CHAPTER TWO
MATERIALS AND METHODS

2.1 Bacterial strains

A multiple antibiotic resistant *S. typhi* (S8) strain, isolated from a local patient admitted to the Kuala Lumpur General Hospital, was used in this study. S8 harbours a conjugative R plasmid that confers $Ac^{R}Cm^{R}Ctm^{R}Sm^{R}Tc^{R}$ (Chong, 1992; Kadambeswaran, 1993).

E. coli DH5α [F^{-}ϕ80*dlacz*ΔM15 *endA1 recA1 hsdR17*($r_k^{-}m_k^{+}$) *supE44 thi-1* λ^{-} *gyrA relA* Δ(*lacZYA-argF*)*U169*] (Woodcock *et al.*, 1989) was used as a host for plasmids and DH5αF'[F'ϕ80*dlacz*ΔM15 *endA1 recA1 hsdR17*($r_k^{-}m_k^{+}$) *supE44 thi-1* λ^{-} *gyrA relA* Δ(*lacZYA-argF*)*U169*] (Liss, 1987) was used as a host for M13. These cells were obtained from Life Technologies, Inc., U.S.A.

2.2 Plasmids and phage

The recombinant plasmids pCLS55 ($Km^{R}Sm^{R}$) and pCL8 ($Ac^{R}Cm^{R}Ctm^{R}Sm^{R}Tc^{S}$), constructed by Mr. Wong H.L. (Wong *et al.*, 1996; Wong, 1999), were used in this study. pST8 was extracted from the multiple antibiotic resistant *S. typhi* S8.

The plasmid cloning vector pUC19 (Ac^{R}) (Vieira and Messing, 1982) was purchased from Pharmacia Biotech., Sweden. pKan (Micklos and Freyer, 1990; Bloom *et al.*, 1995) was obtained from Cabisco Biotech., Carolina Biological Suppl. Co., U.S.A.

The replicative form (RF) of bacteriophage M13mp18 was also obtained from Pharmacia Biotech., Sweden.

2.3 Materials

All chemicals and solvents used were of Analar Grade or of the highest grade available commercially from Aldrich Chemical Co., U.S.A.; BDH Chemicals Ltd.,

England; Boehringer Mannheim, Germany; E. Merck AG, Germany; Fluka BioChemika, Switzerland; GIBCO BRL, U.S.A.; Pharmacia Biotech., Sweden; Riedal-de Haën AG, Germany; Sigma Chemical Co., U.S.A.; United States Biochemical Corp. (USB), U.S.A.; and Williams Ltd., England.

These included boric acid, bromophenol blue, cesium chloride, chloroform, concentrated hydrochloric acid, concentrated sulphuric acid, EDTA disodium salt, ethanol, ethidium bromide, Ficoll 400, glycerol, 8-hydroxyquinoline, isopropanol, phenol, sodium chloride, sodium dodecyl sulphate, sodium hydroxide, Tris-HCl, and other chemicals and solvents.

Acrylamide and N', N-methylenebisacrylamide, for polyacrylamide gels, were from GIBCO BRL, U.S.A. Agarose powder (low EEO) used was SEAKEM LE grade from FMC Corp., U.S.A.

Grade A and Mueller Hinton agar powders were from Becton Dickinson Microbiology Systems, U.S.A. Tryptone was from Oxoid Ltd., England.

Nitrocellulose membrane filters (Type GS, 0.22 μm pore size, 25 mm diameter) were from Millipore Corp., U.S.A. Dialysis tubing Spectra/Por®4 was from Spectron Medical Industries, Inc. Glass beads (5 mm diameter) were from Riedal-de Haën AG, Germany. Centrifuge bottles and tubes were from Nalgene Co., U.S.A. and Quick-seal tubes were from Beckman Instruments, Inc.

Size reference markers, 1 kb DNA ladder, were from GIBCO BRL. Recovery of DNA fragments from agarose gels was performed by using the GENECLEAN II kit obtained from BIO 101, Inc., U.S.A.

Cy5™ AutoRead ™ Sequencing and the Double-stranded Nested Deletion kits were obtained from Pharmacia Biotech., Sweden. The Enhanced Chemiluminescence (ECL) Direct Nucleic Acid Labelling and Detection System used in the Southern hybridization experiments was from Amersham International plc., U.K. Antibiotics used and their sources were listed in Table 1.

Enzymes used in this study are listed in Table 2. Unless otherwise stated, all enzymatic reactions were performed as described by the manufacturers.

Table 1: List of antibiotics

Antibiotic	Salt	Commercial name	Form	Source
Ampicillin (Ac)	Ampicillin sodium	Penbritin	Powder	Beecham Research Lab., England
Chloramphenicol (Cm)	_	_	Powder	Sigma Chemical Co., U.S.A.
Kanamycin (Km)	Kanamycin sulfate	_	Powder	Sigma Chemical Co., U.S.A.
Streptomycin (Sm)	Streptomycin sulfate	_	Powder	Sigma Chemical Co., U.S.A.
Tetracycline (Tc)	Tetracycline hydrochloride	_	Powder	Sigma Chemical Co., U.S.A.

Table 2 : Enzymes used in this study

Enzyme	Source
*Bam*HI	New England Biolabs, Inc.
DNase free-RNase A	Sigma Chemical Co., U.S.A.
*Eco*RI	New England Biolabs, Inc.
*Hin*dIII	New England Biolabs, Inc.
*Kpn*I	New England Biolabs, Inc.
*Pst*I	New England Biolabs, Inc.
*Sal*I	New England Biolabs, Inc.
*Sph*I	New England Biolabs, Inc.
T4 DNA ligase	New England Biolabs, Inc.
*Xba*I	New England Biolabs, Inc.

