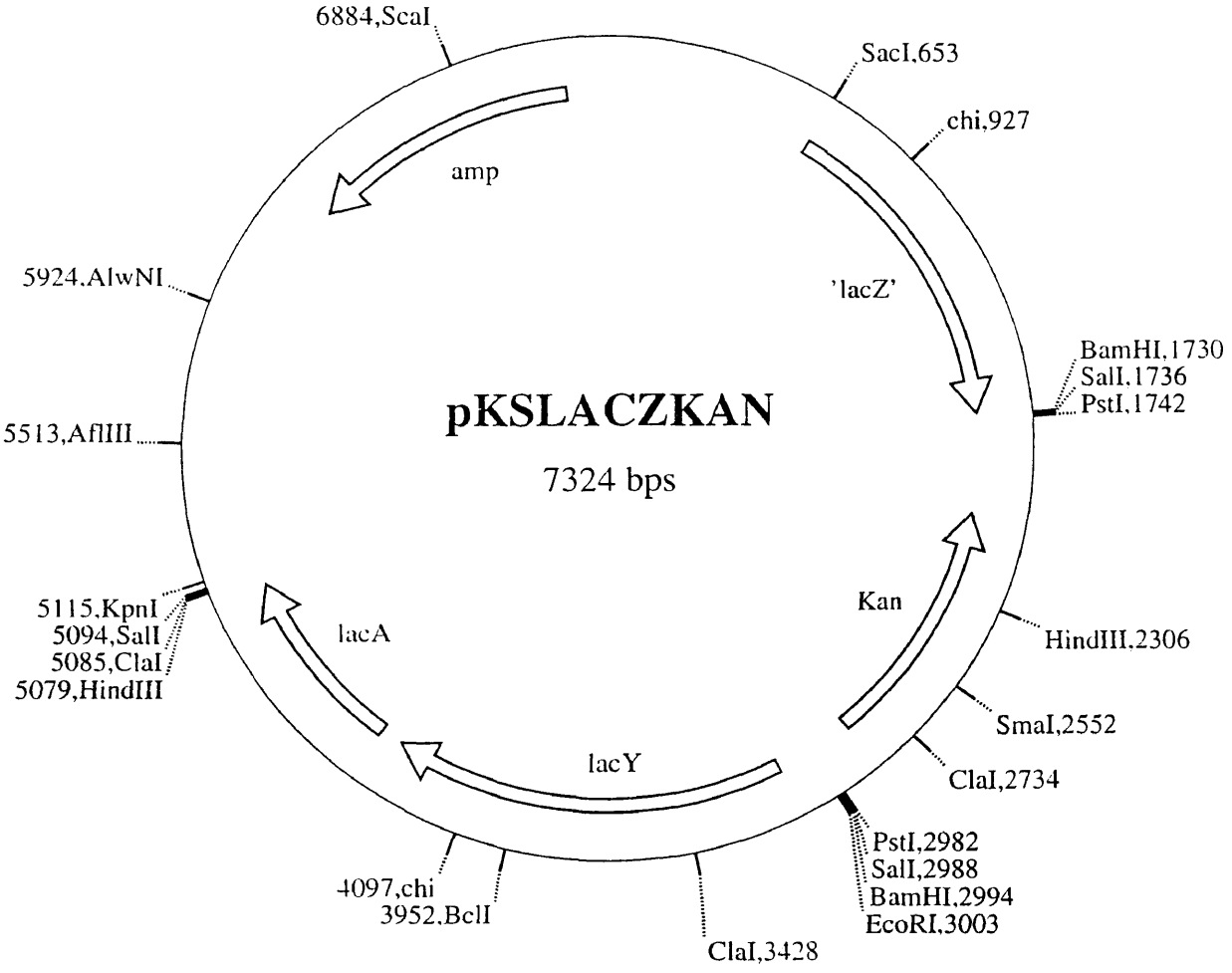
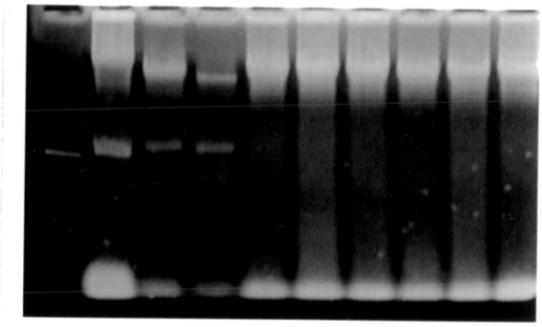


