SUBCLONING, CHARACTERIZATION AND SEQUENCING OF THE STREPTOMYCIN RESISTANCE GENE OF A MULTIPLE ANTIBIOTIC RESISTANCE TRANSPOSON FROM Salmonella typhi

RAMESVARI A/P PARARAJASINGAM PILLAI

A dissertation submitted to the University of Malaya, in partial fulfillment of the requirements for the Degree of Master of Biotechnology

INSTITUTE OF POSTGRADUATE STUDIES AND RESEARCH
UNIVERSITY OF MALAYA

KUALA LUMPUR

June 2000
Dedicated to,

The loving memory of my Daddy (late), ............

the wellspring of wisdom and perseverance and
to my Mummy, .................

for her devotion and her constant demonstrations
of love.
ABSTRACT

_Salmonella typhi_ S8 is resistant to ampicillin (Ac), chloramphenicol (Cm), co-trimoxazole (Ctm), streptomycin (Sm), and tetracycline (Tc). The Ac, Cm, Ctm, and Sm resistance traits have been shown to be mediated by a multiple antibiotic resistance transposon located on a large conjugative plasmid. Transposition of the transposon encoding Ac\(^R\) (ampicillin resistance), Cm\(^R\), Ctm\(^R\), and Sm\(^R\), designated TnX8, was done by Wong (1999) to a recipient replicon, plasmid pUB307. The Ac\(^R\), Cm\(^R\), Ctm\(^R\), and Sm\(^R\) genes were subcloned into plasmid pKan by digesting the recombinant plasmid, designated pCL8 (pUB307::TnX8), with _SalI_. This subcloning strategy generated different recombinant pKans that harbour different antibiotic resistance genes. Plasmid pCLS55 is a recombinant pKan that harbours a _SalI_ insert bearing Sm\(^R\) genes from the multiple antibiotic resistance transposon of _S. typhi_ S8.

The main objective of this study was to subclone and characterizes, by DNA sequencing, the Sm\(^R\) genes derived from the transposon of _S. typhi_ S8, in pCLS55. Successive subclonings into pUC19 and M13mp18 vectors, and construction of deletions localized the Sm\(^R\) genes on a 2.5 kb _EcoRI_ DNA fragment. Southern hybridization, performed before DNA sequencing, confirmed that the 2.5 kb _EcoRI_ Sm\(^R\) inserts carried by M13pSMR13 and M13pSMS16 originated from the plasmid in _S. typhi_ S8.

A total of 2,128 nucleotides was determined based on complete DNA sequencing of the 2.5 kb _EcoRI_ Sm\(^R\) insert in recombinant M13mp18. The nucleotide sequences were analyzed and the gene structure (containing _str_ genes, _strA_ and _strB_) within the 2.5 kb _EcoRI_ Sm\(^R\) insert from pCLS55 was determined. These sequences were identical to the _strA_ and _strB_ genes encoding Sm resistance in plasmid RSF1010 (Scholz _et al._, 1989) and in Tn5393 of _Erwinia amylovora_ (Chiou and Jones, 1993).

The nucleotide sequences obtained in this study indicated that there are three open reading frames (ORF) in the 2.5 kb _EcoRI_ Sm\(^R\) fragment. The first ORF of 267 amino acids was designated ORF A\(^{+}\). The deduced amino acid sequence corresponded to a molecular mass of 29,595 Daltons and has high similarity with the amino acid sequence of Sm phosphotransferase protein A, which is also encoded by RSF1010
and Tn5393. This ORF translation starts at UUG (TTG in the DNA sequence) and this suggests that this gene did not originally come from members of the family Enterobacteriaceae.

The second ORF, designated ORF B', encodes a polypeptide of 278 amino acids; which corresponded to a molecular mass of 30,824 Daltons and has high similarity with the amino acid sequence of Sm phosphotransferase protein B.

The Sm\(^r\) genes were also observed to contain part of the transposase gene belonging to IS26 and its variants. This was observed in the third ORF, ORF C', in the nucleotide sequences obtained. Partial DNA sequencing of the 5.5 kb SalI insert in pCLS55 showed that the two ends of this insert each contained part of the transposase gene of IS26 and its variants.

