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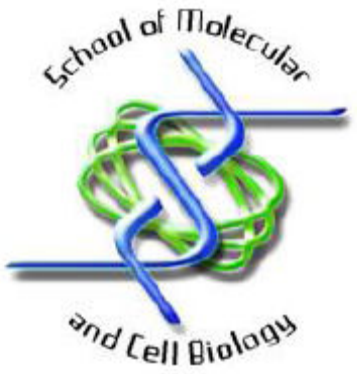
Development of improved cloning vectors for Bacillus and Staphylococcus species

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DEVELOPMENT OF IMPROVED CLONING VECTORS FOR *BACILLUS* AND *STAPHYLOCOCCUS* SPECIES

MSc DISSERTATION

BY

DUDUZILE EDITH NDWANDWE

Submitted in fulfillment of the requirements for the Degree Master of Science in the Faculty of Science, School of Molecular and Cell Biology at the University of the Witwatersrand

DECLARATION

I hereby declare that this research report is my own, unaided work. It is being submitted in fulfillment of the Degree of Masters of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Signature ……..

Date……...

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DEDICATIONS

This work is dedicated to my family at large but most importantly to my late brother Sibusiso Ndwandwe, my mother Lettie Mhlanga, my father Derrick Ndwandwe, my grandmother Ellen Mhlanga, and my younger brother Thabani Ndwandwe.

ABSTRACT

A shuttle vector was constructed which was stably maintained in *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. It was made by ligating *E. coli* positive selection plasmid pEcoR251 and *S. aureus* resistance plasmid pC194 via their respective *Bam*HI and *Xho*II sites. Designated pNDW1, it was shown to be effective in genomic library construction. The number of restriction sites in the *EcoR*I ("suicide") gene was increased by successive addition of *Xba*I and *Xho*I using site-directed mutagenesis. *Spe*I, *Nhe*I and *Avr*II generate DNA fragments with compatible cohesive ends to *Xba*I while *Sal*I digestion gives rise to ends compatible with *Xho*I. Therefore the number of different genomic libraries which can be made using this system is augmented by six. A principal impediment to full exploitation of these shuttle vectors is apparently the severe restriction by *B. subtilis* and *S. aureus* of DNA coming from *E. coli*.

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ABBREVIATIONS

˚C	Degrees Celsius
µg	Microgram
µl	Microliter
bp	Basepair
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylene diamine tetra acetic acid
g	Gram
K_2HPO_4	Dipotassium hydrogen Phosphate
kb	Kilobase
KH_2PO_4	Potassium dihydrogen phosphate
M	Molar
mg	milligram
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Millimolar
ng	Nanogram
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
RNA	Ribonucleic acid
rpm	Revolutions per minutes
SDS	Sodium dodecyl sulphate
TBE	Tris, Boric Acid, EDTA
TE	Tris, EDTA
λ	Lambda

1. INTRODUCTION

1.1 The genus *Bacillus*

Bacillus subtilis is a rod-shaped, spore-forming, non-pathogenic Gram-positive bacterium found in soil and vegetation. They can contaminate food, however, seldom result in food poisoning. They are used on vegetable and soybean seeds as fungicide. Some strains cause rots on potatoes and ropiness in bread. *Bacillus thuringiensis* is a bacterium in the same genus known to be used in insect control. *Bacillus cereus* and *Bacillus licheniformis* can cause food poisoning. *Bacillus anthracis* causes anthrax, an acute and sometimes lethal disease of humans and animals. It has been identified as a potential bioterrorism attack agent (Hughes and Gerberding, 2002).

Bacillus species produce a wide range of hydrolytic enzymes that breakdown complex polymers such as polysaccharides, nucleic acids and lipids, permitting the organism to use these products as carbon sources and electron donors. Many *Bacillus* species produce antibiotics, of which bacitracin, polymyxin, tyrocidine, gramicidin and circulin are examples. Strains of *Bacillus polymyxa* biosynthesize a number of useful and potentially useful compound, including peptide antibiotics, proteases, and a wide variety of carbohydrates-utilizing enzymes, such as β-amylase, β-D-xylanases, pullulanase, glucose isomerase and polygalacturonate lyase (Malloonee and Speckman, 1989). Frequently these enzymes are secreted making them easier to purify. *B. subtilis* has proven highly amenable to genetic manipulation, and has therefore become widely adopted as a model organism for laboratory studies, especially of sporulation, which is a simplified example of cellular differentiation In terms of popularity as a laboratory model organism *B. subtilis* is often used as the Gram-positive equivalent of *Escherichia coli*, an extensively studied Gram-negative rod.

1.2 *Staphylococcus* Spp.

Taxonomically, the genus *Staphylococcus* is in the bacterial family *Staphylococcaceae,* which includes three lesser known genera, *Gamella, Macrococcus* and *Salinicoccus.* The *Listeriaceae* are also a closely related family. *Staphylococcus aureus* forms a fairly large yellow colony on rich medium. *S. epidermidis* has a relatively small white colony. *S. aureus* is often hemolytic on blood agar; *S. epidermidis* is non hemolytic. Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid. The bacteria are catalase-positive and oxidase-negative. *S. aureus* can grow at a temperature range of 15 to 45 degrees and at sodium chloride concentrations as high as 15 percent. Nearly all strains of *S. aureus* produce the enzyme coagulase: nearly all strains of *S. epidermidis* lack this enzyme. *S. aureus* should always be considered a potential pathogen; most strains of *S. epidermidis* are nonpathogenic and may even play a protective role in their host as normal flora. *Staphylococcus epidermidis* may be a pathogen in the hospital environment (Madigan *et al*., 2003). Staphylococci are perfectly spherical cells about 1 micrometer in diameter. They grow in clusters because staphylococci divide in two planes. The configuration of the cocci helps to distinguish staphylococci from streptococci, which are slightly oblong cells that usually grow in chains (because they divide in one plane only). The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci, which are vigorous catalase-producers. The test is performed by adding 3% hydrogen peroxide to a colony on an agar plate or slant. Catalase-positive cultures produce O_2 and bubble at once. The test should not be done on blood agar because blood itself contains catalase (Todar, 2005).

Staphylococcus aureus causes a variety of suppurative (pus-forming) infections and toxicoses in humans. It causes superficial skin lesions such as boils, styes and furunculosis; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis (Madigan *et al*., 2003). *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* causes food poisoning by releasing enterotoxins into food, and

toxic shock syndrome by release of super antigens into the blood stream. *S. aureus* expresses many potential virulence factors: (1) surface proteins that promote colonization of host tissues; (2) invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase); (3) surface factors that inhibit phagocytic engulfment (capsule, Protein A); (4) biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production); (5) immunological disguises (Protein A, coagulase, clotting factor); and (6) membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin; (7) exotoxins that damage host tissues or otherwise provoke symptoms of disease; (8) inherent and acquired resistance to antimicrobial agents.

For the majority of diseases caused by *S. aureus*, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor. However, there are correlations between strains isolated from particular diseases and expression of particular virulence determinants, which suggests their role in a particular disease. The application of molecular biology has led to advances in unraveling the pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced, and many protein toxins have been purified. With some staphylococcal toxins, symptoms of a human disease can be reproduced in animals with the purified protein toxins, lending an understanding of their mechanism of action (Todar, 2005).

Hospital strains of *S. aureus* are usually resistant to a variety of different antibiotics. A few strains are resistant to all clinically useful antibiotics except vancomycin, and vancomycin-resistant strains are increasingly-reported. The term MRSA refers to Methicillin resistant *Staphylococcus aureus*. Methicillin resistance is widespread and most methicillin-resistant strains are also multiple resistant. In addition, *S. aureus* exhibits resistance to antiseptics and disinfectants, such as quaternary ammonium compounds, which may aid its survival in the hospital environment. *S. aureus* responded to the introduction of antibiotics by the usual bacterial means to develop drug resistance: (1) mutation in chromosomal genes followed by selection of resistant strains and (2) acquisition of resistance genes as extra-chromosomal plasmids, transducing particles,

transposons, or other types of DNA inserts. *S. aureus* expresses its resistance to drugs and antibiotics through a variety of mechanisms.

1.3 Plasmids

Plasmids represent an important factor in bacterial evolution. They enable rapid short-term adaptation of the bacterial host to changing environmental conditions and allow amplification of gene by transferring them within one or between many species. Normally, plasmids are nonessential to their hosts, conferring only an energy burden that can slow cell growth. They can, however, be stably maintained in a bacterial population even under non selective conditions. Copy number of a plasmid is maintained by regulation of replication. Low copy number plasmid requires a tighter regulation of regulation and segregation than the high copy number (Kües and Stahl, 1989).

1.4 *Staphylococcus aureus* plasmids

Many small plasmids conferring resistance to different antibiotics have been isolated from *Staphylococcus aureus* (te Riele *et al*., 1986). Since the discovery of staphylococcal plasmids in the early 1960s, three general classes have been identified and characterized. The first *S. aureus* plasmids discovered were of class II. These are of intermediate size and copy number; they encode a combination of β-lactamase and inorganic ion resistances, and they occur in *S. aureus* plus various other species such as *Staphylococcus xylosus* and *Staphylococcus simulans*. Plasmids of class I were discovered shortly afterward. These are of small size (1-5 kb) and high copy number (15-50 copies per cell); they usually encode a single antibiotic resistance, and they occur in many staphylococcal species. Most recently, a number of larger (30-60 kb) plasmids carrying multiple antibiotic resistances have been identified (Novick, 1989).

Class II plasmids are larger (15-30Kb), have a lower copy numbers and carry some combination of resistance to β-lactam antibiotics (β-lactamase), macrolides and a variety

of heavy metal ions such as arsenic, cadmium, lead and mercury (Novick and Roth, 1968). Some of these plasmids are reported to be transposable. Class III consists of a considerably larger (30-60Kb) plasmids that carry a determinant of conjugative transfer, *tra*, plus some combination of resistance markers including gentamycin and penicillin Qa, some are transposable and contain a number of insertion sequence like elements (Gray, 1983). Staphylococcal plasmids are of particular importance in the study of antibiotic resistance of *S. aureus* because using plasmids isolated from this organism might help understand the how these resistant determinants are being transferred to other species and to other genera.

1.5 Plasmid pC194

Plasmid pC194 is one of the several small (2.9kb in size) found in *S. aureus* reported by Iordanescu *et al*. (1978). Plasmid pC194 specifies chloramphenicol-induced resistance to chloramphenicol mediated by the enzyme chloramphenicol acetyl-transferase (CAT), an enzyme that inactivates chloramphenicol by converting it successively to the inactive 3-acetyl and 1, 3-diacetyl derivatives. It has served as a useful vector for analytical cloning of determinants of inducible resistance and replication in conjugation studies of another small plasmid, pE194 (Horinouchi and Weisblum, 1982). It can also serve as a useful system for studies of gene expression control mechanism since the synthesis of CAT appears to be under autogenous control. Plasmid pC194 is of interest because it can replicate in a wide variety of bacterial hosts (Goze and Ehrlich, 1980). It was also found to be maintained in the yeast *Saccharomyces cerevisiae* (Goursot *et al*., 1982). This maintenance of these plasmids in different bacterial species might be attributed to horizontal gene transfer as a normal phenomenon.

1.6 Restriction modification system

The restriction modification system (RM system) is used by bacteria, and perhaps other prokaryotic organisms to protect themselves from foreign DNA, such as bacteriophages. This phenomenon was first noticed in the 1950s. Certain bacteria strains were found to

inhibit (restrict) the growth of viruses grown in other strains. This effect was attributed to sequence-specific restriction enzymes. Bacteria have restriction enzymes, also called restriction endonucleases, which cleave double stranded DNA at specific points into fragments, which are then degraded further by other endonucleases. This prevents infection by effectively destroying the foreign DNA introduced by an infectious agent such as a bacteriophage (**Figure 1.1 B**). Approximately one quarter of known bacteria possess RM systems and of those about one half has more than one type of system (Wilson, 1991a; Wilson, 1991b).

Restriction enzymes only cleave at specific sequences of DNA which are usually 4-6 base pairs long, and often palindromic. Given that the sequences that the restriction enzymes recognize are very short, the bacterium itself will almost certainly have many of these sequences present in its own DNA. Therefore, in order to prevent destruction of its own DNA by the restriction enzymes, the bacterium marks its own DNA by adding methyl groups to it. This modification must not interfere with the DNA base-pairing, and therefore, usually only a few specific bases are modified on each strand (Wilson, 1991b).

A restriction endonuclease recognizes a specific DNA sequence and introduce double-stranded break and the modification enzyme recognizes the same sequence and protects it from the restriction enzyme by methylation **Figure 1.1 A**. It has been hypothesized that several restriction-modification gene complexes in bacteria are not easily replaced by competitor genetic elements because their loss leads to cell death (Naito *et al*., 1995; Handa *et al*., 2001; **Figure. 1C**). This finding led to the proposal that these complexes may actually represent one of the simplest forms of life, similar to viruses, transposons, and homing endonucleases. This selfish gene hypothesis (Naito *et al*., 1995; Kusano *et al.,* 1995; Kobayashi, 2001) is now supported by many lines of evidence from genome analysis and experimentation. After loss of the restriction-modification gene complex, the restriction enzyme and modification enzyme will become increasingly diluted through cell division. Finally, too few modification enzyme molecules remain to defend all the recognition sites present on the newly replicated chromosomes. Any one of the remaining molecules of the restriction enzyme can attack these exposed sites. The chromosome

breakage then leads to extensive chromosome degradation, and the cell dies unless the breakage is somehow repaired. The chromosome breakage may stimulate recombination and generate a variety of rearranged genomes, some of which might survive. The principle of post-segregational killing once established in a cell, the addiction gene complex is difficult to eliminate because its loss, or some sort of threat to its persistence, leads to cell death. Intact copies of the gene complex survive in the other cells of the clone.

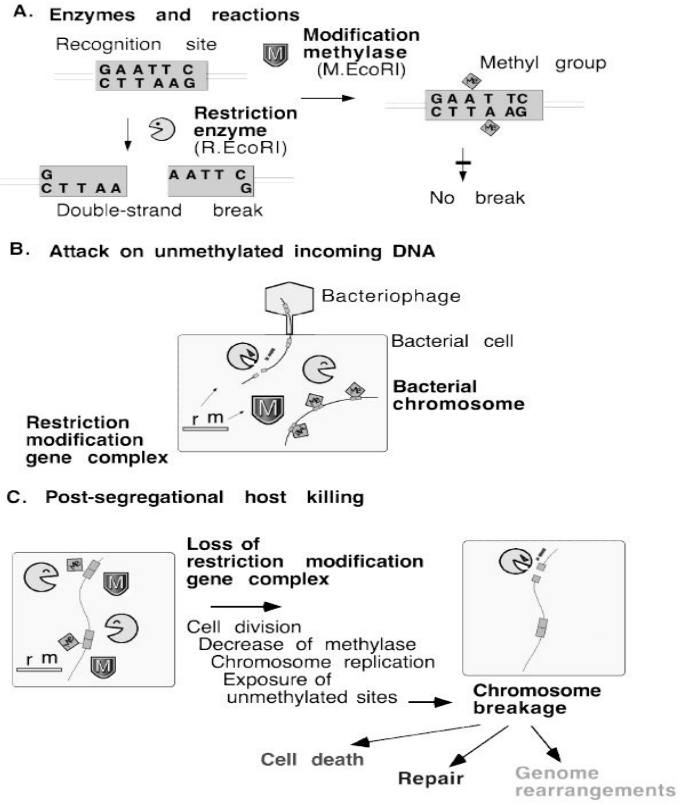


Figure 1.1 Represents the action of a restriction-modification gene complex (Adapted from Kobayashi, 2004). **A** Restriction enzyme (Endonuclease) cleaves unmethylated DNA and modification methyltransferase protects the DNA from cleavage. **B** Attack on incoming DNA. An attack on invading DNA that is not appropriately methylated is likely to be beneficial to the restriction-modification gene complex and to its host. **C** A simple dilution model for post-segregational killing.

1.7 Types of Restriction Modification Systems

There are three kinds of restriction modification systems: type I, type II and type III, all with restriction enzyme activity and a methylase activity. They were named in the order of discovery, although the type II system is the most common. Type I systems are the most complex, consisting of three polypeptides: R (restriction), M (modification), and S (specificity). The resulting complex can both cleave and methylate DNA. Both reactions require ATP, and cleavage often occurs a considerable distance from the recognition site. The S subunit determines the specificity of both restriction and methylation. Cleavage occurs at variable distances from the recognition sequence, so discrete bands are not easily visualized by gel electrophoresis (Wilson and Murray, 1991). Type II systems are the simplest and the most prevalent. Instead of working as a complex, the methyltransferase and endonuclease are encoded as two separate proteins and act independently (there is no specificity protein). Both proteins recognize the same recognition site, and therefore compete for activity. The methyltransferase acts as a monomer, methylating the duplex one strand at a time. The endonuclease acts as a homodimer, which facilitates the cleavage of both strands. Cleavage occurs at a defined position close to or within the recognition sequence, thus producing discrete fragments during gel electrophoresis. For this reason, Type II systems are used in labs for DNA analysis and gene cloning. Type III systems have R and M proteins that form a complex of modification and cleavage. The M protein, however, can methylate on its own. Methylation also only occurs on one strand of the DNA unlike most other known mechanisms. The heterodimer formed by the R and M proteins competes with itself by modifying and restricting the same reaction. This results in incomplete digestion (Wilson and Murray, 1991).

A hypothesis was proposed that the certain type II RM systems may represent selfish genetic elements in the sense that they can maintain and increase their copy number even when they do not confer any advantageous phenotype on their host cells (Kobayashi, 2001; Naito *et al*., 1995). This hypothesis was based on the observation that certain type II RM systems on a plasmid can increase stability of a plasmid by selectively killing cells

that failed to retain the plasmid, thereby causing an increase in their relative frequency in the viable bacterial population. Analysis of bacterial genomes provided evidence that RM systems can move between bacterial genomes, which seems to be consistent with the behavior as selfish DNA elements (Kobayashi, 2001; Naderer *et al*., 2002; Nobusato *et al.*, 2000). The selfish hypothesis was given strong support when the RM system on a chromosome was found to multiply in tandem in a manner dependent on a functional restriction gene (Sadykov *et al*., 2003). This was reminiscent of the induction of the replication of prophage genomes. Type II RM systems show similarities to viruses in their regulation of gene expression (Kobayashi, 2004). When they enter a new host, they have to establish themselves in the host without excessive killing the host cells, just like a temperate bacteriophage establish themselves in the host cells as prophages. It is postulated that the RM gene system express the modification activity before restriction activity to protect the host chromosome by methylation. The methyl-transferases of type II RM systems were shown to function as transcriptional regulators required for coordinated expression of the restriction and the modification enzyme (Som and Friedman, 1993).

In some type II RM systems, a third regulatory protein, called C protein, plays the role of delaying expression of their restriction enzymes (Nakayama and Kobayashi, 1998; Tao *et al*., 1991). After establishment, the type II RM system are expected to tight regulate their gene expression to maintain constant cellular levels of restriction enzyme and modification enzyme to prevent attack on the host, just as a prophages do until critical events such as gene loss happens to trigger the attack. Type II RM systems are of particular importance because the plasmid used in this study has an *EcoR*I endonuclease gene of the EcoRI RM system. The EcoRI system is composed of the *ecoRIR* (R) and *ecoRIM* (M) genes, which encode EcoRI R and M proteins, respectively (Liu *et al*., 2007). The R gene is located upstream of the M gene. It has been proposed that the R and M genes constitutes an operon, in which expression of the two genes is coordinately controlled by a promoter located immediately upstream of the R gene (Liu *et al*., 2007). In addition, a specific promoter for the M gene has been proposed to be present within the R. gene. This postulated M gene-specific promoter, which should allow expression of

the M gene in the absence of the R gene, might play a role for sequential expression of the modification activity and restriction activity when the *EcoR*I gene system enters a new host cell (Liu *et al*., 2007).

1.8 Positive selection vectors

Non-recombinant transformants in DNA cloning experiments are a problem that is especially troublesome when the relative amount of target DNA is low or when the percentage of recombinants must be maximized, as when constructing a genomic library. Several approaches have been taken to resolve this problem such as treatment of the vector with phosphatase prior ligation and cutting the vector with restriction enzymes that produce ends that are non-complementary so that the inserts will have the complementary ends that could easily ligate to the vector. These methods have disadvantages of being subjected to the variable efficiencies of the enzymatic reactions and purifications used to prepare the vector for cloning. A number of positive selection vectors have been generated which employ genetic means to eliminate transformants carrying religated or uncut vector. Such vectors typically rely upon the inactivation of a lethal gene, inactivation of a dominant function conferring cell sensitivity to metabolites, removal of a lethal site or the depression of an antibiotic-resistance function. Positive selection vectors have the advantages of being simpler to use, more efficient, and more reliable than biochemical methods for the selection of recombinants (Kuhn, 1986).

These plasmids have proven useful for the selection and expression of a number of genes from a variety of organism. A number of positive-selection vectors have been constructed for example Cheng and Modrich (1983) have constructed a plasmid that has a functional endonuclease gene and a non functional modification gene. This plasmid is under the control of the P_L promoter of the bacteriophage lambda (λ). When this plasmid is introduced in a host that has a functional methyltransferase activity the endonuclease activity is inhibited. If it is introduced into a lambda lysogen the endonuclease gene is controlled and insertion of DNA into the unique restriction sites on the endonuclease gene abolishes the endonuclease activity (Cheng and Modrich, 1983).

