CHAPTER THREE  RESULTS

3.1 Detection of transposons

3.1.1 Antibiogrammes of E. coli UB1637 transconjugants

As described in Section 2.22, after incubation on Dorset egg slants, E. coli UB5201 that harboured both pUB307 and a plasmid of S. typhi (pST2, pST6, pST8, or pST9) was conjugated with E. coli UB1637. The number and antibiogrammes of E. coli UB1637 transconjugants obtained and the calculated transposition frequency for each selective antibiotic are given in Tables 6a and b. Transpositions of Ac\(^f\), Cm\(^f\), and Ctm\(^f\) markers from each S. typhi plasmid to pUB307 were observed. For each S. typhi plasmid, the transposition frequencies of Ac\(^f\) and Ctm\(^f\) were higher than the transposition frequency of Cm, particularly for pST8 and pST9.

Two major types of E. coli UB1637 transconjugants (Ac\(^f\)Cm\(^f\)Ctm\(^f\)Km\(^f\)Sm\(^f\)Tc\(^f\) and Ac\(^f\)Cm\(^f\)Ctm\(^f\)Km\(^f\)Sm\(^f\)Tc\(^f\)) were obtained between the conjugation of E. coli UB1637 and E. coli UB5201 harbouring the recipient replicon pUB307 and a plasmid of S. typhi (pST2, pST6, pST8, or pST9) when transconjugants were selected on supplemented M9 agar (Section 2.4.3) containing Km, Sm, and Ac, Cm, or Ctm. When transconjugants were selected for Ac\(^f\) or Ctm\(^f\), almost all of them were both Ac\(^f\) and Ctm\(^f\), but only few were Cm\(^f\). When transconjugants were selected for Cm\(^f\), almost all transconjugants tested were also Ac\(^f\) and Ctm\(^f\).

From Tables 6a and b, it can be observed that the difference between the transposition frequency of Cm\(^f\) and Ac\(^f\) or Ctm\(^f\) was greater in UB5201 (pUB307 and pST8 or pST9) than in UB5201 (pUB307 and pST2 or pST6). This difference might be due to the storage of UB5201 (pUB307, pST8) and UB5201 (pUB307, pST9) on
Table 6a: Characteristics of antibiotic resistance transfer from *E. coli* UB5201(pUB307, pST2) and UB5201(pUB307, pST6) to *E. coli* UB1637 at 37°C, after transposition

<table>
<thead>
<tr>
<th>Donor</th>
<th>Phenotype</th>
<th>Selective donor antibiotics</th>
<th>Transposition frequency</th>
<th>No. of transconjugants obtained and examined</th>
<th>No. and resistance phenotype of transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB5201 (pUB307, pST2)</td>
<td>Lac⁺ Nxr Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁻ Te⁻</td>
<td>Km + Sm + Ac</td>
<td>7.60 x 10⁻⁴</td>
<td>42</td>
<td>Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁻ Te⁻</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Sm + Ctm</td>
<td>6.30 x 10⁻⁴</td>
<td>28</td>
<td>Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁻ Te⁻</td>
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<tr>
<td></td>
<td></td>
<td>Km + Sm + Cm</td>
<td>4.17 x 10⁻⁵</td>
<td>44</td>
<td>Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁻ Te⁻</td>
</tr>
<tr>
<td>UB5201 (pUB307, pST6)</td>
<td>Lac⁺ Nxr Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁻ Te⁻</td>
<td>Km + Sm + Ac</td>
<td>3.33 x 10⁻⁵</td>
<td>10</td>
<td>Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁻ Te⁻</td>
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<td></td>
<td></td>
<td>Km + Sm + Ctm</td>
<td>2.78 x 10⁻⁵</td>
<td>12</td>
<td>Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁻ Te⁻</td>
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<td></td>
<td>Km + Sm + Cm</td>
<td>1.98 x 10⁻⁵</td>
<td>30</td>
<td>Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁻ Te⁻</td>
</tr>
</tbody>
</table>
Table 6b: Characteristics of antibiotic resistance transfer from *E. coli* UB5201 (pUB307, pST8) and UB5201 (pUB307, pST9) to *E. coli* UB1637 at 37°C, after transposition

<table>
<thead>
<tr>
<th>Donor</th>
<th>Phenotype</th>
<th>Selective donor antibiotics</th>
<th>Transposition frequency</th>
<th>No. of transconjugants examined</th>
<th>No. and resistance phenotype of transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>UB5201 (pUB307, pST8)</em></td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt; Nx&lt;sup&gt;+&lt;/sup&gt; Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Km + Sm + Ac</td>
<td>8.23 x 10⁻³</td>
<td>99</td>
<td>93 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; 6 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>Km + Sm + Ctm</td>
<td>7.13 x 10⁻³</td>
<td>99</td>
<td>95 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; 4 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Sm + Cm</td>
<td>2.03 x 10⁻⁷</td>
<td><strong>40</strong></td>
<td>38 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; 2 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>UB5201 (pUB307, pST9)</em></td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt; Nx&lt;sup&gt;+&lt;/sup&gt; Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Km + Sm + Ac</td>
<td>4.85 x 10⁻⁵</td>
<td>96</td>
<td>93 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; 3 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Sm + Ctm</td>
<td>4.58 x 10⁻⁵</td>
<td>99</td>
<td>99 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Sm + Cm</td>
<td>5.02 x 10⁻⁷</td>
<td><strong>65</strong></td>
<td>60 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; 4 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; 1 Ac&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note:
* Prepared by Kadambeswaran (1993) and kept on Dorset egg slants for more than 2 years at room temperature

** Total no. of transconjugants obtained and examined
Dorset egg slants for more than 2 years, whereas UB5201 (pUB307, pST2) and UB5201 (pUB307, pST6) were kept on similar slants for 2 weeks.

Another interesting observation was that when the transposition frequency of Cm\(^\text{r}\) was about the same as that of Ac\(^\text{r}\) or Ctm\(^\text{r}\), e.g., in UB5201 (pUB307, pST6), Cm\(^\text{r}\) transconjugants were also obtained, albeit at lower frequencies, e.g., 1/10 and 2/12, when selection was made on Ac\(^\text{r}\) or Ctm\(^\text{r}\).

The results indicated that the Ac\(^\text{r}\) and Ctm\(^\text{r}\) genes in the plasmids of *S. typhi* isolates were located on the same transposon and were co-transposed to pUB307 in *E. coli* UB1637, a recA host. Because *E. coli* UB1637 is resistant to Sm and the plasmid pUB307 is Km\(^\text{r}\)Tc\(^\text{r}\), the transpositions of Sm\(^\text{r}\) and Tc\(^\text{r}\) genes from pST2, pST6, pST8, or pST9 to pUB307 in these transconjugants could not be determined. However, the presence of Lac\(^\text{+}\)Ac\(^\text{r}\)Cm\(^\text{r}\)Ctm\(^\text{r}\)Km\(^\text{r}\)Sm\(^\text{r}\)Tc\(^\text{s}\) transconjugants indicated that the Tc\(^\text{r}\) determinant on pUB307 was inactivated by the insertion of the putative transposon. Therefore, it also indicated that the putative transposon did not carry the Tc\(^\text{r}\) determinant present in the R plasmids of *S. typhi*.

The results of the transposition experiments indicated that the Ac\(^\text{r}\) Ctm\(^\text{r}\) determinants and Cm\(^\text{r}\) determinant were located on different transposons. The Cm\(^\text{r}\) transposon may carry other resistance genes, particularly the Tc\(^\text{r}\) determinant, because all transconjugants selected for Cm\(^\text{r}\) were also Tc\(^\text{r}\), while some Tc\(^\text{s}\) were obtained when the transconjugants were selected for Ac\(^\text{r}\) or Ctm\(^\text{r}\).

### 3.1.2 Plasmid profiles of *E. coli* UB1637 transconjugants

Agarose gel electrophoresis of plasmids extracted from multiresistant *E. coli* UB1637 transconjugants obtained in Section 3.1.1 showed that most of these
transconjugants harboured a ccc plasmid that had a reduced electrophoretic mobility compared to that of pUB307 (Fig. 22a and b). The reduced electrophoretic mobility indicated an increase in the size of pUB307 in these transconjugants. This increase was most likely caused by the transposition of a transposon from each of \textit{S. typhi} plasmids to pUB307. However, some Cm$^r$ transconjugants derived from pST8 and pST9 did not harbour any visible plasmid even though they were resistant to multiple antibiotics, including Km and Tc (Fig. 22b). The R plasmid in these transconjugants might have integrated in the chromosome of the host \textit{E. coli} UB1637.

