CHAPTER FOUR
DISCUSSION AND CONCLUSION

4.1 Discussion

pST8 is a multiple antibiotic resistance plasmid found in the multiple antibiotic resistant *S. typhi* S8 and was found to harbour a multiple antibiotic resistance transposon, Tn*X8*. The data from this study and other related studies by co-workers suggest that Tn*X8* is identical to Tn*5410* (Wong, 1999). To identify the genetic organization of the multiple antibiotic resistance genes in Tn*5410*, the Sm^R genes harboured in this transposon were successfully located, subcloned, characterized, and sequenced in this project.

The analyses done exhibited that the multiple antibiotic resistance transposon, Tn*X8*, found in pST8 harboured two Sm^R genes, *strA* and *strB*, in the 5.5 kb fragment. The identities of these genes were determined by comparing the resultant nucleotide sequences obtained from DNA sequencing done with similar sequences from the GenBank-database which will be discussed in more detail below.

4.1.1 Localization of the Sm^R genes

Previously, pCLS55 (9.7 kb, $Km^RSm^RSu^R$), a pKan (Micklos and Freyer, 1990) recombinant, was constructed by Wong (1999) (the origin of this recombinant was described in Section 1.14). Although, the 5.5 kb *Sal*I fragment from pCLS55 was initially identified as the insert that harbours the Sm^R genes (Wong, 1999), the specific site and relative location of these genes in this large 5.5 kb insert was not known.

Through a series of analyses, which include restriction endonuclease digestions and subcloning into pUC19 and M13mp18, the insert harbouring the Sm^R genes was trimmed down and finally localized in the 2.5 kb *Eco*RI fragment from pCLS55. This fragment was then subjected to further characterization.

4.1.2 Southern hybridization

Southern hybridization was performed on the trimmed 2.5 kb Sm^R *Eco*RI fragment. This was done to confirm and reveal that the 2.5 kb Sm^R *Eco*RI fragment had originated from pST8 of *S. typhi* S8.

The results obtained from the Southern hybridization analysis confirmed that the 2.5 kb Sm^R *Eco*RI was from pST8. The labelled 2.5 kb Sm^R probe hybridized and gave strong signals to the 5.5 kb *Sal*I fragment from pCL8 (pUB307::Tn*X8*, $Ac^RCm^RCtm^RSm^RTc^S$).

The Southern hybridization also demonstrated that the 2.5 kb Sm^R probe also hybridized to the other recombinants, the 5.5 kb *Sal*I fragment from pCLS55 (Km^RSm^R), the 5.2 kb *Sal*I fragment from pSR3 (Ac^RSm^R), the 2.5 kb *Eco*RI fragment from pSR3a (Ac^RSm^R), and the 2.5 kb *Eco*RI fragments from pSMR13 (Sm^R) and pSMS16 (Sm^S), which have pertained to the generation of the trimmed 2.5 kb Sm^R *Eco*RI fragment.

4.1.3 Nucleotide sequences of the 2.5 kb Sm^R genes

Analysis of the nucleotide sequences of the 2.5 kb Sm^R genes obtained showed that these sequences were identical to the *strA* and *strB* genes encoding streptomycin resistance in plasmid RSF1010 (Scholz *et al.*, 1989) and in Tn*5393* of *Erwinia amylovora* (Chiou and Jones, 1993).

The similarities are between the nucleotides 90-890 and 893-1726 (Figure 41) obtained from the template of pSMS16 with the nucleotides 1-1708 and 8676-8684 of RSF1010, respectively (Scholz *et al.*, 1989). The analysis also showed sequence similarity to the nucleotide sequence of Tn*5393* (4928 to 6608 nucleotides) carrying the Sm^R genes in *Erwinia amylovora* (Chiou and Jones, 1993).

In RSF1010, the nucleotides 90-892 belong to the *strA* gene that encodes StrA protein, whilst nucleotides 893-1726 belong to the *strB* gene, which encodes StrB protein (Scholz *et al.* 1989). The *strA* gene downstream of *sulII* encodes aminoglycoside phosphotransferase [APH-(3")] (Kawabe *et al.*, 1978), and the *strB* gene shows homology with a Sm^R gene found in transposon Tn*5* (Scholz *et al.*, 1989). The StrA and StrB proteins are encoded by two ORFs, which are designated ORFs H and I, respectively (Scholz *et al.*, 1989).

Tn*5393* is a class II, Tn*3*-like transposon and is located on plasmid pEa34 from *E. amylovora*. Tn*5393* consists of 6,705 bp with 81 bp terminal inverted repeats

and generates 5 bp duplications of the target DNA following insertion. It has two ORFs specifying resistance to Sm, designated ORFs A and B, identified to encode the Sm^R resistance proteins, StrA and StrB, respectively. The Sm^R genes on Tn*5393* (nucleotides 4298-6705) (Chiou and Jones, 1993) have been identified on the basis of their DNA homology to Sm^R genes in plasmid RSF1010 (nucleotides 19-1737; Scholz *et al.*, 1989).