Kuhn et al. (1986) have also constructed a series of positive selection plasmid vectors. The principle of selection in these plasmids is based on the separation of the EcoRI restriction and modification functions. The vectors carry either a wild-type or mutant endonuclease gene, the expression of either of which is lethal in the absence of active EcoRI methylase. DNA cloned into any of the several unique restriction sites within the endonuclease gene disrupts its lethal function, allowing induced transformed cells carrying recombinant plasmids to survive, while cells carrying non-recombinant plasmids are killed. These vectors have an advantage over the other positive selection vectors because their expression is induced chemically rather than thermally (such in the case of plasmids employing induction by lambda promoters by inactivation of a temperature sensitive lambda repressor). Expression of this vectors constructed by Kuhn et al (1986) is under the control of the *lac* operon.

1.18.1 Plasmid pEcoR251

Plasmid pEcoR251 is about 3.3kb in size. The *EcoR*I endonuclease gene is placed under the control of the P_R promoter of phage lambda (λ) and this plasmid can therefore be maintained in a λ lysogen of *E. coli* since the phage λ infects *E. coli*. This plasmid has an ampicillin resistance gene which can be used to select for transformants containing this plasmid. When a piece of DNA is inserted into the one of the unique restriction sites in the endonuclease gene, the endonuclease gene is disrupted leading to the endonuclease activity being destroyed. This property can be used to select for transformants in a non-lysogen that have incorporated an insert in the endonuclease gene and those that did not incorporate an insert will die due to the expression of a functional EcoRI endonuclease (Dabbs *et al*., 1990).

In a λ lysogen the expression of the suicide gene is under the control of the P_R promoter and the regulation of the expression of the endonuclease gene is by a λ repressor protein called CI857. In a lysogen the CI repressor silences the lytic genes (Svenningsen *et al*., 2005). Plasmid pEcoR251 is constructed by fusing the 180bp fragment of the phage λ chromosome bearing the P_R promoter and the first 96bp of the *cro* gene. The

endonuclease gene is fused in frame with the cro gene and the expression of this gene is under the control of the P_R promoter (Zabeau and Stanley, 1982). In a λ lysogen the prophage expresses a repressor protein that binds to the cro operator region and thus inhibiting the expression of the *EcoR*I endonuclease gene. **Figure 1.2** shows the regulation of the expression of the suicide gene by the repressor protein. In a non lysogen there is no repressor protein being expressed to block transcription of the *EcoR*I suicide gene, hence the death of cell containing this plasmid.

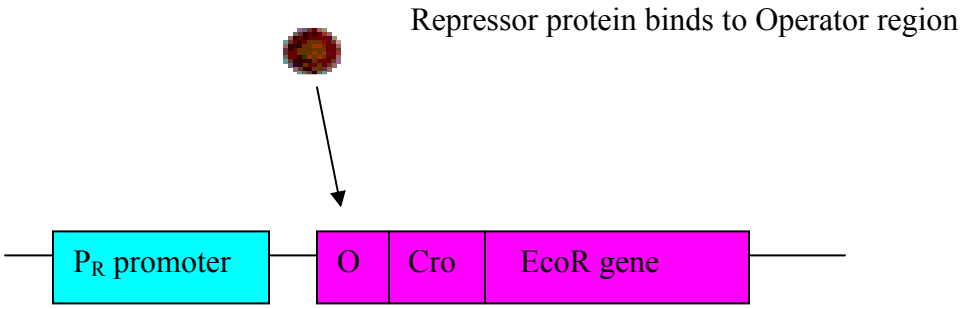


Figure 1.2: Regulation of the expression of the suicide gene. O is the operator region of the *cro* gene

1.9 Regulatory element from lambda phage

P_L and P_R promoters of the λ phage are widely used as promoters for the expression vectors. This type of vectors can be regulated in combination with a λ *c*I857 repressor gene which codes for a temperature-sensitive repressor. At 28-30˚C, this repressor is active and represses the transcription from the λ promoters. At 42˚C, the temperature-sensitive repressor protein is inactive and transcription from the λ promoters is derepressed. A λ prophage can be used as a carrier for the cI857 repressor gene only if the regulatory unit lacks lytic functions resident on prophages. Otherwise cells would be killed after heat induction (Mieschendahl and Müller-Hill, 1985). With this properties of

the λ lysogen together with the properties of the *EcoR*I endonuclease gene provide a better system for selection of recombinants transformants. When cells carrying a plasmid that encodes a suicide gene which is controlled by the P_R promoter are grown on a non-lysogen only recombinant transformants will be observed and when grown in a lysogen the expression of the endonuclease gene is repressed by the temperature-sensitive repressor protein

1.10 Justification and Aim

Antimicrobial resistance in pneumococci, enterococci and staphylococci is a norm. *S. aureus* is a pathogen of greatest concern because of its intrinsic virulence, its ability to cause array of life-threatening infections and its capacity to adapt to different environmental conditions. The mortality rate of *S. aureus* remains approximately 20-40% despite the availability of effective antimicrobials (Mylotte *et al*., 1987) and is now the leading overall cause of nosocomial infections. As more patients with *S. aureus* are treated outside the hospital setting, it is an increasing concern in the community. *S. aureus* isolates from blood samples world wide are increasingly resistant to a great number of antimicrobials. Inevitably this has left fewer effective bactericidal antibiotics to treat infection caused by the multidrug resistant *S. aureus*. Even when new antibiotics are used against *S. aureus* it can develop mechanisms to neutralize the antibiotic. This simply means that new antibiotics have to be discovered or that other ways of treating infection have to be used. The use of bacteriophages as therapeutic agents is probably the way to go. The bacteriophage expresses some proteins that inhibit the growth of the pathogen. So genes responsible for the inhibition of the growth of the bacteria have to be screened. This can be accomplished by having a vector that can be used to construct a genomic library in which the screening can be done. Therefore the aim of this work was to construct a shuttle vector for *E. coli*, *B. subtilis* and *S. aureus* that will facilitate the construction of genomic library in *E. coli* and screening these in *S. aureus* or *B. subtilis*. Also to increase the versatility of the shuttle vector by introducing unique restriction sites.

2. MATERIALS AND METHODS

2.1 Bacterial strains and plasmids used in this work

Strains	Characteristics	Source
Escherichia coli		
MM294-4	*end*A1, *hsd*R17, *gyr*A	E. Dabbs
λ MM294-4	λ lysogen of MM294-4	E. Dabbs
MJ109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*lac-proAB*)/F' [*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Y. Shibayama
B. subtilis		
IA3	*cysE*14, *purA*26, *trpC*2	E. Dabbs
168	(pC194) Cm *trpC2*	*Bacillus* Genetic Genetic Stock Centre (BGSC)
QB944 KIT-1	*cysE14 purA26 trpC2*	BGSC
QB934 KIT-3	*glyB133 metC3 tre-12 trpC2*	BGSC
QB922 KIT-5	*gltA292 trpC2*	BGSC
QB917 KIT-8	*hisA1 thrC5 trpC2*	BGSC
ND1	iri$^-$ mutant of IA3 which is Rifampicin resistant	This work
ND3	Mutant of IA3 which is Streptomycin resistant	This work
B. polymyxa		
ATCC 842	Wild type isolates	BGSC

S. aureus	Microbiology Department (Wits)
Streptomyces pseudogriseolus	M. Chengalroyen

Plasmids	**Characteristics**	**Source**
pDA71	*E. coli-Rhodococcus* shuttle vector	Quan and Dabbs (1993)
pDA71*	*E. coli-Rhodococcus* shuttle vector with the *EcoR*I suicide gene interrupted	E. Dabbs
pUC19	*E. coli* cloning vector with *lacZ´* gene	Fermentas
pC194	*S. aureus* original isolate with Cm^R	BGSC
pEcoR251	*E. coli* suicide vector with Amp^R resistance gene	E. Dabbs
pNDW1	*E. coli-B. subtilis* shuttle vector	This work
pNDW4	pNDW1 with a 1.8Kb *Pst*I fragment	This work
pNDW2	*E.coli-B. subtilis* shuttle vector joined in different restriction sites	This work
pNDW3	*E.coli-B. subtilis* shuttle vector joined in different restriction sites	This work
pNDW5	pNDW1:: *Xba*I restriction site	This work
pDA71-1	pDA71:: XbaI restriction site	This work
pNDW6	pNDW5:: fragment of genomic DNA	This work
pNDW7	pNDW5:: XhoI site	This work
pEcoR251-1	pEcoR251::XhoI site	This work

2.2 Media and growth conditions

Luria-Bertani (LB) (Appendix) media was used for growing *E. coli* and *Bacillus* strains and Brain Heart Infusion (BHI) (Appendix) media was used to grow *S. aureus*. Liquid cultures were achieved by inoculating a single bacterial colony into 5ml LB medium and incubated at 37°C overnight. For short-term storage, *E. coli* strains were kept on LB-agar plates at 4ºC and *Bacillus* strains were kept on LB-agar at room temperature. *S. aureus* was kept on BHI agar plates at room temperature.

2.3 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of antibiotics was determined on LB-agar plates for *E. coli* and *B. subtilis* by agar dilution method. Freshly grown colonies were inoculated into 200µl of sterile distilled water per well of the replicator plate. This was then transferred to the antibiotics plates with a multipoint inoculator. Antibiotic-free plates were used as positive controls. The spot test results were analyzed after incubation at 37°C for 24 hours.

2.4 DNA preparation

2.4.1 *E. coli* bulk plasmid preparation

A single colony was used to inoculate 100 ml of LB with appropriate selective agent for the maintenance of the plasmid. The culture was grown with gentle agitation at 37°C for overnight. Cells were harvested by spinning in a JA-10 rotor (Beckman) at 6 000 revolutions per minutes (rpm) for 10 minutes (min) and then resuspended in 5 ml of solution I (Appendix). A 10 ml of solution II (Appendix) was then added to the cell suspension and mixed gently by inversion. The mixture was left to stand at room temperature for 15 min. Then 7.5 ml of solution III (Appendix) was added and shaken vigorously and then left on ice for 15 min.

The cell debris were removed by spinning in a pre-chilled (4°C) JA-20 rotor at 15 000 rpm for 10 min. The supernatant was transferred to a sterile JA-20 centrifuge tube and the DNA was precipitated with 12ml of isopropanol. The precipitation process was allowed to continue at room temperature for 15minutes. This was followed by centrifugation of 15 000 rpm for 15 min at room temperature. The supernatant was decanted off and the DNA pellet washed with 2ml of ethanol. The ethanol was gently poured off and the DNA pellet was vacuum-dried for 20 min. The DNA was then re-suspended in 4ml TE buffer (Appendix) for 2 hours with gently agitation. Thereafter, 4.1 g of cesium chloride (CsCl) was added and dissolved by gently mixing and then 600µl of 1% ethidium bromide (EtBr) solution was added. The refractive index was adjusted to between 1.387 and 1.389 (0.001 units =100mg CsCl if the index was below or 0.001 units= 100µl TE if the index was above). The mixture was loaded into a Beckman Quick-seal tube using a Pasteur pipette. The tube was sealed, balanced and ultra-centrifuged overnight at 45 000rpm in a Beckman vertical VTi 65.2 rotor. The plasmid DNA was extracted from the tube using a needle attached to a hypodermic syringe. DNA was purified in section **2.5.1.3**

2.4.2 *B. subtilis* bulk genomic DNA preparation

A *B. polymyxa* culture was grown for 8 hours in 200ml LB at 37°C. Cells were harvested by centrifugation in a Beckman JA-10 rotor at 6 000rpm for 10 min at room temperature. The cells were resuspended in 5 ml of TE buffer to which 5mg/ml of lysozyme was added and incubated for 1 hour at 37°C. The cells were centrifuged at 8 000rpm for 5 min in a Beckman JA-20 rotor and resuspended in 4 ml TE buffer to which minute quantity of proteinase K was added. A 1/10 volume of Solution B (Appendix) was added to the cells and the tube incubated at 45°C for 30 min. The viscous solution was transferred to a 50Ti tube and spun at 40 000rpm for 30 minutes in a Beckman L5-50 ultracentrifuge. The supernatant was transferred to a clean JA-20 centrifuge tube and 4.4 g of CsCl was added. The contents of the tube were mixed by inversion of the tube for several times. The solution was centrifuged at 15 000 rpm in a Beckman JA-20 rotor for 15 min. The liquid was decanted, 600µl of 1% EtBr solution was added to it and the refractive index adjusted to between 1.391 and 1.392. The solution was loaded into a Beckman Quick seal

tube and centrifuged for overnight at 45 000 rpm in a VTi65.2 rotor in a Beckman L5-50 ultracentrifuge. The genomic DNA was extracted from the tube using a needle attached to a hypodermic syringe. DNA was purified in section **2.5.1.3**.

2.4.3 *E. coli* mini plasmid preparations

Individual bacterial colony was inoculated into 1ml of LB containing the appropriate selective agent. This was incubated at 37°C with agitation for overnight. The culture was transferred into sterile Eppendorf tube and the cells harvested by microfuging for 1 min. The supernatant was decanted off and the pellet resuspended in 80µl of solution I by vortexing. Then 160µl of solution II was added to the cell suspension and mixed by gently inversion of the tube. The tube was left to stand at room temperature for 15 min. Thereafter, 120µl of solution III was added and the mixture shaken vigorously and kept on ice for 5 min. Cellular debris was removed by microfuging in the cold room (4°C) for 10 min. The supernatant was collected into a new sterile Eppendorf tube and placed in a water bath (42°C) for 2 min to warm the supernatant. Isopropanol (220µl) was added and the precipitation process allowed continuing at room temperature for 5 min followed by centrifuging at room temperature for 5 min. The pellet was washed with 150µl ethanol and vacuum-dried for 20 min. The DNA was then resuspended in 100-150µl of sterile distilled water containing freshly boiled ribonuclease (RNaseA) (1µl of 10mg/ml). A small aliquot was analyzed on an agarose gel.

2.4.4 *B. subtilis* mini plasmid preparation

Single colonies were inoculated in 1ml LB containing appropriate selective agent and grown for overnight at 37ºC. The culture was transferred into sterile Eppendorf tube and the cells harvested by microfuging for 1 min at room temperature. The pellet was re-suspended in 200µl of TE buffer to which lysozyme (5mg/ml) were added. The suspension was incubated for 1 hour in a 37ºC water bath with inversion of the tube in 10 min intervals. Thereafter, 40µl of TE-SDS (10%) was added and the tube mixed by inversion and left to stand at room temperature for 10 minutes. A volume of 40µl of 5 M

KAc (pH 6.0) was then added and the mixture was shaken vigorously and left on ice for 5 min. The mixture was microfuged for 5 min in the cold room (4ºC) and the supernatant was transferred into a fresh Eppendorf tube. DNA was purified by phenol-chloroform extraction in **2.5.1.4** and precipitated by salt and ethanol precipitation in **2.5.1.1.**

2.5 DNA manipulations and cloning techniques

2.5.1 DNA precipitation

2.5.1.1 Salt and ethanol precipitation

DNA was precipitated from aqueous solution with 1/3 volume of 1M NaCl and 2 volumes of ethanol. The mixture was microfuged at 4°C for 20 min. The supernatant was decanted and the remaining liquid removed by blotting on a paper towel. The DNA pellet was vacuum-dried for 10-20 min and resuspended in the appropriate volume of sterile distilled water. RNaseA (10mg/ml) was used to remove contaminating RNA.

2.5.1.2 Isopropanol DNA precipitation

After the addition of isopropanol (220µl), the solution was mixed by inversion and left to stand at room temperature for 5 min. The solution was then centrifuged at room temperature for further 5 min. The pellet was washed with 150µl of 96% ethanol and vacuum-dried for 20 min. The plasmid was resuspended in an appropriate volume of sterile distilled water containing RNaseA.

2.5.1.3 DNA precipitation from CsCl gradient

Ethidium Bromide (EtBr) was removed from the DNA by thorough mixing with 0.1 volume of butanol. EtBr suspended in butanol forms a top layer in the tube, which is removed. This procedure was repeated at least 3 times until there were no traces of EtBr. This left the DNA in CsCl solution. The DNA was stored at -20°C until required. The salt

was removed by adding 2 volumes of sterile distilled water and 2.5 volumes of 96% ethanol and precipitated by centrifugation at 4°C for 20 min. The pellet was vacuum-dried and resuspended in appropriate volume of sterile distilled water.

2.5.1.4 Phenol-chloroform extraction

The DNA solution in water was extracted by addition of 1/3 volume TE-saturated phenol (Appendix), mixed by inversion and microfuged at room temperature for 5 min to separate the organic and aqueous phases. The upper aqueous layer was transferred into a new sterile Eppendorf tube and where necessary, as in the case of extracting DNA from agarose gels, a further phenol step was performed. Then 1/3 volumes of chloroform was then added to the aqueous layer and mixed gently by inversion. The organic and aqueous layers were separated by microfuging at room temperature for 2 min. The upper aqueous layer was transferred into a sterile Eppendorf tube and the DNA was precipitated by the addition of 1/3 volume of NaCl (Appendix) as described in **2.5.1.1.**

2.5.2 Restriction enzyme digestion

Enzymes were obtained from Fermentas, Boehringer Mannheim, New England Biolabs, Amersham or Promega and used according to manufacturer's instructions. The total volume of digestion was 15µl (13.5µl DNA and 1.5µl 10× buffer). The mixture was tapped briefly to ensure even buffer distribution and spun down for a couple of seconds. 0.3-1µl of restriction endonuclease was added and the contents mixed and re-spun briefly. Digestions were incubated at the appropriate temperature for maximal enzyme activity for at least 4 hours. For double digestions an appropriate buffer in which both enzymes showed suitable activity was selected, otherwise the digestions were performed sequentially starting with the enzyme that require a lower incubation temperature.

2.5.3 Ligation of DNA

T4 DNA ligase (Fermentas) was used for all ligations procedures. The total volume for ligation was kept minimal at 20µl. Ligation buffer and the appropriate volume of sterile distilled water were added to the DNA sample, mixed by tapping and microfuged for a couple of seconds. Subsequently, 1µl of ligase was added, remixed and re-spun. Ligation was performed in a water bath at 22°C for 16-22 hours.

2.5.4 Alkaline phosphatase treatment

Calf intestinal alkaline phosphatase (Boehringer Mannheim) was used to prevent the vector from re-ligating to itself. The alkaline phosphatase removes the 5´-phosphate that is necessary for the ligation by DNA ligase. Following the digestion of vector DNA, 1µl of calf intestinal alkaline phosphatase was added to the reaction mixture. Addition of 10× dephosphorylation buffer was added (1/10 volume). The reaction mixture was incubated at 37°C for overnight. Immediately after incubation, the enzyme was removed by a phenol-chloroform DNA extraction as previously described in **2.5.1.1**.

2.6 Gel electrophoresis

2.6.1 Agarose gel electrophoresis

Agarose solutions stock solutions were prepared in 0.5× TBE buffer (Appendix) at concentrations of 0.4%, 0.8% or 1.2% depending on a fragment size to be separated. The solutions were sterilized by autoclaving (121°C, 20 minutes). Fragment sizes ≥10kb were analyzed on 0.4% agarose gels, 2-10kb on 0.8% agarose gel and ≤3kb on 1.2% agarose gel. Gels were prepared by melting the agarose stock solution in a microwave oven. A volume of 25ml of the melted agarose was mixed with 2.5µl of a 1% EtBr solution (Appendix). The mixture was poured in a gel tray with a 12-tooth well comb and allowed to polymerize at 4°C for 20 min. Combs were removed when the gel had completely set.

The electrophoresis buffer was 0.5× TBE mixed with 1% EtBr solution. DNA samples were loaded with 2µl of bromophenol blue tracking dye. GeneRuler ^{TM} DNA 1KB ladder Plus mix (Fermentas) was used in all electrophoretic runs using a Hoefer PS 500xdc power supply. The process was carried out at room temperature, 80V and a current of 21-28mA, until the dye front reached the bottom of the gel. DNA sizes were quantified from standard curve generated from migrations distances of known molecular weight marker sizes run on the same gel. The concentrations were estimated by comparing the intensity of the bands to bands of similar intensity and known concentration. The DNA was visualized and captured using the UVP BioDoc-It^{TM} system.

2.6.2 The freeze-squeeze method of extracting DNA from agarose gels

DNA was digested with appropriate restriction endonuclease and fragments were separated on agarose gel. The fragment of interest was excised from the gel with a scalpel while viewing under long wavelength UV light (366nm). The gel slice was transferred into a sterile Eppendorf tube where it was crushed with a sterile spatula. The crushed slice of gel was kept at -70ºC for 30 min and it was thawed at room temperature. Following thawing it was microfuged for 6 min and the supernatant collected into a sterile tube. A second round of crushing, freezing, thawing and centrifuging was done. The DNA which was suspended in the collected supernatant was purified by phenol-chloroform extraction in **2.5.1.4**. The DNA was re-suspended in appropriate volume of sterile distilled water.

2.7 Transformations

2.7.1 *E. coli* standard $CaCl_2$ transformation

A flask containing 20 ml of pre-warmed LB supplemented with 0.5% glucose (Appendix) was inoculated with 200µl of an overnight culture of *E. coli* MM294-4. The culture was incubated with vigorous shaking at 37ºC until the OD_{600} of 0.2-0.4 had been reached. That OD was generally obtained by incubating for a minimum of 1hour 45 min - 2 hours.