2.4 Media, antibiotic solutions, and stock solutions

2.4.1 Luria-Bertani (LB) medium (Sambrook *et al.*, 1989)

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 media, 1.5 g of Grade A agar per 100 ml was added.

2.4.2 SOB medium

This medium (Hanahan, 1983, 1985) was used to grow *E. coli* cells 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 $MgCl_2$ was added.

2.4.3 SOC medium

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

SOB medium---------- 100 ml

2 M D-glucose---------- 1.0 ml

2.4.4 Mueller Hinton (MH) agar

MH agar powder ---------- 3.8 g

Distilled water to ---------- 100 ml

The agar suspension was autoclaved before use.

2.4.5 B agar (Sambrook *et al.*, 1989)

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 suspension was autoclaved. For soft agar, agar was added to a final concentration of 0.6% (w/v).

2.4.6 2X TY

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.

2.4.7 Antibiotic stock solutions

All antibiotic stock solutions were prepared according to Table 3. Sterilization was by membrane filtration. Working stocks were stored in the dark at 4°C, while the remainder 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 (Table 3).

2.4.8 Solutions for agarose gel electrophoresis

2.4.8.1 Tris-borate EDTA (TBE) buffer, pH 8.3 (Sambrook *et al.*, 1989)

A 10X strength stock solution was prepared as follows:

Tris-base---------- 10.8 g

Boric acid---------- 5.5 g

$Na_2EDTA.2H_2O$---------- 0.93 g

Distilled water to ---------- 100 ml

TBE buffer was used at 0.5X strength for agarose gel electrophoresis. When necessary, the 0.5X TBE buffer was sterilized by autoclaving.

Table 3: Antibiotic stock solutions

Antibiotic	Stock solution (mg/ml)	Solvent used	Vol. (µl) of stock solution per 100 ml medium	Final concentration (µg/ml)
Ampicillin (Ac)	20	distilled water	250	50
Chloramphenicol (Cm)	25	ethanol (95% v/v)	100	25
Kanamycin (Km)	20	distilled water	100	20
Streptomycin (Sm)	20	distilled water	1000	200
Tetracycline (Tc)	4	distilled water	250	10

2.4.8.2 50X Tris–acetate EDTA (TAE) buffer

Tris-base---------- 48.4 g

Glacial acetic acid---------- 11.42 ml

0.5 M EDTA, pH 8.0----------20.0 ml

Distilled water to----------200 ml

TAE buffer was diluted to 1X and sterilized by autoclaving for gel elution of DNA fragments.

2.4.8.3 6X Bromophenol blue (BPB) loading dye

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

Ficoll 400---------- 9.0% (w/v)

Glycerol---------- 40.0 % (v/v)

For elution of DNA fragments, filter-sterilized 6X gel loading dye was used to avoid contaminating DNA.

2.4.9 Common solutions for DNA extraction, cloning, and transformation

The following common solutions used in DNA extraction 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 8.0 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 acetate, pH 5.2; buffered phenol-chloroform (1:1, v/v); Solution I (50 mM D-glucose, 25 mM Tris-HCl, 10 M EDTA, pH 8.0); Solution II (0.2 M NaOH, 1% (w/v) SDS); Solution III (3 M potassium acetate, 1.8 M formic acid), and DNase-free RNase A solution (10 mg RNase A per ml of 10 mM Tris HCl, pH 7.5, 15 mM NaCl) for plasmid extraction by the alkaline lysis method of Birnboim (1983); (0.15 M NaCl., 0.1 M EDTA, pH 8.0); and (1% SDS, 0.1 M NaCl, 0.1 M Tris-HCl, pH8.0).

2.5 Solutions for hybridization experiments

2.5.1 Solutions for Southern blotting

2.5.1.1 Depurination solution

HCl---------- 0.25 M

2.5.1.2 Denaturation solution

NaOH---------- 0.5 M

NaCl---------- 1.5 M

2.5.1.3 Neutralization solution

Tris-HCl, pH 7.5---------- 0.5 M

Na_3-citrate---------- 0.3 M

2.5.1.4 Solutions for hybridization and blot washing

The hybridization and blot washing solutions were prepared according to the protocols suggested by Amersham International plc., U.K.

2.5.1.5 Hybridization buffer

The hybridization buffer was supplied with the ECL Direct Nucleic Acid Labelling and Detection System. NaCl and the blocking agent (supplied) were added to final concentrations of 0.5 M and 5% (w/v), respectively, before using.

2.5.1.6 Primary wash buffer (without urea)

SDS---------- 4.0 g

20X SSC---------- 25 ml

Distilled water to ---------- 1000 ml

The ingredients were dissolved before autoclaving.

2.5.1.7 Secondary wash buffer (2X SSC)

20X SSC---------- 100 ml

Distilled water to ---------- 1000 ml

The buffer was mixed and autoclaved.

2.5.2 Solutions for labelling DNA probes

Solutions for producing labelled DNA probes were supplied with the ECL Direct Nucleic Acid Labelling and Detection System. The system also contained a DNA labelling reagent with positively charged complexes of peroxidase and glutaraldehyde solution for the preparation of labelled probes.

2.5.3 Signal generation and detection

The ECL Direct Nucleic Acid Labelling and Detection System also contained two detection reagents. Detection reagent 1 is a substrate for peroxidase and detection reagent 2 contains luminol. Luminol produces blue light on oxidation and simultaneously couples the reduction of hydrogen peroxidase. It also has an enhancer, which increases and prolongs the output of light. This light was then detected on an X-ray film.