When the plasmids extracted from these \textit{E. coli} UB1637 transconjugants were digested with \textit{PstI} (Fig. 23a and b), extra bands were visible, in addition to bands corresponding to those of pUB307. Recombinant pUB307 derived from different \textit{S. typhi} donor plasmids all gave a similar restriction pattern. This indicated that these transposons derived from different \textit{S. typhi} donors are closely related and thus, the \textit{S. typhi} donors themselves may have a common origin even though they were isolated from patients of two different countries. These results showed that each recombinant extracted from \textit{E. coli} UB1637 transconjugants had acquired additional DNA, the result of transposition.

3.1.3 Antibiogrammes of transformants

Plasmids extracted from different \textit{E. coli} UB1637 transconjugants were used to transform \textit{E. coli} DH5$\alpha$. The number and antibiogrammes of transformants obtained are shown in Tables 7a and b.

When competent \textit{E. coli} DH5$\alpha$ cells were transformed with plasmid extracted from each Cm$^r$ transconjugant, the transformants generated had the same antibiogramme
Figure 22a: Ethidium bromide-stained agarose gel (0.5%, w/v) of pUB307 and recombinant pUB307 from UB1637 transconjugants

Lane 1: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 2: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 3: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 4: pUB307 (Km'Tc')
Lane 5: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 6: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 7: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 8: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 9: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant

Note:

Lanes 1-3: Derivatives of the mating between UB5201 (pUB307, pST2) and UB1637
Lanes 5-9: Derivatives of the mating between UB5201 (pUB307, pST6) and UB1637
Figure 22b: Ethidium bromide-stained agarose gel (0.5%, w/v) of pUB307 and recombinant pUB307 from UB1637 transconjugants

Lane 1: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 2: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 3: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 4: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 5: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 6: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 7: pUB307 (Km'^Tc'^)
Lane 8: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 9: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 10: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 11: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 12: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant
Lane 13: Recombinant pUB307 from Ac'^Cm'^Ctm'^Km'^Sm'^Tc'^ transconjugant

Note:
Lanes 1-6: Derivatives of the mating between UB5201 (pUB307, pST8) and UB1637
Lanes 8-13: Derivatives of the mating between UB5201 (pUB307, pST9) and UB1637
Figure 23a: Ethidium bromide-stained agarose gel (0.5%, w/v) of pUB307 and recombinant pUB307 after PstI digestion

Lanes 1 and 11: 1 kb ladder size markers
Lane 2: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 3: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 4: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 5: pUB307 (Km'Tc')
Lane 6: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 7: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 8: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 9: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant
Lane 10: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugant

Note:
Lanes 2-4: Derivatives of the mating between UB5201 (pUB307, pST2) and UB1637
Lanes 6-10: Derivatives of the mating between UB5201 (pUB307, pST6) and UB1637
Figure 23b: Ethidium bromide-stained agarose gel (0.5%, w/v) of pUB307 and recombinant pUB307 after PstI digestion

Lanes 1 and 11: 1 kb ladder size markers

Lane 2: Recombinant pUB307 from Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant
Lane 3: Recombinant pUB307 from Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant
Lane 4: Recombinant pUB307 from Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant
Lane 5: Recombinant pUB307 from Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant
Lane 6: Recombinant pUB307 from Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant
Lane 7: pUB307 (Km\textsuperscript{r}Tc\textsuperscript{r})
Lane 8: Recombinant pUB307 from Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant
Lane 9: Recombinant pUB307 from Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant
Lane 10: Recombinant pUB307 from Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant

Note:

Lanes 2-7: Derivatives of the mating between UB5201 (pUB307, pST8) and UB1637
Lanes 8-10: Derivatives of the mating between UB5201 (pUB307, pST9) and UB1637
Table 7a: Antibiotic resistance phenotypes of *E. coli* DH5α after transformation with plasmids extracted from different *E. coli* UB1637 transconjugants selected on different antibiotic plates after mating with UB5201 (pUB307 and pST2 or pST6)

<table>
<thead>
<tr>
<th>Transposon source</th>
<th>Selective donor antibiotics</th>
<th>Resistant phenotype of transconjugants</th>
<th>No. of TJ</th>
<th>Presence of plasmid</th>
<th>Selective antibiotic and no. of transformants obtained and examined</th>
<th>No. and resistance phenotype of transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>pST2</td>
<td>Km + Sm + Ac</td>
<td>Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1</td>
<td>+</td>
<td>Ac</td>
<td>5 Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
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<td></td>
<td>Km + Sm + Ctm</td>
<td>Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1</td>
<td>+</td>
<td>Ctm</td>
<td>2 Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
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<td></td>
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<td>Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>+</td>
<td>Cm</td>
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<td>1</td>
<td>+</td>
<td>Ac</td>
<td>2 Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
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<td></td>
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<td>Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1</td>
<td>+</td>
<td>Ac</td>
<td>1 Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Km + Sm + Ctm</td>
<td>Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1</td>
<td>+</td>
<td>Ctm</td>
<td>3 Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1</td>
<td>+</td>
<td>Ctm</td>
<td>2 Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Km + Sm + Cm</td>
<td>Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1</td>
<td>+</td>
<td>Cm</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*Ac</td>
<td>57 Ac&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;d&lt;/sup&gt; Ctm&lt;sup&gt;e&lt;/sup&gt; Km&lt;sup&gt;f&lt;/sup&gt; Sm&lt;sup&gt;g&lt;/sup&gt; Te&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note:
+ : presence
- : absence

TJ: Transconjugants examined for plasmid

* Transformation was repeated with Ac as selective antibiotic but the transformants obtained were not analysed for presence of plasmid
Table 7b: Antibiotic resistance phenotypes of *E. coli* DH5α obtained after transformation with plasmids extracted from different *E. coli* UB1637 transconjugants selected on different antibiotic plates after mating with UB5201 (pUB307 and pST8 or pST9)

<table>
<thead>
<tr>
<th>Transposon source</th>
<th>Selective donor antibiotics</th>
<th>Resistant phenotype of transconjugants</th>
<th>No. of TJ</th>
<th>Presence of plasmid</th>
<th>Selective antibiotic and no. of transformants obtained and examined</th>
<th>No. and resistance phenotype of transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>pST8</td>
<td>Km + Sm + Ac</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>+</td>
<td>Ac</td>
<td>7 Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>+</td>
<td>Ac</td>
<td>2 Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td>Km + Sm + Ctm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>+</td>
<td>Ctm</td>
<td>5 Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>+</td>
<td>Ctm</td>
<td>1 Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td>Km + Sm + Cm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>+</td>
<td>Cm *Ac</td>
<td>87 Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>2</td>
<td>-</td>
<td>Cm *Ctm</td>
<td>0</td>
</tr>
<tr>
<td>pST9</td>
<td>Km + Sm + Ac</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>+</td>
<td>Ac</td>
<td>7 Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>+</td>
<td>Ac</td>
<td>20 Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td>Km + Sm + Ctm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>+</td>
<td>Ctm</td>
<td>4 Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>2</td>
<td>-</td>
<td>Cm *Ac</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>2</td>
<td>-</td>
<td>Cm *Ac</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1</td>
<td>-</td>
<td>Cm *Ac</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: + : presence  
- : absence  
TJ: Transconjugants examined for plasmid  
* Transformation was repeated with Ac or Ctm as selective antibiotic but the transformants obtained were not analysed for presence of plasmid
as their plasmid donor. However, all transformants derived from Cm\(^{r}\) transconjugants that harboured a visible plasmid band in Section 3.1.2 had the same antiibiogramme as their plasmid donor except that they were all Cm\(^{s}\) (Tables 7a and b), indicating that the Cm\(^{r}\) gene was not on the recombinant pUB307. Most likely, the Cm\(^{r}\) transposon had transposed from the recombinant pUB307 to the bacterial chromosome. The absence of plasmid bands in Figure 22b (lanes 6, 11, 12, and 13) in Cm\(^{r}\) transconjugants and absence of Cm\(^{r}\) transformants from Cm\(^{r}\) transconjugants (Tables 7b), again indicating that the Cm\(^{r}\) gene, together with other antibiotic resistance genes, had integrated or transposed into the bacterial chromosome.

