Chapter Four:

4. Creation of a mutant lacking the antimicrobial activity using transposon mutagenesis

4.1 Introduction

Genomic mutations can be regarded as a necessary process required for the adaptation of bacteria. These mutations are classified as deleterious (loss of genes) or beneficial (insertion of useful genes). For instance in, *E. coli* deleterious mutations occur at a frequency of approximately 2 - 8 x 10^{-4} and beneficiary mutations occur at 2 x 10^{-9} per genome per replication (Denamur and Matic, 2006). From this data it suggests that deleterious mutations are more likely to occur than beneficiary (via insertional mutagenesis).According to Drake *et al.* (1998) these mutation rates can be increased by physiological changes (occurs during starvation of the bacteria eliciting the SOS response) whilst other rate increases occur during the replication process such as errors during translation or transcription (Drake *et al.* 1998). It is not only these genetic processes that cause bacterial mutations and ultimately flexibility of the bacterial genome but other mobile genetic elements such as plasmids, bacteriophage and transposons (Hayes, 2003) (to name a few) that are able to change the bacterial genome via insertional mutagenesis.

Transposons are biological elements isolated from gram negative bacteria (Hayes, 2003) consisting of a DNA sequence (Reznikoff, 1993) that randomly inserts into genomic DNA sequences resulting in the formation of mutants (Lodge, Weston –hafer and Berg, 1988). They occur in many kingdoms and are classified into two groups. The class I elements undergo transposition by using an RNA intermediate and reverse transcriptase to convert the RNA to DNA once it has transposed into the genome. The class II elements involve DNA to DNA interaction. They have their own transposase which assists with the direct insertion of DNA into the genome (Hamer *et al.*, 2001) and also contain specific end sequences which are necessary for transposition (Reznikoff, 1993). In this study the Tn5 transposon which is apart of the class II elements was used. This transposon is well characterised (Rezinikoff, 1993;

Reznikoff, 2003; Goryshin *et al.*, 2000) and has been used to transform *Pseudomonas* strains (Dubern *et al.*, 2005; Kuiper *et al.* 2003, Schnider *et al.* 1995).

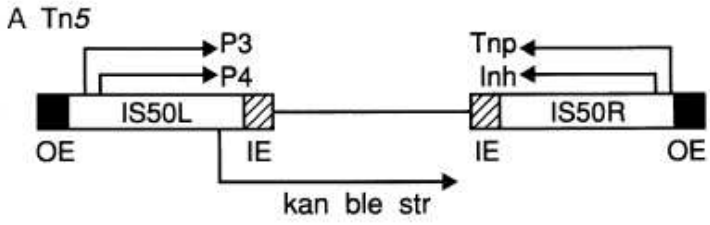

Figure 27: Schematic diagram of the Tn5 transposon as depicted in Reznikoff, 2003. IS50L and IS50R represent the transposable regions which are separated by the antibiotic resistance markers namely kanamycin (kan), bleomycin (ble) and streptomycin (str). The IS50R encodes genes for Transposase (Tnp) for transposition and an inhibitory protein (Inh) to down regulate transposition. P3 and P4 are analogues of the ISR50 region genes which are not active (Reznikoff 1993, Reznikoff 2003 and Goryshin *et al* 2000).

As can be seen in Fig. 27, the Tn5 transposon contains three antibiotic markers which are bordered by the transposable element sequences (IS50L and IS50R) and thereafter the two inverted repeat sequences (19bp) that are essential for the transposition process (Reznikoff, 2003; Goryshin *et al.*, 2000). The inverted repeat regions, outside end (OE) and inside end (IE) contain sequences that are inverts of each other. These sequences enable the two transposons to be circularised (Reznikoff, 1993). Transposition of these elements requires three components, a transposase (encoded by IS50R), DNA of the transposon and donor DNA from a bacterial sequence which includes a target sequence. The 'target sequence' can also be a non-specific sequence which can result in random binding of the transposon (Reznikoff, 2003).

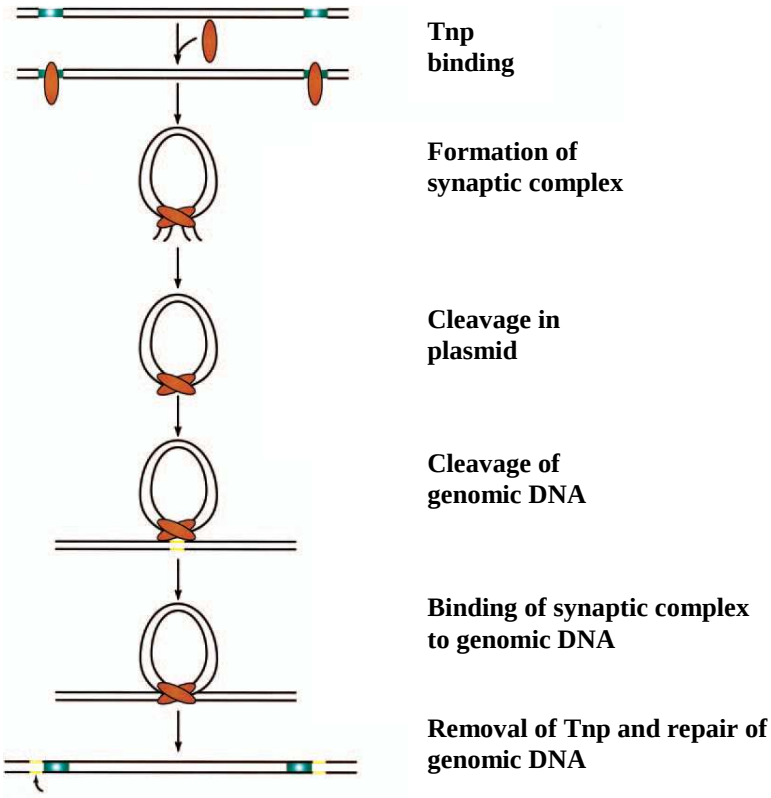

Figure 28: Diagrammatic representation of transposon insertion into the host genome (Reznikoff, 2003).

The Tn*5* transposition event occurs as follows (refer to Fig. 28): Firstly the transposase elements (protein complexes) bind to the end sequences of the inverted repeat region and form a loop which then forms a synaptic complex. The loop formation occurs due to the inverted repeat sequences (regions have sequences that are inverts of each other) which temporarily bind to each other. The plasmid and the target host DNA are then cleaved by the transposase elements between the inverted repeats with respect to the plasmid and cleave randomly in the bacterial genome. The synaptic complex (at the inverted repeat region) binds to the genomic DNA non-specifically and incorporates its own DNA in the strand. Detachment of the transposase protein is believed to be facilitated by host machinery. The hypothesis is that this process is achieved by degradation of the transposase protein by host machinery leading to missing base pairs at the insertion site. The host machinery then replaces these base pairs and the transposition process is then complete (Reznikoff, 1993; Reznikoff, 2003; Goryshin *et al.*, 2000).