2.6 Solutions for M13mp18 subcloning

2.6.1 Xgal

5-bromo-4-chloro-3-indolyl-D-galactoside (Xgal) was dissolved in N-N-dimethylformamide to make 20 mg/ml solution. The solution was stored in a tube, which was wrapped up with aluminium foil to avoid damage by light and kept at 4 or -20°C.

2.6.2 IPTG (0.1 M)

Isopropyl-D-thiogalactoside---------- 0.2383 g

Sterile distilled water to ---------- 10 ml

The solution was mixed and sterilized by membrane filtration. The filtered solution was then divided in small aliquots and stored at -20°C.

2.6.3 20% (w/v) PEG, 2.5 M NaCl

40% (w/v) PEG 8000---------- 50 ml

5 M NaCl ---------- 50 ml

2.6.4 SDS-formamide dye mix (Young, 1984)

BPB---------- 0.1% (w/v)

EDTA, pH 8.0---------- 25 mM

SDS---------- 3% (w/v)

Deionized formamide---------- 60% (w/v)

The dye was mixed thoroughly before it was sterilized by membrane filtration.

2.6.5 Frozen storage buffer (FSB)

KCl---------- 7.4 g

$MnCl_2.4H_2O$---------- 8.9 g

$CaCl_2.2H_2O$---------- 1.5 g

$HACoCl_3$---------- 0.8 g

1 M KAc, pH 7.5---------- 10 ml

Redistilled glycerol---------- 100 g

Distilled water to---------- 1 litre

The ingredients were mixed and the pH was adjusted to 6.4 using 0.1 N HCl. The solution was filtered through a pre-rinsed 0.22 μ membrane, aliquoted into sterile containers, and stored at 4°C.

2.7 Sterilization techniques

2.7.1 Heat sterilization

Canisters containing pipettes were sterilized by dry heat in an oven (Gallenkamp BS Oven-Model OV-160) at 180°C for 2 hr.

2.7.2 Steam sterilization

Media and heat-stable solutions were steam sterilized at 121°C (15 p.s.i.) for 20 min in an autoclave. Universal bottles, Millipore membrane filters, polypropylene centrifuge tubes, glass beads, cocktail toothpicks, Swinnex-25 filter holders, disposable micropippette tips, and microfuge tubes were sterilized for 30 min. Biologically contaminated materials were also autoclaved at 121°C for 30 min before cleaning for re-use or before disposal.

2.7.3 Membrane sterilization

Antibiotic stock solutions and other thermolabile stock solutions were sterilized by filtration through sterile membrane filters (Millipore type GS, 0.22 μm, 25 mm) positioned in sterile Swinnex-25 filter holders.

2.8 Plating techniques

Plating was done by aliquoting 100 μl of a bacterial suspension to an appropriate agar plate. Five to eight sterile glass beads (5 mm diameter) were dropped onto the surface of the agar. Then, the plate was shaken gently to roll the glass beads on the surface, thus spreading the bacterial suspension evenly on the agar surface. The beads were later removed when no free liquid was seen present on the agar surface. The plates were incubated in a 37°C incubator overnight.

2.9 Maintenance and purification of bacterial strains

Bacterial strains were purified by streaking them separately on appropriate agar plates, which were then 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.

2.10 Small scale rapid extraction of plasmid DNA (miniprep)

This protocol was a modification of the alkaline lysis method of Birnboim (1983). Plasmid and replicative form (RF) DNA from *E. coli* DH5α and DH5αF' containing pKan, pCLS55, pUC19, M13mp18 were extracted. Extraction was done after growing the cultures at 37°C with shaking at 200 rpm (Orbital Shaker Incubator,

Model 706, Hotech Instruments Corp., Taiwan) overnight (or 5-6 hr for M13mp18 RF DNA) in 1.5 ml of LB broth, with or without an appropriate antibiotic. The bacterial culture was then transferred into a microcentrifuge tube and all subsequent centrifugations were carried out in a microcentrifuge at 15,000 rpm (Model Sigma 112, B. Braun International, Germany) at room temperature.

The cells were harvested by centrifugation for 1 min, and the supernatant was discarded. The pellet was then resuspended in 100 µl of ice-cold Solution I (Section 2.4.9) and incubated on ice for 5 min. Subsequently, the cells were lysed by adding 200 µl of freshly prepared Solution II (Section 2.4.9). The lysate was incubated on ice for 5 min before 150 µl of Solution III (Section 2.4.9) was added to precipitate SDS and denatured chromosomal DNA.

The mixture above was again incubated on ice for 10-15 min before it was extracted with 200 µl of buffered phenol-chloroform by gentle inversions for 5 min. The emulsion was centrifuged for 5 min in a microcentrifuge to separate the mixture into three phases, i.e., the aqueous phase, inter-phase, and organic phase.

The upper aqueous phase containing the plasmid was transferred to a new 1.5 ml microcentrifuge tube and two volumes of ice-cold absolute ethanol was added and mixed. The mixture was centrifuged for 20 min after storage at –70°C for 1 hr. The supernatant was carefully removed. The pellet was rinsed with ice-cold 70% (v/v) ethanol and then dried under vacuum. The DNA was then dissolved in 20 µl of TE containing DNase-free RNase A (50 µg/ml) and incubated for 15 min at 65°C before incubating at 37°C for 30 min.

The resulting DNA was then used for agarose gel electrophoresis, restriction mapping, transformation, and transfection.