All E. coli DH5\(\alpha\) transformants were Sm\(^{r}\), indicating that the transposon also harboured the Sm\(^{r}\) determinant.

### 3.1.4 Plasmid profiles of transformants

Figures 24a and b show the plasmid profiles of multiresistant E. coli DH5\(\alpha\) transformants that were obtained (Section 2.22.5). Each transformant harboured a ccc plasmid of similar electrophoretic mobility as that of the ccc plasmid extracted from its respective donor. All donor and transformant plasmids migrated slower than pUB307. These results confirmed that the additional antibiotic resistance traits acquired by recombinant pUB307 were transposon-mediated.

### 3.2 Isolation of chloramphenicol resistance transposon

#### 3.2.1 Curing of recombinant pUB307 carrying the Ac\(^{r}\)Ctm\(^{r}\)Sm\(^{r}\) transposon in Ac\(^{r}\)Cm\(^{r}\)Ctm\(^{r}\)Km\(^{r}\)Sm\(^{r}\)Tc\(^{r}\) UB1637

The subinhibitory concentration of EtBr for Ac\(^{r}\)Cm\(^{r}\)Ctm\(^{r}\)Km\(^{r}\)Sm\(^{r}\)Tc\(^{r}\) UB1637 at 42°C was 50 \(\mu\)g/ml (Section 2.23.1). After two cycles of treatment with EtBr
Figure 24a: (Refer to opposite page)
Lane 6, 8, 10, and 12: Derivatives of the plasmid between pUB201 (pUB307, PSt6) and pUB1637

Lanes 1 and 3: Derivatives of the plasmid between pUB201 and pUB307 (pUB307, PSt2) and Note

Plasmids shown in lane 12
Lane 12: Recombinant pUB307 from Ac,Cm,Cm,Km,Rm,Tm, Tn,St derivative plasmid

Plasmid shown in lane 10
Lane 10: Recombinant pUB307 from Ac,Cm,Cm,Km,Rm,Tm, Tn,St derivative plasmid

Plasmid shown in lane 8
Lane 8: Recombinant pUB307 from Ac,Cm,Cm,Km,Rm,Tm, Tn,St derivative plasmid

Plasmid shown in lane 6
Lane 6: Recombinant pUB307 from Ac,Cm,Cm,Km,Rm,Tm, Tn,St derivative plasmid

Plasmid shown in lane 5
Lane 5: pUB307 (Km,Tc)

Plasmid shown in lane 3
Lane 3: Recombinant pUB307 from Ac,Cm,Cm,Km,Rm,Tm, Tn,St derivative plasmid

Plasmid shown in lane 1
Lane 1: Recombinant pUB307 from Ac,Cm,Cm,Km,Rm,Tm, Tn,St derivative plasmid

Transformants, recombinants and their derivatives

Figure 24: Ethidium bromide-stained agarose gel (0.7%, w/v) of pUB307 and
UB1637

Lanes 10, 12, and 14: Derivatives of the main between UB5201 (pUB307, pST6) and

UB1637

Lanes 1, 3, 5, and 7: Derivatives of the main between UB5201 (pUB307, pST6) and

Note

shown in lane 14

Lane 15: Plasmid from acrCm, km rSm, tC, transconjugant derived in plasmid
Lane 14: Recombinant pUB307 from acrCm, km rSm, tC, transconjugant

shown in lane 12

Lane 13: Plasmid from acrCm, km rSm, tC, transconjugant derived in plasmid
Lane 12: Recombinant pUB307 from acrCm, km rSm, tC, transconjugant

shown in lane 10

Lane 11: Plasmid from acrCm, km rSm, tC, transconjugant derived in plasmid
Lane 10: Recombinant pUB307 from acrCm, km rSm, tC, transconjugant

pUB307 (Km rT6)

shown in lane 7

Lane 8: Plasmid from acrCm, km rSm, tC, transconjugant derived in plasmid
Lane 7: Recombinant pUB307 from acrCm, km rSm, tC, transconjugant

shown in lane 5

Lane 6: Plasmid from acrCm, km rSm, tC, transconjugant derived in plasmid
Lane 5: Recombinant pUB307 from acrCm, km rSm, tC, transconjugant

shown in lane 3

Lane 4: Plasmid from acrCm, km rSm, tC, transconjugant derived in plasmid
Lane 3: Recombinant pUB307 from acrCm, km rSm, tC, transconjugant

shown in lane 1

Lane 2: Plasmid from acrCm, km rSm, tC, transconjugant derived in plasmid
Lane 1: Recombinant pUB307 from acrCm, km rSm, tC, transconjugant

Transformants

Recombinant pUB307 from transconjugants and their respective

Figure 24b: Elhidiin bromide-stained agarose gel (0.5%) w/v of pUB307 and
Figure 24b: (Refer to opposite page)
(50 µg/ml) and 42°C, three Km^{5} derivatives were obtained from Ac'Cm'I Ctm'I Km^{5} Sm'I Tc'I UB1637 derived from UB5201(pUB307, pST2) and UB5201(pUB307, pST6), and all of them were Ac'Cm'I Ctm'I Km^{5} Sm'I Tc'I (Table 8).

These Km^{5} cured derivatives did not harbour any plasmid as analysed by agarose gel electrophoresis (Fig. 25). When competent *E. coli* DH5α was transformed with the plasmid extracts of these Km^{5} cured derivatives, no Ac'I or Km'I transformants were obtained. These confirmed that the recombinant pUB307 carrying the Ac'Cm'I Sm'I transposon had been removed from these Km^{5} cured derivatives, and the chromosome of the Ac'Cm'I Ctm'I Km^{5} Sm'I Tc'I *E. coli* UB1637 contained at least a copy of Cm'I transposon with or without other antibiotic resistance genes. The chromosome may also contain another copy of Ac'Cm'I Sm'I transposon.

### 3.2.2 Secondary transposition

When a new copy of pUB307 was conjugally transferred into each of these Ac'Cm'I Ctm'I Km^{5} Sm'I Tc'I cured derivatives (Section 2.23.2), many (>200) Km'I transconjugants were obtained. Plasmids extracted from these Km'I transconjugants (Fig. 25, lanes 9-11) appeared to have a similar size as pUB307 (lane 5).

One Ac'Cm'I Ctm'I Km^{5} Sm'I Tc'I transconjugant from each Km^{5} strain derived from UB5201(pUB307, pST2) and UB5201(pUB307, pST6) was incubated on Dorset egg slant for 2 weeks to allow the putative Cm'I transposon on the chromosome of the Ac'Cm'I Ctm'I Km^{5} Sm'I Tc'I cured *E. coli* UB1637 to transpose onto the newly acquired pUB307. After mating with UB5201, the number and antibiogrammes of the transconjugants obtained and the calculated transposition frequency for each selective antibiotic are shown in Table 8.
Figure 25: Ethidium bromide-stained agarose gel (0.5%, w/v) of plasmids from Ac'Cm'Ctm'Km'Sm'Tc' UB1637 before and after plasmid curing and after receiving a new copy of pUB307 by conjugation

Lanes 1-4: Recombinant pUB307 from Ac'Cm'Ctm'Km'Sm'Tc' transconjugants
Lane 5: pUB307 (Km'Tc')
Lanes 6-8: Absence of plasmid in Km' derivatives of UB1637 cultures mentioned in lanes 1, 2, and 3, respectively, after plasmid curing
Lanes 9-11: Plasmids from Km' derivatives of cultures mentioned in lanes 6, 7, and 8, respectively, after receiving a new copy of pUB307 by conjugation

Note:
Lane 1: Transconjugant from the mating between UB5201 (pUB307, pST2) and UB1637 in the first transposition experiment
Lanes 2 and 3: Transconjugant from the mating between UB5201 (pUB307, pST6) and UB1637 in the first transposition experiment
Lane 4: Transconjugant from the mating between UB5201 (pUB307, pST8) and UB1637 in the first transposition experiment
Table 8: Characteristics of antibiotic resistance transfer after secondary transposition from cured transconjugants that received a new copy of plasmid pUB307 from *E. coli* UB5201