The Sm\(^r\) genes in pCLS55 appeared to be borne on a composite element, flanked by IS26 at its ends. The presence of the composite IS26 element within TnX8 supports the hypothesis that multiple antibiotic resistance transposons evolved by insertion of antibiotic resistance determinants, which are themselves transposable.
**ABSTRAK**

*Salmonella typhi* S8 adalah rintang terhadap ampilisin (Ac), kloramfenicol (Cm), ko-trimoxazole (Ctm), streptomisin (Sm), dan tetrasiklin (Tc). Ciri-ciri kerintangan terhadap Ac, Cm, Ctm, dan Sm adalah diperantarkan oleh suatu transposon pelbagai kerintangan antibiotik yang berlokasi pada suatu plasmid konjugatif yang besar. Transposisi transposon TnX8, transposon yang mengekodkan AcR (kerintangan terhadap ampilisin), CmR, CtmR, dan SmR, kepada replikon penerima iaitu plasmid pUB307 telah dilakukan oleh Wong (1999).


Objektif utama kajian ini adalah untuk mensubklon dan mencirikan, melalui penjaukan DNA, gen-gen SmR dalam pCLS55 yang diterbitkan dari transposon *S. typhi* S8. Pensubklonan berturutan kedalam vektor pUC19 dan sejurusnya kedalam vektor M13mp18, serta penjanaan delesi berjaya melokasikan gen-gen SmR pada satu fragmen *EcoRI* bersaiz 2.5 kb. Sebelum penjaukan DNA, hibridisasi Southern dilakukan dan ia menunjukkan bahawa asalan fragmen SmR *EcoRI* bersaiz 2.5 kb ini adalah sememangnya dari plasmid *S. typhi* S8.

Nukleoitida-nukleoitida berjumlah 2,128 ditentukan berdasarkan penjaukan DNA seluruh fragmen *EcoRI* bersaiz 2.5 kb yang terkandung dalam M13mp18 rekombinan. Jujukan nukleoitida yang diperolehi dianalisis dan struktur gen (yang mengandungi gen-gen *str*, iaitu *strA* dan *strB*) dalam fragmen *EcoRI* bersaiz 2.5 kb daripada pCLS55 berjaya ditentuakan. Jujukan-jujukan ini didapati seiras dengan gen-gen *strA* dan *strB* yang mengekodkan kerintangan terhadap Sm dalam plasmid RSF1010 (Scholz *et al.*, 1989) dan dalam Tn5393 yang termaktub dalam *Erwinia amylovora* (Chiou and Jones, 1993).
Jujukan nukelotida yang diperolehi dalam kajian ini menunjukkan bahawa ada tiga bingkai bacaan terbuka (ORF, "open reading frame") dalam fragmen Sm\(^R\) EcoRI bersaiz 2.5 kb. Bingkai bacaan pertama yang berdesignasi ORF A' mengandungi 267 asid amino dan sejajar dengan berat molekul 29,595 Dalton. Ia mempunyai kesamaan tinggi dengan jujukan asid amino protin A fosfotransferase Sm yang dikodkan oleh RSF1010 dan Tn\(^{5393}\). Translasi ORF ini bermula dengan UUG (TTG dalam jujukan DNA) dan mencadangkan bahawa gen \(strA\) ini tidak berasal daripada ahli famili Enterobacteriaceae.

Bingkai bacaan kedua berdesignasi ORF B' mengekodkan polipetida sebesar 278 asid amino dan sejajar dengan berat molekul 30,824 Dalton. Analisa jujukan asid amino ini sejajar dengan protin B fosfotransferase Sm yang dikodkan oleh gen \(strB\) RSF1010 dan Tn\(^{5393}\). Gen-gen Sm\(^R\) ini didapati juga mengandungi sebahagian gen enzim transposisi IS26 dan variasinya. Ini diperhatikan dalam bingkai bacaan ketiga, yakni ORF C', dalam jujukan nukleotida yang diperolehi dalam kajian ini. Penjuyukan DNA separa juga dilakukan terhadap fragmen \(S_{a}I\) bersaiz 5.5 kb dari pCLS55. Analisa jujukan nukleotid separa yang diperolehi ini juga menunjukkan kehadiran gen enzim transposisi IS26 dan variasinya.