The presence of the antibiotic cassette provides a selection marker which can be used to identify the transformed bacteria and since the sequences before the inverted repeat regions are known, the genes flanking them can be identified (Milcamps *et al.*, 1998). This is achieved by firstly isolating the genomic DNA from the mutants. Thereafter, the DNA is then digested with an enzyme that does not cut within the Tn5 region and the fragments ligated and cloned into *E. coli* DH5α cells. These cells are then screened for kanamycin resistance. Plasmid DNA from the selected clones are isolated and sent for sequencing for identification of the disrupted gene (Milcamps *et al.*, 1998). Hence it was proposed that transposon mutagenesis could be a useful technique to create a *Pseudomonas* αmb mutant lacking in the antimicrobial activity. For the purposes of this study, confirmation of transposon mutagenesis in *Pseudomonas* αmb was a preliminary step towards the identification of the gene or genes that are required for the compounds production.

4.2 Materials and methods:

4.2.1 Bacterial strains:

E. coli DH5α bearing the pRL1063a plasmid which contains the Tn5 transposon was kindly supplied to us by C.P. Wolk from Michigan State University. Culturing of the bacteria was done by inoculation of a colony into LB (50 µg/ml kanamycin) and incubated at 37°C; shaking at 180rpm overnight.

4.2.2 Plasmid DNA extraction from *E. coli* pRL1063a (method adapted from Sambrook *et al.*, 1998):

The bacterial cells of an overnight culture (6ml) of *E. coli* pRL1063a (incubated at 37°C, shaking at 200 rpm) had undergone centrifugation (13000 rpm for 5 minutes) in 1.5 ml eppendorf tubes via a benchtop centrifuge (Minispin, Eppendorf). The pellet was retained whilst the supernatant was discarded. The pellet was suspended in 100 µl of solution I (50mM glucose, 25mM Tris. Cl (pH 8), 10mM EDTA (pH 8)) and incubated at room temperature for five minutes. Thereafter 200 µl of solution II (0.2N

NaOH, 1% SDS) was added to the tubes which were gently inverted a few times before incubating on ice for 10 minutes. After the incubation, 150 µl solution III (5M potassium acetate, glacial acetic acid and sdH_2O) was added to neutralise the solution and the tubes inverted several times. The proteins and chromosomal DNA was then removed (Boyer, 2006) via centrifugation (10 minutes at 13000 rpm at room temperature) and the supernatant transferred to a new tube. Thereafter, the plasmid DNA was precipitated with 350 µl of isopropanol and underwent centrifugation to form a pellet (13000 rpm for 10 minutes at room temperature). The supernatant was discarded and the pellet was then washed with 1.5 ml of ice cold 70% ethanol. The pellet was then suspended in 50µl $_dH_2O$ (sterile distilled water) once the ethanol had fully evaporated (air-dried for 10 minutes). RNase was added to the tube final concentration of 100µg/ml and incubated at 37°C for 45 minutes to remove any excess RNA.

4.2.3 Sodium Acetate precipitation of plasmid DNA (method adapted from Sambrook *et al,* 1989):

A solution of 3M sodium acetate (pH 6.8) was added to the sample to bring to a final concentration of 10%. Thereafter 1ml of 98% ethanol was added and the DNA was precipitated by centrifugation (12000 rpm for 10 minutes).The supernatant was discarded and the pellet was washed with 70% ice cold ethanol (12000 rpm for 5 minutes). The supernatant was discarded and the pellet was suspended in 50µl of deionised sterile water. Purification of plasmid DNA was done using the Wizard® SV Gel and PCR clean-up System (Promega).

4.2.4 Agarose gel electrophoresis:

All DNA samples (plasmid DNA- digested or undigested or genomic DNA) were stained with 2µl of loading dye (Fermentas ready-to-use loading dye) viewed on a 1% agarose gel (10µg/ml EtBr) which was electrophorised at 85V for 1hr 30 minutes; in TAE buffer (30 µg/ml EtBr). For determination of the concentrations of the DNA extractions samples were compared to the 1 kb 'O' range marker (Fermentas). All gels were viewed using the BioDoc-It™ system (UVP, The scientific group).

4.2.5 Restriction digests of plasmid and genomic DNA:

Single and double digests were done using 1µg of plasmid DNA. For single digests, the restriction enzymes Pst I and Bgl II (Fermentas) and they were used in combination for double digests. All digests were made up to a final volume of 20µl in accordance to the Fermentas protocols. The digests were mixed via centrifugation (13 000 rpm for 5 seconds). The digests were incubated at 37°C for 3 hours and the digested DNA was viewed on a 1% agarose gel (as in 4.2.4).

4.2.6 Making of the *Pseudomonas* isolate electro-competent cells:

Bacterial cells from an overnight culture (100ml) of *P. putida* αmb were pelleted via centrifugation (8000rpm for 15 minutes). The supernatant was discarded and the pellet was washed with sterile distilled water. This process was done three times. Thereafter the pellet was washed with 1% glycerol (done three times). The final pellet was suspended in 1ml of 1% glycerol. The colony forming units (cfu/ml) of the culture was determined for the calculation of mutation frequency.

4.2.7 Transposon mutagenesis of the *Pseudomonas* isolate by electroporation:

Approximately 1µg of plasmid DNA was added to 200µlof electro-competent cells in a 0.2 cm *E. coli* Pulser®-cuvette (Biorad). A plasmid free control with only electro-competent cells was included. The cells were pulse electroporated (settings: 2,5kV; 100Ω; 25µF) (van de Broek, 2005) and thereafter immediately suspended into 800 µl of fresh SOB media in a new eppendorf tube. The tubes were then incubated for 2 hours at 28°C on a shaker (80rpm). The cells were separated from the culture via centrifugation (10 000 rpm for 5 minutes) and suspended in 900µl of fresh SOB media which was plated onto LA plates (50µg/ml kanamycin). For the plasmid free electroporated sample (control), the cells were plated onto kanamycin plates. The plates were incubated at 37°C overnight and were periodically checked for colony development (Sambrook *et al.*, 1989). Colonies obtained were streaked once more

onto LA (50µg/ml kanamycin) plates to confirm that they were true colonies which were kanamycin resistant.