<table>
<thead>
<tr>
<th>Transposon source</th>
<th>UB1637 with recombinant pUB307 (before plasmid curing)</th>
<th>Phenotype (before plasmid curing)</th>
<th>No. of cured derivative and phenotype (after plasmid curing)</th>
<th>Selective donor antibiotics</th>
<th>First transposition frequency</th>
<th>Secondary transposition frequency</th>
<th>No. of trans-conjugants examined</th>
<th>No. and resistance phenotype of transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>pST2</td>
<td>UB1637 (pUB307::Tn of S2)</td>
<td>Lac⁺ Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁺ Tc⁺</td>
<td>2 Lac⁺ Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁺ Tc⁺</td>
<td>Km + Ac</td>
<td>7.60 x 10⁻⁴</td>
<td>7.08 x 10⁻⁴</td>
<td>100</td>
<td>74 Ac⁺ Cm⁺ Ctm⁺ Km⁻ Sm⁺ Tc⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25 Ac⁺ Cm⁺ Ctm⁺ Km⁻ Sm⁺ Tc⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 Ac⁺ Cm⁺ Ctm⁺ Km⁻ Sm⁺ Tc⁺</td>
</tr>
<tr>
<td>pST6</td>
<td>UB1637 (pUB307::Tn of S6)</td>
<td>Lac⁺ Ac⁺ Cm⁺ Ctm⁻ Km⁻ Sm⁺ Tc⁺</td>
<td>1 Lac⁺ Ac⁺ Cm⁺ Ctm⁻ Km⁺ Sm⁺ Tc⁺</td>
<td>Km + Ac</td>
<td>3.33 x 10⁻⁵</td>
<td>7.87 x 10⁻⁶</td>
<td>100</td>
<td>100 Ac⁺ Cm⁺ Ctm⁺ Km⁻ Sm⁺ Tc⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100 Ac⁺ Cm⁺ Ctm⁺ Km⁻ Sm⁺ Tc⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Km + Ctm</td>
<td>2.78 x 10⁻⁵</td>
<td>5.74 x 10⁻⁶</td>
<td>100</td>
<td>84 Ac⁺ Cm⁺ Ctm⁺ Km⁻ Sm⁺ Tc⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14 Ac⁺ Cm⁺ Ctm⁺ Km⁻ Sm⁺ Tc⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Km + Cm</td>
<td>1.98 x 10⁻⁵</td>
<td>4.47 x 10⁻⁶</td>
<td>100</td>
<td>100 Ac⁺ Cm⁺ Ctm⁺ Km⁻ Sm⁺ Tc⁺</td>
</tr>
</tbody>
</table>
In the secondary transposition experiment, the transposition frequencies for Ac' and Ctm' from the pST2 derivative were similar to those of the first experiment and were about 10²-fold higher than the transposition frequency for Cm' (Table 8). Consequently, Cm⁵ transconjugants were most abundant when the transconjugants were selected for Ac' or Ctm'.

The results also showed that a copy of Ac'Ctm'Sm' transposon was present in the chromosome of Ac'Cm'Ctm'Km'Sm'Tc' cured E. coli UB1637 and this transposon could transpose to pUB307 independent of the Cm' transposon, which was present in the bacterial chromosome. Therefore, the Cm' transposon could not be isolated.

The transposition frequencies for Ac', Cm', and Ctm' of the pST6 derivative were 10-fold lower than those of the first transposition experiment (Table 8). The difference between the transposition frequencies for Ac', Cm', and Ctm' was relatively small and thus, the transconjugants obtained were mostly Cm', even when they were selected for Ac' or Ctm'. However, in the first transposition experiment, most transconjugants were Cm⁵ when they were selected for Ac' or Ctm'. The discrepancy could be due to the low number of transconjugants obtained in the first transposition experiment in which the mating mixture was plated on only one selective plate for each 10-fold serial dilutions of the mating mixture. In subsequent experiments, for each selective antibiotic, two or three plates were used for each dilution and a wider range of the 10-fold serial dilutions were used. These measures increased the number of transconjugant colonies. The average numbers of the colonies obtained from the plates were used in the calculation of transposition frequencies.

A second attempt was made to isolate the Cm' transposon from the Km⁶ E. coli
UB1637 cured derivatives. These Km\textsuperscript{r} derivatives, which now had acquired a new copy of pUB307 and had been kept on Dorset egg slants for about two months, were separately conjugated with plasmidless \textit{E. coli} UB5201. The transconjugants were selected for Km\textsuperscript{r} and Ac\textsuperscript{r}, Cm\textsuperscript{r}, or Ctm\textsuperscript{r}, as in the first set of secondary transposition experiment.

Two major antibiotic resistant transconjugants were obtained: Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} and Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} (Table 9). The resistance phenotypes were similar to those obtained in the previous transposition experiments. However, in general, the transposition frequencies for Ac\textsuperscript{r}, Ctm\textsuperscript{r}, and Cm\textsuperscript{r} had decreased, some by $10^3$ to $10^4$-fold compared to the corresponding frequencies determined in the first transposition experiment (Table 9). These results were similar to those of pST8 and pST9 derivatives in the first experiment (Table 6b) in which the bacterial cultures had been kept on Dorset egg slants for more than two years. Therefore, incubation of more than two weeks on Dorset egg slant might have caused a drastic decrease in the transposition frequency for Cm\textsuperscript{r}.

An Ac\textsuperscript{r}Cm\textsuperscript{r}Ctm\textsuperscript{r}Km\textsuperscript{r}Sm\textsuperscript{r}Tc\textsuperscript{r} transconjugant harbouring a recombinant pUB307, a derivative of transposon from pST6, was obtained (Table 9). This suggested that the Cm\textsuperscript{r} transposon did not carry Ac\textsuperscript{r}, Ctm\textsuperscript{r}, and Sm\textsuperscript{r} determinants but may or may not carry the Tc\textsuperscript{r} determinant. The presence of Tc\textsuperscript{r} determinant on the Cm\textsuperscript{r} transposon could not be verified because pUB307 also encoded Tc\textsuperscript{r}. Nevertheless, the Cm\textsuperscript{r} transposon probably also carried Tc\textsuperscript{r} because all Cm\textsuperscript{r} transconjugants obtained in all of the transposition experiments carried out so far were also Tc\textsuperscript{r}.
Table 9: Characteristics of antibiotic resistance transfer after the second set of secondary transposition from cured transconjugants that had received a new copy of plasmid pUB307 from E. coli UB5201

<table>
<thead>
<tr>
<th>Transposon source</th>
<th>UB1637 with recombinant pUB307 (after plasmid curing)</th>
<th>Selective donor antibiotics</th>
<th>First transposition frequency</th>
<th>Secondary transposition frequency</th>
<th>Secondary transposition frequency (second set)</th>
<th>No. of trans-conjugants examined</th>
<th>No. and resistance phenotype of transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>pST2</td>
<td>UB1637 (pUB307::Tn of S2)</td>
<td>Km + Ac</td>
<td>7.60 x 10^{-4}</td>
<td>7.08 x 10^{-4}</td>
<td>1.26 x 10^{-7}</td>
<td>100</td>
<td>100 Ac' Cm Ctm' Km' Sm' Tc'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ctm</td>
<td>6.30 x 10^{-4}</td>
<td>6.94 x 10^{-4}</td>
<td>8.00 x 10^{-8}</td>
<td>100</td>
<td>100 Ac' Cm Ctm' Km' Sm' Tc'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Cm</td>
<td>4.17 x 10^{-5}</td>
<td>6.94 x 10^{-6}</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>pST6</td>
<td>UB1637 (pUB307::Tn of S6)</td>
<td>Km + Ac</td>
<td>3.33 x 10^{-5}</td>
<td>7.87 x 10^{-6}</td>
<td>3.92 x 10^{-6}</td>
<td>99</td>
<td>98 Ac' Cm Ctm' Km' Sm' Tc' 1 Ac' Cm Ctm' Km' Sm' Tc'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ctm</td>
<td>2.78 x 10^{-5}</td>
<td>5.74 x 10^{-6}</td>
<td>4.48 x 10^{-6}</td>
<td>99</td>
<td>96 Ac' Cm Ctm' Km' Sm' Tc' 2 Ac' Cm Ctm' Km' Sm' Tc' 1 Ac' Cm Ctm' Km' Sm' Tc'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Cm</td>
<td>1.98 x 10^{-5}</td>
<td>4.47 x 10^{-6}</td>
<td>7.30 x 10^{-8}</td>
<td>100</td>
<td>96 Ac' Cm Ctm' Km' Sm' Tc' 1 Ac' Cm Ctm' Km' Sm' Tc' 2 Ac' Cm Ctm' Km' Sm' Tc' 1 Ac' Cm Ctm' Km' Sm' Tc'</td>
</tr>
</tbody>
</table>
3.3 Plasmid profiles of transconjugants from secondary transposition and transformation of *E. coli* DH5α

Plasmids extracted from the transconjugants obtained after the second set of secondary transposition were analysed by agarose gel electrophoresis (Fig. 26). Except for lane 10 (Fig. 26) that shows two plasmid bands, i.e., one plasmid migrated slower than pUB307 (lane 9) and the other at about the same motility as pUB307, plasmids in other lanes migrated slower than pUB307 (lane 9). Differences in mobility were also observed among plasmids extracted from transconjugants of different phenotypes. In general, transconjugants with more resistance phenotype, particularly Cm\(^\prime\) (e.g., lane 5), showed slower mobility.