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

The method used for large-scale preparation of ccc DNA was a scale-up of the 'miniprep' method (Section 2.10). *E. coli* cells harbouring a plasmid or phage were grown overnight (or 5-6 hr for M13mp18 RF DNA) with shaking at 200 rpm in 200 ml of LB broth, with or without an appropriate antibiotic. The bacterial culture was then incubated on ice for 10 min before it was transferred to a pre-chilled 250 ml centrifuge bottle. The cells were sedimented at 6,000 rpm for 10 min at 4°C in a

Sorvall GSA rotor, and the supernatant was discarded, leaving the cell pellet as dry as possible.

The cells were then resuspended in 4 ml of ice-cold Solution I (Section 2.4.9) by repeated pipetting before incubation on ice for 5 min. Then, 8 ml of freshly prepared Solution II (Section 2.4.9) was added and mixed gently on ice for 5 min. Later, 6 ml of ice-cold Solution III (Section 2.4.9) was added and mixed gently on ice for 10-15 min. Subsequently, 8 ml of buffered phenol-chloroform was added to the ice-cold mixture and swirled gently at room temperature for 10-15 min.

The resultant emulsion was transferred to a 50 ml polypropylene centrifuge tube and centrifuged at 9,000 rpm for 30 min at 20°C in a Sorvall HB-4 swinging-bucket rotor. The upper aqueous layer was transferred to a new 50 ml polypropylene tube and 0.6 to 1 volume of isopropanol was added at room temperature. The solution was left to stand for 10 min at room temperature before centrifugation at 9,000 rpm for 30 min at 20°C in a Sorvall HB-4 swinging-bucket rotor.

The supernatant was removed and the pellet was rinsed with 2 ml of ice-cold 70% (v/v) ethanol. The tube was later centrifuged at 5,000 rpm at 20°C for 5 min in a Sorvall SS34 rotor. The supernatant was discarded and the pellet was dried under vacuum (Speed Vac Concentrator SVC100H, Savant Instruments Inc., U.S.A.) for 10-20 min.

The dried pellet was subsequently dissolved in 1.0 ml of TE containing DNase-free RNase (20 μg/ml) (Section 2.4.9) and incubated at 65°C for 10 min. After 10 min, 2.5 ml of TE was added to the resulting mixture, which was incubated further at 37°C for 30 min.

The extracted DNA was then purified by cesium chloride-ethidium bromide (CsCl-EtBr) density gradient equilibrium centrifugation (Sambrook *et al.*, 1989). 3.5 g of CsCl was dissolved in the extracted DNA solution, which was later transferred to an ultra-clear Quick-seal centrifuge tube by using a Pasteur pipette. The tube was then sealed after filling it with paraffin oil. The density gradients were centrifuged at 18°C, 55,000 rpm for 5 hr in a Beckman VTi65 vertical rotor. At the end of the centrifugation, the tubes were carefully removed from the rotor and secured firmly in an upright position. The DNA bands were viewed by illuminating the tube with a portable long wavelength UV lamp (Model UVGL-15, Ultra-Violet Products, Inc.,

U.S.A.). The lower covalently closed circular (ccc) plasmid band was collected with a 19G hypodermic needle fitted to a 1 ml syringe.

The collected EtBr-CsCl-DNA solution was mixed with five volumes of equilibrated isopropanol in a tube. The tube was left on a rack to allow the mixture to separate into two phases. The upper purple-red organic phase was removed and another five volumes of equilibrated isopropanol was added and mixed vigorously. The mixture was again left on the rack to separate. The lower colourless aqueous phase, containing the ccc DNA, was transferred to a dialysis tubing and dialysed against two changes of 0.8 L of TE buffer at room temperature. The purified DNA was then stored at 4°C after the DNA concentration was measured (Section 2.12). The purified DNA was used for agarose gel electrophoresis, restriction mapping, transformation or transfection, subcloning, double stranded nested deletion, Southern hybridization, single and double stranded DNA sequencing.

2.12 Estimation of DNA concentration

Concentration of nucleic acid solutions was determined by measuring the absorbance of an appropriately diluted solution at 260 nm. 1 A_{260} unit 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, and A_{260}/A_{280} ratio of 1.8 indicates a relatively pure DNA sample (Sambrook *et al.*, 1989).

The quantity of DNA in a nucleic acid preparation can also be estimated by the intensity of the fluorescence emitted by EtBr (Sambrook *et al.*, 1989). After agarose gel electrophoresis and photography of the gel, the intensity of fluorescence of a DNA band of unknown quantity was compared with that of the DNA ladder size markers (Section 2.3) to estimate the quantity of the DNA band.

The amount of fluorescence emitted by the EtBr molecules intercalated into the DNA is proportional to the total mass of the DNA.

2.13 Restriction endonuclease digestions of DNA

Restriction endonucleases were used according to the instructions supplied by the manufacturers. Digestions were carried out in 1.5 ml microcentrifuge tubes in a final reaction volumes of 10 to 100 μl, containing 0.5 to 100 μg of DNA and 1 to 100

units of restriction endonuclease in an appropriate buffer. The reaction mixture was incubated at 37°C (or at 25°C for *Sma*I) for 2 hr to overnight. The extent of digestion was analysed by agarose gel electrophoresis.

2.14 Agarose gel electrophoresis

Single and double-stranded DNA were resolved by electrophoresis in submerged horizontal agarose slab gels (0.5 to 1.0 %, w/v) in 0.5X TBE buffer, pH 8.3 (Section 2.4.8.1), with EtBr (0.5 μg/ml).

DNA samples, e.g., plasmids, double-stranded replicative forms of phages, and DNA fragments generated by restriction endonuclease digestions, were mixed with one-fifth volume of 6X BPB loading dye (Section 2.4.8.3). The mixture was then loaded into the sample well and electrophoresis on agarose gel was performed from cathode to anode at constant voltage of 25 to 120 V at room temperature, depending on the experiment. The electrophoresis was carried out until the tracking dye reached the anode end of the gel.