Analysis of the nucleotide sequences indicated that there are three ORFs in the sequences determined from the 2.5 kb Sm^R fragment (Figure 41). The reading frame of 267 amino acids shown in Figure 41, designated ORF A' and corresponding to a molecular weight of 29,595 Dalton, has high similarity with the amino acid sequence of Sm phosphotransferase protein A which is also encoded by in RSF1010 and Tn*5393*. The translation starts at UUG (TTG in the DNA sequence) given as the start codon and terminates at the TGA codon at nucleotide position 890-892. Although rare in Gram-negative bacteria, occasional instances when UUG acts as a met start codon in *E. coli* have been reported earlier (Reddy *et al.*, 1985; Fling *et al.*, 1988; Ringquist *et al.*, 1992). The observation that the *strA* gene starts with a UUG codon suggests that this gene did not originally come from members of the family *Enterobacteriaceae*.

A plausible ribosome binding sequence, AAGGA, was found upstream of the UUG triplet. Comparison was made between the amino acid sequences obtained from ORF A' and from RSF1010 (Scholz *et al.*, 1989) and Tn*5393* of *Erwinia amylovora* (Chiou and Jones, 1993). The amino acid similarities are 100% to StrA encoded by ORF H of RSF1010 and 99% to StrA encoded by ORF A of Tn*5393*. The discrepancy found in amino acid sequence of the StrA protein from Tn*5393* is explained by neutral substitutions at amino acid positions 156^{th} and 157^{th}. In ORF A', the nucleotides at these amino acid positions (156^{th} and 157^{th}) are T and A, respectively, instead of A and T, which are found in ORF A of Tn*5393* (Chiou and Jones, 1993). Owing to this, the difference in the amino acid sequences of the *strA* gene in RSF1010 and Tn*5393*, are leucine and histidine (amino acid positions 156-157) instead of glycine and leucine (nucleotide positions 5426 and 5429), respectively.

The second ORF, designated ORF B', encodes a polypeptide of 278 amino acids (Figure 41), corresponding to a molecular mass of 30,824 Dalton. Analysis of the amino acid sequences corresponds to the Sm phosphotransferase protein B. The initiation codon is AUG (ATG in DNA sequence; nucleotide positions 893-895). A

comparison of the amino acid sequence indicated that this ORF shares identical amino acid sequence encoded by ORF I of plasmid RSF1010 (Scholz *et al.*, 1989) and ORF B of Tn*5393* (Chiou and Jones, 1993). These results suggest that the nucleotide sequences of ORF A' and ORF B' are more similar to the nucleotide sequences of the *strA* and *strB* genes of RSF1010.

The third ORF, designated ORF C', encodes a polypeptide of 112 amino acids, transcribed in the reverse direction as the other ORFs (Figure 41). When the database was searched for a putative polypeptide encoded by ORF C', similarities of 100, 98, and 97% with part of the transposase of IS*26* (Mollet *et al.*, 1983), IS*176* of *S. typhimurium* plasmid NTP16 (Wrighton and Strike, 1987), and Tn*1525* (Trieu-Cuot and Courvalin, 1984), respectively, were obtained. Hartl *et al.* (1987) suggested that the transposase of IS*26* belongs to a superfamily of transposase that carries a D, D(35)E protein motif. This transposase has also been reported to involve in deletion of sequences flanking IS*26*-like elements reported in several plasmids, e.g., plasmids NTP16 (Wrighton and Strike, 1987), R46 (Hall, 1987), and N3 (Brown *et al.*, 1984).

The nucleotides obtained in this study indicate that the three genes found in the 2.5 kb Sm^R *Eco*RI fragment are probably co-transcribed. Although the promoter regions are not identified in the obtained nucleotide sequences, this assumption can be made due to the presence of the IS*26*-like element flanking these genes. In a report by Lee K-Y *et al.* (1990), they presented evidence that IS*26* elements are involved in the assembly of new antibiotic resistance operon. They also showed that this insertion element provides part of the promoter of the operon. IS*26* has been associated with several antibiotic resistance genes, including *aphA1* in Tn*2680* and other transposons (Mollet *et al.*, 1985) and a *blaT-aacC5* operon in the plasmid pUZ3644 (Martin *et al.*, 1987), and has been assumed from sequencing data to provide part of a hybrid promoter the antibiotic resistance genes (K-Y Lee, 1990). In addition, Prentki *et al.* (1986) suggested that IS*26* is a portable -35 promoter site. To support this, it is suggested that the whole 5.5 kb *Sal*I fragment from pST8 should be sequenced.

4.1.4 IS*26*-like segment

The presence of an IS*26*-like element downstream of the *str* genes suggests that the *str* genes could have transposed into the progenitor of pST8 as an intact element.

IS*26* has unique *Pst*I and *Sal*I sites at positions 159 and 252, respectively (Iida *et al.*, 1983; Mollet *et al.*, 1983). Nevertheless, it is not conclusively known if the IS*26*-like element discovered in the nucleotide sequences obtained from the 2.5 kb *str* genes is identical to IS*26* or its variants because the segment subcloned and sequenced is common to IS*26* and its variants.