When competent cells were transformed with plasmid extracted from each Cm\(^\prime\) transconjugant, the transformants generated had the same antibiogramme as their plasmid donor. Most transformants derived from Cm\(^\prime\) transconjugants were Cm\(^\prime\). Ac\(^\prime\)Cm\(^\prime\)Ctm\(^\prime\)Km\(^\prime\)Sm\(^\prime\)Tc\(^\prime\) transformants were obtained when *E. coli* DH5α cells were transformed with recombinant pUB307 (a derivative of transposon from pST6) extracted from an Ac\(^\prime\)Cm\(^\prime\)Ctm\(^\prime\)Km\(^\prime\)Sm\(^\prime\)Tc\(^\prime\) transconjugant, indicating that resistance traits carried by both the Cm\(^\prime\) and Ac\(^\prime\)Ctm\(^\prime\)Sm\(^\prime\) transposons were not present in these transformants (Table 10). These indicated that the Cm\(^\prime\) transposon had transposed into the bacterial chromosome again and carrying the Ac\(^\prime\)Ctm\(^\prime\)Sm\(^\prime\) transposon with it, thereby resulting in the loss of all transposon-mediated antibiotic resistances in the recombinant pUB307.
Resistase Phenoype

Lanes 11 and 12: Plasmids extracted from two different transconjugants of the same

Resistase Phenoype

Lanes 6 and 7: Plasmids extracted from two different transconjugants of the same

Second set of secondary transposition experiment

First transposition experiment, and made with UB5201 after the

used strain derived from UB5201 (PUB307, Psl6), after the

transposition experiment

made with UB5201 after the second set of secondary
derived from UB5201 (PUB307, Psl2), after the first transposition

Lanes 1-2: Deviations of the mean between PUB307 and PUB1637 control strain

Note:

Lane 14: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 13: Recombinant PUB307 from ACCh, Km, Km, Sm, Tc, transconjugant
Lane 12: Recombinant PUB307 from ACCh, Km, Km, Sm, Tc, transconjugant
Lane 11: Recombinant PUB307 from ACCh, Km, Km, Sm, Tc, transconjugant
Lane 10: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 9: PUB307 (Km, Tc)
Lane 8: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 7: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 6: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 5: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 4: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 3: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 2: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant
Lane 1: Recombinant PUB307 from ACCh, Km, Sm, Tc, transconjugant

Transconjugants obtained after the second set of secondary transposition

Figure 26: Ethidium bromide-stained agarose gel (0.5% W/V) of plasmids from
Figure 26: (Refer to opposite page)
Table 10: Antibiotic resistance phenotypes of *E. coli* DH5α after transformation with plasmids extracted from different *E. coli* UB5201 transconjugants selected on different antibiotic plates after mating with cured derivatives of UB1637 carrying pUB307, after second set of secondary transposition

<table>
<thead>
<tr>
<th>Transposon source</th>
<th>Phenotype of cured derivative with pUB307</th>
<th>Selective donor antibiotics</th>
<th>Resistance phenotype of transconjugants</th>
<th>No. of transconjugants examined for plasmid</th>
<th>Selective antibiotic</th>
<th>No. of transformants obtained &amp; examined</th>
<th>No. and resistance phenotype of transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>pST2</td>
<td>Lac' Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>Km + Ac</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>25</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ctm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>21</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Cm</td>
<td></td>
<td>0</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pST6</td>
<td>Lac' Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>Km + Ac</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>62</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ctm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>182</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Cm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>19</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ac</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>14</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ctm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>36</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Cm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>13</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ac</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>14</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ctm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>50</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Cm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>37</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ac</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>50</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Km + Ctm</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
<td>1 +</td>
<td>Km</td>
<td>41</td>
<td>Ac' Cm' Ctm' Km' Sm' Te'</td>
</tr>
</tbody>
</table>

Note: The transformants were selected for Km', to detect the presence of Cm' transformant.

+ : presence  - : absence
3.4 Cloning of antibiotic resistance genes

3.4.1 Restriction endonuclease digestion of pCL8 and pCL8R

Plasmid pCL8 (Section 2.24.1) was digested separately with BamHI, EcoRI, HindIII, SalI, and SmaI, which were known to cleave pUB307. The restriction patterns were compared with those of pUB307 (Fig. 27).

The results of SmaI digestion of pCL8 (Fig. 27b, lane 8) showed that the Ac'Ctm'Sm' transposon had inserted into a 7.3 kb SmaI fragment containing part of the tetracycline determinant of pUB307, resulting in a SmaI fragment of over 23.1 kb. Therefore, the size of this Ac'Ctm'Sm' transposon was larger than 15.8 kb.

The results of SalI digestion of pCL8 (Fig. 27b, lane 10) showed that pCL8 harboured 2.5, 5.5, and 6.7 kb SalI fragments derived from the Ac'Ctm'Sm' transposon. Plasmid pUB307 harboured a SalI site which is 363 bp from the SmaI site in its Tc' determinant (Fig. 30) (Waters et al., 1983). Therefore, one of the three SalI fragments may be flanked by a pUB307-derived SalI site.

Another recombinant pUB307 carrying the Ac'Ctm'Sm' transposon from pST8 was extracted from an Ac'Cm'Ctm'Km'Sm'Tc' E. coli UB1637 and designated as pCL8R. The Ac'Ctm'Sm' transposon in pCL8R had inserted into a location out of the Tc' determinant. Plasmid pCL8R was digested with SalI for comparison with SalI-digested pCL8 (Fig. 27 and 28). The results of the digestions showed that the 2.5 and 5.5 kb SalI fragments, which were common to both SalI-digested pCL8 and pCL8R, were flanked by transposon-derived SalI sites and the 6.7 kb fragment was flanked by a pUB307-derived SalI site. Therefore, the Ac'Ctm'Sm' transposon was located in between the SalI and SmaI sites.
Figure 27: (a) Ethidium bromide-stained agarose gel (0.7%, w/v) of pCL8, pCL8R, and pUB307 after restriction endonuclease digestions and (b) the same gel after an extended electrophoresis at 1 V/cm for 12 hr

Lanes 1 and 12: 1 kb ladder size markers
Lane 2: *Eco*RI-digested pUB307
Lane 3: *Eco*RI-digested pCL8
Lane 4: *Bam*HI-digested pCL8
Lane 5: *Hind*III-digested pCL8
Lane 6: *λ*- *Hind*III DNA size markers
Lane 7: *Sma*I-digested pUB307
Lane 8: *Sma*I-digested pCL8
Lane 9: *Sal*I-digested pUB307
Lane 10: *Sal*I-digested pCL8
Lane 11: *Sal*I-digested pCL8R
Figure 28: Ethidium bromide-stained agarose gel (0.7%, w/v) of pCL8 and pCL8R after SalI-digestions

Lane 1: 1 kb ladder size markers
Lane 2: pCL8
Lane 3: pCL8R
Figure 29a: Ethidium bromide-stained agarose gel (0.7%, w/v) of plasmid pCL8 after SalI-digestion with 3-fold serial dilutions of SalI

Lane 1: 1 kb ladder size markers

Lanes 2-10: pCL8 digested with 3-fold serial dilutions of SalI
Figure 29b: The arrangement of SalI-digested fragments originated from Ac'Ctm'Sm' transposon on pCL8
Figure 30: Restriction map of plasmid pUB307 with respect to the single EcoRI site.

