

CHAPTER TWO MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and bacteriophages

Table 3 lists some of the bacterial strains used in this study. Four multiple antibiotic resistant *S. typhi* (S2, S6, S8, and S9) isolated from patients admitted to Kuala Lumpur Hospital, were used in this study. S2 and S8 were isolated from two patients from Sungai Buluh, Selangor, whereas S8 and S9 were from two visitors who arrived here from India. S2, S6, S8, and S9 each harboured a single large conjugative R plasmid that confers Ac^rCm^rCtm^rSm^rTc^r (Chong 1992; Kadambeswaran, 1993).

E. coli UB5201 (*recA* Lac⁺ Nx^r Pro^r Met^r) and *E. coli* UB1637 (*recA* Lac⁻ Sm^r His⁻ Trp⁻ Lys⁻) (de la Cruz and Grinsted, 1982), plasmidless or harbouring pUB307 (Km^r Sm^r) (Bennett *et al.*, 1977), were obtained from Dr. P.M. Bennett, University of Bristol, England. Plasmid pUB307, an IncP conjugative plasmid that is transferred at a high frequency at 37°C, was used as the recipient replicon in the transposition experiments. *E. coli* UB5201(pUB307, pST8) and *E. coli* UB5201(pUB307, pST9), prepared by Kadambeswaran (1993) and kept on Dorset egg slants for more than 2 years at room temperature, were used in transposition experiments.

E. coli DH5 α [K12 (r_k⁻ m_k⁻) *relA1*] (Woodcock *et al.*, 1989) and DH5 α F' [F' K12 (r_k⁻ m_k⁻) *relA1*] (Liss, 1987) were from GIBCO BRL, U.S.A. These strains were used as the recipients in transformation and transfection experiments.

Plasmid pKan (Km^r) (Micklos and Freyer, 1990) was used as a cloning vector. *E. coli* MM294 harbouring pKan was from Carolina Biological Company, U.S.A.

The replicative forms (RF) of bacteriophages M13mp18 and M13mp19 were from Pharmacia Biotech, U.S.A.

Table 3: Phenotypes and genotypes of *S. typhi* and *E. coli* strains

Bacterial strain	Phenotype and genotype
<i>S. typhi</i> S2 (pST2)	Lac ⁻ Ac ^r Cm ^r Ctm ^r Sm ^r Tc ^r
<i>S. typhi</i> S6 (pST6)	Lac ⁻ Ac ^r Cm ^r Ctm ^r Sm ^r Tc ^r
<i>S. typhi</i> S8 (pST8)	Lac ⁻ Ac ^r Cm ^r Ctm ^r Sm ^r Tc ^r
<i>S. typhi</i> S9 (pST9)	Lac ⁻ Ac ^r Cm ^r Ctm ^r Sm ^r Tc ^r
<i>E. coli</i> UB1637	recALac ⁻ His ⁻ Lys ⁻ Trp ⁻ Sm ^r
<i>E. coli</i> UB5201	recALac ⁻ Pro ⁻ Met ⁻ Nx ^r
<i>E. coli</i> (pUB307)	Km ^r Tc ^r
<i>E. coli</i> DH5 α	recANx ^r
<i>E. coli</i> DH5 α F [']	recANx ^r

2.2 General materials and reagents

All chemicals and solvents used were of Analar Grade or of the highest grade commercially available from Aldrich Chemical Co., U.S.A.; BDH Chemical Ltd., England; Boehringer Mannheim, Germany; Fluka BioChemika, Switzerland; GIBCO BRL, U.S.A.; Riedel-de-Haen AG, Germany; Sigma Chemical Co., U.S.A.; and United States Biochemical (USB), U.S.A. These included boric acid, bromophenol blue, cesium chloride, chloroform, concentrated sulphuric acid, EDTA disodium salt, ethanol, ethidium bromide, Ficoll 400, glycerol, 8-hydroxyquinoline, isopropanol, phenol, sodium chloride, sodium dodecyl sulphate, sodium hydroxide, Tris-base, Tris-HCl, and other chemicals and solvents. Acrylamide and N,N'-methylenebisacrylamide for polyacrylamide gels were from GIBCO BRL. Agarose powder used was SeaKem LE

grade from FMC Corp., U.S.A.

Agar Grade A, MacConkey agar powder, and yeast extract powder were from Becton Dickinson Microbiology Systems, U.S.A. Tryptone and Iso-Sensitest agar were from Oxoid Ltd., England.

Cellulose acetate membrane filters (0.2 and 0.45 μm pore size) were from Microfiltration Systems, U.S.A. Dialysis tubing Spectra/Por® 4 was from Spectrum Medical Industries, Inc., U.S.A. Glass beads (5 mm diameter) were from Riedal-de-Haen A.G. Centrifuge bottles and tubes were from Nalge Co., U.S.A. and Quick-seal tubes were from Beckman Instruments, Inc., U.S.A.

Size reference markers (1 kb, 100- and 123-bp DNA ladders) were bought from GIBCO BRL and λ -HindIII markers were from Pharmacia Biotech, U.S.A.

2.3 Enzymes, kits, and radiochemicals

T4 DNA ligase and restriction endonucleases used were from New England Biolabs, Inc., except for *SalI* which was from Boehringer Mannheim. Unless otherwise stated, all enzymatic reactions were performed as described by the manufacturers. DNase-free RNase A was from Sigma Chemical Company. Lysozyme was from Boehringer Mannheim.

The ECL Direct Nucleic Acid Labelling Detection System kit used in the Southern hybridization experiments was from Amersham International plc., U.K. The Double-stranded Nested Deletion kit and Cy5TM AutoReadTM Sequencing kit were from Pharmacia Biotech. The SequenaseTM Version 2.0 DNA Sequencing kit was from USB.

5'-[α -³³P]dATP and 5'-[α -³³P]dCTP, with specific activity of 3,000 Ci/mmol, were obtained in aqueous solution from Du Pont NEN Research Products, U.S.A.

2.4 Media

2.4.1 Luria-Bertani (LB) medium (Miller, 1972)

Tryptone.....	1.0 g
Yeast extract.....	0.5 g
NaCl	0.5 g
Distilled water to 100 ml	

All ingredients were dissolved and the resulting solution was autoclaved. For solid medium, 1.2 or 0.65 g of Grade A agar was added to prepare normal or soft agar.

2.4.2 MacConkey agar

This medium was used to differentiate between Lac⁺ and Lac⁻ bacterial strains.

Lac⁺ bacterial colonies appeared red, while Lac⁻ bacterial colonies appeared colourless on this agar.

MacConkey agar powder, 5.0 g, was suspended in 100 ml of distilled water and the resulting mixture was dissolved by heating in a microwave oven before autoclaving.

2.4.3 Supplemented M9 agar (Miller, 1972)

The supplemented M9 agar was prepared by mixing separately autoclaved 90 ml of distilled water containing 1.2 g of Grade A agar and 10 ml of filter-sterilized 10x M9 stock solution, 0.2 ml of 0.05 M CaCl₂, 0.1 ml of 1 M MgSO₄, 1.0 ml of 20% (w/v) glucose, and 0.5 ml of amino acid stock solution.

10x M9 stock solution :

Na ₂ HPO ₄	30.0 g
KH ₂ PO ₄	15.0 g
NaCl.....	2.5 g

NH₄Cl.....5.0 g

Distilled water to 500 ml

The amino acid stock solution consisted of either histidine, lysine, and tryptophan (each 4 mg/ml) or proline and methionine (each 4 mg/ml) depending on experiments. The final concentration of each amino acid in the supplemented M9 agar was 20 µg/ml.