The agarose gel was then 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 deep orange filter and Polaroid Land 665 black and white films. The exposure times varied from 30 sec to 2 min. The gels were also photographed by using the BIO UVP transilluminator and image analyser (UV Products, Inc.), and printed on thermal papers.

For recovery of the DNA fragments by gel-elution and for Southern blotting, the gel tanks, combs and trays were soaked in 0.2 N NaOH for 30 min and rinsed with distilled water before running the gel.

2.15 Recovery of DNA fragments from agarose gels

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

After electrophoresis, the DNA fragments of interest were cut out from the agarose gel with a sterile blade or glass cover slip, and transferred into a dialysis tubing filled with sterile 1X TBE buffer (Section 2.4.8.1). 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 can be seen attached on the inner wall of the dialysis tubing. The DNA was released by rubbing gently against the wall of the tubing. The electroeluted DNA was then extracted twice with buffered phenol-chloroform, ethanol precipitated, and then dissolved in an appropriate volume of TE buffer.

2.15.2 Recovery of DNA fragments from agarose gels by GENECLEAN II kit

The EtBr-stained DNA was viewed by using the long wavelength UV light after agarose gel electrophoresis in 1X autoclaved TAE buffer (Section 2.4.8.2). Then, the agarose gel containing the DNA band of interest was excised with a sterile blade or glass cover slip. The slice of gel was then transferred to a sterile 1.5 ml polypropylene microfuge tube. The tube was weighed to determine the volume of the gel slice (1 gm equals approximately to 1 ml). DNA was recovered from the agarose gel by using the GENECLEAN II kit according to the manufacturer's instructions.

2.16 Ligation of DNA fragments with compatible ends

A series of ligations that covered different insert to vector molar ratios, e.g., 1:1, 2:1, 5:1, were carried out. The amount of the linearized vector was kept constant while that of the insert DNA was varied accordingly.

The insert-vector DNA mixtures were heated at 65°C for 10 min, and then allowed to cool to room temperature for about 20 min.

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 20 µl. After an overnight incubation at 12 to 15°C, the reaction mixture was diluted 50X with sterile deionized water. The diluted mixture was used for bacterial transformation and transfection.

Appropriate negative and positive controls were included during all ligation-transformation and ligation-transfection experiments:

i) linearized vector without ligase: to check background for ligation efficiency

ii) religated linearized vector: to check the efficiency of ligation

iii) uncut vector: this is a cell competency test

2.17 Subcloning of the streptomycin resistance gene(s) (Sm^R) from *PstI*-digested pCLS55 into pUC19

2.17.1 Comparison of pKan and pCLS55 after restriction endonuclease digestions

Plasmid pCLS55 and pKan were digested with several restriction endonucleases as described in Section 2.13. The restriction fragments were analysed by agarose gel electrophoresis to determine the relative locations of restriction sites in pCLS55 and by comparison of the cleavage patterns with that of pKan.

2.17.2 Preparation of *PstI* digested pCLS55 and pUC19

pCLS55 and pUC19 were digested with *PstI* and the digested fragments were resolved on a 0.8% (w/v) agarose gel, followed by gel elution (Section 2.15).

2.17.3 Shotgun subcloning of the fragment harbouring the Sm^R gene(s) into plasmid vector pUC19

After gel elution of the DNA fragments of interest, ligation was performed as shown in Table 4.

Table 4: Protocols for ligation of the *PstI* digested pCLS55 and pUC19

Ligation mixture	Tube (μl)		
	A	B	C
PstI-digested pUC19	1	1	1
PstI-digested pCLS55	1	2	5
10X ligase buffer	1	1	1
T4 DNA ligase	1	1	1
Sterile distilled water	6	5	2
Total volume	10	10	10

2.17.4 Preparation of competent *E. coli* DH5α cells

The $CaCl_2$ method described in Sambrook *et al.* (1989) was used to prepare competent *E. coli* DH5α cells. An overnight 2 ml culture of *E. coli* DH5α cells was

added to 200 ml of LB broth and grown with vigorous shaking at 240 rpm, 37°C for 2-3 hr. The culture was then chilled on ice for 10 min before it was transferred to a pre-chilled 250 ml polypropylene centrifuge bottle. The cell suspension was centrifuged in a Sorvall GSA rotor at 4°C, 6,000 rpm for 5 min.

The supernatant was discarded and the cell pellet was gently resuspended in 50 ml of ice-cold 0.1 M $CaCl_2$. The cell suspension was incubated on ice for 20 min before it was centrifuged again. The supernatant was discarded and the cells were resuspended in 3 ml of ice-cold 0.1 M $CaCl_2$ for immediate use. Alternatively, the cells can be resuspended in 0.1 M $CaCl_2$, 15% (v/v) glycerol and kept frozen in –70°C until needed.

2.17.5 Transformation

About 100 µl of ice-cold competent cells (fresh or frozen) and 1 to 10 µl of plasmid DNA or ligation mixture were mixed gently in a pre-chilled tube. The mixture was incubated on ice for 30 min before heat-shocked at 42°C for 90 sec. Then, 1 ml of LB broth was added to revive the cells. The cell suspension was subsequently incubated at 37°C for 1 hr to allow the expression of the antibiotic resistance genes.

After the incubation period, the cell suspension was briefly spun in a microcentrifuge and 0.1 ml aliquots of each sample were plated on LB agar plates containing Ac and Sm. The control for ligation and the transformation experiments were plated onto two types of plates, i.e., LB containing Ac and Sm, and LB containing Ac. The plates were then incubated overnight at 37°C.