Gen-gen Sm\(^R\) dalam pCLS55 didapati termaktub dalam elemen komposit kerana ia diapiti oleh IS26 pada kedua-dua hujungnya. Kehadiran elemen komposit IS26 dalam Tn\(^{X8}\) mengukuhkan lagi hipotesis bahawa transposon pelbagai kerintangan antibiotik berevolusi melalui insersi penentu-pentu antibiotik, yang mempunyai keupayaan untuk bertransposisi secara sendiri.
ACKNOWLEDGEMENT

First, I would like to express my deepest gratitude to my supervisors, Prof. Dr. Koh Chong Lek and Assoc. Prof. Dr. Sam Choon Kook, who have been ever so supportive during my research and dissertation. Their invaluable advice, constructive criticisms and patient discussions throughout the duration of this project have helped me immensely. They have also provided and offered me enthusiasms and acuity that sharpened my resolve and thinking.

Special thanks and gratitude to Miss Lim Moo Eng for her valuable time and assistance in preparing the diagrams and photographs.

A special note of thanks to my dearest friends and colleagues Ling, May, Dr. Don Gary Benjamin, and Mr. Pathi, Wan Chee, Dr. Fong who constantly gave me the opportunities to share ideas in a large variety of settings and for their friendship.

Among my profound debts is to Wong Hann Ling, who have shared his findings with me, and whose efforts are reviewed and synthesized here.

My indebtedness and gratitude to Kanga, her (Kanga) family, Katherine, and Vinder for their hospitality, encouragement, and assistance in the process of writing this dissertation.

Thanks to staffs of the Institute of Biological sciences and IPSR, especially Mr. Gopal and Mrs. Gan, for offering their assistance to me.

My sincere thanks also to the University of Malaya for financial assistance under the Fellowship and PASCA scheme for funding my study.

A very special note of indebtedness to my family; my mummy, brother and sister, who have provided the cocoon of warmth and love, for their inspirations, encouragement, intellectual and spiritual support to persevere in very trying circumstances. If ever a cliché was true, it would be that without them, this dissertation would never have been possible.

Lastly, my utmost gratification to the LORD for the countless blessings HE bestowed upon me. I thank YOU, LORD.
ABBREVIATIONS

Most of the abbreviations used are standard. However, attention is drawn to the following:

\[\begin{align*}
\mu g & \quad \text{microgram} \\
\mu l & \quad \text{microlitre} \\
\mu m & \quad \text{micrometer} \\
A & \quad \text{adenine (in DNA nucleotide sequence)} \\
Ac & \quad \text{ampicillin} \\
Ac^R & \quad \text{ampicillin resistant or resistance} \\
bp & \quad \text{base pair} \\
BPB & \quad \text{bromophenol blue} \\
C & \quad \text{cytosine (in DNA nucleotide sequence)} \\
Cm & \quad \text{chloramphenicol} \\
Cm^R & \quad \text{chloramphenicol resistant or resistance} \\
CsCl & \quad \text{cesium chloride} \\
Ctm & \quad \text{cotrimoxazole} \\
Ctm^R & \quad \text{cotrimoxazole resistant or resistance} \\
dATP & \quad \text{deoxyadenosine 5'-triphosphate} \\
DNA & \quad \text{deoxyribonucleic acid} \\
EDTA & \quad \text{ethylenediaminetetraacetate} \\
EtBr & \quad \text{ethidium bromide} \\
g & \quad \text{gram} \\
G & \quad \text{guanine (in DNA nucleotide sequence)} \\
h & \quad \text{hour} \\
\text{int} & \quad \text{integron or integrase} \\
IR & \quad \text{inverted repeats} \\
IS & \quad \text{insertion sequence} \\
k & \quad \text{kilobase pair or kilobase} \\
Km & \quad \text{kanamycin}
\end{align*}\]
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>kanamycin resistant or resistance</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mA</td>
<td>milliampere</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MH</td>
<td>Mueller Hinton</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORFS</td>
<td>open reading frame</td>
</tr>
<tr>
<td>ori</td>
<td>origin</td>
</tr>
<tr>
<td>P</td>
<td>promoters</td>
</tr>
<tr>
<td>p.s.i</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>rec</td>
<td>recombination</td>
</tr>
<tr>
<td>RF</td>
<td>replicative form (of M13 DNA)</td>
</tr>
<tr>
<td>RHSs</td>
<td>recombination hot spots</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>Sm</td>
<td>streptomycin</td>
</tr>
<tr>
<td>Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>streptomycin resistant or resistance</td>
</tr>
<tr>
<td>Smz</td>
<td>sulphamethoxazole</td>
</tr>
<tr>
<td>Smz&lt;sup&gt;R&lt;/sup&gt;</td>
<td>sulphamethoxazole resistant or resistance</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride-sodium citrate</td>
</tr>
<tr>
<td>Su</td>
<td>sulfonamide</td>
</tr>
<tr>
<td>Su&lt;sup&gt;R&lt;/sup&gt;</td>
<td>sulfonamide resistant or resistance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>T</td>
<td>thymine (in DNA nucleotide sequence)</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>Tc</td>
<td>tetracycline</td>
</tr>
<tr>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>tetracycline resistant or resistance</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'- tetramethylenediamine</td>
</tr>
<tr>
<td>TGE</td>
<td>transposable genetic element</td>
</tr>
<tr>
<td>Tn</td>
<td>transposon</td>
</tr>
<tr>
<td>T&lt;sub&gt;p&lt;/sub&gt;</td>
<td>trimethoprim</td>
</tr>
<tr>
<td>T&lt;sub&gt;p&lt;/sub&gt;&lt;sup&gt;R&lt;/sup&gt;</td>
<td>trimethoprim resistant or resistance</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) methylamine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>W</td>
<td>watt</td>
</tr>
</tbody>
</table>
# LIST OF CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRAK</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF CONTENTS</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xxiii</td>
</tr>
</tbody>
</table>

## CHAPTER ONE: INTRODUCTION

1.1 *Salmonella typhi*  
1.2 Typhoid fever  
1.3 Diagnosis and treatment of typhoid fever  
1.4 Antibiotics  
  1.4.1 Characteristics of antibiotics  
  1.4.2 Aminoglycosides  
  1.4.3 Streptomycin (Sm)  
  1.4.4 Mechanisms of Sm resistance  
   1.4.4.1 Sm-modifying enzymes  
   1.4.4.2 Alterations in ribosomal Sm binding site  
   1.4.4.3 Mutations interfering with Sm uptake  
1.5 Epidemiology of antibiotic resistance  
1.6 Origin of antibiotic resistance  
1.7 Transfer of antibiotic resistance genes  
1.8 R plasmids  
1.9 Transposable genetic elements (TGE)  
1.10 Transposon Tn21  
1.11 IS elements  
1.12 Integrons
CHAPTER TWO: MATERIALS AND METHODS

2.1 Bacterial strains

2.2 Plasmids and phage

2.3 Materials

2.4 Media, antibiotic solutions, and stock solutions
   2.4.1 Luria-Bertani (LB) medium (Sambrook et al., 1989)
   2.4.2 SOB medium
   2.4.3 SOC medium
   2.4.4 Mueller Hinton (MH) agar
   2.4.5 B agar (Sambrook et al., 1989)
   2.4.6 2X TY
   2.4.7 Antibiotic stock solutions
   2.4.8 Solutions for agarose gel electrophoresis
      2.4.8.1 Tris-borate EDTA (TBE) buffer, pH 8.3
      (Sambrook et al., 1989)
   2.4.8.2 50X Tris-acetate EDTA (TAE) buffer
   2.4.8.3 6X Bromophenol blue (BPB) loading dye
   2.4.9 Common solutions for DNA extraction, cloning, and transformation