2.4.4 Dorset egg slant

Fresh egg mixture (yolk and white).....750 ml

Peptone.....2.50 g

'Lab-Lemco' powder.....2.50 g

NaCl.....1.25 g

Distilled water to 1000 ml

The above ingredients were mixed and the solution was distributed into sterile bijoux bottles by a dispensing filter funnel. Then the medium was inspissated in a slanting position at 80-85°C for 2 hr after which the inspissated slants were sterilized in a steamer for 90 min.

2.4.5 Iso-Sensitest agar

This medium was used for cotrimoxazole sensitivity testing. Iso-Sensitest agar powder, 3.14 g, was suspended in 100 ml of distilled water. The resulting mixture was dissolved by heating in a microwave oven before autoclaving.

2.4.6 SOB medium

This medium (Hanahan, 1983, 1985) was used to grow *E. coli* for transformation experiments.

Tryptone.....	2.0 g
Yeast extract.....	0.5 g
NaCl.....	58 mg
KCl.....	19 mg
Distilled water to 100 ml	

The ingredients were dissolved and the resulting solution was autoclaved. After autoclaving, 1.0 ml of filter-sterilized 1 M MgCl₂ was added.

2.4.7 SOC medium

This medium (Hanahan, 1983, 1985) was used to revive transformed competent cells before they were plated onto selective plates.

SOB medium.....	100 ml
2 M D-glucose.....	1.0 ml

2.4.8 B agar (Messing, 1983)

Tryptone.....	1 g
NaCl.....	0.8 g
1% (w/v) Vitamin B1 Solution.....	0.1 ml
Agar.....	2.0 g
Distilled water to 100 ml.	

The ingredients were mixed and the mixture was autoclaved. For soft agar, agar was added to final concentration of 0.6% (w/v).

2.4.9 2x TY medium

Tryptone.....	1.6 g
Yeast extract.....	1.0 g

NaCl.....0.5 g

Distilled water to 100 ml.

The ingredients were dissolved and the resulting solution was autoclaved.

2.5 Stock solutions and buffers

2.5.1 Antibiotic stock solutions

Table 4 lists the antibiotics used in this study. All antibiotic stock solutions were prepared according to Table 5. Sterilization was by membrane filtration. Working stocks were stored in the dark at 4°C, while the remainders were stored at -20°C. An appropriate volume of each stock solution was added aseptically to a sterile medium to give the required final concentration.

2.5.2 6x Bromophenol blue (BPB) loading dye

BPB.....0.18% (w/v)

Ficoll.....9% (w/v)

Glycerol.....40% (w/v)

The solution was sterilized by membrane filtration. An appropriate volume of 6x BPB loading dye was mixed with each DNA sample in a 1:5 ratio for agarose gel electrophoresis.

2.5.3 6x Bromophenol blue (BPB) stop mix

BPB.....0.15% (w/v)

Ficoll.....9% (w/v)

EDTA, pH 8.0.....60 mM

Glycerol.....40% (w/v)

The 6x BPB stop mix was sterilized by membrane filtration. It was used in

Table 4: List of antibiotics

Antibiotic	Salt	Commercial name	Form	Source
Ampicillin (Ac)	Ampicillin sodium	Bactrin	powder	Beecham Research Lab.
Chloramphenicol (Cm)	-	-	powder	Sigma Chemical Co.
Cotrimoxazole (Ctm)	-	Septrin	liquid	The Wellcome Foundation Ltd.
Kanamycin (Km)	Kanamycin sulfate	-	powder	Sigma Chemical Co.
Nalixidic acid (Nx)	-	-	powder	Sigma Chemical Co.
Streptomycin (Sm)	Streptomycin sulfate	-	powder	Sigma Chemical Co.
Tetracycline (Tc)	Tetracycline hydrochloride	-	powder	Sigma Chemical Co.

Table 5: Antibiotic stock solutions

Antibiotic	Stock solution (mg/ml)	Solvent used	Vol. of stock (μ l) per 100 ml medium	Final conc. (μ g/ml)
Ampicillin (Ac)	20	distilled water	250	50
Chloramphenicol (Cm)	25	ethanol 95% (v/v)	100	25
Co-trimoxazole (Ctm)	16 Tp 80 Smz	distilled water	100	16 Tp 80 Smz
Kanamycin (Km)	20	distilled water	100	20
Nalidixic Acid (Nx)	100	1 N NaOH	50	50
* Streptomycin (Sm)	20 4	distilled water	1000 250	200 10
Tetracycline (Tc)	4	distilled water	250	10

Tp : trimethoprim

Smz : sulfamethaxazole

*Sm : 200 μ g/ml for chromosome-mediated resistance and 10 μ g/ml for plasmid-mediated resistance

stopping enzymatic digestion before agarose gel electrophoresis.

2.5.4 Tris-borate EDTA (TBE) buffer (pH 8.3) (Peacock and Dingman, 1967)

TBE buffer was used at 1 or 0.5x strength for polyacrylamide and agarose gel electrophoresis, respectively. A 10x strength stock solution was prepared as follows:

Tris-base.....	10.80 g
Boric acid.....	5.50 g
Na ₂ EDTA. 2H ₂ O.....	0.93 g
Distilled water to 100 ml.	

TBE buffer was sterilized by autoclaving.

2.6 Common solutions for DNA isolation and cloning experiments

The following common solutions used in DNA isolation and cloning experiments were prepared according to methods described by Sambrook *et al.* (1989): 0.5 M EDTA, pH 8.0; 1 M Tris-HCl, pH 7.5, 8.0, and 8.8 at 25°C; TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0); 10% (w/v) sodium dodecyl sulphate (SDS); 3 M sodium (Na-) acetate, pH 5.2 and 6.0; 7.5 M ammonium (NH₄-) acetate; buffered phenol-chloroform (1:1, v/v); chloroform-isoamyl alcohol (24:1, v/v); 50% (w/v) polyethylene glycol (PEG)-8000; Solutions I (50 mM D-glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0), II (0.2 M NaOH, 1% SDS), and III [3 M potassium (K-) acetate, 1.8 M formic acid] for plasmid isolation by the alkaline lysis method of Birnboim (1983); and DNase-free RNase A solution (10 mg RNase A per ml of 10 mM Tris-HCl, pH 7.5, 15 mM NaCl).

2.7 Sterilization and containment of biological materials

Heat-stable solutions, polypropylene centrifuge tubes and bottles, microfuge tubes, micropipette tips, glass beads, and toothpicks were autoclaved at 121°C (15

p.s.i.) for 20 min before use. Heat-labile solutions were filter-sterilized through 0.2 μ m sterile membrane filters. Pipettes and disposable Pasteur pipettes were sterilized in a dry heat oven at 180°C for 2 hr 30 min. Biologically contaminated materials were autoclaved at 121°C for 30 min before disposal.

2.8 Maintenance and purification of bacterial strains

Bacterial strains were purified by streaking them separately on suitable agar plates which were incubated at 37°C overnight. Single colonies were restreaked before inoculation onto LB agar slants with or without an appropriate antibiotic in bijoux bottles. The bottles were sealed with Parafilm and stored at room temperature.

2.9 Plating techniques

About 0.1 ml of a bacterial suspension was added to the surface of an agar plate. Then, five to eight sterile glass beads (5 mm diameter) were dropped onto the surface of the agar plate. The plate was shaken gently to roll the beads and thus to spread the bacterial suspension over the agar surface. The beads were removed when no free liquid was evident on the agar surface. The agar plate was incubated at an appropriate temperature.