The flask was chilled in an ice-water slurry for 5 min and the cells were harvested in a pre-chilled Beckman JA-20 rotor at 10 000 rpm for 5 min at 4ºC. The supernatant was discarded and the cells re-suspended in 10 ml of ice cold transformation buffer ($CaCl_2$ transformation buffer) (Appendix). The cell suspension was placed on ice for 15 min and re-centrifuged at 10 000 rpm for 5 min. The supernatant was decanted and the cells re-suspended in 1.3 ml of transformation buffer. The cells were left on ice for 2-24 hours in the cold room (4ºC).

Aliquots 100µl of the cell suspension were placed into pre-chilled sterile Eppendorf tubes and about 3µl of plasmid DNA was added and mixed with the cells by bubbling air through. The DNA plus cell suspension was left on ice for 15 min to allow for diffusion. The cells were then heat-shocked at 42ºC for 90 seconds. A volume of 1ml of pre-warmed LB was added to the cells after heat shocking and incubated for 60- 90 min at 37ºC to allow phenotypic expression of the resistance genes. The cells were then spread onto LB-agar containing an appropriate selective agent and further incubated for overnight at 37ºC. Colonies were visible after the overnight incubation for *E. coli* strains.

2.7.2 *B. subtilis* electroporation

A pre-culture of *B. subtilis* IA3 grown on LB was inoculated into 5ml of LB supplemented with 10.3% sucrose and grown for 4 hours at 37ºC. After incubation 1ml of the culture was microfuged for 1 minutes and the supernatant was decanted. The cells were re-suspended in ice-cold SHMG buffer (Appendix). The cells were washed twice in SHMG buffer and re-suspended in 500µl of the same buffer. A volume of 100µl of the cell suspension was transferred into a pre-chilled sterile Eppendorf tube and 5µl of the DNA was added, mixed by bubbling air through. The mixture was placed on ice for 15 min to allow diffusion. The mixture was then transferred into pre-chilled Bio-Rad electroporation cuvette and electroporated using capacitance of 25µFD, voltage of 2.0kV and resistance of 200Ω and the time constant was recorded. After the electroporation 1ml of warm LB was added to the cells immediately and transferred into Bjorn bottles and incubated at 37ºC with shaking for overnight to allow phenotypic expression. After

incubation the cells were spread on selective media and further incubated for 2 days for growth to be observed.

2.7.3 Protoplast transformation of *B. subtilis* and *S. aureus*

This is a method described by Cohen and Cheng (1979) with modification by Zhang et al. (2005). Mid-log phase culture, freshly grown in LB at 37ºC to OD_{600} of 0.6 were harvested and resuspended in 500µl volume of SMMP buffer (Appendix). Lysozyme was added to a final concentration of 10mg/ml and incubated for 2 hours at 37ºC with gently agitation. The treated cells were then harvested and washed once by resuspending them gently in 500µl SMMP buffer and pelleted the second time. The washed protoplast was brought to 500µl volume with SMMP buffer. An amount of 1pg to 5µg of DNA in 7.75µl of TE buffer was mixed with equal volume of 2X SMM buffer (Appendix) in a sterile Eppendorf tube. 77.5µl of the protoplast suspension was added to the DNA, followed by the addition of 232µl of 40% PEG (w/v) solution (Appendix) and the contents of the tube gently mixed. After 2 min of exposure to PEG, 775µl of SMMP buffer was added to the mixture to dilute the PEG. Protoplast was recovered by centrifugation for 10 min and the resuspended in 200µl of SMMP buffer. The resuspended protoplast was incubated for 2 hours at 30ºC to allow phenotypic expression carried by the plasmid. After incubation the suspension was spread on DM3 regeneration plates (Appendix) which were supplemented with 15µg/ml of chloramphenicol and incubated overnight at 37ºC.

2.7.4 Electroporation of *S. aureus*

S. aureus cells were prepared for electroporation by inoculating a fresh colony into 5ml of BHI broth and incubated overnight at 37ºC. The overnight culture was then diluted 100X on BHI broth and grown at 37ºC until the OD_{600} was <0.5. The cells were washed twice in electroporation buffer (Appendix) at 4ºC. An aliquot of 100µl of the *S. aureus* cells was transferred into an Eppendorf tube and 5-10µl of DNA was added. This was mixed by bubbling air through and left to stand on ice for 10 min to allow diffusion. The

mixture was then transferred into a pre-chilled electroporation cuvette and electroporated (25µF, 2.0kV and 200Ω). BHI broth was added immediately to the electroporated cells and incubated for 1 hour at 37ºC for the expression of the antibiotic resistance markers. After incubation the cells were plated on BHI agar plates supplemented with an appropriate selectable marker and incubated for overnight.

2.8 Preparation of DNA for sequencing

DNA was prepared using the *E. coli* mini plasmid preparation method in **2.4.3**. There were modifications done on this procedure. After the cell debris was precipitated and the supernatant collected into a new tube, the DNA was extracted by the phenol-chloroform extraction method in **2.5.1.4** to remove any protein and other cellular components that might be in the supernatant. The DNA was then precipitated with isopropanol as in **2.5.1.2**. After DNA had been prepared the concentration and purity of the DNA was analyzed on agarose gel. Sequencing performed by Inqaba Biotechnology Industries (Pty) Ltd

2.9 Mutagenesis

2.9.1 *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis

A volume of 300µl of an overnight bacterial culture was microfuged for 30 seconds and the supernatant was discarded. The pellet was re-suspended in 1ml of Tris-Maleate buffer (pH 9) and washed twice in the same buffer. After washing, the pellet was re-suspended in 1ml of Tris-Maleate buffer where 50µl of NTG stock solution (1mg/ml) was added. Immediately after the addition of NTG solution the cells were microfuged for 30 seconds and the supernatant discarded. The cells were washed twice in Phosphate buffer (pH 7) because neutral pH completely inhibits the NTG reaction. The cells were inoculated into 5ml of LB and grown overnight at 37ºC. A volume of 100µl of the grown culture was spread on selective media and further incubated for overnight at 37ºC.

2.9.2 Site-directed mutagenesis

Numerous methods have been developed or exploited to mutate DNA. Initially all approaches focused on the generation of random mutations in chromosomal DNA such as those induced by X-rays and chemicals. While these methods of random mutagenesis provided a valuable tool for classical gene studies, they were limited by their inability to target the mutation to a specific gene or genetic element. Site-directed mutagenesis is widely used in the study of gene and protein functions. A Phusion^{TM} Site-Directed Mutagenesis Kit was used in this study. This is a PCR-base site-directed mutagenesis which uses two primers, one which is a mutagenic primer directed to the target site and the other one is non-mutagenic which aligns anywhere in the plasmid.

2.10 Counter-selection for auxotrophic mutants

A mutated culture from **2.9.1** grown overnight was washed 3× with 10× stock III buffer or sterile distilled water to remove traces of rich media. The washed cells were then resuspended in 1 ml of stock III buffer. It was then diluted 50× in Stock III buffer and grown until mid-log phase. Ampicillin was added to a concentration such that it does not kill the latent cell which should be the auxotrophs. After the addition of ampicillin the culture was grown overnight followed by diluting the culture to 10^{-5} and 100µl of the diluted culture was spread on LB agar plates. The plates were incubated overnight at 37˚C. After incubation the developed colonies were patched on minimal media and rich media and grown incubated overnight. The colonies that grow on rich media but not on minimal media were identified as auxotrophic mutants. Their nutritional requirements were determined by patching the auxotrophic mutants in minimal media supplemented with different auxotrophic requirement.

2.11 Rifampicin inactivation test

A mutated culture from **2.9.1** which was rifampicin resistant was dilutes 100X in fresh LB and grown for 2 hours at 37ºC. After the 2 hours of growth rifampicin was added to a

final concentration of 20µg/ml and further incubated for overnight. Uninoculated LB was used as a control. LB agar plates with half the concentration of agar were used and were supplemented with 200µg/ml of streptomycin. ND3 (streptomycin resistant mutant) was used as an overlay organism by spreading it on the ½ LB agar plates supplemented with streptomycin. Once the overlay organism had dissolved properly in the plates wells were made by using the back of a Pasteur pipette. A volume of 80µl of the overnight culture was inoculated into the well made. The plates were kept at 4ºC for 4-6 hours to allow diffusion of the inoculated culture followed by incubation at 37ºC for overnight. After incubation the zones of inhibitions are measured.

2.12 Plasmid curing

B. subtilis 168 (pC194) was cured of its plasmid using EtBr. Cells were inoculated into 5ml of LB and grown overnight without antibiotic. The overnight culture was diluted 100× in fresh LB and different concentrations of EtBr were used (0.01, 0.02 and 0.03%). A no EtBr control was used. The cells were allowed to grow overnight followed by another 100× dilution in LB without a selective agent and grown overnight. The overnight culture was diluted to the 10^{-5} spread on LA plate with no selective agent and incubated for overnight. The colonies were observed and they were patched on LA plate supplemented with 15µg/ml of chloramphenicol (Cm) and also on LA plate with no antibiotic. The plates were incubated at 37˚C for overnight and the colonies that could not grow on the LA plate with Cm were successfully cured of the plasmid.

Table 2.1: Internet sequence analysis programs used

Program	**Web address**
BLAST	http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi
NEBcutter	http://tools.neb.com/BEBcutter2/index.php
FASTA	http://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi
NEBcutter (silent mutagenesis)	http://tools.neb.com/NEBcutter2/silmutlist.php

3. RESULTS

3.1 *Bacillus-Staphylococcus-E. coli* shuttle vector construction

Plasmid pEcoR251 has a *Bam*HI site downstream of the *EcoR*I endonuclease gene (**figure A6.5**) and this site does not fall within any known important regions of the plasmid. Plasmid pC194 has an *Xho*II site which is just after the chloramphenicol resistance determinant (**figure A6.1**) and does not interfere with the expression of this gene. Plasmid pC194 was digested with *Xho*II and pEcoR251 was digested with *Bam*HI and these were ligated together using the complementary single stranded ends to give pNDW1 (**Figure 3.2)**. This construct should be maintained in *B. subtilis* and *E. coli* since it had the origin of replication for Gram positives and Gram negatives. It was transformed into *E. coli* λ lysogen and a non-lysogen to determine the functioning of the suicide gene. Plasmid pEcoR251 was used as a control because it was desired that the construct function in a similar manner due to the suicide gene. Transformants were obtained in the lysogen but not non-lysogen confirming that *EcoR*I was functional. Plasmid DNA from these transformants was digested with informative restriction enzymes and the size expected was confirmed and the plasmid orientation determined (**Figure 3.1**).

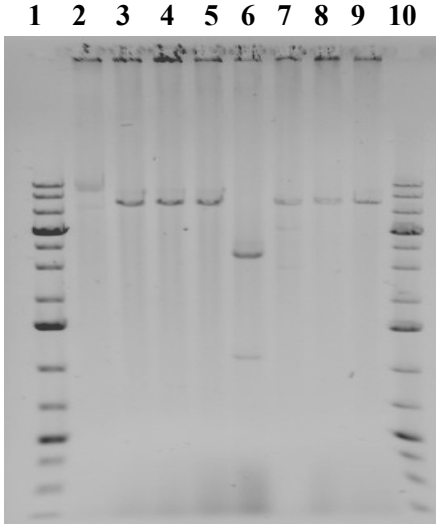


Figure 3.1: Restriction digests of pNDW1. Lane 1 and 10 are DNA markers, 2 is undigested, 3 is *Pst*I, 4 *Swa*I, 5 *Stu*I, 6 *Hind*III, 7 *Bgl*II, 8 *EcoR*I and 9 *Sfu*I.

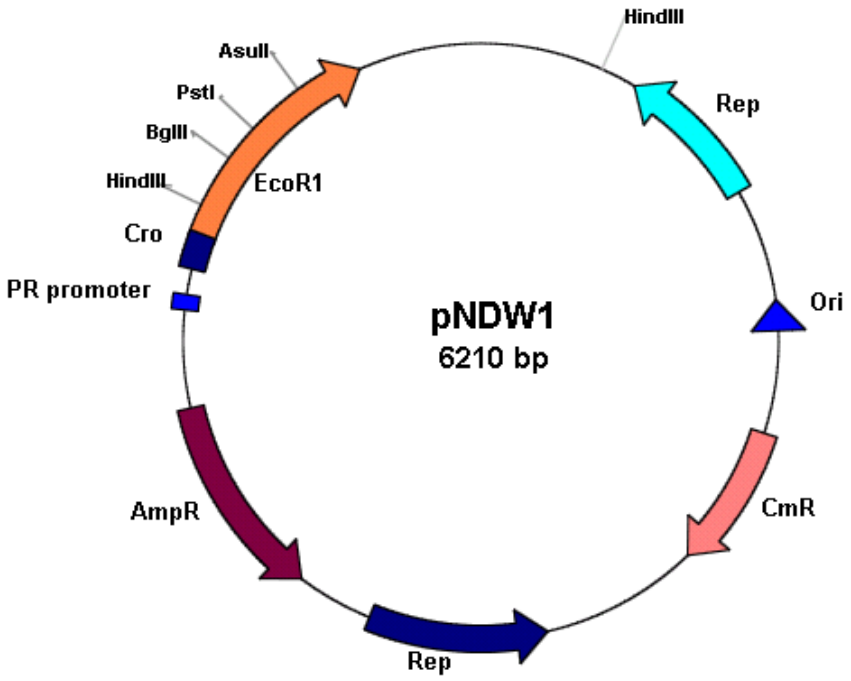


Figure 3.2: Restriction map of plasmid pNDW1.

3.1.1 Transformation of *B. subtilis* with pNDW1

Attempts were made to transform pNDW1 into *B. subtilis* IA3 but these were unsuccessful. To investigate why, two new constructs were made by joining pC194 (*Xho*II) with pEcoR251 (*Bgl*II): pNDW2 and the other one by joining pC194 (*Hind*III) with pEcoR251 (*Hind*III): pNDW3. Both constructs had the suicide gene interrupted and in contrast to pNDW1 transformed a non-lysogen and a lysogen of *E. coli* with similar frequency, behaving in the same manner as pDA71* used as a control. Digestions of miniprep DNA of these constructs were done to confirm their sizes and their orientations (**Figure 3.3 (B)**). Plasmid pNDW1 was used to clone a *Pst*I 1.8Kb fragment isolated from a *Norcadioform brasiliensis Pst*I library, encodes the glucoslytransferase responsible for rifampicin resistance in Norcadioforms, into the *Pst*I site on the suicide gene. This construct was named pNDW4 and mini DNA preparations of this construct were done to confirm the presence of an insert (**Figure 3.3 (A)**). Plasmids pNDW2, pNDW3 and pNDW4 were successfully transformed into *B. subtilis* confirming that the failure with pNDW1 was not because the construct was unable to replicate but because the suicide

gene was expressed in that organism. Restriction maps of pNDW2 and pNDW3 are shown in **Figure 3.4.**

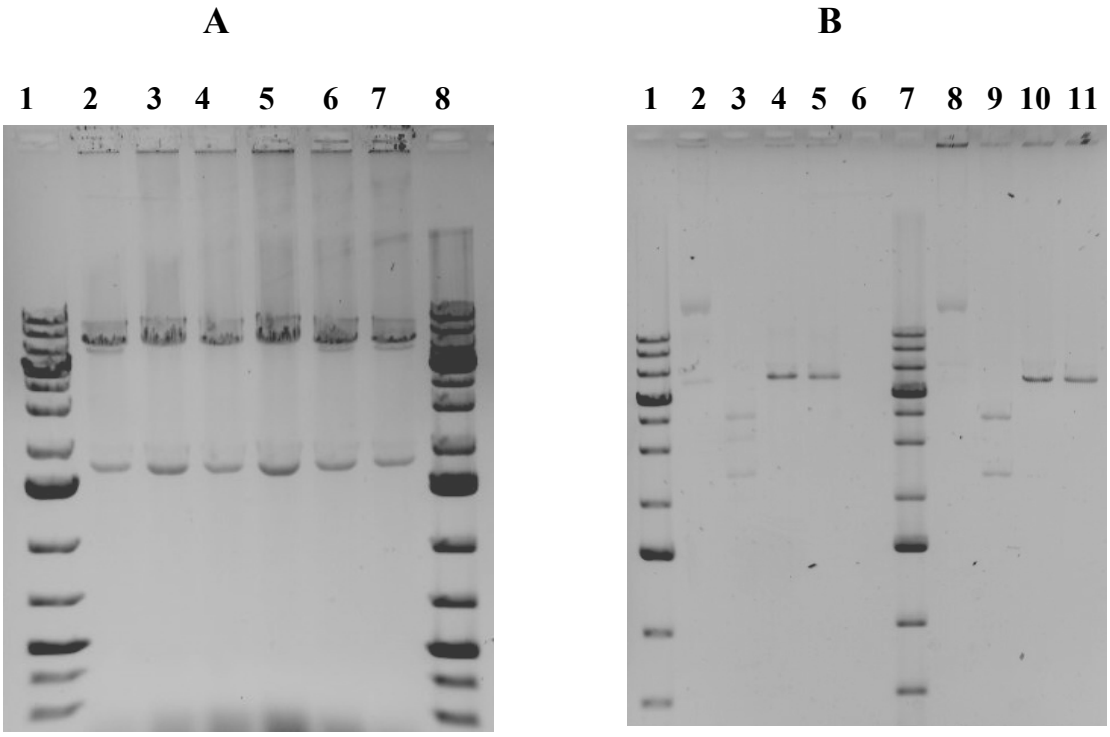


Figure 3.3: **(A)** Lanes 2-7: pNDW4 digested with *Pst* I to release the inserts which are the lower bands. Lanes 1 and 8 are DNA markers. **(B)**: Lanes 2-5: pNDW2, lane 2 is undigested, lane 3 is *Hind*III digest, lane 4 *Pst*I digest and lane 5 is *Stu*I digest. Lanes 8-10 is pNDW3, lane 8 is undigested, lane 9 is *Hind*III digest, lane 10 is *Pst*I digest and lane 10 is *Stu*I digest. Lanes 1 and 7 are DNA markers.

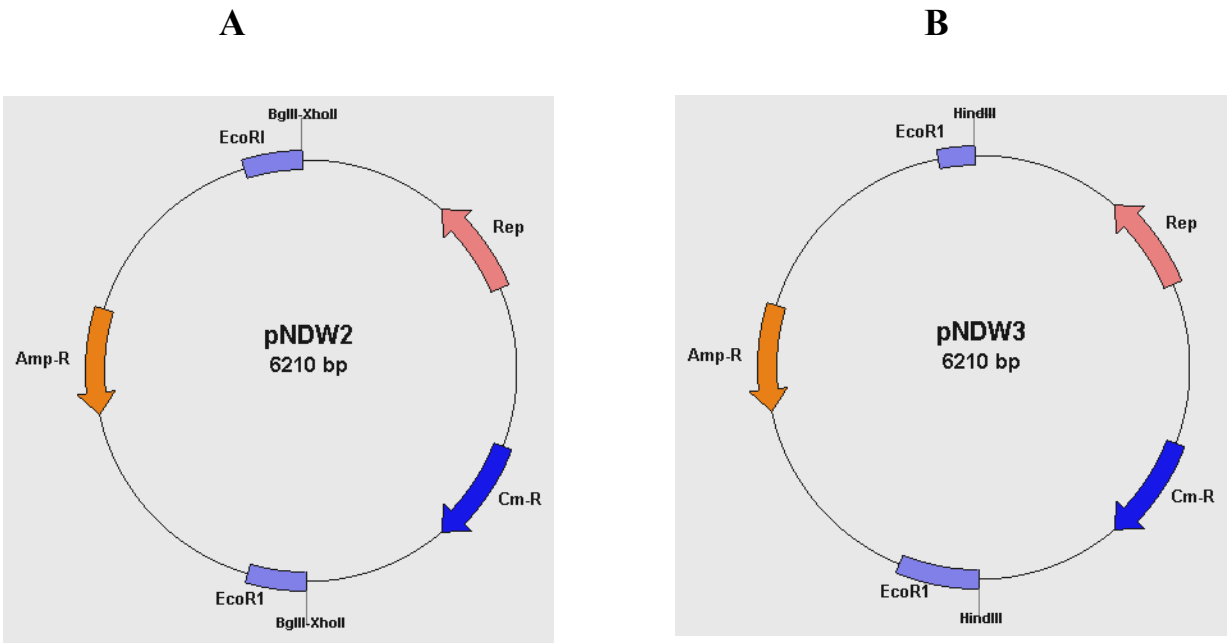


Figure 3.4: Plasmid map of constructs indicating the way in which the suicide gene was disrupted, **(A)** Restriction map of plasmid pNDW2, **(B)** Restriction map of plasmid pNDW3.