2.5 Solutions for hybridization experiments
   2.5.1 Solutions for Southern blotting
      2.5.1.1 Depurination solution
      2.5.1.2 Denaturation solution
      2.5.1.3 Neutralization solution
      2.5.1.4 Solutions for hybridization and blot washing
      2.5.1.5 Hybridization buffer
      2.5.1.6 Primary wash buffer (without urea)
      2.5.1.7 Secondary wash buffer (2X SSC
2.5.2 Solutions for labelling DNA probes 46
2.5.3 Signal generation and detection 46

2.6 Solutions for M13mp18 subcloning 46
2.6.1 Xgal 46
2.6.2 IPTG (0.1 M) 47
2.6.3 20% (w/v) PEG, 2.5 M NaCl 47
2.6.4 SDS-formamide dye mix (Young, 1984) 47

2.7 Sterilization techniques 47
2.7.1 Heat sterilization 47
2.7.2 Steam sterilization 48
2.7.3 Membrane sterilization 48

2.8 Plating techniques 48

2.9 Maintenance and purification of bacterial strains 48

2.10 Small scale rapid extraction of plasmid DNA (miniprep) 48

2.11 Large-scale extraction and purification of covalently closed circular (ccc) DNA 49

2.12 Estimation of DNA concentration 51

2.13 Restriction endonuclease digestions of DNA 51

2.14 Agarose gel electrophoresis 52

2.15 Recovery of DNA fragments from agarose gels 52
2.15.1 Electroelution (Sambrook et al., 1989) 52
2.15.2 Recovery of DNA fragments from agarose gels by the GENE CLEAN II kit 53

2.16 Ligation of DNA fragments with compatible ends 53

2.17 Subcloning of the streptomycin resistance gene(s) (SmR) from PstI digested pCLS55 into pUC19 54
2.17.1 Comparison of pKan and pCLS55 after restriction endonuclease digestions 54
2.17.2 Preparation of PstI digested pCLS55 and pUC19 54
2.17.3 Shotgun subcloning of the fragment harbouring the SmR gene(s) into plasmid vector pUC19 54
2.17.4 Preparation of competent *E. coli* cells  
2.17.5 Transformation  
2.17.6 Analysis of transformants

2.18 Southern blotting and hybridization

2.18.1 Southern transfer of DNA (Southern, 1975) from agarose gel to nylon membrane

2.18.2 Direct labelling of DNA probes

2.18.3 Southern hybridization

2.18.4 Post-hybridization membrane washing

2.18.5 Signal generation and detection

2.19 Subcloning of the 2.5 kb *EcoRI* DNA fragment harbouring the Sm<sup>R</sup> genes into M13mp18

2.19.1 Preparation of the *EcoRI* digested M13mp18

2.19.2 Gel-elution of the 2.5 kb *EcoRI* DNA fragment harbouring the Sm<sup>R</sup> genes

2.19.3 Preparation of competent *E. coli* DH5αF<sup>+</sup> cells

2.19.4 Ligation of the *EcoRI* digested M13mp18 with the 2.5 kb *EcoRI* DNA fragment harbouring the Sm<sup>R</sup> genes

2.19.5 Transfection of *E. coli* DH5αF<sup>+</sup> with the RF DNA of M13mp18

2.19.6 Complementary or C-test to confirm the opposite orientations of inserts in recombinant M13mp18

2.19.7 Purification of single-stranded template DNA of recombinant M13mp18

2.20 Subcloning of the 5.5 kb *SalI* DNA fragment from pCLS55 into *SalI* digested pUC19

2.20.1 Preparation of the *SalI* digested pCLS55 and pUC19

2.20.2 Ligation of the 5.5 kb *SalI* digested DNA fragment with *SalI* digested pUC19

2.20.3 Transformation

2.20.4 Analysis of transformants
2.21 DNA sequencing
   2.21.1 Non-isotopic automated DNA sequencing
   2.21.2 Polyacrylamide gel electrophoresis
   2.21.3 Analysis of nucleotide sequences