2.10 Bacterial conjugation

A single colony of a donor or a recipient strain was inoculated into 2 ml of LB broth and incubated at 37°C overnight. After incubation, 0.1 ml of each culture was inoculated into 2 ml of LB broth and agitated at 220 rpm, 37°C for 4 to 5 hr. After these cultures had reached late exponential phase, 0.2 ml of donor and 0.2 ml of recipient cultures were thoroughly mixed in a 1.5 ml microfuge tube and 0.1 ml of this mixture was transferred onto an LB plate. This mating mixture was incubated at 25 or 37°C for 5 hr.

After mating, the surface of the agar was flooded with 1 ml of LB broth or 0.85% (w/v) NaCl with a pipette and the cells were suspended. Aliquots, 0.1 ml each, of this dense cell suspension were plated onto appropriate MacConkey or supplemented M9 plates containing the appropriate antibiotics.

Controls, containing either donor or recipient cells alone, were also subjected to the same treatment to check for background mutation frequencies. Neither the normal donor nor the normal recipient cells should grow on the selective media.

2.11 Small scale preparation of plasmid and RF DNA (miniprep)

This protocol was a modification of the alkaline lysis method of Birnboim (1983). Plasmid and replicative form (RF) of bacteriophage M13mp18 and M13mp19 were extracted from *E. coli* grown overnight (or 5 hr for RF M13mp18 and M13mp19 DNA) in 1.5 ml of LB broth with or without the appropriate antibiotic. The bacterial culture was transferred into a microcentrifuge tube and all subsequent centrifugations were carried out in a microcentrifuge at 15,000 rpm (Model Sigma 112, B. Braun Biotech International, Germany) at room temperature.

The cells were harvested by centrifugation for 1 min. The pellet was resuspended in 100 μ l of ice-cold Solution I (Section 2.6). After incubation on ice for 5 min, the cells were lysed by adding 200 μ l of freshly prepared Solution II (Section 2.6). The lysate was incubated on ice for 5 min before 150 μ l of Solution III (Section 2.6) was added to precipitate SDS and denatured chromosomal DNA.

After incubation on ice for 15 min, the mixture was extracted with buffered phenol-chloroform and centrifuged. The aqueous phase was ethanol precipitated as in Section 2.18. The pellet containing nucleic acids was dissolved in 30 μ l of TE buffer with

DNase-free RNase A (50 µg/ml). The solution was incubated at 65°C for 15 min and then at 37°C for 30 min.

2.12 Large scale preparation of plasmid and RF DNA

The method used for large-scale preparation of plasmid and RF DNA was a scale-up of the 'miniprep' method (Section 2.11). Plasmid was extracted from 200 ml of an overnight bacterial culture in LB broth by the alkaline lysis method of Birnboim (1983) and purified by cesium chloride-ethidium bromide (CsCl-EtBr) density gradient equilibrium centrifugation (Sambrook *et al.*, 1989). The centrifugation was carried out with a Beckman VTi 65 vertical rotor at 55,000 rpm, 18°C, for 5 hr.

After the centrifugation, DNA bands were observed under long wavelength (366 nm) UV light. The lower ccc plasmid band was carefully withdrawn with a syringe by puncturing the quick-seal centrifuge tube at the side. EtBr was removed from the plasmid solution by extracting with isopropanol saturated with water and NaCl. The resulting colourless plasmid solution was dialysed against two changes of 1.6 litres of TE buffer at room temperature.

All recombinant and non-recombinant plasmids and RF DNA of bacteriophages M13mp18 and M13mp19 used in cloning and Southern hybridization experiments were also purified as above.

2.13 Preparation of plasmid from *S. typhi*

Plasmid was extracted from *S. typhi* by the method of Hansen and Olsen (1978) with some modifications. The plasmid was used in the Southern hybridization experiment (Section 2.25).

The plasmid was extracted from 250 ml of an overnight culture grown at 37°C in

LB broth containing Ac, Cm, Ctm, Sm, and Tc. The overnight culture was harvested by centrifugation at 6,000 rpm for 10 min at room temperature. The pellet was suspended in 6 ml of 25% (w/v) sucrose in 50 mM Tris-HCl (pH 8.0). The cells were lysed by addition of 1 ml of lysozyme (10 mg/ml) in 0.25 M Tris-HCl (pH 8.0). The mixture was incubated on ice for 5 min. Then, 2.5 ml of 0.25 M EDTA (pH 8.0) was added and the mixture was incubated at room temperature for another 5 min. Lysis was completed by the addition of 2.5 ml of 20% (w/v) SDS in TE buffer. Then, 0.75 ml of 3 M NaOH was added and the mixture was mixed gently for 3 min. This was followed by the addition of 6 ml of 2 M Tris-HCl (pH 8.0). Three microlitres of 20% (w/v) SDS in TE buffer was added, followed by 6 ml of ice cold 5 M NaCl. The mixture was thoroughly mixed and incubated at 4°C overnight.

The flocculent precipitate was pelleted by centrifugation at 18,000 rpm, 4°C for 30 min. The DNA in the supernatant was precipitated by adding 40% (w/v) PEG-8000 in 0.1 M phosphate buffer (pH 7.0) and incubation at 4°C overnight. The precipitated DNA was pelleted by centrifugation at 7,000 rpm, 4°C for 10 min. The pellet was dissolved in TE buffer with RNase A (10 mg/ml) and the ccc plasmid was purified by CsCl-EtBr density gradient equilibrium centrifugation, as described in Section 2.12.

2.14 Estimation of DNA concentration

The concentration of each nucleic acid solution was obtained by measuring the absorbency of an appropriately diluted solution at 260 nm. The relation $A_{260} = 1$ corresponds to approximately 50 µg of double-stranded DNA/ml and 33 µg of single-stranded DNA/ml. Absorbance measurements at 260 and 280 nm were used to estimate the purity of DNA solutions; a A_{260}/A_{280} ratio of 1.8 indicates a relatively pure DNA

sample.

2.15 Restriction endonuclease digestion of DNA

Restriction endonucleases were used according to the instructions of the manufacturers. Digestions were carried out in 1.5 ml microfuge tubes in a final reaction volume of 10 to 100 μ l, containing 0.5 to 100 μ g of DNA and 1 to 100 units of endonuclease in an appropriate buffer. The reaction mixture was incubated from 2 hr to overnight at 37°C (or 25°C for *Sma*I). The extent of digestion was analysed by agarose gel electrophoresis (Section 2.16).

2.16 Agarose gel electrophoresis

Single and double-stranded DNA was resolved by electrophoresis in submerged horizontal slab gels (0.5 to 1.5%, w/v) in 0.5x TBE buffer (Section 2.5.4) with EtBr (0.5 μ g/ml).

DNA samples were mixed with one-fifth volume of 6x BPB loading dye (Section 2.5.2) or stop mix (Section 2.5.3). Electrophoresis was performed from cathode to anode at constant voltage of 25 to 120 V at room temperature, depending on experiments, until the tracking dye reached the anode end of the gel.

Then, the agarose gel was viewed on a 302 nm UV transilluminator (Model TM-36, UV Products, Inc.) and photographed with a Polaroid MP-4 Land camera fitted with a yellow filter and Polaroid Land 665 black and white films. The exposure times varied from 30 sec to 1 min 30 sec.

Before gel tanks, trays and combs were used for elution (Section 2.17) or Southern blotting (Section 2.25), they were soaked in 0.2 M NaOH for 30 min and then rinsed with distilled water.