3.1.2 Transformation of *S. aureus* with pNDW1

Transformation of *S. aureus* by pNDW1 using electroporation was successful using the conditions of Wada and Watanabe (1998). pC194 was used as a control since this plasmid was originally isolated from *S. aureus*. Transformants were observed with both but for pNDW1 the efficiency was very low. Transformants were not expected for pNDW1 if the suicide gene was expressed in this organism. DNA preparation of *S. aureus* transformants was re-transformed back into *E. coli* λ lysogen and non-lysogen to check the status of the suicide gene. Transformants were only observed on the λ lysogen showing that the suicide gene is functional. DNA preparation from 20 transformants was done and digested with *Pst*I to check the size of the plasmid (**Figure 3.5)**

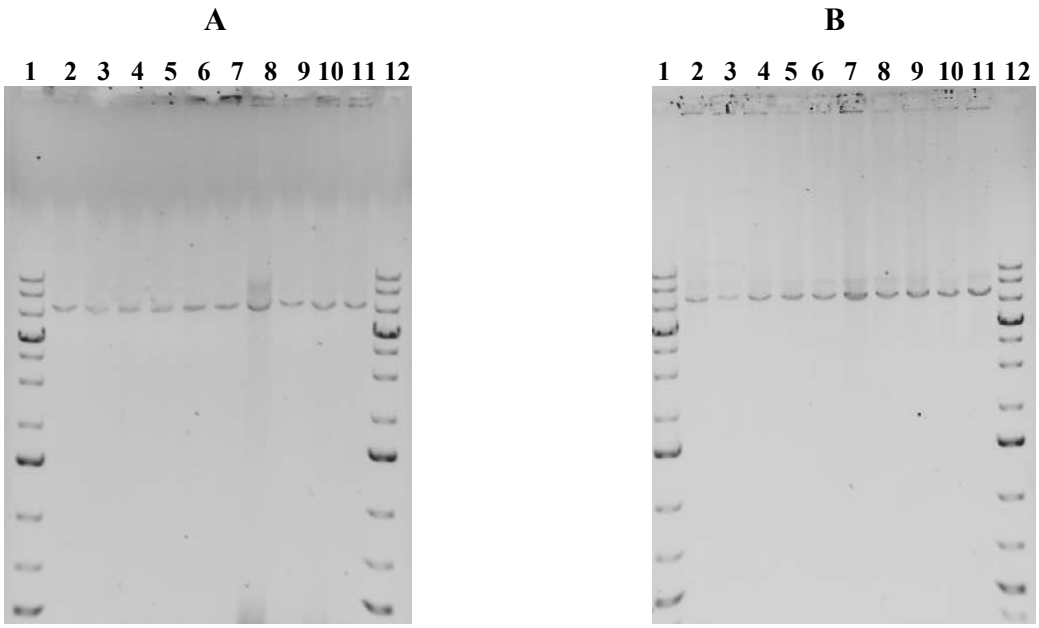


Figure 3.5: Plasmid preparation of pNDW1 from λ lysogen. (**A**) 10 of the 20 transformants. Lane 1 and 12 are molecular weight markers. Lanes 2-11 pNDW1 from different transformants digested with *Pst*I. (**B**) The other 10 transformants. Lanes 1 and 12 are molecular weight markers. Lanes 2-11 pNDW1 from transformants digested with *Pst*I.

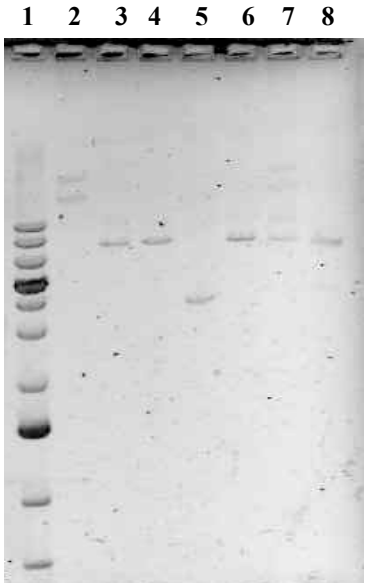


Figure 3.6: pNDW1 from *S. aureus*. Lane 1 is molecular weight marker. Lane 2 is undigested, lane 3 is *Eco*RI, lane 4 is *Stu*I, lane 5 is *Hind*III, lane 6 is *Swa*I, lane 7 is *Spe*I and lane 8 is *Pst*I.

DNA from the clone of **Figure 3.5 (B)** lane 11 was further digested to compare this pNDW1 to the pNDW1 used to transform *S. aureus* (**figure 3.6**). Since the two are indistinguishable I concluded that *EcoR*I is not expressed in *S. aureus*, in contrast to *B. subtilis*.

3.2 Introduction of new sites into *EcoR*I

Introduction of additional restriction sites in the suicide gene of pNDW1 would increase its versatility in genomic library construction and subcloning. Candidate restriction sites were chosen using the following criteria:

1) The enzyme has a 6 bp palindromic recognition sequence.
2) The restriction site should be unique in the vector.
3) The site should towards the 5′ end of *EcoR*I (first 600bp).
4) The enzyme should generate cohesive rather than blunt ends for better ligation.

The Silent Mutagenesis Program on NEBcutter website was used to determine which restriction sites can be introduced and where mutation should be made on the suicide gene sequence. This program gives possible mutations that can be introduced into the sequence without changing the amino acid sequence of the protein. All possible mutations were analyzed and 11 candidates identified. **Table 3.1** lists these. The bases that are coloured and underlined red are the mutated bases.

TABLE 3.1: Candidate Additional Restriction Sites in *EcoR*I

Enzyme	Specificity	Position	Mutated bases underlined
*Avr*II	C CTAG G	**228**	**211** AAAAAAATTGACCCTGACCTAGG CGGTACTTTATTTGTTTCA **252**
*Bcl*I	T GATC A	**396**	**379** TTAGTTGGGAAAAGAGGTGATCA AGATTTAATGGCTGCTGGT **420**
*Cla*I	AT CG AT	**23**	**40**TCTAATAAAAAACAGTCAAATCGAT TAACTGAACAACATAAG **45**

*Cla*I	AT CG AT	**217**	**199** AATGAAGCTTTAAAAAAAATCGAT CCTGATCTTGGCGGTACT **240**
*Cla*I	AT CG AT	**258**	**241** TTATTTGTTTCAAATTCATCGAT CAAACCTGATGGTGGAATT **282**
*Spe*I	A CTAG T	**120**	**103** GTTGGTGAGGTTTCAAAACTAGT AAAGAAAGCTCTTAGCAAC **144**
*Spe*I	A CTAG T	**321**	**304** GGTGAATGGAGAGTTGTACTAGT TGCTGAAGCCAAACACCAA **345**
*Xba*I	T CTAG A	**504**	**487** TTTCCTTACGTCCTTTTTCTAGA GGGGTCTAACTTTTTAACA **528**
*Xho*I	C TCGA G	**255**	**238** ACTTTATTTGTTTCAAACTCGAG CATCAAACCTGATGGTGGA **279**
*Xho*I	C TCGA G	**505**	**487** TTTCCTTACGTCCTTTTCCTCGAG GGGTCTAACTTTTTAACA **528**

*Spe*I is present in pC194 so was excluded. Genomic DNA of *B. polymyxa* was digested with these restriction enzymes to see which might be suitable in cloning *Bacillus* genomic DNA. **Figure 3.7** shows the gel image of this.

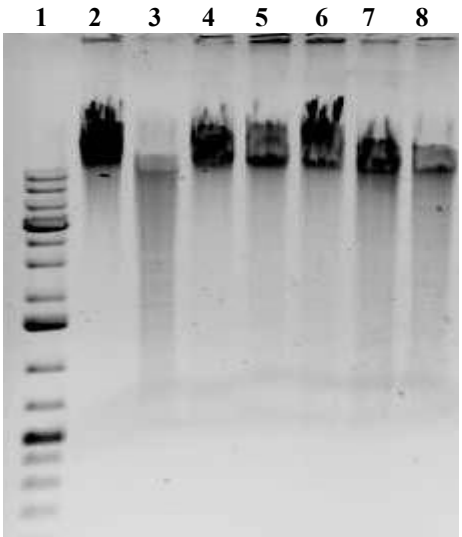


Figure 3.7: *B. polymyxa* genomic DNA, lane: 2 undigested; 3-8 digested with: 3 *Cla*I, 4 *Xho*I, 5 *Avr*II, 6 *Sal*I, 7 *Pst*I, and 8 *Xba*I.

These results (figure 3.7) were useful in narrowing down which restriction site should be introduced into pNDW1 suicide gene. *Cla*I digested the genomic DNA very frequently and so was unlikely to be useful when creating a genomic library because it is preferable to have the entire open reading frame of a particular gene rather than having pieces of the gene scattered all over the library. Also *Cla*I partial digestions of *Bacillus* genomic DNA might be inserted into the *Sfu*I site already present in the suicide gene. *Avr*II, *Sal*I and *Xho*I were possible candidates as they digested the genomic DNA generating large fragments. *Pst*I was used as a control because it has been observed that *Pst*I would be a suitable enzyme to create a genomic library of *B. polymyxa* genomic DNA (**figure 3.37**). *Xba*I seemed to digest genomic DNA comparably to *Pst*I so I decided this would be the best choice.

3.2.1 Introduction of an *Xba*I site in pNDW1

To empirically confirm that *Xba*I sites were absent in pC194 and pEcoR251 (as predicted from published sequences) they were digested with this enzyme. As shown in **Figure 3.8**. *Xba*I did not cut either plasmid.

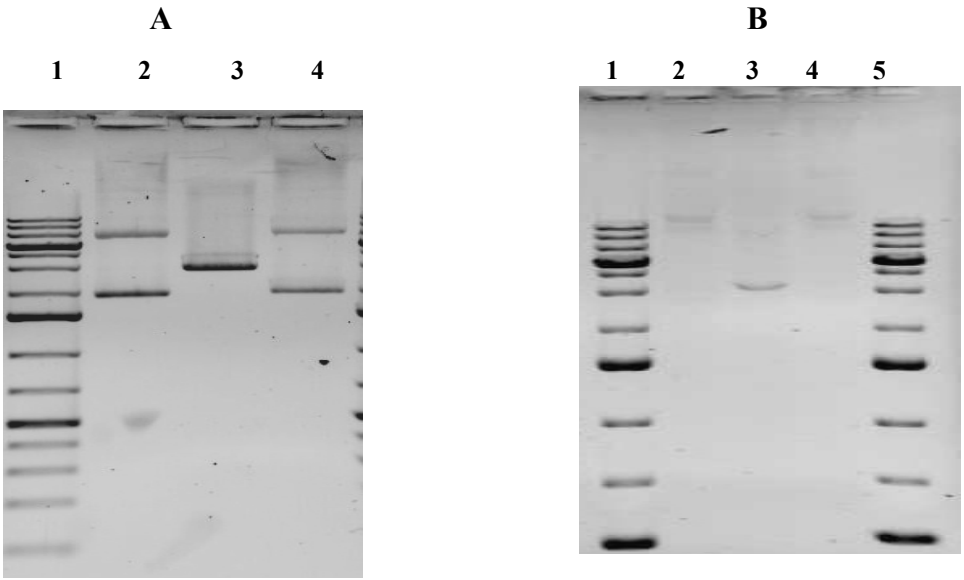


Figure 3.8:(**A**) Lane 1 is the molecular weight markers, lane 2 is pC194 undigested, lane 3 is pC194 digested with *Spe*I and lane 4 is pC194 digested with *Xba*I. (**B**) Lanes 1 and 5

are molecular weight markers, lane 2 pEcoR251 undigested, lane 3 *Pst*I digest and lane 4 is *Xba*I digest.

Having concluded that *Xba*I was the best restriction site to introduce into the pNDW1 suicide gene a site-directed mutagenesis kit was used to introduce the mutation required. The following primers were used

5′-TACGTCCTTTT**TC**TAGAGGGGTCTA-3′ and a reverse primer
5′-AGGAAAGTGGGCTCTCAGAGAGCA-3′.

TABLE 3.2 Mutagenic reaction mixture for *Xba*I site

Components	Volume/50µl reaction	Final concentration
Sterile dH_2O	36.7µl	
5X Phusion HF buffer	10µl	1X
10mM dNTPs	1µl	200µM each
Mutagenic primer	0.25µl	0.5µM
Reverse primer	0.25µl	0.5µM
DNA template (0.4ng/µl)	1.25µl	10pg
Phusion hot start DNA Polymerase (2U/µl)	0.5µl	0.02U/µl

TABLE 3.3 Cycling conditions for mutagenic reaction for *Xba*I site

Cycle step	Temperature	Time	Number of cycles
Initial Denaturation	98ºC	30 Sec	1
Denaturation	98ºC	10 sec	

Annealing	67ºC	30 sec	25
Extension	72ºC	4 min	
Final Extension	72ºC	5 min	

The PCR reaction was successful because a band of amplification was observed corresponding to the template size (pNDW1) (**Figure 3.9**). A no template control was used.

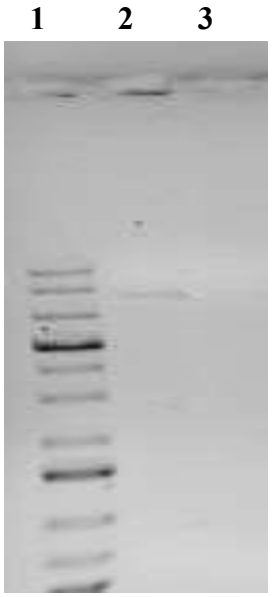


Figure 3.9: 5µl of the PCR product ran on 0.6% agarose gel. Lane 1 is Molecular weight marker, Lane 2 is the PCR product and lane 3 is the control reaction.

The PCR product was a linear plasmid molecule therefore it was circularized with Quick T4 DNA ligase provided in the kit. The circularized plasmid was then transformed into *E. coli* MM294-4 λ lysogen. DNA mini preparation of 10 transformants was done and digested with *Xba*I to screen for plasmid which had acquired the *Xba*I site. A single clone had the *Xba*I site (**Figure 3.10** lane 3). To confirm that the plasmid was pNDW1 with a new restriction site *Xba*I, restriction digestions were done on the DNA in lane 3 **Figure**

3.10. **Figure 3.11** presents the gel image of the digestions and confirmed that the *Xba*I restriction site was introduced on pNDW1.

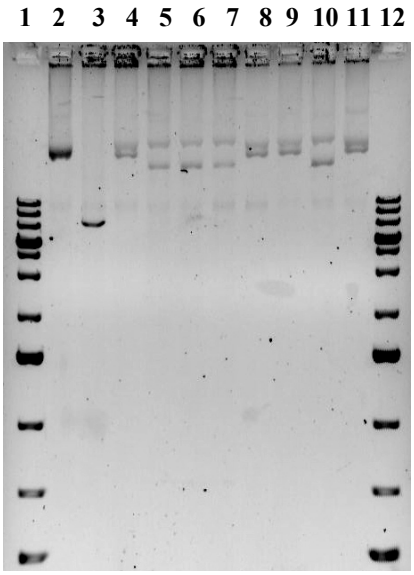


Figure 3.10: Lanes 1and 12 are molecular weight markers, lanes 2-11 are the DNA preparations digested with *Xba*I.

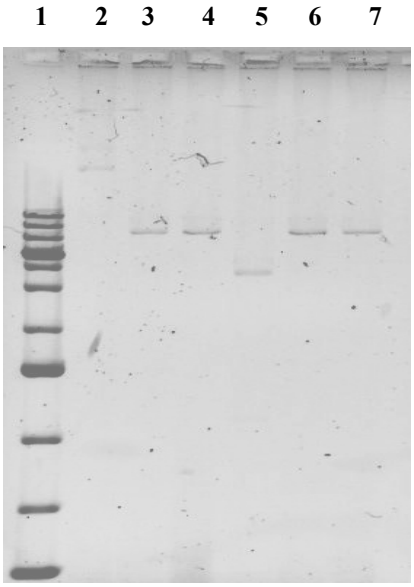


Figure 3.11: Lane 1 is the molecular weight marker, lane 2 is the undigested DNA, lane 3 is *Spe*I digest, lane 4 is *Pst*I digest, lane 5 is *Hind*III digest, lane 6 is *Eco*RI digest and lane 7 is *Xba*I digest.

All of the enzymes used to digest the DNA preparation linearized the plasmid except for *Hind*III which should give two fragments. **Figure 3.11** confirmed that the *Xba*I restriction site was introduced in plasmid pNDW1 which was named pNDW5. The other restriction enzymes used indicated that *Xba*I linearized the plasmid as did the other enzymes. To check if the suicide gene was still functional after the introduction of the new site, pNDW5 was transformed into *E. coli* MM294-4 λ lysogen and *E. coli* MM294-4 non-lysogen. pDA71* and pNDW1 were controls.

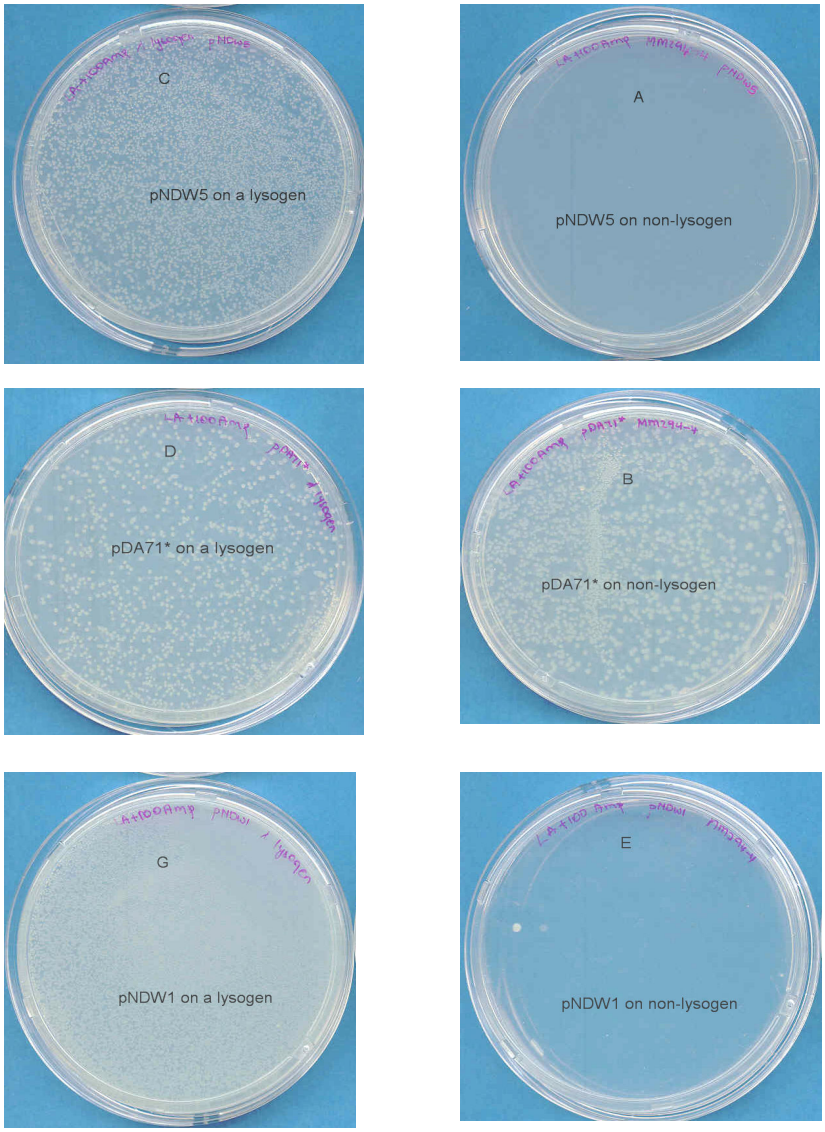


Figure 3.12: *E. coli* transformed with pNDW1, pNDW5 and pDA71* showing the behavior of pNDW5 in relation to pNDW1 and pDA71*.

The function of the suicide gene was maintained, as shown by the transformants on plate C but not A (**Figure 3.12)**. DNA preparations of pNDW5 on plate C (**Figure 3.12**) were done and the DNA was digested with *Xba*I to check if the transformants have plasmid pNDW5 (**Figure 3.14**). **Figure 3.13** is a map of pNDW5 showing the new *Xba*I site between restriction sites *Bgl*II and *Pst*I on the *EcoR*I endonuclease gene.

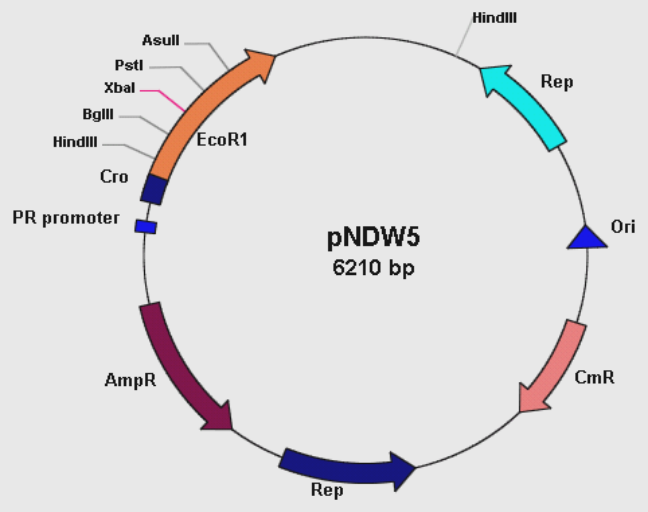


Figure 3.13: Restriction map of plasmid pNDW5.