2.22 Flowcharts
   2.22.1 Flowchart of experiments to subclone the Sm\textsuperscript{R} gene(s) into pUC19
   2.22.2 Flowchart of experiments to construct a restriction map of the 5.5 kb insert and to locate Sm\textsuperscript{R} gene(s) to a smaller fragment
   2.22.3 Flowchart of experiments to subclone the Sm\textsuperscript{R} gene(s) into M13mp18 vector, sequence the Sm\textsuperscript{R} gene(s), and analyses of the nucleotide sequences

CHAPTER THREE: RESULTS

3.1 Comparison between pKan and pCLS55
3.2 Restriction patterns of pKan and pCLS55
3.3 Complete digestion of pCLS55 and pUC19 with \textit{PstI}
3.4 Elution of the excised fragments from pCLS55 and linearized pUC19
3.5 Shot-gun subcloning of the Sm\textsuperscript{R} gene(s)
3.6 Plasmid profiles of \textit{Ac}\textsuperscript{R}Sm\textsuperscript{R} transformants
3.7 Restriction patterns of recombinant pUC19 after \textit{PstI} and \textit{SalI} digestions
3.8 Complete digestion of pSR3 and pSR4 with \textit{SalI}
3.9 Elution of the excised fragments from \textit{SalI}-digested pSR3 and pSR4
3.10 Subcloning of the Sm\textsuperscript{R} DNA fragment from pSR3
3.11 Plasmid profiles of the \textit{Ac}\textsuperscript{R}Sm\textsuperscript{R} transformants
3.12 Restriction analyses of recombinant pUC19 obtained after circularization of the 5.2 kb \textit{SalI} fragment from pSR3
3.13 Complete digestion of pSR3a and RF DNA of M13mp18 with EcoRI
3.14 Subcloning of the 2.5 kb EcoRI fragment harbouring the SmR genes into M13mp18
3.15 Screening of the recombinant M13mp18
3.16 Confirmation of the opposite orientation of inserts in recombinant M13mp18 RF DNA
3.17 Southern hybridization with recombinant plasmids and phages
3.18 Isolation of single-stranded DNA from M13pSMR13 and M13pSMS16
3.19 Nucleotide sequences from M13pSMR13 and M13pSMS16
3.20 Analysis of the nucleotide sequence from M13pSMR13 and M13pSMS16
3.21 Subcloning of the 5.5 kb fragment from SalI-digested pCLS55 harbouring SmR gene into SalI-digested pUC19
3.22 SalI-digestion of the recombinant pUC19
3.23 Isolation and sequencing of double-stranded pSR55
3.24 Partial nucleotide sequences from pSR55
3.25 Analysis of the nucleotide sequences from pSR55

CHAPTER FOUR: DISCUSSION AND CONCLUSION
4.1 Discussion
4.1.1 Localization of the SmR genes
4.1.2 Southern hybridization
4.1.3 Nucleotide sequences of the 2.5 kb SmR genes
4.1.4 IS26-like segment
4.2 Conclusion