2.17 Recovery of DNA fragments from agarose gels

2.17.1 Electroelution (Sambrook *et al.*, 1989)

After electrophoresis, the DNA fragments of interest were cut out from the agarose gel with a sterile blade and transferred into a dialysis tubing filled with sterile 1x TBE buffer (Section 2.5.4). The tubing was then clipped at both ends and immersed parallel to the electrodes, in the horizontal electrophoresis tank. Electroelution was carried out at 110 to 120 V for 30 to 45 min to elute DNA out from the gel into the 1x TBE buffer. Under long wavelength UV light, the EtBr-stained DNA appeared to be stucked on the inner wall of the dialysis bag. The DNA was released by rubbing gently against the wall of the tubing. The electroeluted DNA was extracted twice with buffered phenol-chloroform, ethanol precipitated and then dissolved in an appropriate volume of TE buffer.

2.17.2 Elution of DNA fragments by centrifugation (Heery *et al.*, 1990)

After electrophoresis, a slice of gel containing the DNA fragment of interest was cut and transferred to a punctured sterile 1.5 ml microfuge tube. The microfuge tube was plugged with about 2 to 3 mm of siliconized sterile glass-wool. This was placed on top of another microfuge tube in a two-tiered system and centrifuged at 6,000 rpm for 10 min in a High Speed Centrifuge (Savant Instruments, Inc., U.S.A.). The eluate collected at the bottom of the tube contained the DNA fragment. After the DNA was extracted with buffered phenol-chloroform and ethanol precipitated, it was dissolved in an appropriate volume of sterile distilled water (Section 2.18).

2.18 Phenol-chloroform extraction and ethanol precipitation of DNA solution

Phenol-chloroform extraction and ethanol precipitation of DNA solution were

performed as described by Sambrook *et al.* (1989). DNA solution was extracted with an equal volume of phenol-chloroform (1:1, v/v) (equilibrated to pH 8.0 with Tris-HCl) and the upper aqueous phase was recovered by centrifugation at 15,000 rpm for 15 min. The DNA was precipitated by adding 0.1 volume of 3 M Na-acetate, pH 5.2, and 2 volumes of cold absolute ethanol and incubating at -70°C for 30 min or at -20°C overnight. Precipitated DNA was collected by centrifugation at 15,000 rpm for 15 min. Then the pellet was rinsed with cold 70% (w/v) ethanol and dried under vacuum. The DNA was dissolved in TE buffer or distilled water.

2.19 Ligation of DNA fragments with compatible ends

Ligation of DNA fragments with sticky ends was performed with 1 to 3 Weiss units of T4 DNA ligase and 1x ligation buffer (supplied with the ligase) in a total reaction volume of 10 to 15 μ l.

Insert DNA was combined with 50 to 200 ng of cut vector DNA in molar ratios of 3:1, 1:1, and 1:3. The insert-vector DNA mixtures were heated at 65°C for 10 min, and then cooled to room temperature, before the addition of T4 DNA ligase and 1x ligation buffer. After an overnight incubation at 15°C, the reaction mixture was diluted 50 to 100x with sterile deionized water. The diluted mixture was used for bacterial transformation and transfection.

The general control carried out during all ligation-transformation and transfection experiments were:

- i) linearized vector: to check if any vector was uncut;
- ii) religated linearized vector: to check the efficiency of ligation;
- iii) uncut vector: to check the competency of the competent cells.

2.20 Transformation and transfection of competent *E. coli* by the CaCl₂ heat-shock method

The CaCl₂-heat shock method described in Sambrook *et al.* (1989) with slight modification was used. An overnight culture of *E. coli*, 2 ml, was inoculated into 200 ml of LB broth. The cells were grown with shaking at 250 rpm, 37°C for 2 to 3 hr. The culture was chilled on ice for 10 min, transferred to a cold 250 ml centrifuge bottle, and centrifuged at 6,000 rpm, 4°C for 5 min. The supernatant was discarded and the pelleted cells were gently resuspended in 80 ml of ice-cold 0.1 M CaCl₂. The cell suspension was incubated on ice for 15 to 20 min before being pelleted again. The supernatant was discarded again and the cells were resuspended in 8 ml of ice-cold 0.1 M CaCl₂ for immediate use. Alternatively, the cell suspension was incubated on ice for 12 to 24 hr before use.

Ice-cold competent cells, 100 µl, and 1 to 10 µl of plasmid or ligation mixture were mixed in a pre-chilled tube. The mixture was incubated for 30 min on ice and then heat-shocked at 42°C for 90 sec. LB broth, 1 ml, was added and the cells were incubated at 37°C for 30 to 60 min to allow expression of antibiotic resistance genes. Aliquots of each sample, about 0.1 ml, were plated on LB plates with one or more appropriate antibiotics. The plates were incubated at 37°C for 12 to 24 hr. [Note: Because *E. coli* cells infected with M13 and its derivatives are not lysed by them (Messing, 1983), recombinant M13mp18 and M13mp19 DNA can be transfected into competent *E. coli* cells and the resultant transfectants selected as colonies on selective plates.]

2.21 Transfection of competent *E. coli* DH5 α F' with the RF DNA of bacteriophages M13mp18 and M13mp19

Transfection of the RF DNA of bacteriophages M13mp18 and M13mp19 into competent *E. coli* DH5 α F' was performed as described by Messing (1983).

A small volume of the transfection mix, usually 10, 50, or 140 μ l, was added to 3.0 ml of 0.6% (w/v) B-soft agar containing some early log phase *E. coli* DH5 α F' cells, Xgal, and IPTG. The final concentrations of Xgal and IPTG were 0.27 mg/ml and 0.33 mM, respectively.

The mixture was overlaid on a B-soft agar plate and incubated. Turbid plaques should appear on a lawn of bacteria: 'blue' plaques indicated non-recombinant bacteriophages, whereas 'white' were usually recombinants.

2.22 Detection of transposons

The protocol of Bennett and Grinsted (1988) was followed.

2.22.1 Conjugal transfer of plasmids from *S. typhi* into *E. coli* UB5201 at 25°C

Plasmids pST2 and pST6 from *S. typhi* S2 and S6, respectively, were separately transferred into plasmidless *E. coli* UB5201 through a 5 hr conjugation at 25°C (Section 2.10). [Note: Conjugation was carried out at 25°C because pST2 and pST6 are IncHI plasmids that exhibit temperature-sensitive mode of transfer, i.e., higher transfer frequencies at 25°C than at 37°C.]

For each of the two conjugation mixtures, the resultant *E. coli* transconjugants, each harbouring a plasmid of *S. typhi*, were selected by plating on MacConkey agar containing Nx, Ac, Cm, Ctm, and Tc.

Red colonies [e.g., UB5201 (pST2 or pST6)] growing on the selective plates were tested by toothpicking the colonies onto LB plates containing individual antibiotic, Nx, Ac, Cm, Ctm, Sm, or Tc. This was done to confirm that the *E. coli* transconjugants were resistant to each of the selective antibiotics through the acquisition of the plasmid of *S. typhi* and did not survive because of antagonistic effect of multiple antibiotics used.

2.22.2 Conjugal transfer of plasmid pUB307 from *E. coli* UB1637 into *E. coli* UB5201 harbouring plasmid of *S. typhi* at 37°C

E. coli UB5201 harbouring a plasmid of *S. typhi* (pST2 or pST6) obtained in Section 2.22.1 was conjugated with *E. coli* UB1637 containing plasmid pUB307 (Km^rTc^r). The aim was to transfer pUB307 from UB1637 into *E. coli* UB5201 harbouring pST2 or pST6. The transfer was achieved through a 5 hr conjugation at 37°C (Section 2.10). [Note: Conjugation was carried out at 37°C because pUB307 is transferred at a higher frequency than pST2 or pST6 at 37°C.] For each of the two conjugation mixtures, the resultant transconjugants were first selected on MacConkey agar containing Nx, Ac, Cm, Ctm, Km, Sm, and Tc.