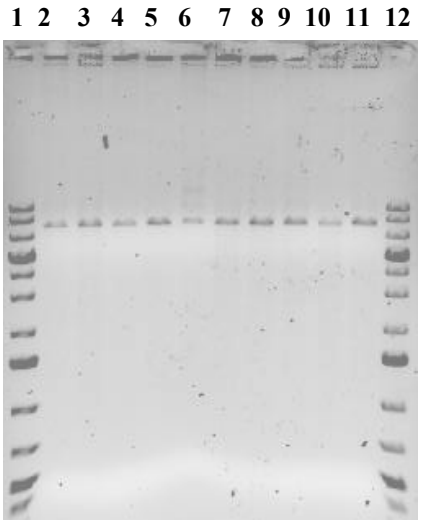


Figure 3.14: Plasmid DNA of randomly picked transformants digested with *Xba*I. Lanes 1 and 12 are molecular weight markers. Lanes 2-11 are DNA digests.

DNA of clones from plate C **Figure 3.12** was digested with *Xba*I and was also digested with *Xba*I. **Figure 3.15** shows the gel image of the digestions. This confirmed that the site was present in pNDW5 but not its parent pNDW1.

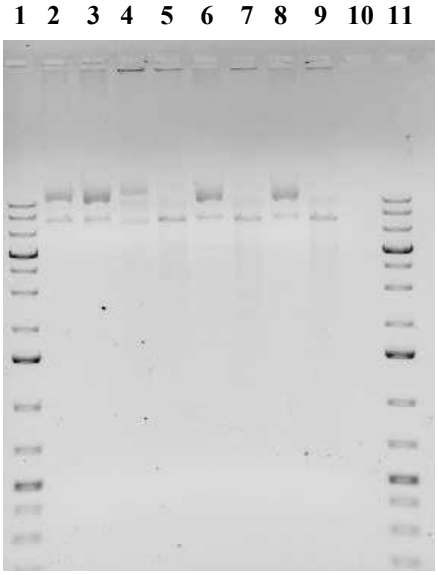


Figure 3.15: pNDW1 and pNDW5 digested with *Xba*I. Lane 1and 11 are molecular weight markers. Lanes 2 and 4 is undigested pNDW1 and pNDW5, respectively. Lanes 3, 6 and 8 are pNDW1 digested with *Xba*I. Lanes 5, 7 and 9 are pNDW5 digested with *Xba*I.

pNDW5 was double digested with *Bgl*II and *Pst*I to cut out the 175bp fragment that had incorporated the *Xba*I restriction site which was cloned into pUC19 pre-incubated with calf intestinal phosphatase via the *Bam*HI and *Pst*I restriction sites. pUC19 was used because the multiple cloning sites is flanked with sequencing primers. This construct was sent to Inqaba Biotechnical industries (Pty) Ltd for sequencing to confirm the presence of the *Xba*I site. Aligning the mutated and unmutated sequences using the Basic local alignment search tool (BLAST) showed the predicted CT→ TC base change (red and underlined)

```
AGAATATATCAGAGATAGCGAATTTTATGCTCTCTGAGAGCCACTTTCCTTACGTCCTTT           60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AGAATATATCAGAGATAGCGAATTTTATGCTCTCTGAGAGCCACTTTCCTTACGTCCTTT  62


TTCTAGAGGGGTCTAACTTTTTAACAGAAAATATCTCAATAACAAGACCAGATGGAAGGG  120
|  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TCTTAGAGGGGTCTAACTTTTTAACAGAAAATATCTCAATAACAAGACCAGATGGAAGGG  122

TTGTTAATCTTGAGTATAATTCTGGTATATTAAATAGGTTAGATCGACTAA  171
|||||||||||||||||||||||||||||||||||||||||||||||||||
TTGTTAATCTTGAGTATAATTCTGGTATATTAAATAGGTTAGATCGACTAA  173
```

Figure 3.16: The sequence alignments of the *Bgl*II-*Pst*I fragment of both the mutant pNDW5 (top sequence) and its parent pNDW1 (bottom sequence).

Genomic DNA of *B. polymyxa* digested with *Xba*I was ligated to *Xba*I-digested pNDW5 and transformed into *E. coli* MM294-4. Minipreps of 10 randomly chosen transformants were digested with *Xba*I to determine if they contain the recombinant pNDW5 plasmid. **Figure 3.17** shows the presence of inserts of various sizes as one would expect.

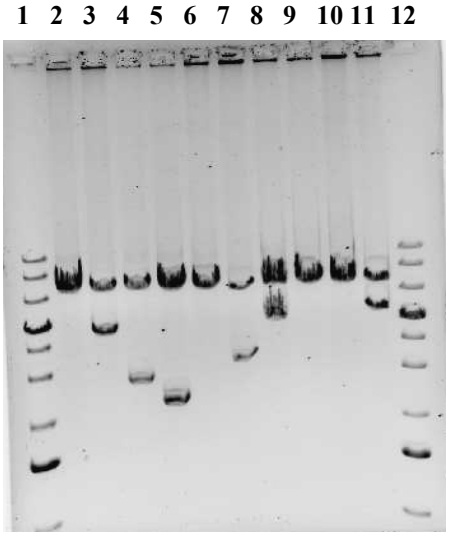


Figure 3.17: pNDW5 clones digested with *Xba*I to release inserts from the plasmid. Lanes 1 and 12 are molecular weight markers. Lanes 2-11 are plasmid DNA (pNDW5 clones) from transformants randomly chosen and digested with *Xba*I.

Different clones of pNDW5 (**figure 3.17)** did not transform *S. aureus* or *B. subtilis* but when a pool of clones was used transformants were observed in both hosts. This suggests that the individual clones that were used to transform *S. aureus* and *B. subtilis* had big fragment sizes (more than 1.5Kb) that could have resulted in low transformation efficiency and these host strains might have a restriction modification system resulting in no transformants.

3.2.2 Introduction of more new sites

*Cla*I, *Avr*II and *Xho*I were other candidate restriction sites to be introduced as described in **section 3.2**. However digesting parent plasmids pC194 and pEcoR251 with *Cla*I indicated that pC194 had two sites for this enzyme (**Figure 3.18**). *Avr*II, *Xba*I and *Spe*I are isocaudomers (i.e. generate identical single-strand ends). *Xho*I was selected as it cut *B. polymyxa* genomic DNA generating mainly large fragments (**Figure 3.7**) and digestion of pC194 and pEcoR251 confirmed absence of the *Xho*I site in both (**Figure 3.18**).

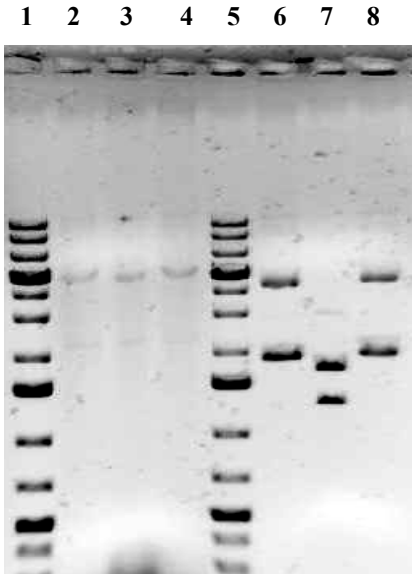


Figure 3.18: pEcoR251 and pC194 digestions. Lanes 1 and 5 are molecular weight markers. Lane 2 is undigested pEcoR251, lane 3 is *Cla*I digested pEcoR251 and lane 4 is *Xho*I digested pEcoR251. Lane 6 is undigested pC194, lane 7 is *Cla*I digested pC194 and lane 7 is *Xho*I digested pC194.

Introduction of the *Xho*I site was done by site-directed mutagenesis using the following primers: Mutagenic primer 5′-TTGTTTCAAA**C**TC**G**AGCATCAAACCT-3′ and reverse primer 5′-ATAAAGTACCGCCAAGATCAGGGTCA-3′.

TABLE 3.4 Mutagenic reaction mixture for *Xho*I site

Components	Volume/50µl reaction	Final concentration
Sterile dH_2O	37.3µl	
5X Phusion HF buffer	10µl	1X
10mM dNTPs	1µl	200µM each
Mutagenic primer	0.25µl	0.5µM
Reverse primer	0.25µl	0.5µM
DNA template (0.7ng/µl)	0.7µl	10pg
Phusion hot start DNA Polymerase (2U/µl)	0.5µl	0.02U/µl

TABLE 3.5 Cycling conditions for mutagenic reaction for *Xho*I site

Cycle step	Temperature	Time	Number of cycles
Initial Denaturation	98ºC	30 Sec	1
Denaturation	98ºC	10 sec	25
Annealing	67ºC	30 sec	
Extension	72ºC	4 min	
Final Extension	72ºC	5 min	

A PCR reaction was performed using the reaction and cycling conditions in **tables 3.4** and **3.5** above. Five micro liters of the PCR product was ran on a 0.4% agarose gel to check if amplification of the template had occurred (**Figure 3.19)**.



Figure 3.19: PCR product ran on 0.4% agarose gel. Lane 1 is the molecular weight marker, lane 2 is the 5µl of the DNA template digested with *Xba*I, lane 3 is 5µl of the PCR product and lane 4 is the PCR control with no DNA template.

The PCR product was ligated with T4 DNA ligase (Fermentas) and introduced into *E. coli* λ lysogen. Ten transformants were chosen and DNA digested with *Xho*I (**Figure 3.20)**.

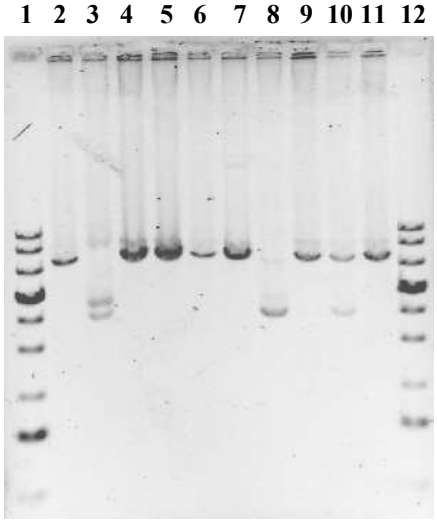


Figure 3.20: DNA preparations digested with *Xho*I. Lanes 1 and 12 are molecular weight markers. Lanes 2-11 is the DNA preparation digested with *Xho*I.

Each of these DNAs was introduced into *E. coli* λ lysogen and *E. coli* non-lysogen to check the function of the suicide gene. Only DNAs from lanes 2, 3 and 11 indicated that the suicide gene was still functional as indicated by the presence of transformants on *E. coli* λ lysogen. DNA preparations for 16 transformants of the DNAs 2, 3 and 11 were selected from each plate and labeled 1-16. The DNA preparations were digested with *Xho*I and run on 0.6% agarose gel.

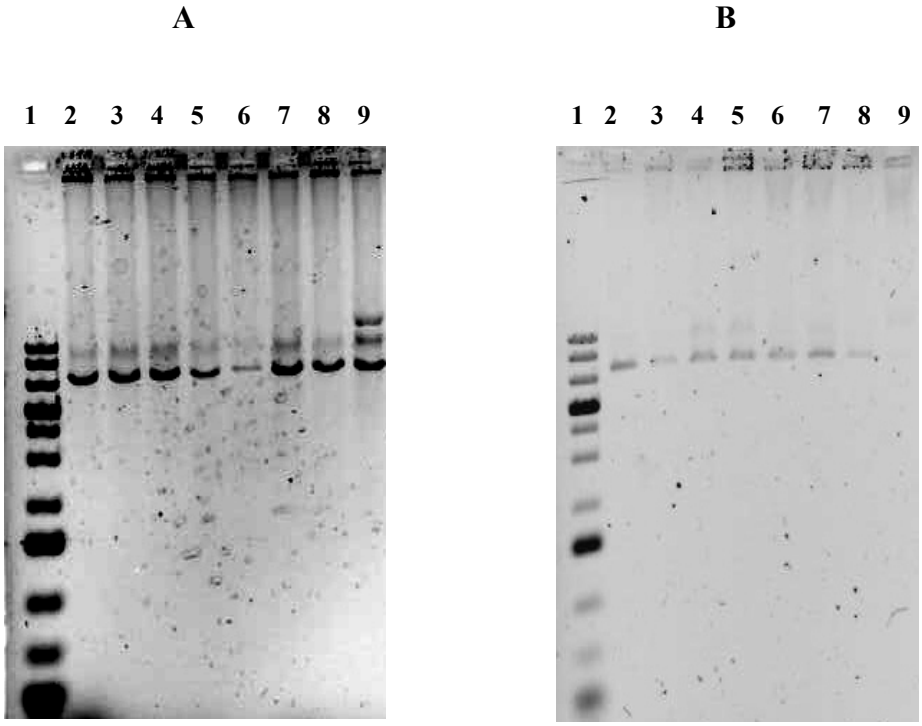


Figure 3.21: DNA preparations 1-16 digested with *Xho*I. (**A**) lane 1 is molecular weight marker and lanes 2-9 is DNA 1-8, respectively. (**B**) Lane 1 is molecular weight marker and lanes 2-9 are DNA 9-16, respectively.

DNA of the clone in lane 8 (**Figure 3.21 A**), named pNDW7, and from lane 2 (**Figure 3.21 B**), named pNDW8 were digested with selected restriction enzymes. Plasmid pNDW7 had similar restriction pattern to pNDW5 in **figure 3.11** but pNDW8 had different restriction digestion pattern because when digested with *Hind*III it indicating a partial digest.

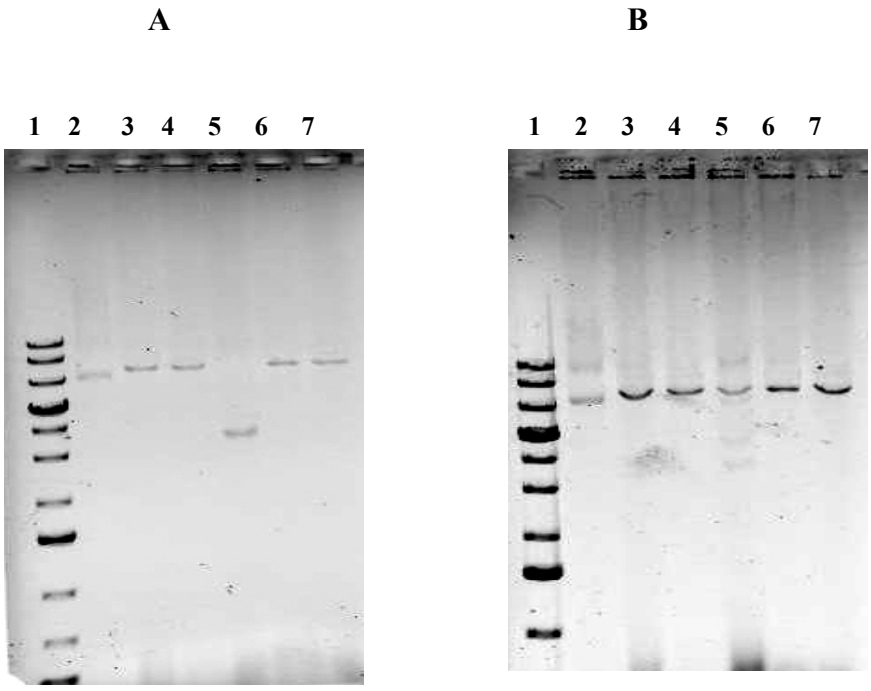


Figure 3.22: Restriction digestions of pNDW7 and pNDW8. (A) Is pNDW7. Lane 1 is molecular weight marker. Lane 2 is undigested DNA, lane 3 is *Xho*I digest, lane 4 is *Stu*I digest, lane 5 is *Hind*III digest, lane 6 is *Xba*I digest and lane 7 is *Eco*RI digest. (B) Is pNDW8. Lane 1 is molecular weight marker. Lane 2 is undigested DNA, lane 3 is *Xho*I digest, lane 4 is *Stu*I digest, lane 5 is *Hind*III digest, lane 6 is *Xba*I digest and lane 7 is *Eco*RI digest.

To check if pNDW7 has a functional suicide gene, it was transformed into *E. coli* λ lysogen and *E. coli* non-lysogen. Controls used were pDA71* and pNDW5. **Figure 3.23** indicated that transformants were observed on lambda lysogen (**image B**) not on a non-lysogen (**image C**) confirming this was so. **Figure 3.25** is the plasmid map of pNDW7

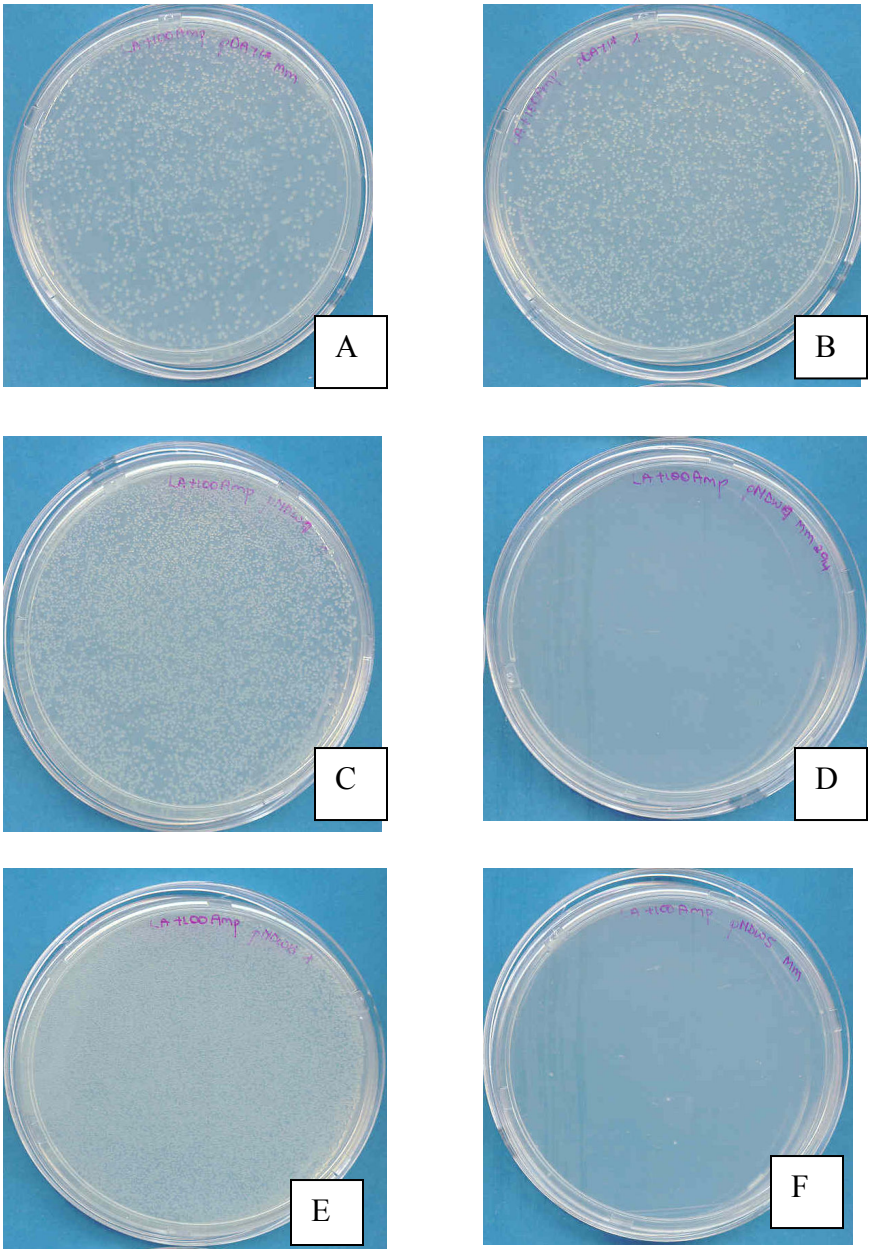


Figure 3.23: Transformation plates indicating the behavior of pNDW7 on a lysogen and a non-lysogen. Image A and B is pDA71* in non-lysogen (A) and in λ lysogen (B). Image C and D is pNDW7 on a non-lysogen (D) and on a lysogen (C). Image E and F is pNDW5 on lysogen (E) and on a non-lysogen (F).

To confirm the *Xho*I site was specific to pNDW7, DNA preparations from plate C **figure 3.23** were digested with *Xho*I and pNDW5 was also digested with *Xho*I (**Figure 3.24)**.

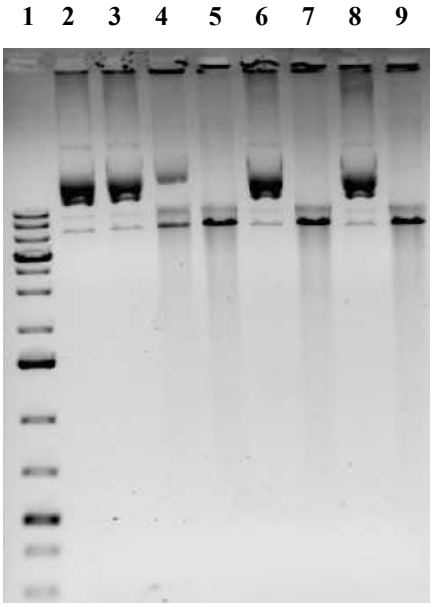


Figure 3.24: Digestions of pNDW5 and pNDW7. Lane 1 is the molecular weight marker. Lane 2 is pNDW5 undigested, lane 3 is pNDW7 undigested, lane 4 is pNDW5 digested with *Xho*I, lane 5 is pNDW7 digested with *Xho*I. Lane 6 is pNDW5 digested with *Xho*I, lane 7 is pNDW7 digested with *Xho*I, lane 8 is pNDW5 digested with *Xho*I and lane 9 is pNDW7 is digested with *Xho*I.