REFERENCES
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Structure of streptomycins</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Chemical structures of the aminoglycosides</td>
<td>9</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Action of aminoglycoside-inactivating enzymes</td>
<td>11</td>
</tr>
<tr>
<td>Figure 4</td>
<td>A schematic diagram displaying the possible route of acquisition of antibiotic resistance genes by bacteria under the selective pressure of antibiotics used</td>
<td>15</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Evolutionary relationships of Tn21 and Tn21-related transposons</td>
<td>20</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Two modes of transposition operative in bacteria</td>
<td>22</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Physical maps showing the regions common to Tn21, R46, and R388</td>
<td>24</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Structure of relevant Km(^R) transposons</td>
<td>26</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Sequence alignment of the termini of IS26R, IS1, and IS102/IS903</td>
<td>28</td>
</tr>
<tr>
<td>Figure 10</td>
<td>General structure of sulI-associated integrons</td>
<td>30</td>
</tr>
<tr>
<td>Figure 11</td>
<td>General structure of integrons</td>
<td>32</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Cloning vectors M13mp18 and M13mp19</td>
<td>34</td>
</tr>
<tr>
<td>Figure 13</td>
<td>The orientation of inserted DNA fragments harbouring antibiotic resistance gene(s) in M13mp18</td>
<td>36</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Summary of the principles of the ECL Direct Nucleic Acid Labelling and Detection System</td>
<td>57</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Principles of the C-test</td>
<td>62</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Ethidium bromide-stained 0.5% (w/v) agarose gel of pHKan and pCLS55</td>
<td>70</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Ethidium bromide-stained 0.7% (w/v) agarose gel of SalI-digested pHKan and SalI-digested pCLS55</td>
<td>71</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Ethidium bromide-stained 0.7% (w/v) agarose gel of pCLS55 digested with various restriction</td>
<td></td>
</tr>
</tbody>
</table>
endonucleases

Figure 19: Ethidium bromide-stained 0.8% (w/v) agarose gel of PstI-digested pKan and PstI-digested pCLS55

Figure 20: The restriction map of pKan (after Micklos and Freyer, 1990)

Figure 21: The physical map of pCLS55

Figure 22: Ethidium bromide-stained 0.8% (w/v) agarose gel of PstI-digested pCLS55 and PstI-digested pUC19

Figure 23: Ethidium bromide-stained 0.5% (w/v) agarose gel of ccc pUC19, pCLS55, and pUC19 recombinants extracted by the alkaline lysis method of Birnboim (1983) from E. coli DH5α transformants

Figure 24: Ethidium bromide-stained 0.7% (w/v) agarose gel of PstI-digested recombinant plasmids isolated from 10 AcR SmR transformants

Figure 25: Strategy used to subclone and locate the DNA fragment harbouring the SmR gene(s)

Figure 26: Ethidium bromide-stained 0.5% (w/v) agarose gel of SalI-digested recombinant plasmids isolated from 10 AcR SmR transformants

Figure 27: Construction of pSR3 and pSR4

Figure 28: Ethidium bromide-stained 0.5% (w/v) agarose gel of ccc pUC19, pSR3, and recombinant plasmids extracted from E. coli DH5α transformants harbouring circularized 5.2 kb SalI fragment from pSR3 (pSR3a-e)

Figure 29: Ethidium bromide-stained 0.5% (w/v) agarose gel of SalI-digested recombinant pUC19 derived from circularized 5.2 kb SalI fragment from pSR3 (pSR3a-e)

Figure 30: Ethidium bromide-stained 0.7% (w/v) agarose gel of EcoRI-digested recombinant pUC19 (pSR3a-e) derived from circularized 5.2 kb SalI fragment from pSR3
Figure 31: Ethidium bromide-stained 0.6% (w/v) agarose gel of gel-eluted EcoRI-digested M13mp18 and the 2.5 kb EcoRI fragment harbouring Sm<sup>R</sup> gene(s) from pSR3a

Figure 32: Ethidium bromide-stained 0.6% (w/v) agarose gel of recombinant M13mp18 RF DNA exhibiting Sm<sup>R</sup> and Sm<sup>S</sup> phenotypes

Figure 33: Ethidium bromide-stained 0.6% (w/v) agarose gel of EcoRI-digested recombinant M13mp18 RF DNA

Figure 34: Ethidium bromide-stained 0.6% (w/v) agarose gel of recombinant M13 after the C-test

Figure 35: Construction of M13pSMR13 and M13pSMS16

Figure 36: Hybridization with the 2.5 kb EcoRI-digested fragment probe

Figure 37: Ethidium bromide-stained 0.7% (w/v) agarose gel showing the single-stranded DNA of M13mp18(lane 1), M13pSMR13 (lane 2), and M13pSMS16 (lane 3)