The size of pUB307 (53.7 kb) used above is derived from the size of the IncPα plasmids (R18, R68, RK2, RP1, and RP4) (60.1 kb) compiled by Pansegrau et al. (1994) with a deletion of a fragment of 4.1x10^6 M W (about 6.4 kb) in between the EcoRI site and the Tc' gene in RP1 that formed pUB307 (Bennett et al., 1977; Grinsted et al., 1977, 1979)
The arrangement of the 2.5 and 5.5 kb SalI fragments was investigated by partial digestions of pCL8 (Fig. 29a). Taken together with the results of previous restriction digestions, the arrangement of transposon-derived SalI fragments in pCL8 was deduced as shown in Fig. 29b. The SalI sites appeared to be suitable for cloning the antibiotic resistance genes.

3.4.2 Shotgun cloning of antibiotic resistance genes

The number and antibiograms of transformants obtained from the transformation of competent E. coli DH5α with the ligation products of SalI-digested pCL8 and SalI-digested pKan are shown in Table 11. A typical plasmid from each type of antibiotic resistant transformant resolved on agarose gel is shown in Figure 31. When competent E. coli DH5α was transformed with recombinant plasmid extracted from each type of antibiotic resistant transformant, many (>200) secondary transformants with an antibiotic resistance phenotype identical to that of the primary transformant were obtained.

3.4.3 Restriction pattern of recombinant pKan after SalI digestion

Figure 32 shows that all SalI-digested recombinant pKan plasmids [Km'Ac' clone (lane 5), Km'Sm' clone (lane 8), Km'Ac'Sm' clone (lanes 6 and 9)] generated a 4.2 kb DNA fragment that corresponded to that of SalI-digested pKan (Fig. 32, lane 2; Fig. 33). In addition, all Ac' clones also generated a 2.5 kb fragment and all Sm' clones a 5.5 kb fragment. These fragments corresponded to similar fragments from SalI-digested pCL8 (Fig. 32, lane 3). From these results, it was possible to deduce that the Ac' and Sm' genes were located on the 2.5 and 5.5 kb SalI fragments of pCL8, respectively. Recombinant pKan plasmids carrying the 2.5 kb Ac' and 5.5 kb Sm' inserts were
Table 11: Phenotypes of transformants from cloning of antibiotic resistance genes from *SalI*-digested recombinant pUB307 derived from pST8

<table>
<thead>
<tr>
<th>Phenotype of recombinant pUB307 derived from pST8</th>
<th>Phenotype conferred by plasmid vector pKan</th>
<th>Selective antibiotic</th>
<th>No. of transformants obtained and examined</th>
<th>No. and resistance phenotype of transformants</th>
<th>No. of transformants examined for plasmid</th>
<th>Size (kb) of DNA fragments from <em>SalI</em>-digested recombinant pKan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac&lt;sup&gt;i&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Ctm&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Te&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ac&lt;sup&gt;i&lt;/sup&gt;</td>
<td>21 Ac&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>4</td>
<td>2.5, 4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 Ac&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt; Sm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>5</td>
<td>2.5, 4.2, 5.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 Ac&lt;sup&gt;i&lt;/sup&gt; Ctm&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2</td>
<td>2.5, &gt;12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Ctm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>23</td>
<td>21 Ctm&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>4</td>
<td>&gt;12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Ctm&lt;sup&gt;i&lt;/sup&gt; Sm&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2</td>
<td>2.5, &gt;12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>27</td>
<td>18 Sm&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>6</td>
<td>4.2, 5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 Ac&lt;sup&gt;i&lt;/sup&gt; Sm&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>6</td>
<td>2.5, 4.2, 5.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 31: Ethidium bromide-stained agarose gel (0.7%, w/v) of ccc plasmids pKan, pUB307, pCL8, and recombinant pKan and pUB307 obtained from ligation of *SalI*-digested pCL8 and pKan

Lane 1: pKan (Km')
Lane 2: pUB307 (Km'Tc')
Lane 3: pCL8 (Ac'Cm'Ctm'Km'Sm'Tc')
Lane 4: Recombinant pKan [pCLA25] (Ac'Km')
Lane 5: Recombinant pKan (Ac'Km'Sm')
Lane 6: Recombinant pUB307 (Ac'Ctm'Km')
Lane 7: Recombinant pKan [pCLS55] (Km'Sm')
Lane 8: Recombinant pKan (Ac'Km'Sm')
Lane 9: Recombinant pUB307 [pCLC600] (Ctm'Km')
Lane 10: Recombinant pUB307 (Ctm'Km'Sm')

Note:

Plasmids in lanes 6, 9 and 10 were presumed to be derived from self-ligation of *SalI*-digested pCL8 fragments.
Figure 32: Ethidium bromide-stained agarose gel (0.7%, w/v) of pKan, pUB307, pCL8, and recombinant pKan and pUB307 after SalI digestion

Lane 1: 1 kb ladder
Lane 2: pKan (Km')
Lane 3: pUB307 (Km'Tc')
Lane 4: pCL8 (Ac'Cm'Ctm'Km'Sm'Tc')
Lane 5: Recombinant pKan [pCLA25] (Ac'Km')
Lane 6: Recombinant pKan (Ac'Km'Sm')
Lane 7: Recombinant pUB307 (Ac'Ctm'Km')
Lane 8: Recombinant pKan [pCLS55] (Km'Sm')
Lane 9: Recombinant pKan (Ac'Km' Sm')
Lane 10: Recombinant pUB307 [pCLC600] (Ctm' Km')
Lane 11: Recombinant pUB307 (Ctm'Km'Sm')

Note:
Plasmids in lanes 7, 10 and 11 were presumed to be derived from self-ligation of SalI-digested pCL8 fragments.
Figure 33: Restriction map of plasmid pKan (Bloom et al., 1996)
designated pCLA25 and pCLS55, respectively.

All Ctm' clones, irrespective of whether they carried additional antibiotic resistance gene or not, did not harbour recombinant plasmids that contained the 4.2 kb fragment of *SalI*-digested pKan, even though they were Km'. Instead, they generated two large (>12 kb) *SalI*-digested fragments which were similar to those of *SalI*-digested pCL8 and pUB307. Figure 34 shows that *PstI*-digested recombinant plasmids from Km'Ctm' and Km'Ac'Ctm' transformants generated fragments that corresponded to *PstI*-digested pUB307 and pCL8. Therefore, these Ctm' recombinant plasmids were probably resulted from the self-ligation of *SalI*-digested pCL8 and the Km' carried by these recombinant plasmids probably was derived from pUB307. Since in pCL8, the Ac'Ctm'Sm' transposon was inserted in between the *SalI* and *SmaI* sites (Fig. 29b) in the *tetR* gene of pUB307, the Ctm' determinants of the Ac'Ctm'Sm' transposon were most likely located near to the *SmaI* site. One of these Ctm' clones that only carried Km' and Ctm' was designated pCLC600.

Plasmid pCLC600 was digested partially with *Sau3A*I (Fig. 35) and ligated to *BamHI*-digested pKan. When competent *E. coli* DH5α was transformed with the ligation products, five Km'Ctm' transformants were obtained. Many (>200) Km'Ctm' secondary transformants were obtained when competent *E. coli* DH5α was transformed with plasmid extracted from each of these five Km'Ctm' clones.

Recombinant plasmids extracted from these five Km'Ctm' transformants were designated pCLC1, pCLC2, pCLC3, pCLC4, and pCLC5. When these recombinant plasmids were analysed by agarose gel electrophoresis (Fig. 36), all of them were larger than pKan but smaller than pUB307 and pCLC600, except for pCLC1 (lane 4). Results
Figure 34: Ethidium bromide-stained agarose gel (0.7%, w/v) of pCL8, pCLC600, pKan, and pUB307, after *Pst*I digestion

Lane 1: 1 kb ladder
Lane 2: pCL8
Lane 3: pCLC600 (Ctm'Km')
Lane 4: pKan
Lane 5: pUB307
Lane 6: λ- *Hind*III markers
Figure 35: Ethidium bromide-stained agarose gel (1.0%, w/v) of plasmid pCLC600, after digestion with 3-fold serial dilutions of Sau3AI

Lane 1: 1 kb ladder

Lanes 2-7: pCLC600 with 3-fold serial dilutions of Sau3AI [ranging from 1U/15μl (lane 2) to 4.11x10^{-3} U/15μl (lane 7)]

Lane 8: pCLC600 (undigested)

Lane 9: λ-HindIII markers

Note:
The concentration of Sau3AI [1.23 x 10^{-3} U/15μl (lane 6)] was choosen for the 10-fold scale-up partial digestion
Figure 36: Ethidium bromide-stained agarose gel (0.8%, w/v) of ccc plasmids extracted from Ctm' transformants

Lane 1: pKan
Lane 2: pCL8
Lane 3: pCLC600
Lane 4: pCLC1
Lane 5: pCLC2
Lane 6: pCLC3
Lane 7: pCLC4
Lane 8: pCLC5
of *HindIII* digestion of these recombinant plasmids also showed that the recombinant pKan in these plasmids had acquired additional DNA owing to the cloned Ctm\(^r\) determinants (Fig. 37).