4.2.8 PCR screening of *Pseudomonas* αmb transformants for Tn5:

PCR was used as a diagnostic tool to determine if the transposition event of Tn5 into *Pseudomonas* αmb had occurred. The pRL1063a plasmid sequence was obtained from the NCBI website (accession no: U55385) and two sets of primers (forward and reverse- shown in Table 6) were created. Primers A and B correspond to the alternate ends of the kanamycin resistance gene sequence to confirm that kanamycin resistance was due to the plasmid sequence and not a spontaneous mutation. To dispute the possibility of the mutant acquiring intrinsic or spontaneous resistance and show that the entire plasmid had inserted, primers C and D which amplify sequences found between the transposase and Streptomycin region (Fig. 29) were also created. If the transposition event had occurred successfully in *Pseudomonas* αmb, this region should also be present; hence the kanamycin resistance would be due to the incorporation of pRL1063a into the genome and would confirm the transposition event. The primers were also tested against the plasmid alone, which would show amplification for all primers (positive control).

Table 6: Primer sequences and positions corresponding to the pRL1063a plasmid

Primer	Primer sequence	Position of primer in the plasmid	Region amplified	Reference
A	5’ gattgaacaagatggattgcac 3’ (forward)	3451 to 3472	Kan	
B	5’ tcagaagaactcgtcaagaagg 3’ (reverse)	4243 to 4221	Kan	
C	5’ ggtcaccgat ctcacctgg 3’ (forward)	5481 to 5500	Tnp-Sm	
D	5’ aagatctgatcaagagacaggg 3’ (reverse)	6320 to 6299	Tnp-Sm	
E	5’aggaggtcacatggaatatcagat 3’(forward)	7758 to 7781	IS50L	Milcamps *et al*, 1993
F	5’tactagattcaatgctatcaatgag 3’ (reverse)	110 to 86	IS0R	Milcamps *et al*, 1993

Key: Kanamycin (Kan), Transposase (Tnp), Streptomycin (Sm) and Inverted repeats (IS50R and IS50L)

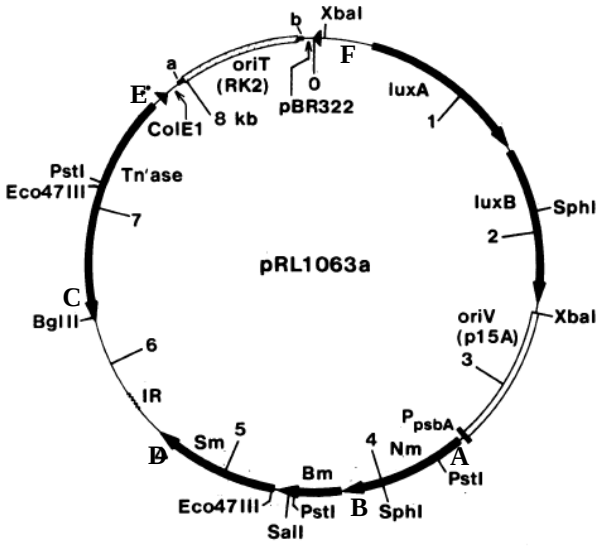

Figure 29: Diagram of the pRL1063a plasmid (Tn5- 1063) from Wolk *et al.*, (1991) showing where the primers bind. The inverted repeats region lie on either side of the oriT region (origin of replication for *E. coli*), IS50R depicted by (▶) and IS50L depicted by the (◀). The three antibiotic markers, Neomycin (Nm) which confers resistance to kan, bleomycin (Bm) and streptomycin (Sm) are between the inverted repeats. The transposase (Tn'ase) gene lies left to the IS50R region. The restriction enzymes shown do not cut within the transposon region. Primers were designed to amplify the Nm resistance marker (A-B), the region between T'nase and the Sm marker (C-D) and sequencing primers (E and F) amplify from either side of the IS50 region.

4.2.9 Screening of the *Pseudomonas* αmb-Tn5 transformants:

Transformants were screened for the lack of antimycobacterial activity by streaking the mutants onto 7H10 *M. smegmatis* IP's and LA *M. smegmatis* IP's (as mentioned in chapter two). The plates were incubated at 37°C overnight and viewed the next day.

4.2.10 Identification of the disrupted gene in the *Pseudomonas* αmb-Tn5 mutants:

The recovery of the interrupted gene was done using an adapted protocol from Milcamps *et al.,* 1998.