FIGURE 9

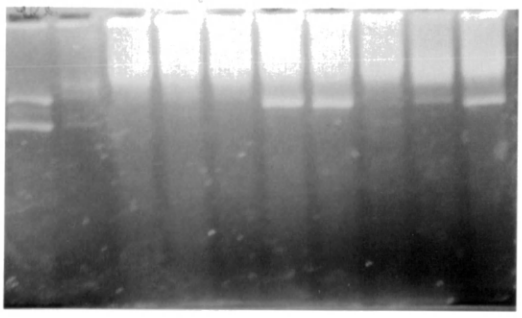
RAPID SCREENS OF TRANSFORMANTS

Rapid Screen of pCD11LAC Recombinants:



Key:
Lane 1 - pCD11LAC plasmid prep, Lane 2 - Rapid screen of S17-Lambda pir/pCD11LAC, Lanes 3-4 - Rapid screens of blue BN102/pCD11LAC transformants, Lanes 5-10 - Rapid screens of white BN102/pCD11LAC transformants

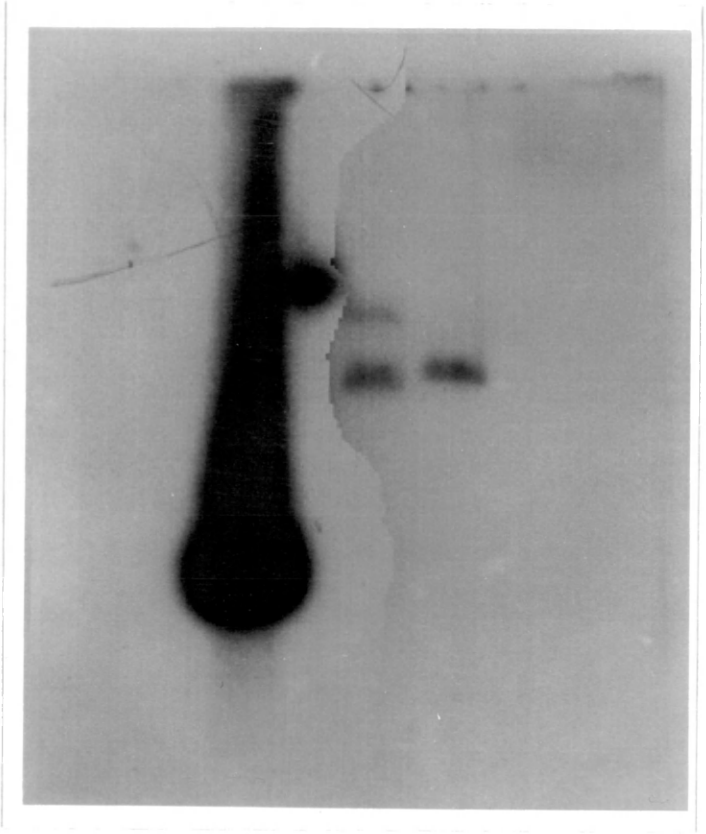
Rapid Screen of Linear DNA Vector Recombinants:



Key:
Lane 1 - pKSLACZKAN plasmid prep, Lane 2 - Rapid screen of NM522/pKSLACZKAN transformant, Lane 3-5 - Rapid screens of white BN102/Linear DNA Vector transformants, Lanes 6-7 - Rapid screens of blue BN102/Linear DNA Vector transformants, Lane 8 - Rapid screen of white BN102/Linear DNA Vector transformant, Lanes 9-10 - Rapid screen of Blue BN102/Linear DNA Vector transformants

FIGURE 10

SOUTHERN BLOT OF TRANSFORMANTS



Lane 1 - pCD11LAC EcoRI digest, Lane 2 - BN102/pCD11LAC (white transformant) genomic BstEII digest, Lane 3 - BN102 genomic BstEII digest, Lane 4 - BN102/Linear DNA Suicide Vector (white transformant) genomic BstEII digest
(Note: this photograph is a combination of two different exposures of the southern blot.)