2.17.6 Analysis of transformants

Transformant colonies growing on the agar plates containing Ac and Sm were purified by toothpicking them onto LB agar plates containing Ac and Sm.

Plasmids were extracted from these Ac^RSm^R transformants by using the miniprep method of Birnboim (1983) (Section 2.10) and compared with pUC19 by agarose gel electrophoresis. Recombinant pUC19 plasmids were identified and digested with *Pst*I and *Sal*I, and analysed by agarose gel electrophoresis.

2.18 Southern blotting and hybridization

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

After agarose gel electrophoresis, the gel containing DNA samples was photographed, and its size measured. The gel was then soaked in the depurination solution (Section 2.5.1.1) with gentle agitation on a Belly Dancer® (Stovall Life Science, Inc., U.S.A.) for 10 min. The acid solution was decanted and the gel was rinsed with distilled water before it was soaked in the denaturation solution (Section 2.5.1.2) with gentle agitation for 30 min. The solution was decanted and the gel was again rinsed briefly with distilled water. The gel was then soaked in neutralization solution (Section 2.5.1.3) with gentle agitation for another 30 min. After that, the gel was rinsed with distilled water.

A piece of nylon membrane (Hybond-N) was cut with both dimensions slightly larger (about 2 mm) than the gel and soaked in distilled water before soaking in 2X SSC (Section 2.5.1.7). The DNA fragments on the gel were transferred to the nylon membrane by vacuum blotting at room temperature for 30 min. After the transfer, the gel was stained with EtBr (0.5 μg/ml) and viewed using a longwave UV light to check if the transfer was complete. The nylon membrane was then rinsed with 2X SSC and blot dried on a piece of 3MM paper. The dried membrane was then wrapped in cling film and the DNA fragments were fixed to the membrane by UV irradiation on a 302 nm transilluminator for 5 min.

2.18.2 Direct labelling of DNA probes

The ECL Direct Nucleic Acid Labelling and Detection System was used to produce labelled DNA probes. This was done accordingly to the protocols outlined by the manufacturer, Amersham International plc., UK. The principles behind the ECL Direct Nucleic Acid Labelling and Detection system are shown in Figure 14.

The eluted DNA insert, about 100 ng in 100 μl, was boiled for 5 min and immediately cooled on ice for another 5 min. Later, 10μl of DNA labelling reagent (Section 2.5.2) was added and mixed thoroughly before 10 μl of glutaraldehyde solution (Section 2.5.2) was added. The final content was briefly centrifuged in a microcentrifuge (Centrifuge 5412, Eppendorf, Germany) and incubated for 10 min at 37 °C. The labelled DNA was then used for hybridization.

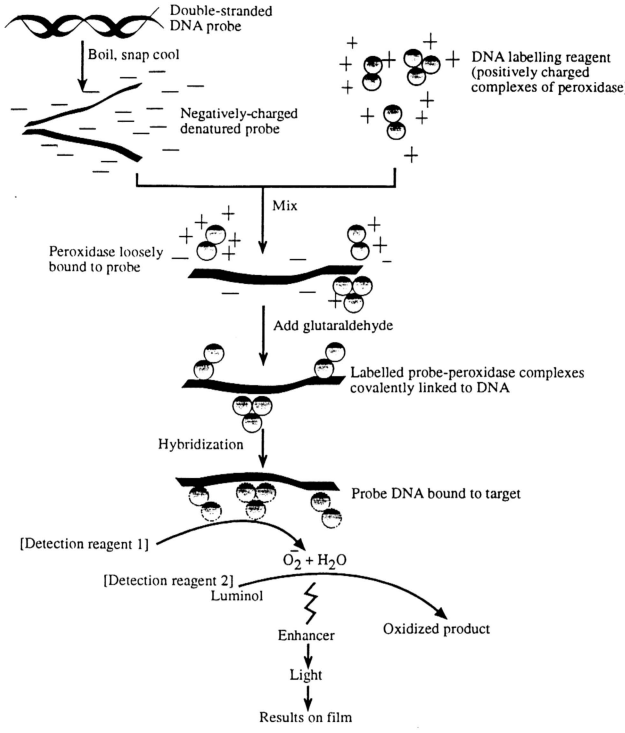


Figure 14: Summary of the principles of the ECL Direct Nucleic Acid Labelling and Detection System. (From the manual of the ECL Direct Nucleic Acid Labelling and Detection System, Amersham International plc., UK.)

2.18.3 Southern hybridization

The Southern blot was placed in an Auto blot bottle [35 mm (diam.) x 100 mm (long)], filled with hybridization buffer (Section 2.5.1.5) to a volume of 0.25 ml/cm^2 membrane. The bottle was fitted to a rotisserie of an Autoblot Micro Hybridization Oven (Bellco Glass, Inc., U.S.A.). Pre-hybridization was carried out at 42°C for 1 hr through continuous gentle rolling. When placing the membranes on to the surface of the hybridization solution, the membrane must be allowed to saturate completely, to expel air from the membrane, before submerging. The labelled DNA probe was added to the pre-hybridization buffer, avoiding placing it directly onto the membrane, and mixed gently. Adding the labelled probe directly onto the membrane can cause high background to develop.

The incubation was continued at 42°C overnight, with continuous gentle rolling. It is important that the temperature of 42°C is not exceeded during hybridization; higher temperatures are deleterious to the enzyme component of the DNA labelling reagent.