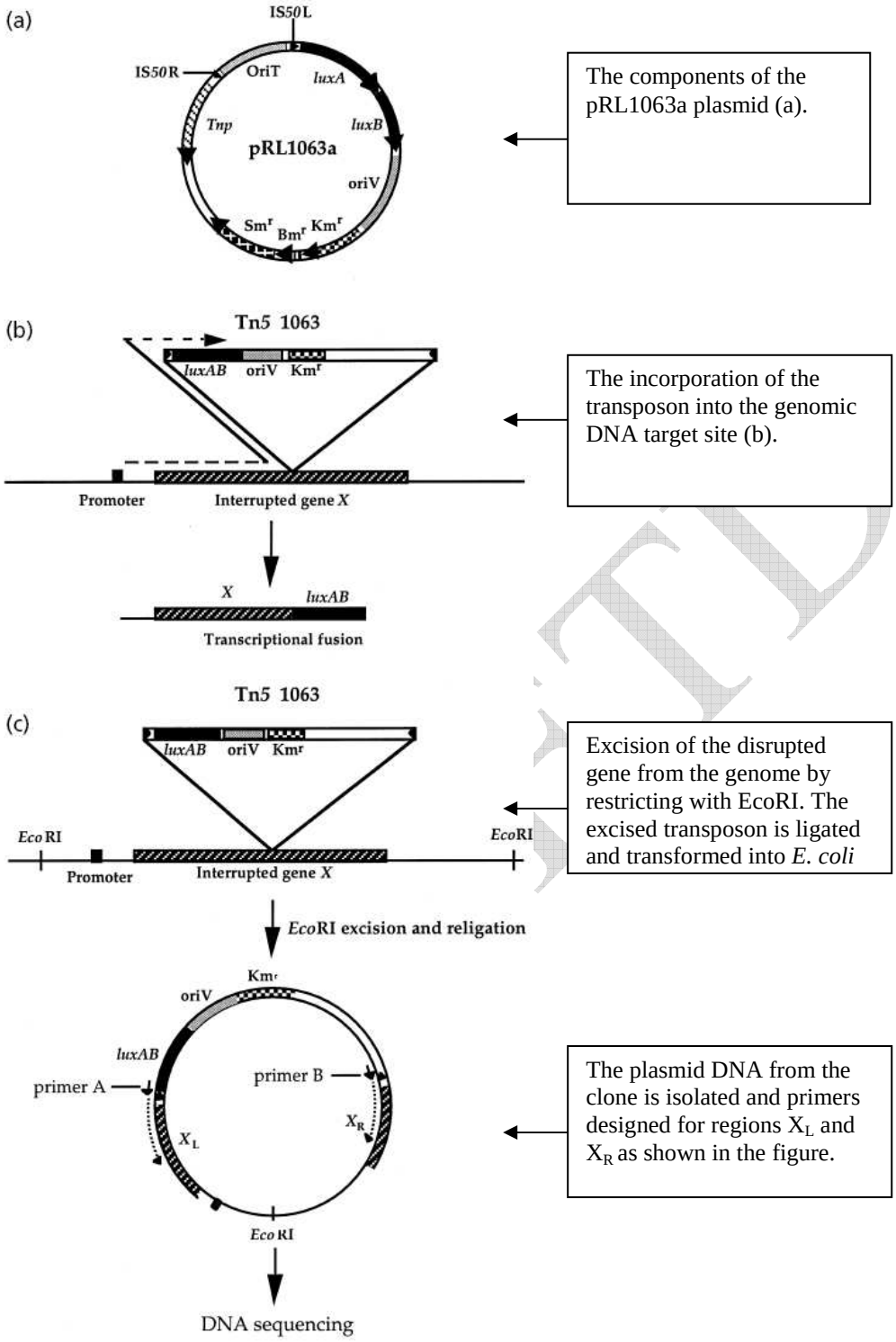

Figure 30: Diagrammatic representation of the process undertaken to identify the disrupted genes. Diagram shown is that seen in Milcamps *et al*, 1998. The primers X_L and X_R correspond to primers E and F (in Table 6) respectively.

4.2.10 a. 1) Extraction of genomic DNA:

Genomic DNA extractions of *Pseudomonas* αmb kanamycin mutants were carried out as follows: 20ml LB (50µg/ml kanamycin) was inoculated with a single colony of the mutant and incubated at 37°C overnight (180rpm). A cell pellet was formed via centrifugation (13 400rpm for 5 minutes) and the supernatant discarded. The cells were suspended in 600µl of TE buffer which was supplemented with a small amount of lysozyme. The tubes were incubated for 15 minutes at 37°C. Thereafter, 120µl of 10% SDS (in TE buffer) was added and the tubes inverted to mix. The tubes were then incubated for 10 minutes at room temperature after which 120µl of 5M KAc (p.H 6.0) was added. The tubes were inverted vigorously to mix and left on ice for 5 minutes. The proteins were then removed via centrifugation at 4°C (13 400rpm for 10 minutes) and the supernatant fraction was retained. A phenol- chloroform extraction was performed on the extracted DNA followed by salt-ethanol precipitation of genomic DNA.

4.2.10 a. 2) Purification of genomic DNA:

Genomic DNA was purified by firstly using the phenol – chloroform extraction as follows: Phenol was added to the extracted genomic DNA in a ratio of 1:2 (phenol: total volume of DNA extract) and the tube as inverted for 1 minute. The suspension was centrifuged (13000 rpm for 5 minutes) to separate the two phases. The top phase was transferred to a new 1.5ml eppendorf tube, to which chloroform in a 1:2 ratio (chloroform: total volume of phase) was added. The tube was then inverted several times to mix and the phases were separated via centrifugation (13000 rpm for 30 seconds). The upper phase was removed and placed into a new eppendorf tube to which 1M NaCl (10% total concentration) and 96% ethanol (20% of total volume) were added. The DNA was then precipitated by centrifugation at 4°C (13 000 rpm for 15 minutes). The supernatant was removed and the pellet was air dried for one hour. The DNA was suspended in 50µl of sterile distilled water.

4.2.10 b) Self circularisation of genomic DNA:

Excision of the transposon from genomic DNA was achieved by digestion of the DNA with EcoRI (see 4.1.5). The enzyme does not cut the transposons (Wolk *et al.*, 1991; Milcamps *et al.*, 1998) hence the chromosomal DNA will be flanking the inverted repeat regions once the plasmid is excised. Self circularisation of digested fragments was achieved by ligating 5µl of the DNA (from the digestion of 1 ug of DNA) in a total volume of 50µl (10% ligation buffer, 1µg/ul T4 DNA ligase (Fermentas) and 39µl dH_20). The ligations were mixed by centrifugation for 5 seconds in the benchtop minispin(Eppendorf) and incubated at 14°C for 16 hours.

4.2.10 c) *E. coli* DH5α calcium chloride- mediated transformation:

A 1% inoculation of 20ml LB from a 5ml pre-culture of *E. coli* DH5α which was inoculated via a single colony was incubated for 2 hours ($O.D_{600nm}$ 0.3-0.5) at 37°C and shaken at 180rpm. The culture was placed in ice water for 5 minutes and the cells precipitated via centrifugation in the JA20 Beckman centrifuge for 10 mins at 5000 rpm. Cells were then suspended in 10ml of PIPES buffer and precipitated as before. Thereafter cells were suspended in 1.3ml of PIPES buffer and incubated on ice for 1 hour. The ligated DNA samples (as well as the following controls: undigested - unligated genomic DNA and digested genomic DNA) were incubated on ice for 10 minutes before adding 100µl of $CaCl_2$ competent cells to them. The cells were heat shocked for 90 seconds at 42°C after which 500µl of pre-warmed LB was added. The tubes and incubated at 37°C for 90 minutes with the tubes open. The contents of tubes were then spread onto LA plates (kanamycin 50µg/ml) and incubated overnight at 37°C (adapted method from Sambrook *et al*., 1989).

4.2.10 d) Sequencing of the *E. coli* DH5α clones:

The plasmid DNA was extracted from the clone *E. coli* M1 using the ToPure™ plasmid miniprep extraction purification kit (Gene tech). Sequencing of the respective plasmids was done by Inqaba biotech (Sanger method). The following sequencing

primers were used: Primer E (X_L): 5'aggaggtcacatggaatatcagat 3' (forward-7758 to 7781) and Primer F (X_R): 5'tactagattcaatgctatcaatgag 3' (reverse- 110 to 86) (Milcamps *et al.*, 1998).