Red colonies [e.g., UB5201 (pST2 or pST6, pUB307)] growing on the selective plates were tested by toothpicking the colonies onto LB plates containing individual antibiotic Nx, Ac, Cm, Ctm, Km, Sm, or Tc. This was done to confirm the presence of both *S. typhi* plasmid and pUB307 in *E. coli* UB5201 [e.g., UB5201 (pST2 or pST6, pUB307)]. The *S. typhi* plasmid would serve as the potential transposon donor and pUB307 as the recipient replicon.

2.22.3 Transposition to pUB307 and analysis of transconjugants for putative transposon markers

From Section 2.22.2, $Nx'Ac'Cm'Ctm'Km'Sm'Tc'$ *E. coli* UB5201 harbouring pUB307 and pST2 or pST6 were obtained. Each of them was stored on Dorset egg slants for two weeks at room temperature for transposition event to take place. At the end of two weeks, each $Nx'Ac'Cm'Ctm'Km'Sm'Tc'$ *E. coli* UB5201 (pUB307 and pST2 or pST6) was used as a donor and conjugated with a plasmidless recipient *E. coli* UB1637 (Section 2.10).

Similar conjugation experiments were also carried out between UB1637 and UB5201(pUB307 and pST8 or pST9), prepared by Kadambeswaran (1993) and kept on Dorset egg slants for more than 2 years at room temperature.

After the conjugation, the cells were resuspended in 1 ml of 0.85% (w/v) NaCl. A series of 1 in 10 dilutions of the suspensions were made in 0.85% (w/v) NaCl. Subsequently, 0.1 ml aliquots of the undiluted suspensions and the serial dilutions were plated (Section 2.9) on (A) supplemented M9 agar containing amino acids (histidine, lysine, and tryptophan), Sm, and Km, and on (B) supplemented M9 agar containing amino acids (histidine, lysine, and tryptophan), Sm, Km, and an antibiotic, e.g., Ac, Cm, or Ctm, appropriate for the putative transposable resistance. The plates were incubated at 37°C for 36 hr. The transposition frequency was expressed as the number of transconjugants exhibiting pUB307- and transposon-mediated antibiotic resistance traits per ml of the mating mixture divided by the number of transconjugants exhibiting pUB307-mediated antibiotic resistance traits per ml of the same mating mixture, i.e., the number of transconjugants per ml of the mating mixture growing on (B) divided by that

growing on (A).

Transconjugants exhibiting pUB307- and transposon-mediated antibiotic resistance traits were toothpicked onto MacConkey agar containing Sm, Km, Ac, Cm, or Ctm. True transconjugants, which were white colonies on these selective MacConkey plates, were toothpicked onto LB plates with appropriate antibiotics to determine their antibiogrammes. The plates were incubated at 37°C for 18 hr. *E. coli* UB1637 was also toothpicked onto each selective plate as a control.

2.22.4 Comparison of pUB307 and recombinant pUB307 carrying putative transposon derived from plasmids of *S. typhi*

Plasmids pUB307 and recombinant pUB307 carrying putative transposon derived from plasmids of *S. typhi* (pST2, pST6, pST8, or pST9) were extracted by the miniprep method (Section 2.11) and analysed by agarose gel electrophoresis (Section 2.16). The same plasmids were also extracted by a large-scale preparation (Section 2.12), then digested with restriction endonuclease *Pst*I, and analysed by agarose gel electrophoresis. An increase in size and changes in the restriction pattern of pUB307 would indicate the acquisition of foreign DNA, i.e., putative transposon.

2.22.5 Transformation and analysis of transformants

Competent *E. coli* DH5 α was transformed with recombinant plasmids carrying putative transposon by the CaCl₂-heat shock method (Section 2.20). The transformed cells were plated on LB plates containing Ac, Ctm, Cm, or Km depending on the resistance phenotype of the transconjugant from which the plasmid was extracted. All the plates were incubated at 37°C for 18 hr.

Transformant colonies growing on different selective plates were toothpicked

onto LB plates, each containing Ac, Cm, Ctm, Sm, Tc, or Km, to determine the acquisition of unselected resistance traits. *E coli* DH5 α was also toothpicked onto each test plate as control.

Plasmids were extracted from some transformants and analysed by agarose gel electrophoresis together with pUB307 and the plasmids that were used in the transformation.

2.23 Isolation of chloramphenicol resistance transposon

2.23.1 Curing of recombinant pUB307 carrying the Ac^rCtm^rSm^r transposon in Ac^rCm^rCtm^rKm^rSm^rTc^r transconjugants

Co-treatment of heat (42°C) and an intercalating agent, ethidium bromide (EtBr) (Stanisich, 1988), was used to remove the recombinant pUB307 carrying Ac^rCtm^rSm^r transposon from Ac^rCm^rCtm^rKm^rSm^rTc^r transconjugants obtained from the matings between UB1637 and UB5201 (pUB307, pST2), UB1637 and UB5201 (pUB307, pST6), and UB1637 and UB5201 (pUB307, pST8).

An experiment was carried out to determine the subinhibitory concentration of EtBr to be used for curing the transconjugants at the growth temperature of 42°C. An overnight culture of Ac^rCm^rCtm^rKm^rSm^rTc^r transconjugant, 20 μ l, was inoculated under dim light into a series of 2 ml of LB broth containing Cm and increasing concentrations of EtBr ranging from 50 to 300 μ g/ml. The cultures were incubated at 42°C for 16 hr in darkness. Twenty microlitres of each culture that showed sign of growth in the highest concentration of EtBr was subcultured into 2 ml of LB broth containing Cm and the same concentration of EtBr. The subcultures were incubated at 42°C overnight in darkness. Ten-fold serial dilutions of each overnight culture were

prepared in LB broth and 0.1 ml aliquots of the appropriate dilutions were plated on LB plates containing Cm.

Following an overnight incubation at 37°C, 100 single colonies were toothpicked onto LB plates containing Km or Cm to detect Km^s cured derivatives. The Km^s cured derivatives were toothpicked onto LB plates containing appropriate antibiotic to determine their antibiogramme. Small-scale plasmid preparation was performed on these Km^s derivatives. The extracts were used to transform *E. coli* DH5α and the transformants were selected on LB plates containing Km (Section 2.20).

2.23.2 Secondary transposition

2.23.2.1 Conjugal transfer of pUB307 from *E. coli* UB5201 into the Km^s cured derivatives of Ac^rCm^rCtm^rKm^rSm^rTc^r *E. coli* UB1637

Through a 5 hr mating at 37°C (Section 2.10), plasmid pUB307 (Km^rTc^r) was transferred from *E. coli* UB5201 into the Km^s cured derivatives of Ac^rCm^rCtm^rKm^rSm^rTc^r UB1637. Transconjugants were selected on MacConkey agar containing Ac, Cm, Ctm, Sm, Tc, and Km. A white single colony from the selective plate was purified by restreaking onto a fresh MacConkey plate containing the selective antibiotics. Plasmids were extracted from the transconjugants and analysed by agarose gel electrophoresis. The newly transferred pUB307 in the resultant transconjugants would serve as the recipient replicon in the transposition of potential donor transposon from the chromosome of *E. coli* UB1637 host.