It is also not known whether the *str* genes from pCLS55 are flanked by this element. Hence, the 5.5 kb *Sal*I fragment was subcloned into pUC19 and was partially sequenced. Analysis of these partial sequences flanking the *str* genes revealed these genes are flanked by direct repeats of elements identical to IS*26*. Figure 46 represents the gene cluster comprising the *str* genes found in the 5.5 kb *Sal*I fragment from pCLS55. The sequences corresponding to the ends of the *str* genes insertion were also determined.

The mechanism of integration of the *str* genes flanked by IS*26* elements in Tn*X8* could be explained as below. It can be postulated that insertion of the Sm^R genes into Tn*X8* proceeds by means of transposition mediated by the IS*26* elements as proposed by Hänni *et al.* (1982) for Tn*2672*. This integration has been found to involve preferential insertion into specific target regions (Mollet *et al.*, 1985). These distinct regions contain a sequence preferred by transposase, i.e., AT-rich flanked by GC pairs, and bear some resemblance to the termini of the element. This non-homologous recombination is catalyzed by an element-encoded transposase, and is distinguishable from homologous recombination mediated by the *rec* system of the host (Mollet *et al.*, 1985).

Elements of the IS*26*-type have been found as modules of several other transposons such as Tn*2680* (Mollet *et al.*, 1983) and Tn*1525* (Trieu-Cuot and Courvalin, 1984), mediating Km^R, and Tn*4352*, mediating neomycin resistance, and Tn*5091*, mediating Tp^R (Sundström *et al.*, 1995). IS*26* has been referred to as IS*6* (Mollet *et al.*, 1983), IS*15Δ* (Trieu-Cuot and Courvalin, 1984), as well as IS*46* and IS*160* (Galas and Chandler, 1989).

4.2 Conclusion

From the results obtained from this work, we can conclude that the 5.5 kb *Sal*I fragment from pST8 contained two *str* genes, *strA* and *strB*, which have 100% homology to the DNA and amino acid sequences of the *str* genes of RSF1010.

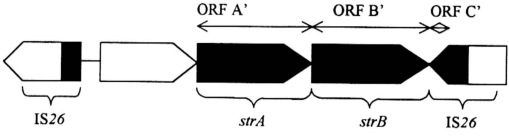

Figure 46: Proposed genetic organization of the 5.5 kb *SalI* fragment from pCLS55. The blackened regions in the arrow box indicate that the identities of the genes in the region have been determined by DNA sequencing. Note: *strA* and *strB*, genes encoding Sm^R.

Besides this, we have also identified that the 5.5 kb *Sal*I fragment is flanked by IS*26*-like elements. The presence of these elements has contributed to the presence of the *Sal*I sites at both of the 5.5 kb fragment. The insertion of the IS*26* elements in the *str* genes caused these genes to be mobilizable. The close association of ISs with antibiotic resistance genes has strongly suggested an active role for the sequences in the evolution or mobility of antibiotic resistance genes.

The rapid spread of antibiotic resistance traits among pathogenic bacteria seems to depend on the recombinational insertion of resistance genes into plasmids and transposons. With the data collected from this study, it can be postulated that Tn*5410* has acquired the *str* genes into its *mer* operon site by random integration. This transposition seemed to have caused the inactivation of the expression of Hg^R. Wong (1999) has reported that *S. typhi* S8 does not confer resistance to Hg.

Tn*5410* has evolved by further acquisition of Tn*5086* (Lye, 1997), the Tn*2*-like (Wong, 1999), and the *strA* and *strB* genes. There are two repeated copies of IS*26*-like element flanking the 3' and 5' ends of the *strA* and *strB* genes in an inverted orientation and one copy of the IS*26*-like element flanking the 3' end of the Tn*2*-like element in opposite orientation (Wong, 1999). Functional analysis of the IS*26*-like element mediated cointegrates shows that both insertion sequences are active and able to form cointegrates. Resolution of these cointegrates requires the presence of the host *rec* system. The presence of the composite IS*26*-like element within Tn*5410* supports the hypothesis that multidrug resistance transposons evolved by insertion of antibiotic determinants, which are transposable.

DNA similar to or homologous with the *strA* and *strB* genes of RSF1010 has been detected in many streptomycin-resistant Gram-negative bacteria isolated from animals, including humans (Rotger *et al.*, 1986). There are also findings that bacteria from plants have the streptomycin resistance genes found in bacteria from human and veterinary clinics extends to the importance of this resistance determinant.

The transposition of the Sm^R genes from plasmids such as RSF1010 to various mobilizable plasmids by insertion sequences, e.g., IS*26*-like elements, could have played an important role in the widespread distribution of the *str* genes among Gram-negative bacteria and generating multiple antibiotic resistance transposons.

Besides this, the presence of a number of IS elements in various bacteria suggests that the IS elements may be desirable agents in evolution, causing DNA

rearrangements that allow bacteria to more readily adapt to new environmental conditions, such as the presence of antimicrobial drugs.