Plasmids pCLC2, pCLC4, and pCLC5 were digested separately with *EcoRI*, *BamHI*, *EcoRI-BamHI* (Fig. 38), and *EcoRI-HindIII* (Fig. 39) and analysed by agarose gel electrophoresis. The restriction endonuclease digestions showed that these three plasmids could be linearized by digestion with either *EcoRI* or *BamHI* (Fig. 38). It should be noted that the *EcoRI* site in each recombinant plasmid was derived from the pKan vector (Fig. 33). In each of these Ctm\(^r\) recombinant pKan (Fig. 38), one of the vector-derived *BamHI* ends was destroyed during the ligation of *BamHI*-digested pKan and *Sau3AI* partially-digested pCLC600. In pCLC2, the *BamHI* site that was destroyed, was distal to the Km\(^r\) gene of pKan (Fig. 33) because its 4.3 kb insert could be excised by *EcoRI-BamHI* digestion (Fig. 39, lane 1). In pCLC4 and pCLC5, their intact *BamHI* site was close to the *EcoRI* site, thus generating only one visible band from the *EcoRI-BamHI* digestion (Fig. 38, lanes 10 and 11).

*HindIII* digestion of pCLC2 generated two fragments of 3.8 and 4.7 kb. This indicated that the 4.3 kb insert of pCLC2 contained at least one *HindIII* internal site. This *HindIII* site may be a suitable site for subcloning of the Su\(^r\) and Tp\(^r\) determinants that collectively conferred Ctm\(^r\).

The antibiotic resistance phenotype conferred by each *SalI* fragment on pCL8 is shown in Fig. 40. The 2.5 kb Ac\(^r\) *SalI* fragment of pCL8 was the smallest fragment carrying an antibiotic resistance gene and it was further characterized.
Figure 37: Ethidium bromide-stained agarose gel (0.8%, w/v) of recombinant plasmid from Cm’ transformant after HindIII digestion

Lane 1: pCLC1
Lane 2: pCLC2
Lane 3: pCLC3
Lane 4: pKan
Lane 5: 1 kb ladder
Lane 6: pCLC4
Lane 7: pCLC5
Figure 38: Ethidium bromide-stained agarose gel (0.8%, w/v) of plasmids extracted from Ctm' transformants after EcoRI, BamHI, and EcoRI-BamHI digestion.

Lanes 1 and 12: 1 kb ladder
Lane 2: EcoRI-digested pKan
Lane 3: EcoRI-digested pCLC2
Lane 4: EcoRI-digested pCLC4
Lane 5: EcoRI-digested pCLC5
Lane 6: BamHI-digested pCLC2
Lane 7: BamHI-digested pCLC4
Lane 8: BamHI-digested pCLC5
Lane 9: EcoRI-BamHI-digested pCLC2
Lane 10: EcoRI-BamHI-digested pCLC4
Lane 11: EcoRI-BamHI-digested pCLC5
Figure 39: Ethidium bromide-stained agarose gel (0.8%, w/v) of EcoRI-BamHI digested pCLC2 after 16 hr electrophoresis at voltage of 21V

Lane 1: EcoRI-BamHI-digested pCLC2
Lane 2: undigested pCLC2
Lane 3: EcoRI-digested pKan
Lane 4: 1 kb ladder
Figure 40: Restriction map of plasmid pCL8
3.5 Hybridization of labelled 2.5 kb Ac' DNA fragment to pST8 of *S. typhi*

*S8 and recombinant pUB307 carrying Ac'Ctm'Sm' transposon*

For the confirmation of the origin of the 2.5 kb Ac' *SalI*-insert from pCLA25, Southern hybridization with the labelled insert as the probe was performed. Plasmids pST8, pUB307, pCL8, pKan, and pCLA25 (Fig. 41) were separately digested with *SalI* and resolved on an agarose gel as shown in Fig. 42a. The DNA fragments of *SalI*-digested plasmids on the agarose gel were transferred onto a nylon membrane under vacuum. After hybridization for 16 hr, the membrane was washed under high stringency condition. The signal generated by chemiluminescence was detected by exposure to an X-ray film. Figure 42b shows that the 2.5 kb *SalI*-insert from pCLA25 hybridized strongly to itself (lane 6), and a 2.5 kb fragment of *SalI*-digested pCL8 (lane 4), and weakly to a fragment of similar size of *SalI*-digested pST8 (lane 2). This confirmed that the 2.5 kb Ac' *SalI*-insert in pCLA25 was cloned from the Ac'Ctm'Sm' transposon on pCL8 which was derived from pST8.

The 2.5 kb probe also hybridized fairly strongly to the 1.6 kb fragment of the 1 kb ladder (Fig. 42, lane 1) and weakly to the 4.2 kb *SalI*-linearized pKan vector (Fig. 42, lanes 5 and 6). In addition, faint signal was detected on the 5.5 kb Sm' *SalI*-fragment of pCL8 (Fig. 42, lane 4).

3.6 M13 cloning and construction of nested deletion libraries

The ligation mixtures comprising the 2.5 kb Ac' *SalI* fragment from pCLA25 and *SalI*-linearized M13mp19 (Fig. 43) were used to transfect competent *E. coli* DH5αF'. Many (<200) white Ac' transfectant colonies were obtained from each ligation mixture. When RF DNA were extracted from five transfectants and analysed by agarose gel
Figure 41: Ethidium bromide stained agarose gel (0.5%, w/v) of ccc plasmids pKan, pCLA25, pUB307, pCL8, and pST8

Lane 1: pKan
Lane 2: pCLA25
Lane 3: pUB307
Lane 4: pCL8
Lane 5: pST8
Figure 42: Hybridization of labelled 2.5 kb $Ac^r$ fragment probe to pST8 and pCL8

(a) Ethidium bromide-stained agarose gel (0.5%, w/v) of:

Lane 1: 1 kb ladder
Lane 2: $SalI$-digested pST8
Lane 3: $SalI$-digested pUB307
Lane 4: $SalI$-digested pCL8
Lane 5: $SalI$-digested pKan
Lane 6: $SalI$-digested pCLA25

(b) Autoradiography of gel (a) after Southern hybridization with labelled 2.5 kb $Ac^r$ fragment probe retrieved from $SalI$-digested pCLA25.
Figure 43: Restriction map of bacteriophage M13 vectors M13mp18 and M13 mp19 (Sambrook et al., 1989). The figure shows the sites at which a number of restriction enzymes cleave the double-stranded RF M13mp18 and M13mp19. The vectors differ from one another only in the orientation of the polycloning site.
electrophoresis (Section 2.26.1), all of them were found to be larger than the RF DNA of M13mp19. Subsequently, one Ac<sup>c</sup> transfectant, designated M13mp19-Ac<sup>c</sup>(2.5) (Fig. 44, lane 5), was chosen and its RF DNA was purified for nested deletion (Section 2.26.2). Fig. 45 shows the progress of nested deletions of M13mp19-Ac<sup>c</sup>(2.5) RF DNA.

After unidirectional nested deletions, deleted derivatives of M13mp19-Ac<sup>c</sup>(2.5) RF DNA were used to transfect *E. coli* DH5αF<sup>’</sup>. Many (>200) colorless plaques were obtained. Two hundred and fifty plaques were individually toothpicked onto LB plates and LB plates containing Ac. Single-stranded DNA were extracted from these Ac<sup>c</sup> transfectants and compared with one another. Single-stranded DNA were also extracted from Ac<sup>s</sup> transfectant and compared with one another. Therefore, a series of progressively deleted clones of M13mp19-Ac<sup>c</sup>(2.5) was obtained.