Figure 38: Complete nucleotide sequence obtained by using M13pSMR13 as the template and three primers: the M13 universal primer and two oligonucleotide primers, R13 5'-CGTCCGCCATCTGTAATGCGT-3' (nucleotide positions 690 to 713 in M13pSMR13) and R132 5'-GCGAAGGCGCGCTCTGCTTCTACT-3' (nucleotide positions 1456 to 1479 in M13pSMR13)

Figure 39: Complete nucleotide sequence obtained by using M13pSMS16 as the template and three primers: the M13 universal primer and two oligonucleotide primers, R16 5'-CGGCTCGGAACAGCAGATCGCTAT-3' (nucleotide positions 708 to 731 in M13pSMS16) and R162 5'-GAAGGCGCGCTCTGCTTCTACT-3' (nucleotide positions 1246 to 1269 in M13pSMS16)

Figure 40: Comparison of the M13pSMR13 (top sequence) and M13pSMS16 (below sequence) nucleotides

Figure 41: Complete nucleotide sequence of the 2.5 kb EcoRI
Figure 42: (A) and (B) show the 2.5 kb EcoRI fragment harboured by M13pSMR13 and M13pSMS16, respectively.

Figure 43: Ethidium bromide-stained 0.5% (w/v) agarose gel of ccc pUC19 and recombinant pUC19 (with 5.5 kb SalI-digested fragment inserted into SalI-digested pUC19) from E. coli transformants extracted by the alkaline lysis method of Birnboim (1983).

Figure 44: Ethidium bromide-stained 0.6% (w/v) agarose gel of SalI-digested recombinant plasmids isolated from AcR SmR transformants.

Figure 45: Partial nucleotide sequence from pSR55 by using the M13 universal primer.

Figure 46: Proposed genetic organization of the 5.5 kb SalI fragment from pCLS55.
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>List of antibiotics</td>
<td>41</td>
</tr>
<tr>
<td>Table 2</td>
<td>Enzymes used in this study</td>
<td>41</td>
</tr>
<tr>
<td>Table 3</td>
<td>Antibiotic stock solutions</td>
<td>44</td>
</tr>
<tr>
<td>Table 4</td>
<td>Protocols for ligation of the <em>PstI</em> digested</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>pCLS55 and pUC19</td>
<td></td>
</tr>
<tr>
<td>Table 5</td>
<td>Protocols for ligation of the 2.5 kb <em>EcoRI</em> DNA</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>fragment harbouring the Sm&lt;sup&gt;R&lt;/sup&gt; genes with <em>EcoRI</em>-digested M13mp18</td>
<td></td>
</tr>
<tr>
<td>Table 6</td>
<td>Fragments produced by single or double restriction</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>endonuclease digestions of pCLS55</td>
<td></td>
</tr>
<tr>
<td>Table 7</td>
<td>Results of the shotgun cloning of the four DNA</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>fragments (5.75, 2.23, 0.92, and 0.8 kb) obtained</td>
<td></td>
</tr>
<tr>
<td></td>
<td>from <em>PstI</em>-digested pCLS55 into pUC19</td>
<td></td>
</tr>
<tr>
<td>Table 8</td>
<td>Results of the subcloning of the Sm&lt;sup&gt;R&lt;/sup&gt; DNA</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>fragment from pSR3 and pSR4</td>
<td></td>
</tr>
<tr>
<td>Table 9</td>
<td>Results of ligation of the 2.5 kb <em>EcoRI</em> fragment from pSR3a with <em>EcoRI</em>-digested RF DNA of M13mp18</td>
<td>94</td>
</tr>
<tr>
<td>Table 10</td>
<td>Results showing the number of colonies growing on LB</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>agar plates containing Sm after an overnight incubation at 37°C of cells from 30 randomly selected plaques/ colonies from tube A or B</td>
<td></td>
</tr>
<tr>
<td>Table 11</td>
<td>Results of the subcloning of the 5.5 kb <em>SalI</em> fragment from pCLS55 into pUC19</td>
<td>118</td>
</tr>
</tbody>
</table>