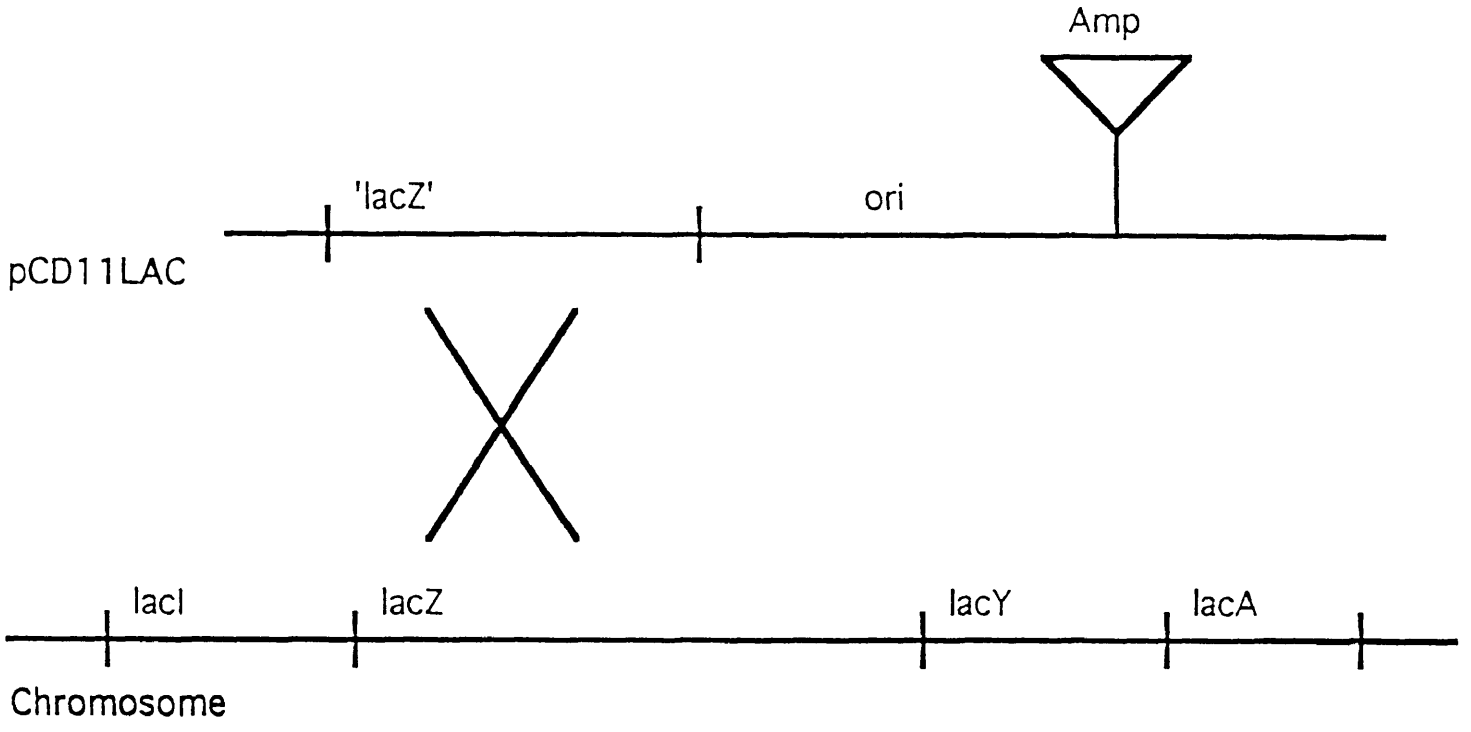


FIGURE 11

RECOMBINATION OF THE R6K BASED SUICIDE PLASMID pCD11LAC

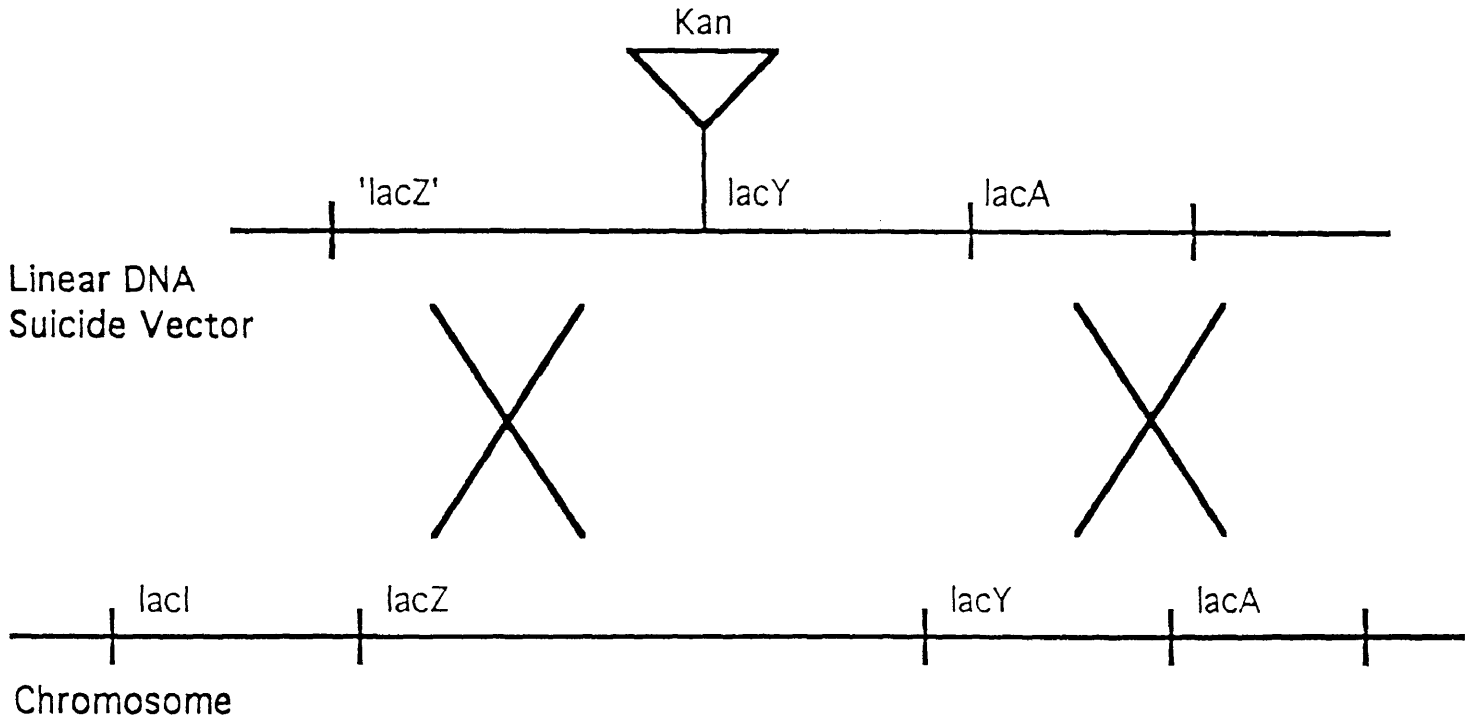


FIGURE 12

RECOMBINATION OF THE LINEAR DNA SUICIDE ELEMENT

BASED ON pKSLACZKAN

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