4.3 Results:

4.3.1 Confirmation of plasmid pRL1063a:

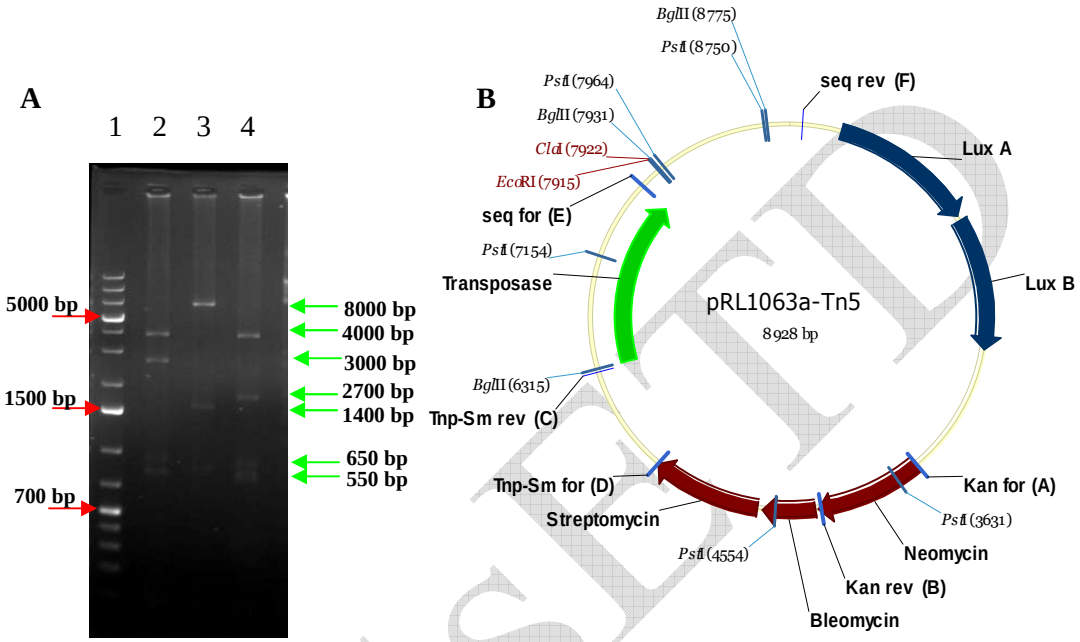

Figure 31: A) 1% agarose gel of restriction digest and B) map of pRL1063a plasmid. The lanes were loaded in the following order and the approximate band sizes (in base pairs) of the fragments are shown in brackets: 1) 1kb 'O' range Fermentas Marker, 2) Pst I (4000, 3000, 2700, 600), 3) Bgl II (8000, 1400, 600) 4) Pst I and Bgl II (4000, 1900, 1800, 650, 550). The red arrows shows the molecular marker fragment sizes in base pairs (bp) and the green arrows represents band sizes found in the respective digests. B) Restriction enzyme sites on plasmid pRL1063a. The restriction site map was created using Vector NTI version 11 (Invitrogen). The respective primer sites are shown in green and the selectable marker region for kanamycin is shown in red. One cutter restriction enzyme sites are depicted in red i.e EcoRI and Cla I. The predicted fragment sizes were determined using a virtual restriction digest program, Nebcutter and the results for the digests are shown in Tables 12-14 (Appendix B).

Restriction digests of pRL1063a was done in order to confirm that the plasmid was in fact pRL1063a. From Figure 31A above, the size and number of the fragments obtained for the respective digests shown in the gel yielded the corresponding fragments sizes that were to be expected. The Pst I enzyme had cut the plasmid 5

times whilst BglII had cut the plasmid 4 times and the double digest using these enzymes cut the plasmid 5 times. These results correspond to the restriction map in Figure 31B which shows the 5 restriction enzyme sites for PstI and 4 restriction enzyme sites for BglII. Since the plasmid restriction profile fitted to the sequence of pRL1063a attained from the NCBI website (Figure 31B above) the plasmid received concluded to be pRL1063a which contained the Tn5 transposon.

4.3.2 Transposon mutagenesis of the *Pseudomonas* isolate by electroporation

To identify the gene that was responsible for the antimycobacterial activity, an attempt to create a mutant lacking the production of the antimycobacterial compound using transposon mutagenesis was done. Transposon mutagenesis with pRL1063a allows for the random insertion of the Tn5 transposon into open reading frames or promoters thus resulting in the disruption of the genes. It was hypothesised that this transposon could disrupt the gene responsible for the production of the compound with antimycobacterial activity. Transformants by this technique is determined by selection on kanamycin plates. For the purposes of this study, transformants which possessed kanaymicin resistance (Kan^R) was referred to as Kan^R colonies. In Table 7 below the total number of transformants obtained from the electroporations experiments is shown. The Kan^R colonies were screened for antimycobacterial activity and the results are shown in Table 7. The Kan^R colony frequency and Kan^R revertant frequencies were calculated for each experiment. All experiments were done by keeping the plasmid DNA concentration, competent cells and incubation times consistent.

Table 7: Results of electroporation experiments.

Transformants				Frequencies		
Total no.	**2nd Kan^R selection**	**Anti-mycobacterial activity**		**Kan^R frequency[a]**	**Total no. of revertants**	**Revertant frequency[b]**
		(-)	**(+)**			
1765	59	0	59	5.9×10^{-8}	0	0
169	34	3	31	3.4×10^{-8}	3	2.24×10^{-11}
1	1	0	1	1×10^{-6}	0	0

Key: Each row in the table represents an individual electroporation event which was carried out using the same protocol. (a) was calculated by dividing the no. of transformants by cfu/ml of *Pseudomonas* αmb culture , (b) was calculated using the poisson equation, the (-) represents the absence of antimycobacterial activity and (+) represents the presence of antimycobacterial activity.

4.3.3 Confirmation of mutants via PCR and preliminary characterisation of mutants:

In order to determine if the kanamycin resistance was due to the presence of the transposon and not merely to acquired resistance; primers were designed to amplify the kanamycin region and the transposase-streptomycin region located in the Tn5 transposon. If the transposon has been integrated into the bacterial genome then amplification of both these regions should occur thus yielding a positive result (see Table 8). Eight Kan^R colonies were randomly chosen for the detection of the kanamycin marker. As can be seen from the table below, five Kan^R transformants including the *E. coli* DH5 α pRL1063a control showed positive amplification thus showing the presence of the kanamycin marker. However of the 5 Kan^R colonies only 3 (including the positive control) showed amplification for the transposase-streptomycin region. The Kan^R colony 5U showed a positive result for the kanamycin region but a negative for the transposase- streptomycin (tnp-strep) region. Taking this into account, suggests that half the transposon had integrated with isolate 5U, whilst the amplification for both regions confirmed the presence of the plasmid but dispute the possibility of self- replication within *Pseudomonas* αmb.

Table 8: Results of the diagnostic PCR done to confirm the presence of the Tn5 transposon

Bacteria	Amplification of PCR primers		Presence of anti-mycobacterial activity
	Tnp- Strep region	Kanamycin region	
***M1**	+	+	+
5U	–	+	–
A1	–	–	+
B1*	+	+	+
C1	–	–	+
C2	–	–	+
C3*	+	+	+
C4	–	–	+
****E. coli* DH5α pRL1063a**	+	+	–
***Pseudomonas* αmb**	–	–	+
E. coli DH5α	–	–	–

Key: A positive amplification is depicted by (+) and no amplification is depicted by (–).

Note: the data is of collective pcr experiments done on various occasions. However, the controls were consistent in all pcr reactions.