2.23.2.2 Transposition to pUB307 and analysis of transconjugants for putative transposon markers

The resultant UB1637 transconjugants from Section 2.23.2.1 were separately

streaked onto Dorset egg slants and stored at room temperature for 2 weeks for transposition events to take place. After 2 weeks, the donor culture UB1637 was conjugated with a plasmidless *E. coli* UB5201, as described in Section 2.10.

The resultant UB5201 transconjugants were analysed as in Section 2.22.3, except the selective supplemented M9 agar contained proline, methionine, Nx, Km, and an antibiotic, e.g., Ac, Cm, or Ctm, appropriate for the putative transposable resistance. True transconjugants were red colonies on selective MacConkey plates containing Nx, Km, and Ac, Cm, or Ctm. The antibiogrammes of the transconjugants were also determined by toothpicking them onto LB plates containing an appropriate antibiotic and incubated at 37°C overnight.

The experiment was repeated after the UB1637 transconjugants had been kept on Dorset egg slants for two months.

2.23.2.3 Comparison of pUB307 and recombinant pUB307 carrying putative transposons derived from the chromosome of cured derivatives of Ac^rCm^rCtm^rKm^rSm^rTc^r *E. coli* UB1637

Plasmid pUB307 and recombinant pUB307 carrying putative transposon were extracted from UB1637 transconjugants and analysed as described in Section 2.22.4.

2.23.2.4 Transformation and analysis of transformants

Recombinant plasmids carrying putative transposon from Section 2.23.2.3 were used to transform competent *E. coli* DH5 α and the transformants were analysed as described in Section 2.22.5.

2.24 Cloning of antibiotic resistance genes

2.24.1 Restriction endonuclease analysis of recombinant pUB307 carrying the Ac^rCtm^rSm^r transposon

A recombinant pUB307 carrying the Ac^rCtm^rSm^r transposon from pST8 was designated as pCL8 and it was extracted from an Ac^rCm^sCtm^rKm^rSm^rTc^s *E. coli* UB1637. It was then digested separately with *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, and *Sma*I. It was also digested partially with *Sal*I to determine suitable restriction sites for cloning of antibiotic resistance genes.

All restriction endonuclease digestions were analysed by agarose gel electrophoresis (Section 2.16).

2.24.2 Shotgun cloning of antibiotic resistance genes from recombinant pUB307 carrying Ac^rCtm^rSm^r transposon into plasmid vector pKan

Plasmid pCL8 and plasmid vector pKan were separately digested with *Sal*I. After digestion, 5 µl of each digestion mixture was analysed by agarose gel electrophoresis for completion of digestion. The digested DNA was extracted twice with buffered phenol-chloroform, precipitated and dissolved in 20 µl of sterile deionized water (Section 2.18). Different molar ratios of inserts and vector were ligated as described in Section 2.19.

2.24.3 Transformation and analysis of transformants

After ligation, the ligation mixtures were diluted 50-fold in sterile deionized water and used to transform competent *E. coli* DH5α by the CaCl₂-heat shock method (Section 2.20). The transformed cells were selected on LB plates containing Km and Ac, Ctm, or Sm. Transformants growing on each selective plate were toothpicked onto LB

plates with Km and Ac or Sm and Iso-Sensitest plates with Km and Ctm to determine the acquisition of unselected resistance traits.

Recombinant pKan from each type of transformants with different resistance phenotypes was extracted by the miniprep method (Section 2.11) and compared with pKan by agarose gel electrophoresis. The recombinant pKan was used in secondary transformation of competent *E. coli* DH5 α to confirm the acquisition of new resistance phenotype from the transposon of *S. typhi*.

The recombinant pKan was also digested with *SalI* and compared with *SalI*-digested pKan and *SalI*-digested recombinant pUB307 carrying Ac^rCtm^rSm^r transposon by agarose gel electrophoresis.

2.24.4 Subcloning of Ctm^r gene from *Sau3AI* partially-digested recombinant pUB307 carrying Ctm^r gene

A recombinant pUB307 (designated pCLC600) extracted from a Km^rCtm^r transformant in Section 2.24.3 was partially-digested with *Sau3AI* to subclone the Ctm^r determinants into pKan.

Trial partial digestions were first carried out with *Sau3AI* to optimize the conditions to generate fragments of desired lengths. A fixed quantity (300 ng) of the recombinant plasmid DNA was digested separately with 3 fold serial dilutions of *Sau3AI* (ranging from 1U/15 μ l to 4.11 x 10⁻³ U/15 μ l) at 37°C for 15 min. Each digestion was terminated by adding a one-fifth volume of 6x BPB stop mix and incubating the reaction mixture at 65°C for 15 min and analysed by agarose gel electrophoresis (Section 2.16). The concentration of *Sau3AI* which gave DNA fragments of the required sizes was determined.

A ten fold scale-up partial digestion was then performed with the previously determined concentration of *Sau3AI* at 37°C for 15 min. The reaction was stopped by phenol-chloroform extraction and then the partial digests were ethanol precipitated and dissolved in sterile deionized water.

The vector pKan was digested with *Bam*HI. After the extent of digestion was analysed by agarose gel electrophoresis, the digestion mixture was extracted with phenol-chloroform, ethanol precipitated and dissolved in deionized water.

Different molar ratios of inserts and vector were ligated as described in Section 2.19.

2.24.5 Transformation and analysis of Ctm^r transformants

After ligation, the ligation mixtures were used to transform competent *E. coli* DH5 α cells by the CaCl₂-heat shock method (Section 2.20). Transformed cells were selected on LB plates containing Ctm and Km. Transformants growing on the plates were toothpicked onto Iso-Sensitest plates containing Ctm.

Recombinant pKan from transformants were extracted by the miniprep method and compared with pKan by agarose gel electrophoresis (Section 2.16). Each recombinant pKan was used in secondary transformation of competent *E. coli* DH5 α to confirm the presence of Ctm^r gene. The plasmids were also digested with *Bam*HI, *Eco*RI, or *Sal*I and analysed by agarose gel electrophoresis.

2.25 Southern blotting and hybridization

2.25.1 Solutions for Southern blotting

Depurination solution (0.25 M HCl)

Denaturation solution (0.5 M NaOH, 1.5 M NaCl)

Neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5)

20x SSC (blotting buffer) (3 M NaCl, 0.3 M Na-citrate)

2.25.2 Solutions for hybridization and blot washing

Hybridization buffer [supplied with the ECL Direct Nucleic Acid Labelling and Detection System kit; 0.5 M NaCl and 5% blocking reagent (also supplied) were added before use]

Primary wash solution (without urea) [0.5x SSC, 0.4% (w/v) SDS]

Secondary wash solution (2x SSC)

2.25.3 Vacuum blotting

After electrophoresis and photography of DNA samples on the agarose gel, the gel was soaked in depurination solution with gentle agitation on a Belly Dancer (Stovall Life Science, Inc., U.S.A.) for 10 min. The gel was rinsed briefly with distilled water and soaked in denaturation solution for 30 min with gentle agitation. The gel was rinsed again with distilled water and soaked in neutralization solution with gentle agitation for another 30 min. The gel was rinsed with distilled water.

A piece of nylon membrane (Standard Nylon 66) was cut to the size of the gel and soaked in distilled water, and then in 2x SSC. The DNA fragments on the gel were transferred onto this nylon membrane by vacuum blotting (Olszewska and Jones, 1988) on a vacuum blotter (Trans-Vac TE80, Hoefer Scientific Instruments). A vacuum of 10 inches-Hg was applied. During the vacuum blotting, the gel was covered with a sponge soaked with 20x SSC. After blotting for 25 min, the membrane was rinsed with 2x SSC and air dried.