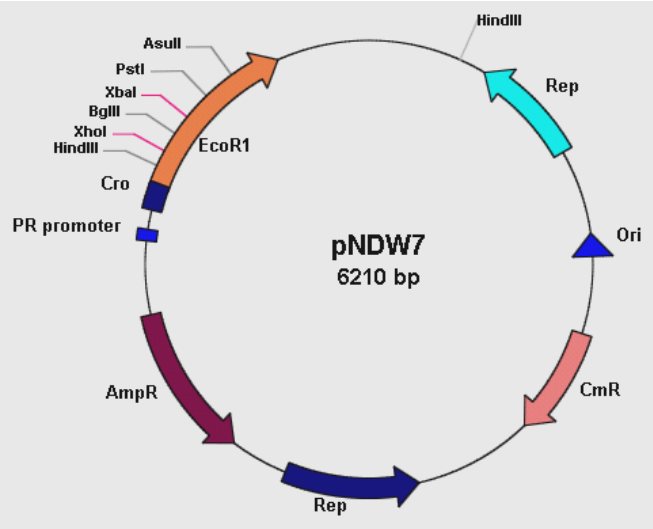


Figure 3.25: Restriction map of plasmid pNDW7.

B. polymyxa genomic DNA was digested with *Xho*I and ligated into pNDW7 digested with the same enzyme and transformed into *E. coli* MM294-4: surprisingly, no colonies

were observed. Genomic DNA of *Streptomyces pseudogriseolus* was then used and was digested with *Xho*I (**Figure 3.26**). This genomic DNA was cloned into *Xho*I pNDW7 digests and transformed into *E. coli* MM294-4. Colonies were observed and their plasmids screened for inserts and indicated that genomic DNA digested with *Xho*I can be cloned into the *Xho*I site of pNDW7 (**Figure 3.27**).

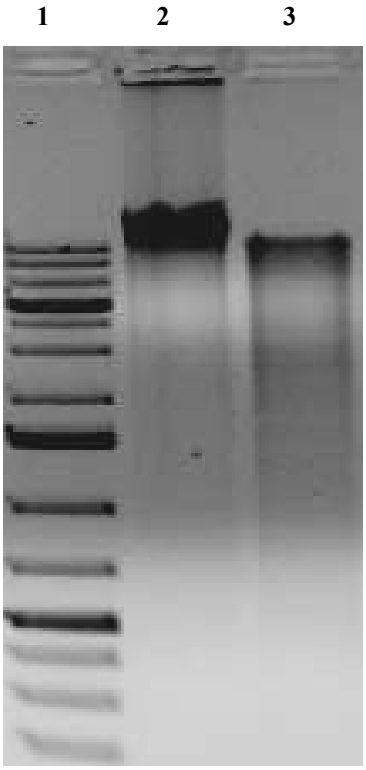


Figure 3.26: *Streptomyces pseudogriseolus* genomic DNA. Lane 1 is molecular weight marker, lane 2 is genomic DNA undigested and lane 3 is genomic DNA digested with *Xho*I.

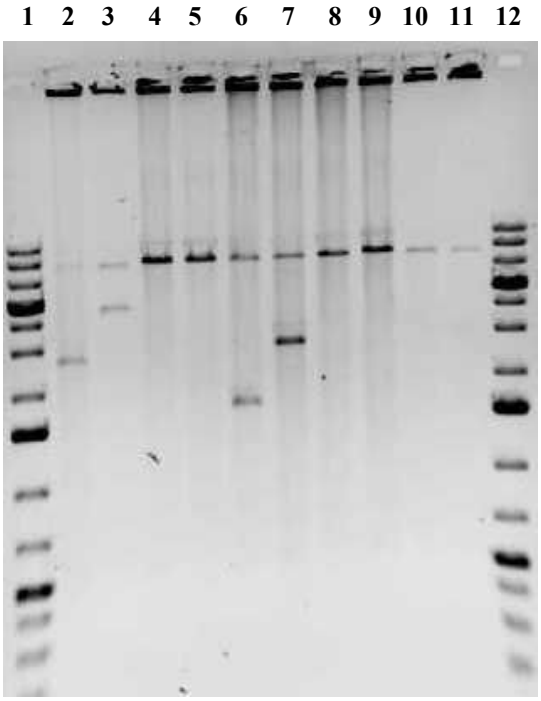


Figure 3.27: DNA preparations of transformants digested with *Xho*I. Lane 1 and 12 are molecular weight markers. Lanes 2-10 is pNDW7 clones digested with *Xho*I.

The *Hind*III-*Bgl*II fragment from pNDW7 was cloned into pUC19 treated with alkaline phosphatase via the *Hind*III and *Bam*HI sites. Sequencing confirmed the presence of an *Xho*I site at the expected position but in addition there was a three base deletion (**figure 3.28)**. This is in-frame and predicted to result in a valine residue deletion. However the endonuclease activity of the *EcoR*I endonuclease gene was intact.

```
AAAAATTGACCCTGATCTTGGCGGTACTTTATTT---TCAAACTCGAGCATCAAACCTGA  57
|||||||||||||||||||||||||||||||||    ||||| || ||||||||||||||
AAAAATTGACCCTGATCTTGGCGGTACTTTATTTGTTTCAAATTCCAGCATCAAACCTGA  63

TGGTGGAATTGTAGAGGTCAAAGATGATTATGGTGAATGGAGAGTTGTACTTGTTGCTGA  117
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGGTGGAATTGTAGAGGTCAAAGATGATTATGGTGAATGGAGAGTTGTACTTGTTGCTGA  123
```

Figure 3.28: Sequence alignment of the *Hind*III-*Bgl*II fragments of the mutated and a non mutated sequence of the suicide gene. The top sequence is the mutated sequence and the bottom sequence is the non-mutated sequence.

The *Hind*III-*Bgl*II fragment of the original *Eco*RI endonuclease gene from plasmid pEcoR251 was sent for sequencing to check if the deletion was present prior to the mutagenesis or not.

```
AAAAAATTGACCCTGATCTTGGCGGTACTTTATTTGTTTCAAATTCCAGCATCAAACCTG  67
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AAAAAATTGACCCTGATCTTGGCGGTACTTTATTTGTTTCAAATTCCAGCATCAAACCTG  69

ATGGTGGAATTGTAGAGGTCAAAGATGATTATGGTGAATGGAGAGTTGTACTTGTTGCTG  127
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ATGGTGGAATTGTAGAGGTCAAAGATGATTATGGTGAATGGAGAGTTGTACTTGTTGCTG  129

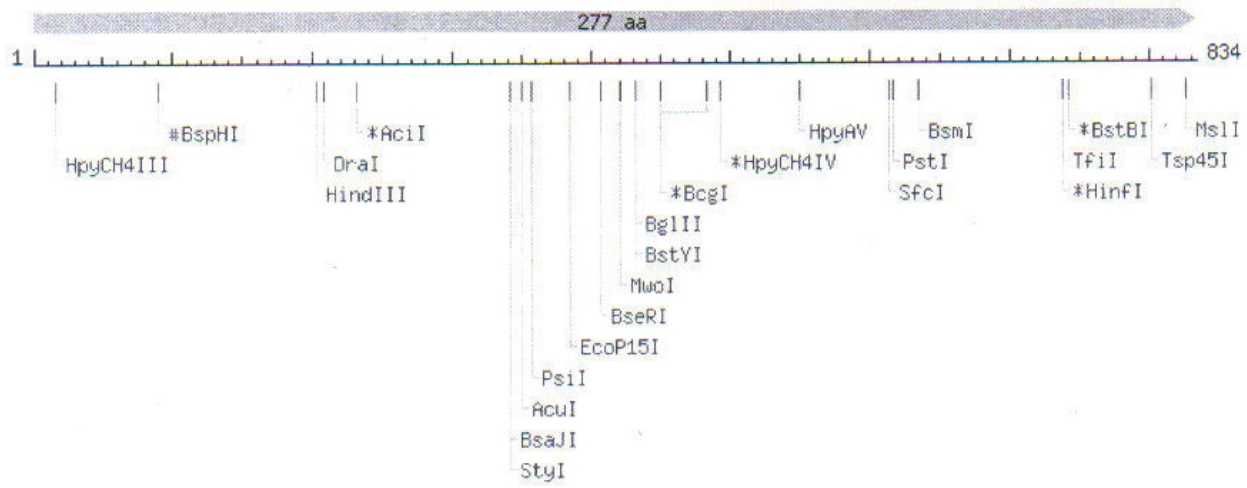
AAGCCAAACACCAAGGTAAAGATATTATAAATATAAGGAATGGTTTGTTAGTTGGGAAAA  187
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AAGCCAAACACCAAGGTAAAGATATTATAAATATAAGGAATGGTTTGTTAGTTGGGAAAA  189

GAGGAGATCAAGATTTAATGGCTGCTGGTAATGCTATCGAA  228
|||||||||||||||||||||||||||||||||||||||
GAGGAGATCAAGATTTAATGGCTGCTGGTAATGCTATCGAA  230
```

Figure 3.29: Sequence alignment of the original *Eco*RI *Hind*III-*Bgl*II fragment aligned to the known sequence of *Eco*RI endonuclease gene. Top sequence (from pEcoR251) and bottom sequence (known sequence).

A deletion was not present in the *Eco*RI gene from pEcoR251 (**Figure 3.29**). The deletion apparently arose during the mutagenesis procedure. The mutated *Eco*RI gene sequence was put on the NEBcutter website to confirm the presence of newly introduced sites. The output from the website indicated that the new sites were (**figure 3.30)**.

A



B

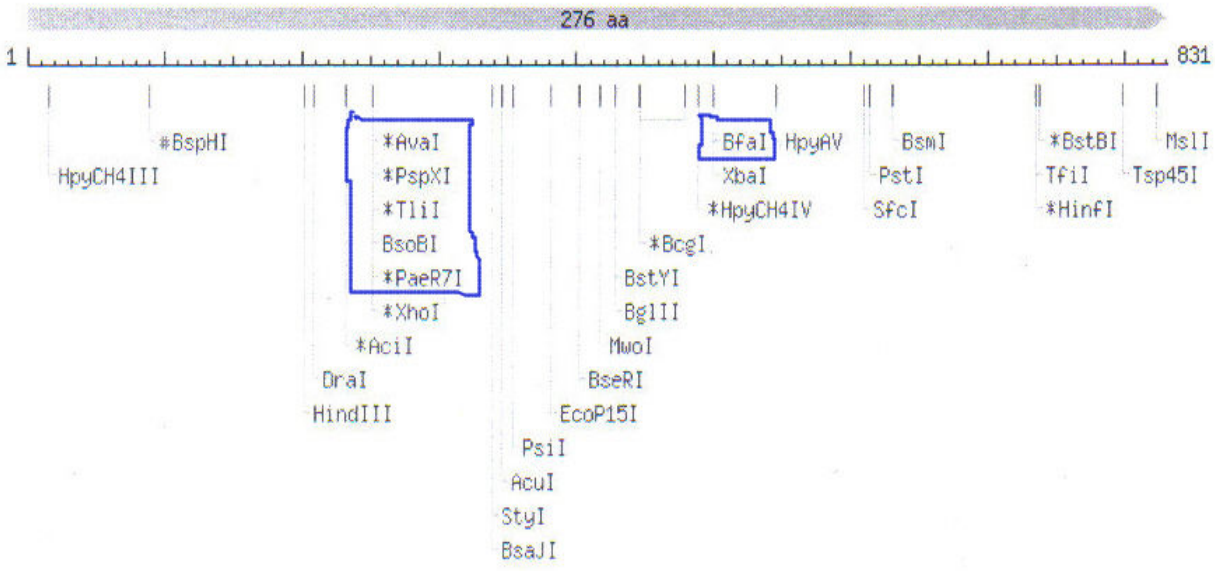


Figure 3.30: Predictions of restriction sites on original and mutated *EcoR*I endonuclease gene. (A) Is the original *EcoR*I sequence. (B) Is the mutated *EcoR*I sequence, the blue box indicates the new restriction sites predicted by NEBcutter program.

3.2.3 Introduction of the *Xba*I restriction site into pDA71

Plasmid pDA71 is an 8.8Kb *E. coli-Rhodococcus* shuttle vector which has an ampicillin resistant determinant expressed in Gram-negatives and a chloramphenicol resistant determinant expressed in Gram-positives; it has been successfully used to clone more than thirty genes of medical or bioremediative interest (Dabbs *et al*., 1995). Introduction of *Xba*I restriction sites in pDA71 allows the use of *Spe*I, *Nhe*I and *Avr*II restriction sites to construct genomic libraries. To introduce the *Xba*I restriction site into it the 175 bp *Bgl*II-*Pst*I fragment from pNDW5 was used to substituted with the same fragment from pDA71. Plasmid pDA71 was double digested with *Bgl*II and *Pst*I and phosphatased. Ligations were transformed into *E. coli* λ lysogen. DNA minipreps were done of 10 transformants were digested with *Xba*I. **Figure 3.31** shows the gel image of the transformants.

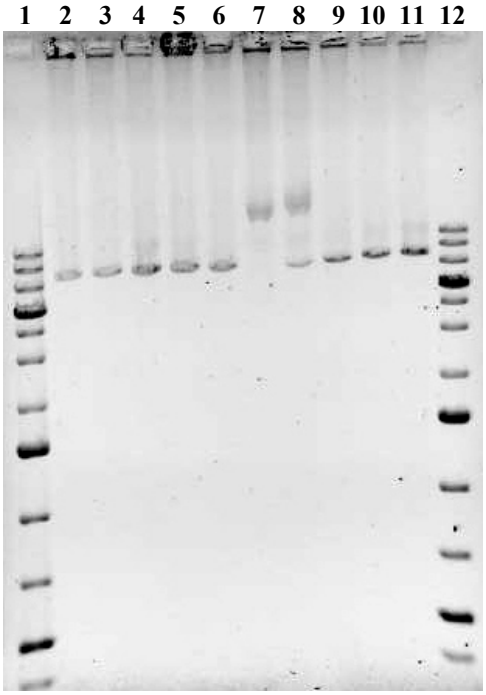


Figure 3.31: DNA preparations digested with *Xba*I to screen for the presence of *Xba*I site in pDA71. Lane 1 and 12 are molecular weight markers. Lanes 2-11 are DNA preparations digested with *Xba*I.

The DNA preparation on lane 3 **Figure 3.31** was further digested with a couple of restriction enzymes in order to determine whether it is a correct plasmid (**Figure 3.32**) and was named pDA71-1. *Stu*I digestion result indicated that it is a correct plasmid because of the presence of two *Stu*I site giving one smaller fragment and one large fragment.

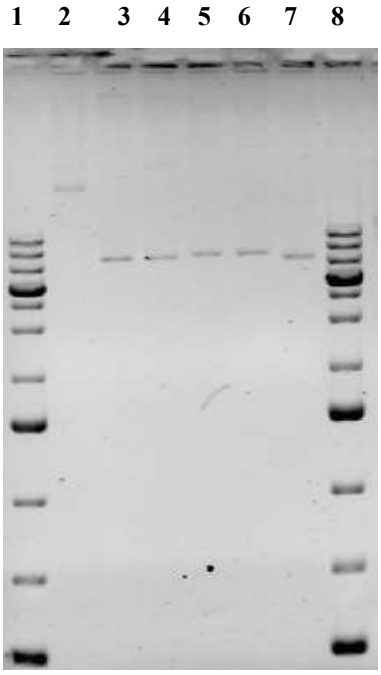


Figure 3.32: Digestion of the pDA71-1 that contains *Xba*I. Lanes 1 and 8 are molecular weight markers; lane 2 is undigested pDA71-1, lane 3 is *Hind*III digest, lane 4 is *Bgl*II digest, lane 5 is *Sfu*I digest, lane 6 is *Xba*I digest and lane 7 is *Stu*I digest.

pDA71-1 was transformed into *E. coli* λ lysogen and non-lysogen together with pNDW5, pDA71* to determine if the suicide gene was functional (**Figure 3.33**).

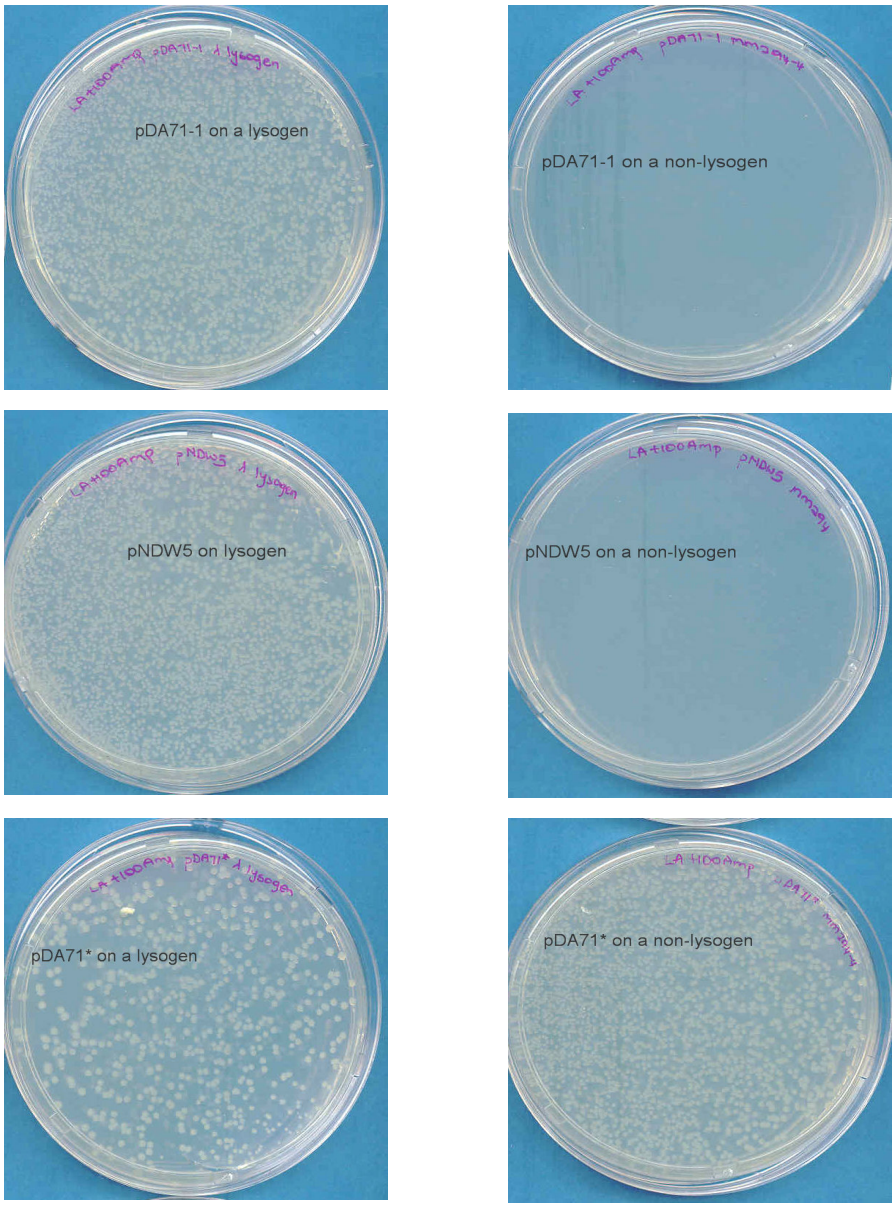


Figure 3.33: Transformation plates indicating the behavior of pDA71-1.

The plates in **figure 3.33** indicates that the suicide gene is functional hence the transformants on a lysogen. DNA was inserted into the *Xba*I site of pDA71-1 to determine if this site is useful in cloning DNA and can be released from the plasmid by digestion with *Xba*I. Plasmid map of pDA71-1 is presented in **Figure 3.35**. Genomic DNA of *B. polymyxa* was digested with *Xba*I and ligated into *Xba*I digested pDA71-1.

This was transformed into *E. coli* MM294, selected for ampicillin resistance, and transformants screened for the presence of the plasmid that has an insert (**Figure 3.34**).

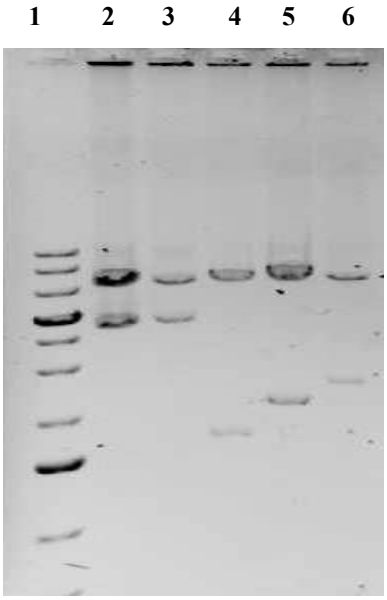


Figure 3.34: plasmid DNA of pDA71-1 clones digested with *Xba*I to release cloned DNA. Lane 1 is the molecular weight marker and lanes 2-6 are plasmid DNA with inserts released.