4.3.4 Determination of self replication of pRL1063a in the kanamycin *Pseudomonas* αmb mutants:

Since the amplification of the both the kanamycin and Tnp-Strep region showed no consistency amongst the mutants, it was hypothesised that the plasmid could have not integrated into the genome but instead be retained as a self-replicating plasmid. To determine if this was the case, it was reasoned that if the transposon had integrated into the bacterial genome, isolation of the pRL1063a plasmid from genomic DNA would result in the extraction of the plasmid with the inclusion of sequences from the genome. The presence of genomic DNA within the extracted pRL1063a plasmid would confirm that the transposition event had occurred. Therefore, the genomic

DNA of the Kan^R colonies was isolated and the pRL1063a plasmid was extracted and cloned into *E. coli* DH5α so that the plasmid could be sequenced. Three mutants from the independent electro-transformation experiments were chosen to be cloned namely, B1, C3 and M1.

E. coli DH5α cells were transformed using approximately 1µg of digested and ligated genomic DNA for the respective mutants and the control *E. coli* DH5α: pRL1063a. Other controls for this experiment included undigested and unligated genomic DNA as well as digested DNA of the respective mutants and bacterial strains. The restriction digests of the genomic DNA of all Kan^R colonies were confirmed via electrophoresis (data not shown). From the bacterial counts in Table 8, it can be seen that the digested and ligated genomic DNA of the *E. coli* DH5α has produced a high number of clones in comparison to the undigested – unligated genomic DNA and digested genomic DNA. The Kan^R colonies M1 and C3 had also shown more transformants on the digested and ligated colonies in comparison to the digested or undigested and unligated transformants with exception of B1 which has the greatest number of clones from digested genomic DNA. The clones obtained from the undigested – unligated DNA could be due to shearing of bacterial DNA during the extraction of genomic DNA. Since the numbers are low in comparison to the control it can be assumed that the plasmid is not self replicating within the *Pseudomonas* cells. If they were replicating, there would have been a much higher number of colonies present on the kanamycin plates.

Table 9: Bacterial counts of the clones of the respective mutants found on the kanamycin plates

Bacterial Clones	**Treatment of genomic DNA**		
	Undigested and unligated	**Digested**	**Digested and ligated**
***E. coli* B1**	4	7	2
***E. coli* C3**	1	0	7
****E. coli* M1**	1	3	7
***E. coli* DH5α pRL1063a**	105	192	205

* The clones obtained from the digested and ligated treated genomic DNA was viable upon propagation.

However, it was disconcerting that there were colonies found on the digested DNA or undigested – unligated DNA. Therefore those colonies were inoculated into LB (kanamycin 50µg/ml) and incubated overnight to see if they were true colonies. The only colonies that were viable in LB (kanamycin 50µg/ml), were from *E. coli* DH5α pRL1063a (all the different treatments) and *E. coli* M1 which was obtained from digested and ligated genomic DNA. The clones that failed to grow were regarded as satellite colonies or cell debri. Furthermore plasmid extractions were attempted from the Kan^R colonies to see if the plasmid was replicating at a low rate but no plasmid DNA was found to be present in any of the Kan^R colonies (A1, B1, C3, M1) tested.

4.3.5 Restriction pattern of the plasmid of *E. coli* DH5α: M1 clone:

To determine if the clone *E. coli* DH5α:M1 contained genomic DNA from *Pseudomonas* αmb, a restriction digest of the plasmids extracted from clone was compared to the restriction digest pattern of the control, pRL1063a which was extracted from *E. coli* DH5 α: pRL1063a. If there was genomic DNA present in the plasmid of the clone, then the plasmid should be larger than the control and the restriction profile should be different. As can be seen in Fig.13, the banding patterns of the DNA fragments of the respective digests look similar when comparing the digested plasmid DNA of the *E. coli* DH5α:M1 clone to the control *E. coli* DH5 α: pRL1063a.

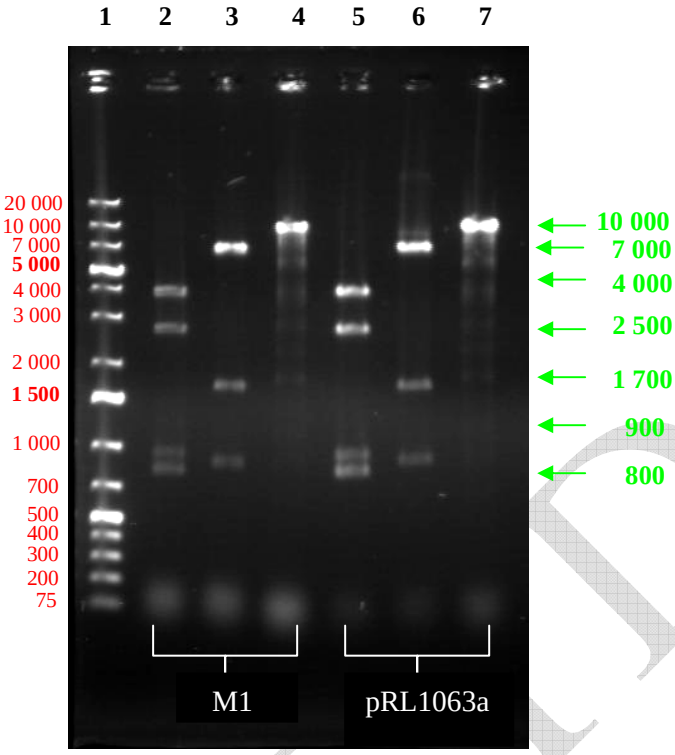

Figure 32: 1% agarose gel showing the restricted plasmid from *E. coli* DH5α: M1 vs *E. coli* DH5α: pRL1063a. Lanes have been loaded with 4µl of 1kb 'O' range Fermentas marker (Lane 1) and 8µl of sample each with 2µl of loading dye respectively. The lanes have been loaded in the following order 1: Fermentas 'O' range 1 kb marker, 2: Pst I digest of M1, 3: Bgl II digest of M1, 4: EcoRI digest of M1, 5: Pst I digest of pRL1063a, 6: Bgl II digest of pRL1063a, 7: EcoRI digest of pRL1063a.

The restriction profile of the plasmid attained from the clone M1 is almost identical to that of the control plasmid pRL1063a (Fig.32). Therefore this strongly suggests that the plasmid does not contain genomic DNA from the *Pseudomonas* αmb. To confirm this suggestion, sequencing of the pRL1063a and the plasmid of the clone M1 was done.

4.3.6 Comparison of sequences from clone M1 and pRL1063a:

Plasmid DNA extracted from the clone *E. coli* M1 was sequenced by Inqaba biotech (Sanger method). Sequences attained from using the primers (primer E and primer F) were aligned with the pRL1063a plasmid using Vector NTI (Version 10). The sequence alignment showed correlation of sequences between position 7780 and 8020 (Appendix C, Fig.). These sequences included the inverted repeat region. Since the

EcoRI site found (7915) between the inverted repeat region showed no presence of foreign DNA to plasmid pRL1063a, it can be concluded that the transposon has not inserted into the genome. This was concluded since foreign DNA would be present near this site because it is closest to where the genomic DNA would have been incorporated into.