2.18.4 Post-hybridization membrane washing

After hybridization at 42°C overnight, the membrane was washed under high stringency with primary wash buffer (Section 2.5.1.6), twice, each for 10 min at 55°C. Then, the membrane was washed twice for 5 min at room temperature with secondary wash buffer (Section 2.5.1.7). After washing, the moist membrane was used for signal generation and detection of target-probe (Section 2.18.5).

2.18.5 Signal generation and detection

Detection reagents I and II (supplied with the System) were thoroughly mixed, 500 µl each, to give the final volume equivalent to 0.125 ml/cm^2 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 gently in cling film to ensure the air pockets are smoothed out.

The wrapped membrane was exposed to a piece of X-ray film (Hyperfim-ECL, Amersham International plc., UK) 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, rinsed with water, fixed with 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. All steps above were carried out in a dark room.

2.19 Subcloning of the 2.5 kb *Eco*RI DNA fragment harbouring the Sm^R genes into M13mp18

2.19.1 Preparation of the *Eco*RI digested M13mp18

M13mp18 RF DNA purified in Section 2.11 was digested with *Eco*RI. The linearized M13mp18 was resolved on a 0.7% (w/v) agarose gel and then gel-eluted following the procedures mentioned in Section 2.15.

2.19.2 Gel-elution of the 2.5 kb *Eco*RI DNA fragment harbouring the Sm^R genes

The DNA fragment harbouring the Sm^R genes was digested with *Eco*RI and the excised 2.5 kb fragment, harbouring the Sm^R gene, was gel-eluted as described in Section 2.15.

2.19.3 Preparation of competent *E. coli* DH5αF' cells

The very high transformation and transfection frequencies protocol of Hanahan (1983, 1985) was used here to prepare competent *E. coli* DH5αF' cells. An overnight culture of *E. coli* DH5αF', 2 ml, was added to 200 ml of SOB broth and grown with vigorous shaking at 250 rpm, 37°C for 2 hr (until the OD_{550} is 0.45-0.55). The culture was chilled on ice for 10-15 min before transferring to a pre-chilled 250 ml polypropylene centrifuge tube. The cell suspension was centrifuged in a Sorvall GSA rotor at 4°C, 3,500 rpm for 12-15 min.

The supernatant was discarded and the cell pellet was gently resuspended in cold FSB (Section 2.6.5) to 1/3 of the original culture volume. The cell suspension was incubated on ice for 10-15 min before pelleted again. The supernatant was discarded again and the cells were resuspended in cold FSB to 1/12.5 of the original culture volume. DMSO was added to the cell suspension to 3.5% (v/v) (i.e., 7 µl of DMSO per 200 µl of cell suspension) and the tubes were swirled for 5-10 seconds before incubating them on ice for 5 min. A second equal aliquot of DMSO was added to the cell suspension to give it a final concentration of 7% of DMSO. The tubes are

kept on ice for 10-15 min before aliquoting 200 µl of the cell suspension into prechilled 1.5 ml microcentrifuge polypropylene tubes. The tubes are kept frozen in -70°C until needed.

2.19.4 Ligation of the *Eco*RI digested M13mp18 with the 2.5 kb *Eco*RI DNA fragment harbouring the Sm^R genes

Ligation of the *Eco*RI-digested M13mp18 with the 2.5 kb *Eco*RI DNA fragment harbouring the Sm^R genes was performed according to the Table 5. All of the procedures and conditions for the ligation were carried out as described in Section 2.16. The ligation mixtures were used to transfect competent *E. coli* DH5αF' cells prepared as described in Section 2.19.3.

Table 5: Protocols for ligation of the 2.5 kb *Eco*RI DNA fragment harbouring the Sm^R genes with *Eco*RI-digested M13mp18

Ligation mixture	Tube (µl)	
	A	B
2.5 kb *Eco*RI DNA fragment harbouring the Sm^R genes	5	5
*Eco*RI-digested M13mp18	3	5
10X ligase buffer	1.5	1.5
T4 DNA ligase	1.5	1.5
Sterile distilled water	4	2
Total volume	15	15

2.19.5 Transfection of *E. coli* DH5αF' with the RF DNA of M13mp18

Transfection of the RF DNA of M13mp18 into competent *E.* coli DH5αF' cells was performed as outlined by Messing (1983).

An aliquot of the transfection mix, usually 10, 50, or 150 µl, was added to 3 ml of 0.6 % (w/v) B-soft agar containing some early log phase *E.* coli DH5αF' cells, Xgal, and IPTG. The final concentrations of Xgal and IPTG were 0.27 mg/ml and 0.33 mM, respectively. The mixtures was overlaid on B-agar plates and incubated.

Isolated plaques will be selected on the basis of their colourless aspect to grow the recombinant phages and perform sequencing reactions.

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

Single-stranded recombinant M13mp18 DNA containing complementary sequences inserted in opposite orientations are capable of hybridizing or annealing to one another to form a dimer structure resembling the figure-8 (Howarth *et al.*, 1981; Messing, 1983). This hybrid structure has molecular weight higher than that of either of the parental recombinant DNA (Messing, 1983), and can be detected by agarose gel electrophoresis. This is the complementary or C-test (Messing, 1983). The principle of the C-test is shown in Figure 15.

Confirmation of recombinant M13mp18 clones harbouring complementary inserts in two directions was performed by mixing 10 µl of each supernatant of the recombinant M13mp18 clones. The mixture was then combined with 1.5 µl of 5 M NaCl and 5 µl of SDS-formamide-dye (Section 2.6.4). The mixture was heated at 68°C for an hour and cooled to room temperature for 30 min. A control was set up in which 20 µl of the supernatant from the recombinant M13mp18 culture was treated likewise.