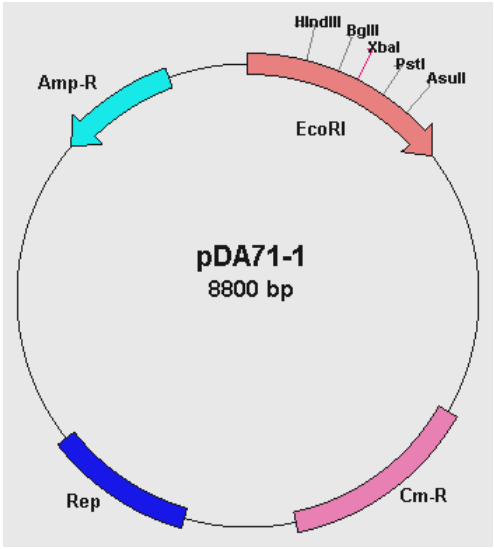


Figure 3.35: Restriction map of plasmid pDA71-1.

3.2.4 Introduction of *Xho*I site into plasmid pEcoR251

Plasmid pEcoR251 is 3.3 kb in size and is useful in the construction of *E. coli* positive selection shuttle vectors. It can provide a Gram-negative replicon for the shuttle vector and a positive selection gene. The *Hind*III-*Bgl*II fragment of the plasmid pNDW7 (**Figure 3.25**) was used as a source of the *Xho*I site. Plasmid pEcoR251 was digested with the same enzymes and phosphatased. The *Hind*III-*Bgl*II fragment from pNDW7 was used to substitute a *Hind*III-*Bgl*II fragment of pEcoR251. Transformants minipreps were screened for the presence of an *Xho*I site. Eight transformants were tested and all of them were digested with *Xho*I indicating that the site was present (**figure 3.36**). Four of the DNA preparations of the 8 transformants were transformed into *E. coli* λ lysogen and a non lysogen to check which of these preparations had a functional suicide gene.

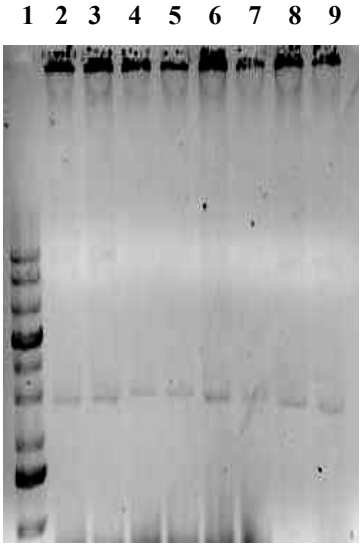


Figure 3.36: DNA preparation of transformants digested with *Xho*I. Lane 1 is molecular weight marker. Lanes 2-9 is *Xho*I digests.

Only DNA from lane 2 (**Figure 3.36**) had a functional suicide gene and was further digested with restriction enzymes to confirm if it is pEcoR251 with a new site. **Figure 3.37** presents the gel image of the digestions.

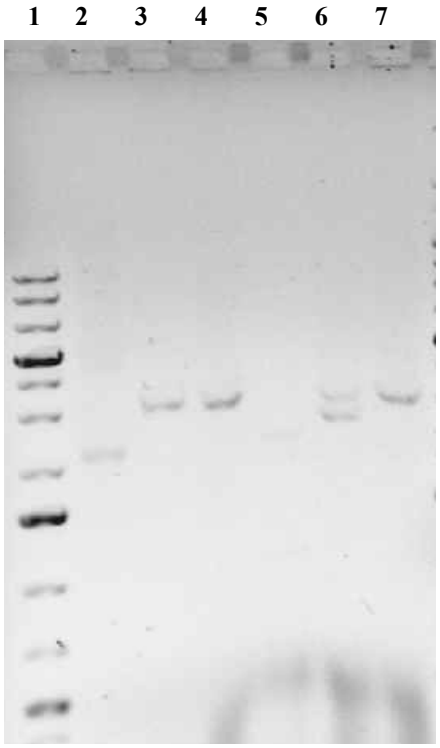


Figure 3.37: pEcoR251 with the new restriction site. Lanes 1 and 8 are molecular weight markers. Lane 2 is undigested, lane 3 is *Hind*III digest, lane 4 is *Bgl*II digest, lane 5 is *Bam*HI-*Eco*RI digest, lane 6 is *Sfu*I digest and lane 7 is *Xho*I digest.

This plasmid was then transformed into *E. coli* λ lysogen and a non-lysogen to check the function of the suicide gene. Plasmids pDA71* and pEcoR251 (original) were used as controls. **Figure 3.38** represents the behavior of the modified pEcoR251 which was named pEcoR251-1.

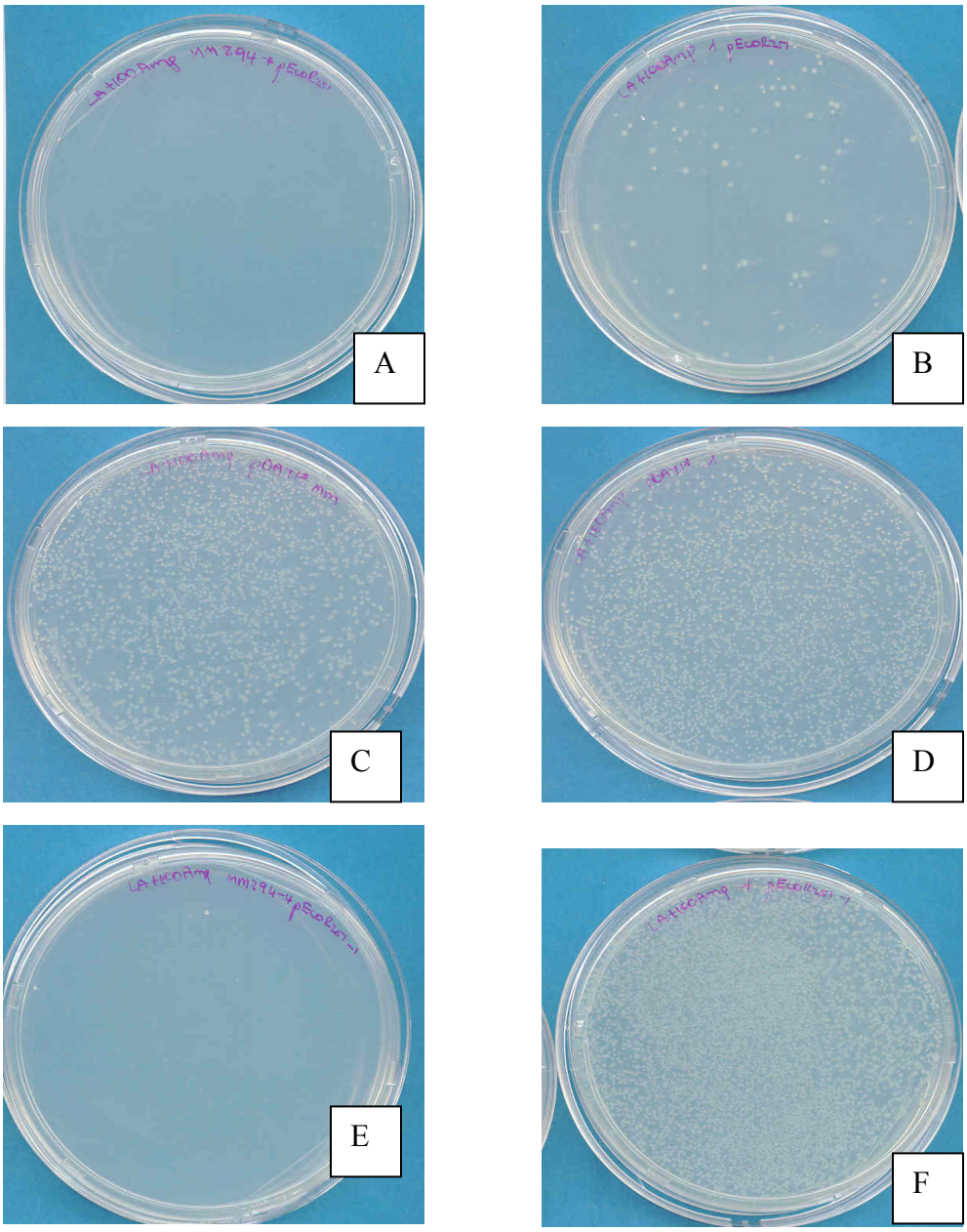


Figure 3.38: Transformants indicating the behavior of the modified pEcoR251. Image A and B is pEcoR251 on a non-lysogen and a lysogen, respectively. Image C and D is pDA71* on a non-lysogen and a lysogen, respectively. Image E and F is pEcoR251-1 on a non-lysogen and a lysogen, respectively.

3.3 Inactivation of rifampicin by *Bacillus* species

Preliminary experiments were done to confirm that one of the shuttle vectors I had made could be used to clone *Bacillus* genes. Members of this genus were shown by Dabbs et al. (1995) to have the ability to inactivate rifampicin. *B. polymyxa* was a species that can inactivate rifampicin by glycosylation. Initially this strain was going to be used to screen for this inactivation gene then the focus of work changed and it was used for cloning by complementation in an auxotrophic mutants of *B. subtilis*.

3.4 Genomic library construction

A genomic library was constructed using plasmid pNDW1. This was done to check if this pNDW1 can be useful in the construction of a genomic library. The library was going to be used to check if there was any fragment of *B. polymyxa* genomic DNA that can complement the auxotrophic mutants, the KIT strains, in **section 2.1**. Genomic DNA of *B. polymyxa* was digested with restriction endonucleases to determine which restriction endonuclease will be used to construct a genomic library.

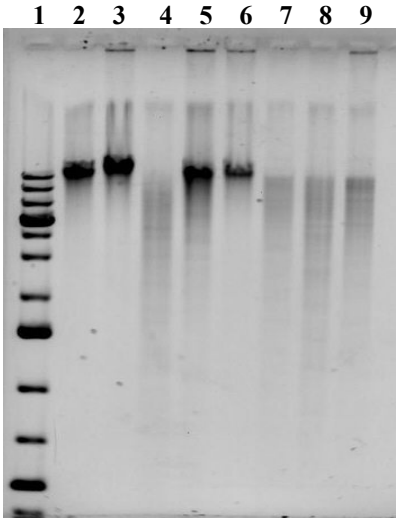


Figure 3.39: Genomic DNA digestions. Lane 1 is molecular weight marker, lane 2 is undigested DNA, lane 3 is *Bgl*II digest, lane 4 is *Hind*III digest, lane 5 is *Pst*I digest, lane 6 is *Nar*I digest, lane 7 is *Cla*I digest, lane 8 is *Sfu*I digest and lane 9 is *Bcl*I digest.

The restriction endonuclease chose was the one that does not digest the genomic DNA too frequently as that could lead to pieces of gene scattered all over a genomic library. It is desirable to have a restriction endonuclease that does not cut genomic DNA frequently because the possibility of getting the entire gene in a plasmid is high. **Figure 3.39** indicates that *Bgl*II does not cut the genomic DNA as there is no difference between the *Bgl*II digest and the undigested genomic DNA. *Hind*III, *Cla*I, *Sfu*I and *Bcl*I cut the genomic DNA too frequently with that they were not used for the library construction. *Pst*I and *Nar*I were found chosen as they do not digest the genomic DNA too frequently. *Pst*I was of best choice because a *Pst*I site is present in pNDW1 and it would be easy to excise the fragment cloned into that site by simple digestion with *Pst*I.

3.4.1 Vector calibration

The amount of vector to be used for library construction was determined. This was to determine the minimum amount required to produce the least amount of transformants caused by mutation and plasmid re-ligating to itself. An aliquot of 200µl was digested with *Pst*I and after digestion the DNA was purified by phenol-chloroform extraction. Ligation reaction were set up with different amount of plasmid, 2µl of T4 DNA ligase buffer and 1µl of T4 DNA ligase was added. The volume was adjusted to 20µl and was incubated at 22ºC for 16-22 hours. The *E. coli* MM294-4 was then transformed with the ligation mixture. The amount of vector which gave the least amount transformants was chosen.

3.4.2 Genomic DNA calibration

The amount of genomic DNA to be used with the plasmid amount determined above, was also determined by first digesting the genomic DNA with *Pst*I and setting up ligations reaction to 20µl using 2µl of T4 DNA ligase buffer and 1µl of T4 DNA ligase. The only difference in this part was that the amount of vector determined above was kept constant and different amounts of the genomic DNA were used. The ligation mixtures in an

vector: insert ratio of 1:3 were transformed into *E. coli* MM294-4. The amount of genomic DNA that gave the most amounts of transformants was chosen.

3.4.3 Construction of the genomic library in *E. coli*

The library was constructed in a highly transformable *E. coli* strain MM294-4 using the *B. subtilis*-*E. coli* shuttle vector constructed in this study (pNDW1). The *Pst*I library gave inserts that were ≥1Kb (**figure 3.40**). The average number of transformants obtained per ligation reaction was desirable, 586 transformants were obtained. The statistical calculation of the probability (P) that any fragment of the genome would be represented in the library was done using the formula of Clarke and Carbon, 1976:

$$\mathbf{N = In\ (1-P) / In\ [1-(a/b)]}$$

where N=number of clones
P= probability (95%)
a= average insert size
b= genome size (assumed to be 4.5×10^6)

Using the formula above the number of clones required to represent the library was determined to be 6 608 clones.

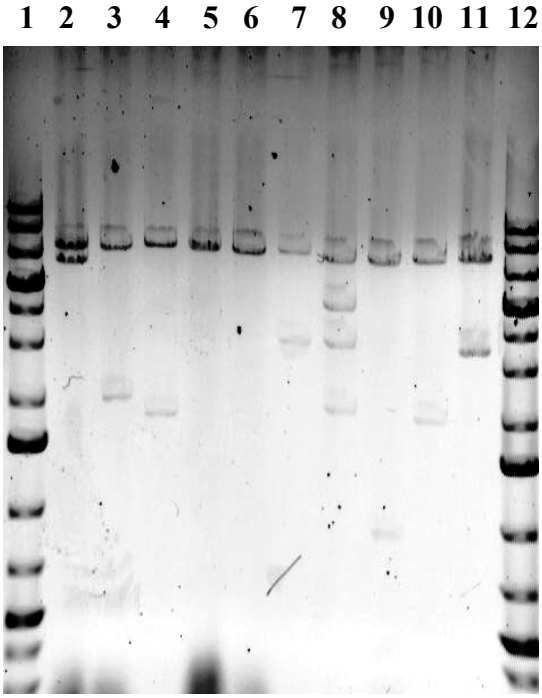


Figure3.40: Independent clone digested with *Pst*I from library construction. Lane 1 and 12 are molecular weight markers. Lanes 2-11 are clones digested with *Pst*I to determine the insert sizes.

4. DISCUSSION

Plasmids isolated from *Staphylococcus aureus* also replicate in *Bacillus subtilis*. That led to development of gene cloning technology in *B. subtilis* aided by the construction of vectors that are maintained in both *B. subtilis* and *Escherichia coli*. Gene cloning directly in *B. subtilis* as a primary host is relatively inefficient so it is preferable to conduct cloning steps with a shuttle plasmid in *E. coli* then transfer the recombinant plasmids into *B. subtilis* (Bruckner, 1992; Sullivan *et al*., 1984). Vectors of this type allow the use of well-developed techniques for the isolation and manipulation of DNA in *E. coli*. The resulting hybrid molecules can then be introduced into *B. subtilis* by transformation. Gryzan *et al*. (1980) have also shown that bi-functional plasmids are of great importance because the generation of hybrid plasmids by direct transformation of ligation mixtures into *B. subtilis* is very inefficient probably due to the inability of plasmid monomers to transform competent *B. subtilis* (Canosi *et al*., 1978). Most plasmids functioning in both *B. subtilis* and *E. coli* are based on the use of the rolling cycle plasmid isolated from S. *aureus*. In this study I have also adopted the same approach, using plasmid pC194.

This was joined to plasmid pEcoR251 which carries an *EcoR*I endonuclease gene, giving a positive selection shuttle vector. The positive selection is due to insertional inactivation of the *EcoR*I endonuclease gene providing a simple method for selecting recombinant plasmids. *E. coli-B. subtilis* shuttle vectors have been previously constructed, and selection in these plasmids is by insertional inactivation of a resistant marker (Gryczan *et al*., 1980; Sullivan *et al*,. 1984)). This selection procedure can be time consuming as transformants have to be screened for loss of a resistant marker as an indication that foreign DNA has been inserted. Vieira and Messing (1982) constructed shuttle plasmids that contain a fragment of the *lacZ* gene which is functional in α-complementation. Upstream of this is an array of unique restriction sites and insertion of DNA into any of these blocks expression, causing a loss of α-complementation and resulting in a Lac^- phenotype. Transformants containing inserts are readily detected directly by blue/white screening on medium supplemented with the chromogenic substrate X-Gal. With pNDW1 one has the advantage of only getting recombinant transformants because those

that do not contain an insert will be killed by the endonuclease activity of the suicide gene.

Shuttle vectors based on plasmids such as pUB110, pC194 and pE194 replicate stably in *B. subtilis*, but addition of recombinant DNA may confer structural instability and sometimes segregational stability. The molecular basis for this is the mode of replication. These plasmids replicate by a rolling circle mechanism producing single-stranded DNA as an intermediate, and short direct repeats within this single-stranded DNA may lead to the deletion of one of the repeats (Bron *et al*., 1987; Nguyen *et al*., 2005). Instability is also observed when a relatively small foreign DNA fragment results in structural and/or segregational stability (Grkovic *et al*., 2003). However, I found that plasmid pNDW1 and others I made did not show any evident structural instability. With pNDW1 the *Bacillus subtilis* and *Staphylococcus aureus* cells carrying the plasmid or clones of the plasmid were selected for chloramphenicol resistance, but this phenotype was only observed when the cells were incubated overnight. This suggested that the enzyme chloramphenicol acetyltransferase was expressed at low levels such that when allowed to grow overnight the level of expression would be enough to cause the aforementioned phenotype.

Plasmid pNDW1 without any insert was able to replicate in *S. aureus* suggesting that the suicide gene was not expressed in this species. That is in contrast to *B. subtilis* and *E. coli*. When pNDW4 and the same plasmid carrying a *Pst*I *B. polymyxa* genomic library were used to transform *S. aureus*, transformants were observed at a high efficiency indicating that disruption of the suicide gene gives rise to more transformants. This means that this plasmid can be used in *S. aureus* and selection of recombinant transformants would be challenging because the suicide gene is not expressed in this host.

A previous limitation of the EcoR-based system was that there were only four useful unique restriction sites: *Hin*dIII, *Bgl*II, *Pst*I, and *Sfu*I. I improved the shuttle vector I made by introducing two new restriction sites for cloning and subcloning purposes, *Xba*I and *Xho*I. The former generates the same single-stranded ends as *Avr*II, *Spe*I, and *Nhe*I. The latter produces the same single-stranded ends as *Sal*I. The *Xba*I site was introduced

into the suicide gene between the *Bgl*II and *Pst*I sites and the *Xho*I was introduced between *Hind*III and *Bgl*II site. These are both relatively early in the gene, desirable since studies have shown that positive selection by insertion of DNA towards the 3′ end is relatively inefficient (E. Dabbs, personal communication). This may be because such a fusion protein still possesses endonuclease activity. *Bacillus* genomic DNA was successfully cloned into pNDW5 at the *Xba*I site with pNDW7 and *Xho*I transformants were not obtained, probably because of the large size of inserts. *Streptomyces pseudogriseolus* genomic DNA digested with *Xho*I was used and transformants were obtained. It should be borne in mind that the *B. subtilis* and *S. aureus* recipients used in this work have fully functional restriction systems (Seeber *et al*., 1990; Waldron, 2006). *B. subtilis* has the *BsuM* restriction modification system. This system recognizes the sequence CTCGAG the same as that of *Xho*I (Jentsch, 1983). The DNA cloned into plasmid pNDW7 has the *Xho*I site flanking the insert and nucleic was coming from *E. coli* which lacks the appropriate modification system means that the DNA is likely to be attacked by the *BsuM* restriction endonuclease. There are other restriction modification systems in *B. subtilis* such as the *BsuF* (isoschizomer of *Hpa*II), *BsuR* (isoschizomer of *Hae*III) and the *BsuB* (*Pst*I isoschizomer) (Jentsch, 1983). There are sites for all these in the plasmid pNDW1 in *S. aureus* there is the *Sau*1 type I restriction modification system as well as the *Sau*3AI type II restriction modification system which recognizes the GATC sequence (Seeber *et al*., 1990; Waldron, 2006). Future work could productively address elimination of these activities by gene disruption.

Introduction of these sites was confirmed by sequencing and with *Xba*I the expected base change was observed. When sequencing was done for the *Xho*I site, the *Hin*dIII-*Bgl*II fragment of the suicide gene of pNDW7 had in-frame deletion of a codon, predicted to remove a valine. The corresponding sequence from pEcoR251 revealed that the deletion was not present. Therefore it has arisen during the mutagenesis procedure. Removal of this valine did not affect the protein as the phenotype was observed when the behavior of pNDW7 was determined on a lysogen and a non-lysogen. This was consistent with the studies done by Wolfers and co-workers (1986) that the Glu111, Glu 144 and Arg 145 are essential for the nucleolytic activity of the EcoRI. They made mutants in which the Glu

111 is replaced by Gln, Glu 144 replaced by Gln and Arg 145 replaced by Lys which lost most of their endonucleolytic activity. This deletion arose concomitantly with the mutation introduced to the *EcoR*I sequence though usually with *Taq* polymerase it is a one nucleotide deletion not a codon.