4.4 Discussion:

The Tn5 transposon has been exploited as a tool for mutagenesis. Mutagenesis using the transposon is not simple, with various things to take into consideration such as micro-organism compatibility and the required goals. According to a study done by Abdel-Salam and Klingmuller (1987), the Tn5 transposon has a high transposition efficiency and a low insertional specificity (Abdel-Salam and Klingmuller, 1987). This transposon was chosen since it inserts specifically in high G/C rich sequences (Lodge, Weston- Hafer and Berg, 1988) and since most *Pseudomonas* species have G/C contents which range from 58% to 66.6% (Joarder *et al*. 2005; Paulsen *et al*. 2006, Stover *et al.* 2000), this technique could work with this isolate. Furthermore since the *Pseudomonas* αmb has been shown to reside amongst other *Pseudomonas* strains with respect to it 16s rDNA sequences and other studies involving *Pseudomonas* strains have used the transposable element with success (Dubern *et al*. 2005), creation of a mutant lacking the production of the antimycobacterial compound via transposon mutagenesis with Tn5 was attempted. In order to achieve to achieve this objective, the feasibility of this technique with this strain had to be determined first.

To confirm that the plasmid was in fact pRL1063a a restriction digest using Pst I , Bgl II and Pst I and Bgl II respectively was done. The bands on the gel for the respective digestions conformed to that of the plasmid map (see appendix B) that was constructed on Vector NTI (Invitrogen) using the sequence attained from NCBI (U55385). Since this was satisfactory the isolation of the plasmid followed. Despite the ability of the pRL1063a Tn5 plasmid to replicate in *E. coli* DH5α, isolation of the plasmid proved to be quite difficult. Once isolation was achieved, the pRL1063a plasmid was electroporated into the *Pseudomonas* αmb cells and selected for

kanamycin resistance. Electroporation experiments (three in total) yielded 1935 potential kanamycin mutants. The mutation rates ranged from 1 x 10^{-6} to 5.9 x 10^{-8} was still low in accordance to the number of colonies to be expected as the transformation efficiencies in other *Pseudomonas* ranges from approximately 2 x 10^{-7} to 9 x 10^{-6} (Cuppels, 1986). However these frequencies related were achieved using triparental mating. These colonies were then further streaked onto LA kanamycin (50µg/ml) plates to ensure that the colonies were true and not merely satellite colonies. It was found that only 94 were viable on the kanamycin plates. The 94 kanamycin mutants were then further streaked onto 7H10 *M. smegmatis* IP's to screen for the lack of the production of the inhibition zone. Of the 94 kanamycin mutants, only 3 showed no inhibitory activity on the IP's.

Taking into consideration that a high number of colonies (greater than 50%) reverted from kanamycin resistant to kanamycin susceptible, it was surprising to note that reversion frequencies found in other studies were extremely low. For example, Anderson and Mills noted a reversion rate of less than 10^{-8} (Anderson and Mills, 1985) and other studies involving Azospirillium lipoferum which showed a reversion rate ranging from 2 – 20 x 10^{-8} (Abdel-Salam and Klingmuller, 1987) and this is consistent with our reversion rate which was 2.24 x 10^{-11} per cell division. Hence, an investigation into whether the kanamycin resistance was due to the transposon, acquired resistance or satellite colonies was investigated.

The reversion of approximately 800 colonies could have been the result of satellite colonies. This usually arises when many colonies which contain the plasmid with the kanamycin resistance lower the concentration of the antibiotic in their vicinity. This allows for other colonies to grow around the transformant thus forming a cluster. However a recent study done by Adam *et al* 2008 investigated the reasons for the formation of satellite colonies in *E. coli* when creating ampicillin, tetracycline and nalidixic acid mutants. The study revealed that these colonies do not possess stable mutations to degrade the antibiotic, but rather have different gene expression profiles which select for phenotypic changes that favour antibiotic tolerance (Adam *et al*. 2008). This type of resistance is referred to an epigenetic mode of resistance and is identified by a high reversion rate of greater than 50%. This was most likely what had occurred in the screening of the *Pseudomonas* αmb kanamycin mutants. The satellite

phenomena has been shown to occur in *P. aeruginosa* with respect to the generation of resistance to carbenicillin (Day *et al.* 1984) The possibility of acquired resistance was excluded since the control plates did not show the presence of any colonies.

So in order to be certain that resistance to the kanamycin in the 94 mutants was due to the insertion of the transposon, primers were designed to amplify regions within the transposon. Two sets of primers (refer to Table 6) the first set being, primer A and B were designed to amplify the kanamycin region and primers C and D were designed to amplify the region between the streptomycin and the transposase region. The kanamycin resistance marker that resides in Tn5 encodes for an aminoglycoside 3-*O*-phosphotransferase (neomycin phosphotransferase). Amplification of these regions would be indicative of the presence of the plasmid, however, the actual transposition event could not be confirmed with only the pcr result. From the 8 mutants that were tested using the primer sets (Table. 8), only three were positive for both primers. It was later found that of the 4 mutants lacking the antimycobacterial compound 3 had reverted to their original phenotype, i.e kanamycin susceptible and the production of the inhibitory compound after propagation in broth culture. Surprisingly upon testing isolate 5U (one of the 4 mutants lacking antimycobacterial activity) using the pcr diagnostic, amplification of the kanamycin region did occur but this was not the case with the str-tnp region. Isolates A1, C1, C2 and C4 did not amplify for both primers therefore these primers could possibly be satellite colonies as previously described. In some studies, the transposons (IS50L and IS50R) have been shown to transpose independently of each other. It was found that in *Pseudomonas syringae* pv syringae PS9020 and *Pseudomonas syringae* pv phaseolicola PP7010 the IS50 region transposed from the Tn5 and was responsible for the kanamycin resistance. The problem was that the transposon was not responsible for the kanamycin resistance (Anderson and Mills. 1985). This could be the reason for the reversion of the phenotype with respect to isolate 5U. In some cases inactivation of the Tn5 transposon was observed, for instance Tn5 mutants were used to transform Bean *Rhizobia* to test their stability in temperature stress conditions. It was found that the Tn5 elements were present within the transformants but did not show the presence of the antibiotic activity. It was concluded that transposon was inactivated when it was exposed to high temperatures (40°C) (Pillai and Pepper, 1991). The possibility lies that the *Pseudomonas* mutant 5U was under propagated under stressful conditions

when grown in broth and even though the PCR results showed positive for the kanamycin region, it was unable to grow in the LB that was supplemented with kanamycin. However this still does not explain why the isolate was not viable.