An Ac<sup>c</sup> deleted recombinant clone harbouring the smallest M13mp19-Ac<sup>c</sup> RF DNA (Fig. 44, lane 4) was chosen and its RF DNA was designated M13mp19-Ac<sup>c</sup>(1.8). The RF DNA of M13mp19-Ac<sup>c</sup>(1.8) was purified and double digested with *Eco*RI and *Hind*III to release its 1.8 kb Ac<sup>c</sup> insert. The insert was eluted, purified, and ligated with *Eco*RI- and *Hind*III-digested M13mp18 RF DNA. The ligated mixture was transfected into competent *E. coli* DH5αF<sup>’</sup> and many (>200) plaques were obtained. Fifty transfectant plaques were toothpicked onto LB plate containing Ac.

One of these Ac<sup>c</sup> recombinant clones was designated as M13mp18-Ac<sup>c</sup>(1.8) (Fig. 44, lane 3). Its RF DNA was purified for unidirectional nested deletions (Section 2.26.4). Fig. 46 shows the progress of nested deletions of M13mp18-Ac<sup>c</sup>(1.8) and Fig. 47 shows the single-stranded DNA extracted from a series of progressively deleted clones of M13mp18-Ac<sup>c</sup>(1.8). A deleted recombinant M13mp18-Ac<sup>c</sup>(1.8) that harboured the
Figure 44: Ethidium bromide-stained agarose gel (0.7%, w/v) of restriction endonuclease-digested recombinant RF DNA of M13mp18 and M13mp19

Lane 1: 1 kb ladder
Lane 2: EcoRI-digested M13mp18-Ac° (1.4)
Lane 3: SalI-digested M13mp18-Ac° (1.8)
Lane 4: SalI-digested M13mp19-Ac° (1.8)
Lane 5: SalI-digested M13mp19-Ac° (2.5)
Lane 6: SalI-digested M13mp19
Figure 45: Ethidium bromide-stained agarose gel (0.7%, w/v) showing progressive deletions of RF DNA of M13mp19-Ac(2.5) after 0 to 28 minutes of digestion with exonuclease III (lanes 2 to 16)

Lanes 1 and 18: 1 kb ladder

Lane 17: EcoRI-linearised RF DNA of M13mp19
Figure 46: Ethidium bromide-stained agarose gel (0.7%, w/v) showing progressive deletions of RF DNA of M13mp18-AcE(1.8) after 0 to 28 minutes of digestion with exonuclease III (lanes 2 to 16).

Lanes 1 and 18: 1 kb ladder

Lane 17: SalI-linearised RF DNA of M13mp18-AcE(1.8)
Figure 47: Ethidium bromide-stained agarose gel (0.7%, w/v) of single-stranded DNA from transfectants derived from RF DNA of M13mp18-Ac’ (1.8) after nested deletions (lanes 1 to 17)

Lane 18: single-stranded DNA of M13mp18
smallest Ac' insert (about 1.4 kb) was designated M13mp18-Ac'(1.4) (Fig. 44, lane 2).

3.7 Confirmation of the opposite orientations of inserts of recombinant

M13mp18 and M13mp19

The opposite orientations of DNA inserts in recombinant M13mp18 and M13mp19 were confirmed by the complementary test (C-test). In this test, ssDNA of M13mp18-Ac'(1.8) and M13mp19-Ac'(1.8) were hybridized as described in Section 2.26.5.

These complementary inserts in recombinant M13mp18 and M13mp19 hybridized or annealed to form a figure of ‘8’ which exhibited a slower electrophoretic mobility (Fig. 48, lanes 3 and 4). Figure 48 also shows that the ssDNA of M13mp18 (lane 1), recombinant M13mp18 (lane 2), and recombinant M13mp19 (lanes 5 and 6) could not hybridize to itself and remained as a monomer.

Results from the C-test confirmed that the two complementary strands of 1.8 kb Ac' DNA inserts had been cloned in opposite orientations to yield M13mp18-Ac'(1.8) and M13mp19-Ac'(1.8).

3.8 Autoradiographs of sequencing reactions

Recombinant single-stranded DNA from recombinant M13mp18 and M13mp19 were purified and DNA inserts in the deleted recombinant M13mp18 and M13mp19 derivatives were sequenced individually. After development, autoradiographs of the sequencing reactions were placed on a light box and the sequences recorded. Figure 49 shows examples of autoradiographs showing DNA bands from which DNA sequences were read.

Some of the processed sequence data obtained from automatic DNA sequencing
Figure 48: Ethidium bromide-stained agarose gel (0.5%, w/v) of single stranded recombinant M13mp18 and M13mp19 after the C-test

Lane 1: M13mp18
Lane 2: M13mp18-Ac′(1.8)
Lanes 3 and 4: M13mp18-Ac′(1.8) + M13mp19-Ac′(1.8)
Lanes 5 and 6: M13mp19-Ac′(1.8)
Figure 49: Photograph of autoradiographs of DNA sequencing gels produced by using Sequenase™ Version 2.0 DNA Sequencing Kit with 5’-[α-33P]dATP. Sequencing reactions with:
(a) M13mp18-Ac’(1.8)
(b) M13mp19-Ac’(1.8)
are shown in Fig. 50a, b, and c.

3.9 Analysis of nucleotide sequences

Figure 51 shows the nucleotide sequence that corresponds to the sense strand (i.e., the DNA strand with the same sequence as that in the mRNA) of ampicillin resistance determinant. Both strands of 1814 nucleotides were sequenced except for nucleotides 1 to 553, the sequence of which was based on one strand sequencing. Comparison of the nucleotide sequences obtained against sequences from Genbank and EMBL showed three distinct segments (Fig. 52). The first 351 nucleotides were identical to nucleotides 351 to 1 of IS26R (Mollet et al., 1983) and its variants (Trieu-Cout and Courvalin, 1984; Ouellette et al., 1987; Nucken et al., 1990) (Table 12). Nucleotides 352 to 1795 showed 100 and 99.6% in sequence identities to Tn2 (Chen and Clowes, 1987) and Tn3 (Heffron et al., 1979), respectively, at nucleotides 3514 to 4957. The truncated Tn2-like sequences differed from the Tn3 sequences by 5 base substitutions as shown in Fig. 50; three of them occurred within the Acf TEM-1 β-lactamase gene. The junction between the IS26-like and Tn2-like sequences occurred between nucleotides 351 and 352 (Fig. 53). The third segment contained 19 nucleotides (1796 to 1814) that were identical to nucleotides 2089 to 2071 of plasmid RSF1010 (Scholz et al., 1989).
Figure 50a: Photograph of processed DNA sequencing data obtained by using Cy5\textsuperscript{TM} Autoread\textsuperscript{TM} Sequencing Kit. Differences in nucleotide sequences obtained and Tn3 highlighted above are as follows:

(a) \#2 - * position 754 [G] \rightarrow Tn3 (non-coding region) \*position 3916 [A]

\#3 - * position 805 [T] \rightarrow Tn3 (TEM-1 Ac\textsuperscript{f}) \*position 3967 [C]

\#4 - * position 1015 [T] \rightarrow Tn3 (TEM-1 Ac\textsuperscript{f}) \*position 4177 [C]

Note:

* According to nucleotide sequences in Fig. 51

\* According to Heffron \textit{et al.} (1979)
Figure 50b and c: Photograph of (a) processed DNA sequencing data obtained by using Cy5\textsuperscript{TM} Autoread\textsuperscript{TM} Sequencing Kit. Differences in nucleotide sequences obtained and Tn3 highlighted above are as follows:

(b) \#1 - *position 419 [A] \rightarrow Tn3 (\textit{tnpR}) *position 3581 [G]

(c) \#5 - *position 1183 [C] \rightarrow Tn3 (\textit{TEM-1 Ac'}) *position 4345 [G]

Note:

* According to nucleotide sequences in Fig. 51

* According to Heffron \textit{et al.} (1979)
Figure 51: Partial nucleotide sequence of DNA insert of pCLA25 showing the three distinct segments. Inverted repeat sequences of Tn2-like and IS26-like sequences are in underlined italics.
Figure 52: The three segments on the 2.5 kb Ac′ insert of M13mp19-Ac′ (2.5)

Note:
The “unknown” segment was deleted in M13mp19-Ac′ (1.8) and M13mp18-Ac′ (1.8)
Figure 53: The junction of IS26-like segment and Tn2-like segment. Inverted repeat of the IS26-like segment is in bolded-italics. Homologous sequences between the terminus IS26-like segment and Tn2 near the putative junction of the two segments sequenced in pCLA25 are underlined.