The nylon membrane (blot) was wrapped in a cling film and the DNA samples

on the membrane were fixed by UV irradiation on a 302 nm transilluminator for 5 min.

2.25.4 Direct labelling of DNA probes

Electroeluted insert DNA (Section 2.17.1) was labelled directly with the enzyme horseradish peroxidase by the ECL Direct Nucleic Acid Labelling and Detection System according to the protocols outlined by the manufacturer Amersham International plc.

Electroeluted DNA probe, about 100 ng in 10 μ l, was boiled for 5 min and immediately cooled on ice for 5 min. Then, 10 μ l of DNA labelling reagent (supplied) was added and mixed, before the addition of 10 μ l of glutaraldehyde solution (supplied). The mixture was thoroughly mixed and briefly centrifuged in a microcentrifuge. The mixture was incubated at 37°C for 10 min.

2.25.5 Southern hybridization

The Southern blot was placed in an Autoblot bottle [35 mm (diameter) x 100 mm (long)], filled with hybridization buffer in a volume of 0.25 ml/cm² membrane. The bottle was fitted to a rotisserie of an Autoblot Micro Hybridization Oven (Bellco Glass, Inc., Vineland, U.S.A.). Prehybridization was carried out at 42°C for 1 hr with gentle rolling. The labelled DNA probe was added to the hybridization buffer. Care was taken to avoid adding it directly on the membrane, and incubation was continued at 42°C overnight, with gentle rolling.

The membrane was washed under high stringency with primary wash buffer, twice, each for 10 min at 55°C. Then, the membrane was washed twice for 5 min at room temperature with secondary wash buffer. After washing, the moist membrane was used for signal generation and detection of target-probe (Section 2.25.6).

2.25.6 Signal generation and detection

Detection reagents I and II (both supplied), 500 μ l each, were thoroughly mixed to give a final volume equivalent to 0.125 ml/cm² membrane. The mixture was added onto the face of the blot containing the DNA and incubated at room temperature for 1 min. Excess detection mixture was drained off and the membrane was wrapped in cling film carefully, avoiding the formation of air pockets.

The wrapped membrane was exposed to a piece of X-ray film (Hyperfilm-ECL, Amersham International plc) in a Kodak X-Omatic cassette for 10 to 15 min at room temperature. The X-ray film was later developed manually in Kodak GBX Developer and Replenisher for 3 min, fixed in Kodak GBX Fixer and Replenisher for 3 min, and rinsed again with water. The film was air dried before the result was documented by photography.

2.26 Cloning with M13mp18 and M13mp19 vectors and construction of nested deletion libraries

2.26.1 Subcloning of a 2.5 kb Ac^r DNA fragment into M13mp19

A recombinant pKan carrying a 2.5 kb Ac^r insert (cloned in Section 2.24.2) and RF DNA of M13mp19 were separately digested with *Sal*I. The excised 2.5 kb insert DNA of *Sal*I-digested recombinant pKan was purified by electroelution (Section 2.17.1). After the *Sal*I-digested RF DNA of M13mp19 was extracted twice with buffered phenol-chloroform and ethanol precipitated, it was dissolved in 20 μ l of sterile deionized water (Section 2.18).

Different molar ratios of insert and vector were ligated as described in Section 2.19. The ligation mixtures were used to transfect competent *E. coli* DH5 α F' (Section

2.20) and transfectant colonies were selected on LB plates with Ac. Subsequently, five isolated colonies growing on LB plates with Ac were separately inoculated into 1.5 ml of LB broth containing an overnight culture of *E. coli* DH5 α F' that had been diluted 1 in 50 with LB broth. The cultures were grown at 37°C with shaking for 5 hr. RF DNA of recombinant M13mp19 was prepared as described in Section 2.11 and analysed by agarose gel electrophoresis (Section 2.16).

One of the Ac^r transfectants was selected for large scale preparation of RF DNA of the recombinant M13mp19 (Section 2.12).

2.26.2 Construction of nested deletion library I

The double-stranded Nested Deletion kit from Pharmacia Biotech was used to construct the nested deletion library. All reaction conditions were performed according to the manufacturer's instructions except the exonuclease III digestion was carried out at 37°C instead of 30°C.

Before the exonuclease digestion, the recombinant M13mp19 RF DNA was digested with *Kpn*I and *Xba*I. The *Kpn*I and *Xba*I ends were, respectively, proximal and distal to the universal sequencing primer site of M13mp19 vector.

After recircularization by blunt-end ligation as described in the instruction manual of the kit, the ligation mixtures were used to transfect competent *E. coli* DH5 α F' prepared according to Hanahan (1985). The transfection mix was prepared as described in Section 2.21 without Xgal, IPTG, and vitamin B1.

Plaques obtained were individually toothpicked onto LB plates and LB plates containing Ac. The same toothpicks were also used to infect a diluted overnight culture of *E. coli* DH5 α F' as described in Section 2.26.1. RF DNA of the deleted recombinant

M13mp19 derivatives were prepared by the miniprep method (Section 2.11) and the viral suspensions in the broth were kept aside.

Ten microlitres of each viral suspension was mixed with 1 μ l of 2% (w/v) SDS and 3 μ l of 0.1% (w/v) bromophenol blue. The size of each deleted recombinant M13mp19 derivative was analysed by agarose gel electrophoresis.

The entire procedure for constructing the nested deletion library was summarized in Fig. 21a and b. A deleted recombinant M13mp19 derivative [M13mp19-Ac^r(1.8)] carrying the smallest Ac^r insert (1.8 kb) was purified by single plaque isolation and its RF DNA was prepared in large scale as described by Sambrook *et al.* (1989).

2.26.3 Subcloning of a 1.8 kb Ac^r DNA fragment into M13mp18

M13mp19-Ac^r(1.8) obtained in Section 2.26.2 was digested simultaneously with restriction endonucleases *Eco*RI and *Hind*III to release the 1.8 kb Ac^r insert. The insert DNA fragment was purified by elution (Section 2.17.2) and force-cloned into M13mp18 as described in Section 2.26.1, except that the deleted M13mp19 derivative and the vector M13mp18 were digested with *Eco*RI and *Hind*III. Clones obtained were confirmed by toothpicking onto LB plates containing Ac. RF DNA of the clones was isolated and analysed by agarose gel electrophoresis. Finally, after purification by single plaque isolation, large scale RF DNA was prepared from the Ac^r recombinant M13mp18 clone.

2.26.4 Construction of nested deletion library II

Construction of nested deletion library II by using the RF DNA of the recombinant M13mp18 from Section 2.26.3 was performed as described in Section

2.26.2. However, before the exonuclease III digestion, the recombinant M13mp18 RF DNA was digested with *Hind*III and *Sal*I. The *Hind*III and *Sal*I ends were, respectively, proximal and distal to the universal sequencing primer site of the M13mp18 vector. The recessed 3' *Hind*III end was filled-in with thionucleotide to protect this end from exonuclease III digestion. Ligation and transfection were performed as described in Section 2.26.2.

The entire procedure for constructing nested deletion library II was summarized in Figure 21.

2.26.5 Complementary test (C-test) to confirm the opposite orientations of inserts in recombinant M13mp18 and M13mp19

Single-stranded recombinant M13mp18 and M13mp19 DNA containing complementary sequences inserted in opposite orientations are capable of hybridizing or annealing to the one another to form a dimer structure resembling the figure-8 (Howarth *et al.*, 1981; Messing, 1983). This hybrid structure has a molecular weight higher than that of either of the parental recombinant DNA and can be detected by agarose gel electrophoresis. This is the C-test (Messing, 1983).