Predictions of restriction sites on the double mutant *EcoR*I gene indicated that there were additional restriction sites in addition to those introduced (**Figure 3.30**). However, these are for enzymes that are not useful for cloning as they do not have a definite recognition sequence. The restriction enzymes *Ava*I, *Psp*XI, *Bso*BI do not have unique recognition sequences: 5′-CYCGRG-3′, 5′-VCTCGAGB-3′, and 5′-CYCGRG-3′, respectively.

I used my mutant plasmids to increase the versatility of related vectors. The *Bgl*II-*Pst*I fragment of pNDW5 was used to introduce *Xba*I into pDA71 (giving pDA71-1) to increase the number of restriction sites to use for cloning. The *Hind*III-*Bgl*II fragment of pNDW7 was used to introduce the *Xho*I site into pEcoR251. The *Xba*I site in pDA71-1 was shown to function effectively in cloning of genomic DNA.

Preliminary experiments were undertaken to check if pNDW1 can be used in library construction and that a *Bacillus* gene can be cloned using this system. Auxotrophic mutants I made using NTG and counter-selection method did not show specific auxotrophic requirement. The *Pst*I genomic library was electroporated into QB917 KIT-8 strain to complement the histidine and threonine auxotrophy but transformants were not observed. Protoplast transformation was successful but the efficiency was low. This emphasized again the desirability of generating restriction-deficient mutants of *B. subtilis*.

In conclusion, I constructed a shuttle vector with a positive selection (suicide) function replicating in *E. coli*, *B. subtilis* and *S. aureus* and showed cloning of DNA can be done by insertional inactivation allowing positive selection of recombinant transformants. I improved it by introducing two unique restriction sites allowing cloning of DNA digested by any of six additional restriction enzymes. These were used to introduce new restriction

sites into pEcoR251 and pDA71 Future work might productively focus on constructing non-restricting strains of *S. aureus* and *B. subtilis*.

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6. APPENDICES

APPENDIX A: MEDIA

Luria Bertani (LB) broth

Tryptone	3g
Yeast extract	1.5g
NaCl	1.5g
dH_2O	300ml

LB- agar

Tryptone	3g
Yeast extract	1.5g
NaCl	1.5g
Agar	4.5g
dH_2O	300ml

Brain Heart Infusion (BHI) Broth

BHI	3.7g
dH_2O	100ml

BHI agar

BHI	3.7g
Agar	1.5%

dH_2O	100ml

Spizizen's medium (Minimal medium for *B. subtilis*)

$(NH_4)_2SO_4$	2g
K_2HPO_4	14g
KH_2PO_4	6g
$Na_2H\ (C_3H_5O\ (COO)_3$	1g
dH_2O	1000ml

Glucose to final concentration 0.5% and auxotrophic requirement added after autoclaving

Minimal agar prepared by autoclaving 1.5% agar separately, then adding the salts prepared separately and glucose.

10X Stock III buffer

$K_2HPO_4.3H_2O$	91.7g
KH_2PO_4	26.8g
$MgSO_4$	1.0g
dH_2O	1L

Store at room temperature with chloroform added

LBS (LB plus 10.3% sucrose)

Tryptone	1g
Yeast extract	0.5g
NaCl	0.5g

Sucrose	10.3g
dH_2O	100ml

DM3 regeneration medium

4% Agar	200ml
1M Sodium succinate (pH 7.3)	500ml
5% Casamino acids	100ml
10% Yeast extract	50ml
3.5% K_2HPO_4 and 1.5% KH_2PO_4	100ml
20% Glucose	25ml
1M $MgCl_2$	20ml
Filter sterilized 2% Bovine Serum Albumin	5ml

All the components add up to a liter

APPENDIX B: SOLUTIONS

A6.1 Solutions used for plasmid preparations from *E. coli*

Solution I

Glucose	50mM
Tris-HCl	25mM
EDTA	10mM

pH 8.0

Solution II

NaOH	0.2M
SDS	1.0%

Solution III

Potassium acetate	5M, pH 4.8
Glacial acetic acid	11.5ml
dH_2O	88.5ml

Ribonuclease

10mg/ml solution in sdH_2O, boiled at 95ºC before use

A6.2 Solutions for plasmid preparations from Gram positives

TE buffer

0.5M EDTA	0.2ml, pH 8.0
1M Tris-HCl	1.0ml, pH 8.0
dH_2O	100ml

TE-SDS (10%)

SDS	10g
TE buffer	100ml

A6.3 Solutions used for transformations

$CaCl_2$ Transformation buffer

Tris-HCl	20mM
$CaCl_2$	100mM

pH 7.6-8.0

20% Glucose

Glucose	4g
dH_2O	20ml

Sucrose, HEPES, $MgCl_2$ and Glycerol (SHMG) buffer

Sucrose	85.56g
HEPES	0.24g
$MgCl_2$	0.20g
Glycerol	10% v/v
dH_2O	1 liter

pH 7.0

A6.4 Solutions for protoplast transformation of *B. subtilis* and *S. aureus*

2× Sucrose, maleate, $MgCl_2$ (SMM)

Sucrose	17.12g
Maleic acid	0.232g

Magnesium chloride	0.40g
dH_2O	50ml

pH adjusted to 6.5 with NaOH

4× Pennassay Broth (adapted from El-Helow *et al*., 1997)

Glucose	0.08g
Beef extract	0.12g
Yeast extract	0.12g
Peptone	0.40g
NaCl	0.28g
K_2HPO_4	0.29g
KH_2PO_4	0.11g
dH_2O	20ml

SMM plus Penassay broth buffer (SMMP)

Equal volumes of 4× Penassay broth and 2× SMM buffer

Polyethylene glycol (PEG) 40% w/v

PEG	40g
2× SMM buffer	50ml
dH_2O	100ml

A6.5 Solution for DNA analysis

Agarose gels

Agarose	0.8g (0.4%), 1.6g (0.8%) or 2.4g (1.2%)
5X TBE	20ml
sdH_2O	180ml

5X TBE buffer

Tris base	54.0g
Boric acid	27.5g
0.5M EDTA	20ml, pH 8.0
dH_2O	1000ml

Bromophenol blue tracking dye

30% glycerol (w/v) in TE and 0.0025% bromophenol blue

Running buffer (0.5x TBE)

5X TBE	50ml
dH_2O	450ml

0.5M EDTA pH 8.0

EDTA	18.6g
dH_2O	1L

Adjust pH to 8.0 with NaOH

1M Tris-HCl pH 8.0

Tris-HCl	15.8g
dH_2O	100ml

TE-saturated Phenol

Phenol	14g
TE buffer	10ml

1M NaCl

NaCl	5.8g
dH_2O	100ml

A6.6 Solutions for mutagenesis

10X A-N buffer

K_2HPO_4	70.0g
KH_2PO_4	26.8g
$Na_3C_6H_5O_7$ (tri-sodium citrate)	5.0g
$MgSO_4$	1.0g
dH_2O	1L

Tris-maleic acid buffer

200mM Tris

50mM Maleic acid

Titrate until desired pH obtained

Nitrosoguanidine (NTG) 1mg/ml stock solution

NTG 0.01g

Tris-maleate buffer (pH4.3) 1ml

Microwave for 30 Sec until it has dissolved and do not allow to boil

Use within three days

Table A6.1 Antimicrobial agents

Agent	Stock concentration (mg/ml)	Solvent	Supplier
Ampicillin	100	30%sdH_2O 70% ethanol	Sigma
Chloramphenicol	20	ethanol	Boehringer
Kanamycin	10	sdH_2O	
Nalidixic Acid	10	30% sdH_2O 70% ethanol	Sigma-Aldrich
Rifampicin	10	methanol	Sigma
Streptomycin	20	ethanol	Boehringer

Table A6.2 List of Chemicals

Name of the Chemical	Supplier
Agarose	Bio-Rad
Acetic acid	Merck
BHI	Oxoid
Boric acid	Saarchem
Bromophenol blue	Sigma
Butanol	uniLAB
Chloroform	Saarchem
Calcium chloride	Saarchem
Casamino acids	Difco
EDTA	Sigma
Ethidium bromide	Sigma
Ethanol	Saarchem
Glucose	Fluka Biochemika
Glycerol	Merck
Glycine	Merck
Hydrochloric acid	Merck
HEPES	uniLAB
Isopropanol	uniLAB
Magnesium chloride	Sigma-Aldrich

Maleic acid	BDH analytical reagents
Potassium acetate	Sigma
Dipotassium hydrogen Phosphate	Merck
Potassium dihydrogen phosphate	AnalaR-analytical reagents
Phenol	Saarchem
Polyethylene glycol	Fluka
Sodium hydroxide	Saarchem
SDS	Boehringer Mannheim
Tryptone	Oxoid
Tris	Saarchem
Technical agar	Oxoid
Yeast extract	Oxoid

APPENDIX C: PLASMIDS AND MOLECULAR WEIGHT MARKER

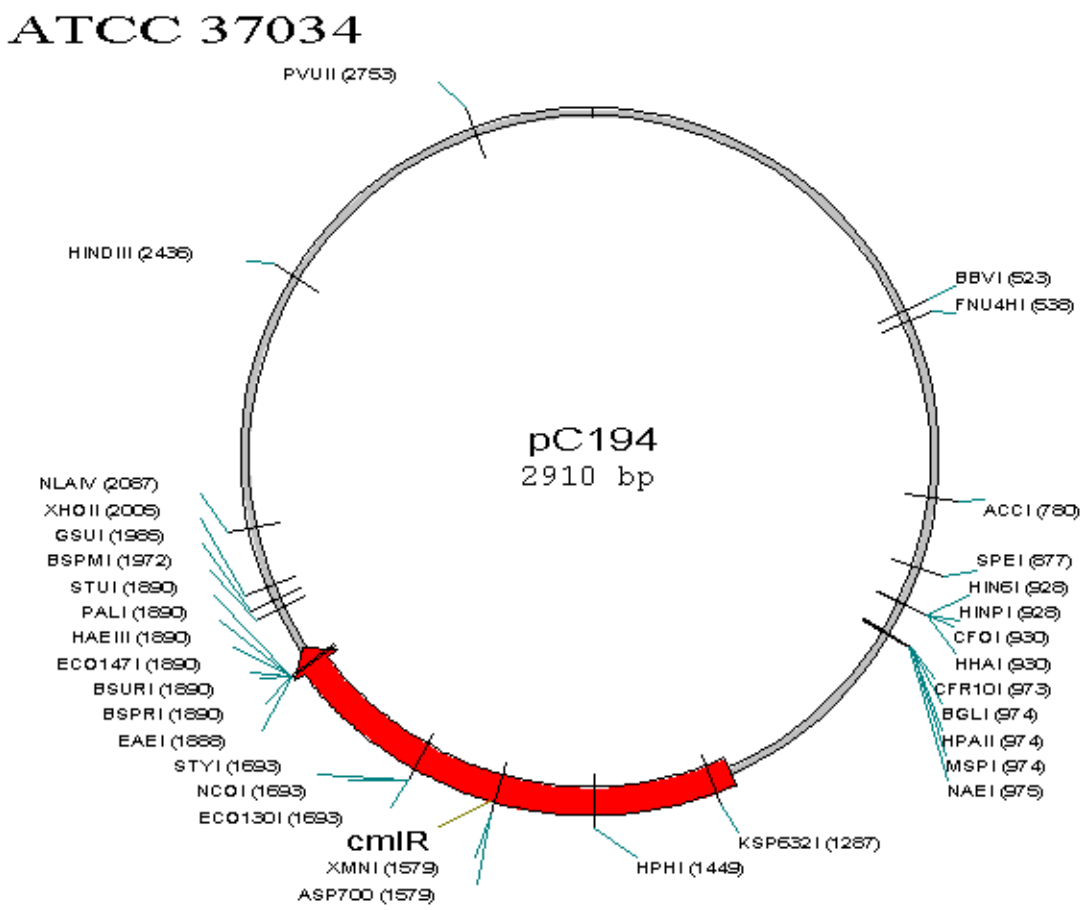


Figure A6.1: Plasmid map of pC194.

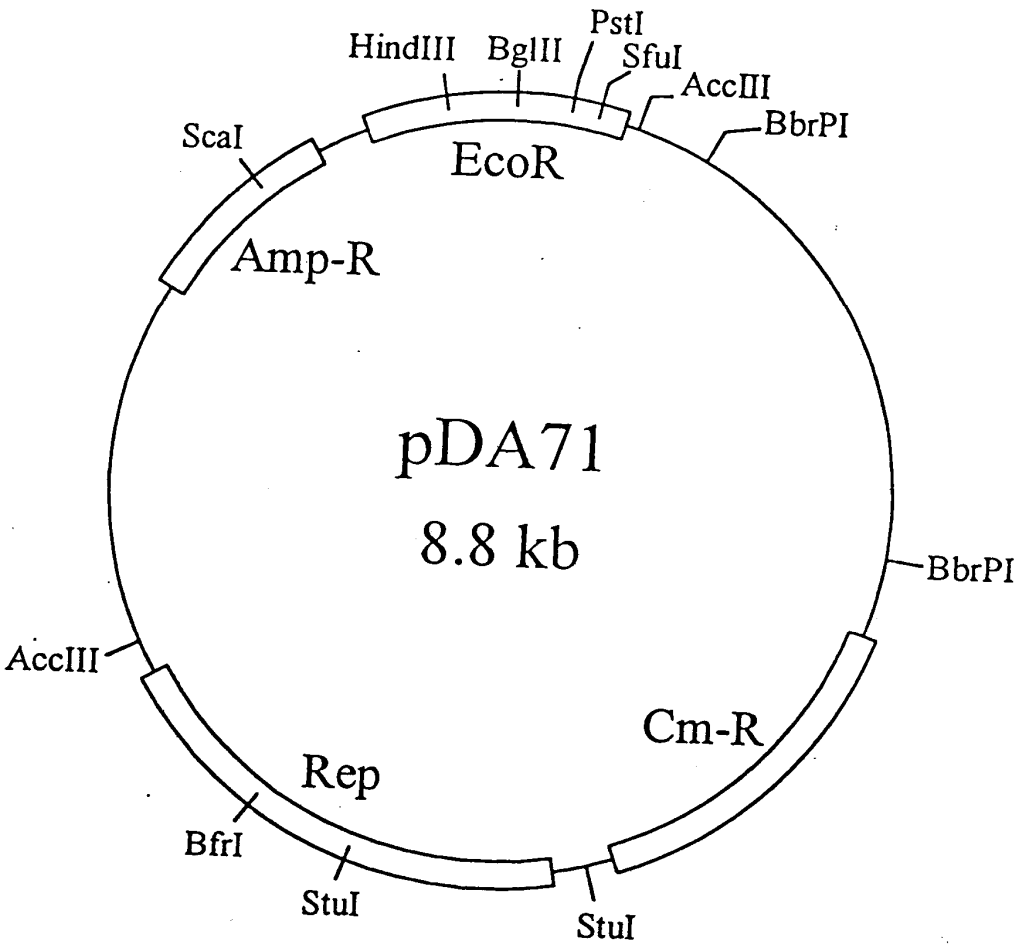


Figure A6.2: Plasmid map of pDA71 (adapted from http://seq.yeastgenome.org/vectordb/vector.html).

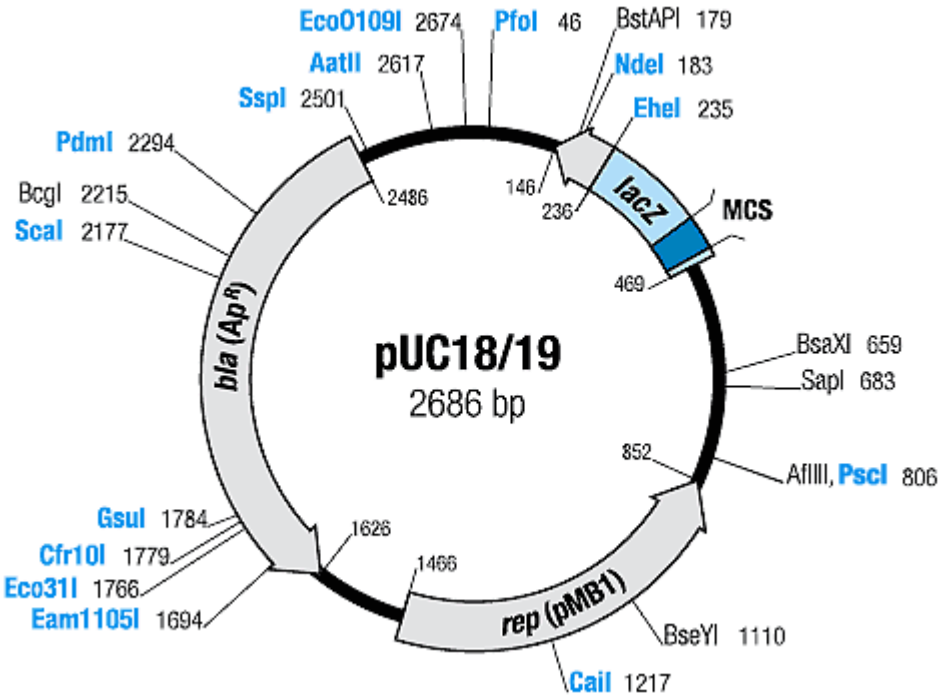


Figure A6.3: Plasmid map of pU18/19 (http://www.fermentas.com/techinfo/nucleicacids/mappuc1819.htm).

Multiple Cloning Sites (MSC) of pU18/19

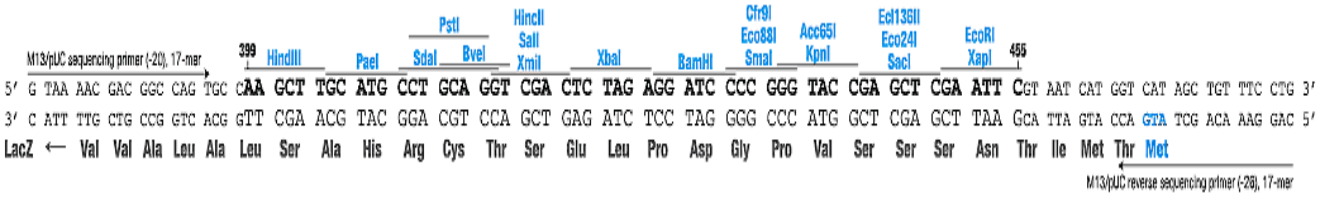


Figure A6.4: Multiple cloning site of pUC18 and the MCS of pUC19 is the reverse of pUC18.

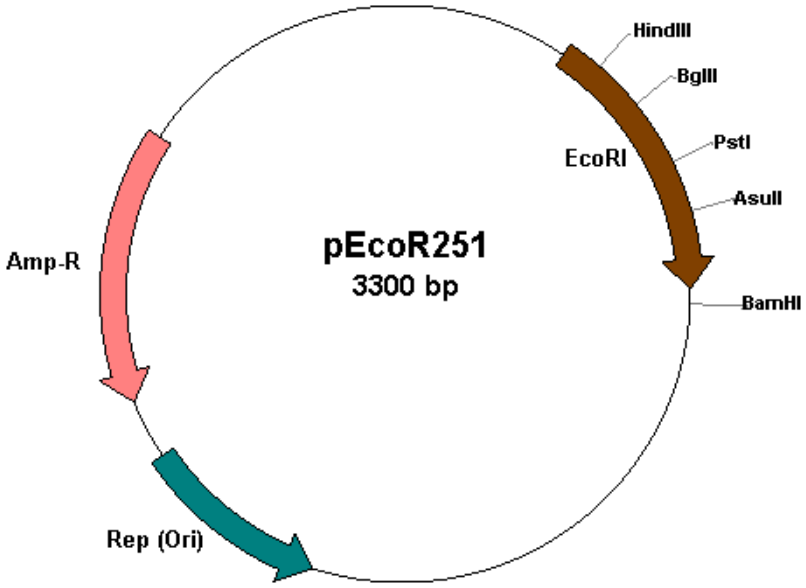


Figure A6.5: Plasmid map of pEcoR251.

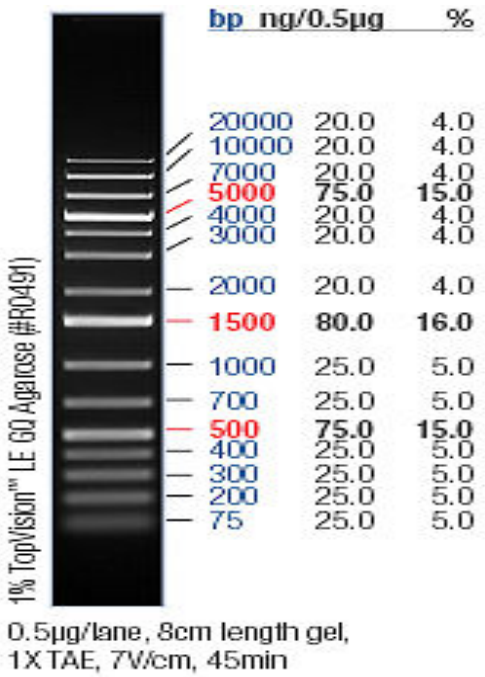


Figure A6.6: 1KB ladder plus, Fermentas (http://www.fermentas.com/catalog/electrophoresis/generulers.htm).