The pcr diagnostic results did not confirm if a transposition event had occurred but only that the plasmid pRL1063a was present. In fact there was a possibility that since the mutants showed positive results which were similar to that of the *E. coli* DH5α pRL1063a, suggested that the Tn5 derivative could reside within the mutants as a self replicating plasmid. The Tn5 plasmid contains a GATC sequence in the transposase promoter which upon being methylated prevents the activation of the enzyme within *E. coli* hence the transposon derivative is able to reside within the bacterium without integrating into the genome (Wolk *et al.* 1991). The positive amplification for both primers did not distinguish between self replicating plasmids and integrated plasmids, hence there was a possibility that the Tn5 derivative was self replicating within the mutants.

According to Reznikoff (1993), the ratio of two proteins namely Tnp (a transposase-active form) and Inh (a transposase inhibitor) are responsible for the success of a transposition frequency thus integration into the genome. The promoters for these regions overlap and are regulated by two host proteins namely Dam (adenine methylase) and DnaA. The transcript which encodes for Tnp (T1) overlaps the transcript encoding for Inh (T2) therefore when T1 is being expressed both proteins (Tnp and Inh) are synthesised (Reznikoff 2003; Reznikoff, 1993). However when the DNA is methylated (binds to a GATC site in T1), the Inh protein is only synthesised resulting in the down regulation of Tnp (Reznikoff, 1993). Further down regulation is achieved by Inh which acts a negative inhibitor by binding to Tnp forming inactive Inh:Tnp dimers (Reznikoff, 2003). This down regulation of Tnp is of great importance since a highly active Tnp would result in genetic instability (due to the high transposition frequencies of transposition) and cell death (Reznikoff, 2003). Therefore Dam methylation plays an important role in the transposition event. It has been found that not all bacterial organisms possess Dam methylation systems like *E. coli*. Some gram negative bacteria have been reported to lack Dam methylation systems such as *P. aeruginosa* and *P. putida* strains (Barbeyron, Kean and Foterre, 1984; Yee and Smith, 1990). Hence the consequence of not having a Dam methylation system when

using the pRL1063a plasmid could result in cell death of transformants or a decrease in integration of the transposon into the genome.

Furthermore, the plasmid pRL1063a (containing the Tn5 derivative), was modified to suit transposition conditions for the cyanobacterium Anaebena. One of these modifications included the removal of two GATC sites required for Dam methylation which were replaced with sequences from pRZ1107 (Wolk, 1991). According to Wolk (1991) this was done to ensure that methylation did not decrease transposition in *Anaebena*. Perhaps the removal of these sequences could have decreased or abolished transposition frequency in *Pseudomonas* αmb. Despite the reports of *Pseudomonas* strains without the presence of Dam methylation systems, other *Pseudomonas* strains have been mutated using triparental mating. Perhaps this particular method should be attempted with this strain.

There was no evidence found in the literature to state that integration of the Tn5 mutant did not occur in *Pseudomonas*, however we hypothesised that if it was self-replicating then transformation using digested, and genomic DNA would produce the same number of transformants as the digested and ligated DNA in a $CaCl_2$ mediated transformation. From the results (refer to Table 8) it was clearly shown that for the digested and ligated DNA (1µg) which was originally isolated from the *E. coli* DH5α pRL1063a strain was able to produce approximately 4×10^5 colonies. Since the same amount of DNA from the mutants was used and number of colonies for the digested and ligated DNA, undigested and unligated DNA and digested and unligated DNA of the mutants was a 100 times lower, this implied that i) the plasmid does not replicate within *Pseudomonas* but is maintained, or ii) self replication could be occurring but at an extremely low copy number.

From the literature, the origin of replication regions (ori T and oriV) found in plasmid pRL1063a are originally from plasmid RK2 which is functional in both *Pseudomonas* and *E. coli*. In fact with respect to oriT, the segment ensures that the plasmid is transferred into the host cell and is used in *Pseudomonas* successfully in plasmid RK2. The oriV region is also able to be active in various gram negative bacteria including *E. coli* and *Pseudomonas* (Shah *et al.* 1995). The genetic elements that are needed for the functioning of the ori regions entail both plasmid and host proteins

such as a replication initiator proteins encoded by the plasmid (TrfA) and for e.g DnaA, helicases and DNA polymerase encoded by the bacteria (Shah *et al*, 1995). These proteins allow for the initiation and replication of the plasmid within a bacterial host. Initiation of replication is activated by the binding of TrfA to OriV (Kruger, Rakowski and Filutowiscz, 2004). There are two TrfA proteins and the shorter form is only allows for replication in certain bacteria (Helinski, 2004). DnA a host protein has also been identified as a protein essential for the plasmid replication. OriV contains two binding sites (boxes) for DnaA between iteron 4 and 5 (binding sites for TrfA) which is essential for replication in *E. coli*. DnaA is highly conserved between several bacterial species since *E. coli*, *Pseudomonas aeruginosa* and *P. putida* all contain the same sequence required for the DnaA binding (Shah *et al*., 1995). The oriV segment has four DnaA boxes that affect the replication process of RK2 (Kruger, Rakowski and Filutowiscz, 2004). DnaA box I was essential for replication of the plasmid RK2 in *E. coli* K12 and *P. putida* KT2440, however, this was not the case with *P. aeruginosa* PAO1161 (Shah *et al*, 1995). With the latter it was found that the DnaA box II allowed the RK2 plasmid to be maintained and possible replicate at a very low copy numbers which cannot be detected in plasmid extractions. Therefore it seems reasonable to suggest that this could be the occurring within this particular *Pseudomonas* strain since we see replication in *E. coli* DH5α in high copy numbers but in *Pseudomonas* αmb: M1 transformant no plasmid DNA was obtained from the plasmid extractions done (data not shown).

The plasmid from clone M1 was isolated, digested with restriction enzymes and compared to the control pRL1063a (Fig.32). The restriction profile of the two plasmids showed similar banding patterns. Since no difference in plasmid size could be inferred from the gel, the plasmids were sent off for sequencing to Inqaba biotech to determine if foreign DNA is present within the plasmid of clone M1. Primers used for sequencing (Table 6, primers E and F) were attained from Milcamps *et al*, 1993. Sequencing data revealed that the M1 clone did not possess any foreign DNA so it failed to integrate into the genome. Thus no transposition event had occurred which strongly suggests that the plasmid is either replicating at a very low copy number or is maintained within the bacteria.

Cloning of other mutants still needs to be done as well as sequencing of those clones in order to understand what is occurring in other Kan^R mutants. At this point in time the transformation efficiencies shown in the Tn5 mediated transformation of *Pseudomonas amb* are promising but transposition of Tn5 is not guaranteed.