Confirmation of a recombinant M13mp18 and a recombinant M13mp19 harbouring complementary inserts in opposite orientations was achieved by mixing 10 μ l of supernatant containing the recombinant M13mp18 virus particles with 10 μ l of another supernatant containing the recombinant M13mp19 virus particles. The mixture was then combined with 1.5 μ l of 5 M NaCl and 5 μ l of SDS-formamide-dye (Section 2.5.4). The mixture was heated at 68°C for an hour and cooled to room temperature for 30 min. A control set up in which 20 μ l of the supernatant from the recombinant

M13mp18 culture was treated likewise.

The C-test reaction mixture and the control were analysed by agarose gel electrophoresis (Section 2.16). The presence of a slower migrating DNA band in the C-test reaction mixture would indicate hybridization had occurred and each recombinant bacteriophage DNA molecule contained an inserted sequence complementary to that of the other in opposite orientations.

2.26.6 Purification of single-stranded template DNA of recombinant M13mp18 and M13mp19

Cells of an isolated plaque were transferred into 1.5 ml of an overnight culture of *E. coli* DH5 α F' that had been diluted 1 in 50 with 2x TY medium. After the culture was grown for 5 to 6 hr at 37°C with shaking, it was harvested by centrifugation.

The virus particles were recovered from the supernatant by PEG precipitation, and single-stranded bacteriophage M13mp18 and M13mp19 DNA was purified by extractions with TE-saturated phenol followed by phenol-chloroform, as described by Sambrook *et al.* (1989).

The purified single-stranded DNA was dissolved in 20 μ l of TE buffer. It was used for agarose gel electrophoresis (Section 2.1.16) and DNA sequencing (Section 2.27).

2.27 DNA sequencing

2.27.1 Manual Sanger dideoxy chain termination sequencing (Sanger *et al.*, 1977)

The SequenaseTM version 2.0 DNA Sequencing kit from United States Biochemical, U.S.A., was used to determine the nucleotide sequences of cloned inserts. Radioisotope 5'-[α -³³P]dATP or 5'-[α -³³P]dCTP was used in each sequencing reaction.

Single-stranded recombinant M13mp18 and M13mp19 templates carrying inserts of opposite orientations were used for sequencing.

The template-primer annealing and sequencing reactions were performed according to the manufacturer's instructions. The sequencing mixture was used immediately or stored at -20°C until polyacrylamide gel electrophoresis. The mixture was denatured before loading into the sequencing gel.

2.27.2 Preparation of a 6% (w/v) polyacrylamide - 8 M urea sequencing gel

A 6% (w/v) polyacrylamide sequencing gel (0.4 mm thick) was prepared in 1x TBE and 8 M urea as a denaturing agent, according to instructions given in the Instruction Manual, M13 Cloning and Dideoxy Sequencing, GIBCO BRL. Polymerization was initiated with 0.05% (v/v) TEMED and 0.06% (w/v) NH_4 -persulphate.

2.27.3 Polyacrylamide gel electrophoresis

The gel sandwich was secured to a sequencing gel electrophoresis system (Model S2, GIBCO BRL) and the upper and lower reservoirs were filled with 1xTBE buffer. The sample wells formed with sharktooth comb (0.4 mm thick) were flushed with the buffer. A pre-run was performed at constant power of 60 W for 30 min to heat up the gel.

Freshly denatured sequencing reaction mixes (G, A, T, and C reactions), 3 to 4 μl , were loaded into the appropriate wells in the GATC order. The gel was electrophoresed at constant power of 60 W (30 to 45 mA and 1,500 to 1,900 V) until the bromophenol blue dye reached the bottom of the gel.

For second and third loadings (to read a longer DNA sequence), the electrophoresis was stopped when the xylene cyanol dye migrated 2/3 length of the gel for the first and second runs, respectively. Freshly denatured sequencing reaction mixes

were reloaded and electrophoresed.

For reading DNA sequences beyond 350 nucleotides, an 88 cm (gel height) adjustable sequencing gel electrophoresis system (Model SA, GIBCO BRL) was used. The pre-run was performed at constant power of 80 W for 30 min. The gel was electrophoresed at constant power 80 W (25 to 30 mA and 3,000 to 3,800 V) for 12 to 14 hr. Freshly denatured sequencing reaction mixes, 3 to 4 μ l, were loaded in the GATCGTAC order.

2.27.4 Autoradiography of sequencing gels

After electrophoresis, the gel was dried on a dryer (Savant Instrument, Inc.) under vacuum at 80°C for 2 hr.

After drying and cooling, the gel was exposed to a piece of X-ray film (HyperfilmTM - MP, Amersham International plc) in a Kodak X-Omatic film cassette with intensifying screens for 1 to 2 days at room temperature.

The exposed film was developed in Kodak GBX Developer and Replenisher, fixed in Kodak Fixer and Replenisher, and air-dried.

2.27.5 Automated DNA sequencing

The Cy5TM AutoReadTM Sequencing kit from Pharmacia Biotech was also used to determine the nucleotide sequence of cloned inserts. The template-primer annealing and sequencing reactions were performed according to the manufacturer's instructions. The sequencing mixture was used immediately or stored at -20°C until polyacrylamide gel electrophoresis. The mixture was denatured before loading into the sequencing gel.

2.27.5.1 Preparation of 6% (w/v) polyacrylamide -7 M urea sequencing gel

A 6% (w/v) polyacrylamide sequencing gel (0.5 mm thick) was prepared in 1.5x

TBE and 7 M urea as denaturing agent. Polymerization was initiated with 0.05% (v/v) TEMED and 0.05% (w/v) NH₄-persulphate. The gel cassette supplied with the DNA sequencer was assembled according to the user manual of ALFexpress of DNA Sequencer, Pharmacia Biotech.

2.27.5.2 Polyacrylamide gel electrophoresis

The gel electrophoresis conditions and post-run processing of raw data of the automated DNA sequencer were controlled through an AM V3.0 software.

After the gel cassette was mounted onto the DNA sequencer, the thermostatic water circulator was connected to the gel cassette, and the upper and lower reservoirs were filled with 0.5x TBE buffer. After the temperature of the gel cassette was stable at 55°C, the sample wells formed by the 0.5 mm comb were flushed with the buffer. No pre-run was required.

Freshly denatured sequencing reaction mixes, 5 to 10 µl, were loaded in the ACGT order. The running parameters were set at 1,500 V, 40 mA, and 25 W. The electrophoresis was performed for 700 to 1000 min at sampling intervals of 2 sec.

2.27.6 Analysis of nucleotide sequences

The sequences from the autoradiographs (Section 2.27.4) were read from the first band at the bottom of the gel. This gives the sequence in a 5' to 3' direction from the primer.

For the automated DNA sequencing, the Cy5TM fluorescently labelled DNA fragments were detected by a linear array of photodiodes behind the gel on the sequencer during electrophoresis. The raw data were processed by the AM V3.0 software. The processed data could be read from the 5' to 3' direction, 3' to 5' direction, and the

inversed complements of either direction.

The nucleotide sequences obtained from manual and automated DNA sequencing were analysed by means of computer aided software, DNA InspectorTM IIe (Textco, Inc., New Hampshire, U.S.A.) and WebANGIS GCG, and compared with sequences from the EMBL- and Genbank-databases.