The C-test reaction mixture and the control were analysed by agarose gel (0.7%, w/v) electrophoresis (Section 2.14). The presence of a slowly migrating DNA band in the C-test reaction mixture would confirm that hybridization had occurred and each recombinant phage DNA molecule contained an inserted sequence complementary to that of the other in opposite orientations.

2.19.7 Purification of single-stranded template DNA of recombinant M13mp18

Supernatant, 1 ml, containing phage particles was mixed with 1.5 ml of an overnight *E. coli* DH5αF' cell culture that had been diluted 1 in 50 with 2X YT medium. The mixture was left in room temperature for 10 min before inoculating it into 250 ml of LB broth. The culture was then grown in for 5-6 hr at 37°C with aeration. The phage particles were recovered from the supernatant by PEG precipitation and single-stranded recombinant M13mp18 DNA was purified by extraction with TE-saturated phenol followed by phenol-chloroform, as described by

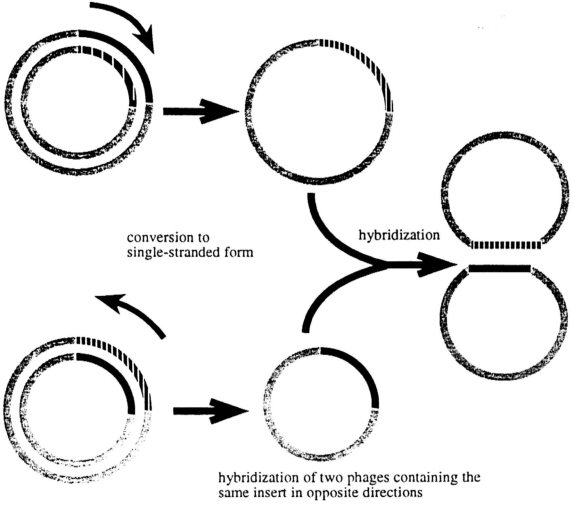


Figure 15: Principles of the C-test. (From Sambrook *et al.*, 1989.)

Sambrook *et al.* (1989).

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

2.20.1 Preparation of the *SalI* digested pCLS55 and pUC19

pCLS55 and pUC19 were digested with *SalI* and the digested fragments were resolved on a 0.8% (w/v) agarose gel, followed by gel elution (Section 2.15).

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

After gel elution of the DNA fragments, ligation of the *SalI*-digested pUC19 and the 5.5 kb *SalI* digested DNA fragment from pCLS55 was performed as shown in table 4 (Section 2.17.3). The ligation mixtures were used to transform competent *E. coli* DH5α cells prepared as described in Section 2.17.4.

2.20.3 Transformation

Transformation protocol for the above mentioned ligation mixtures was outlined in Section 2.17.5.

2.20.4 Analysis of transformants

Transformants colonies growing on the agar plates containing Ac and Sm were purified by toothpicking them onto LB agar plates containing both Ac and Sm.

Plasmids were extracted from these $Ac^{R}Sm^{R}$ transformants by using the miniprep method of Birnboim (1983) (Section 2.10) and compared with pUC19 by agarose gel electrophoresis. Recombinant pUC19 plasmids were identified and digested with *SalI*, and analysed by agarose gel electrophoresis.

2.21 DNA sequencing

2.21.1 Non-isotopic automated DNA sequencing

The Cy5™ Autoread™ Sequencing kit from Pharmacia Biotech was used to determine the nucleotide sequence of either double stranded or single stranded DNA 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 attached to the ALFexpress DNA Sequencer (Pharmacia Biotech., U.S.A.).

In addition, double stranded DNA sequencing was also performed on recombinant pUC19 by using the Thermo sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP from Amersham International plc., UK. Cycle sequencing of the double stranded DNA were performed on Perkin Elmer GeneAmp PCR system 9600 or system 2400.

M13 universal and reverse Cy5-labelled primers, which were provided by the supplier, were used in the sequencing reactions above. Subsequent sequencing reactions were performed using four other unlabelled primers that were synthesised by Genosys, U.S.A. Before the sequencing reactions were carried out, the unlabelled synthesised primers were incorporated with Cy5-labelled dATP in a brief labelling step.

The 24-mer oligonucleotides internal primers used to complete the sequence of both strands are: (R13) 5'-CGTCCGCCATCTGTGCAATGCGTC-3' (nucleotide positions 691 to 714 in Figure 38), (R132) 5'-CGAAGGCGCGCTCTGCTTCATCT-3' (nucleotide positions 1457 to 1480 in Figure 38), (R16) 5'CGGCTCGGAACAGCAGATCGCTAT-3' (nucleotide positions 708 to 731 in Figure 39), and (R162) 5'-CCTGCCTTCTGCCCTTCTCCCGAT-3' (nucleotide positions 1246 to 1269 in Figure 39).

2.21.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed on a 0.5mm thick polyacrylamide-7 M urea sequencing gel prepared accordingly to the manufacturer's instructions. As an alternative, polyacrylamide gel, Long Ranger™ gel (FMC Bioproducts, U.S.A.), was also used particularly to get extended length of readable sequences. The gel electrophoresis conditions and post-run processing of raw data of the automated DNA sequencer were controlled through ALFwin 1.10 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 1X TBE buffer. After the temperature of the gel cassette was stable at 52°C, the sample wells formed by the 0.5 mm comb were flushed with the buffer.

Freshly denatured sequencing reaction mixes, 5-10 µl, were loaded in the ACGT order. The running parameters were set at 1500 V, 60 mA, and 30 W. The electrophoresis was performed for 800 min at sampling interval of 2-3 sec.

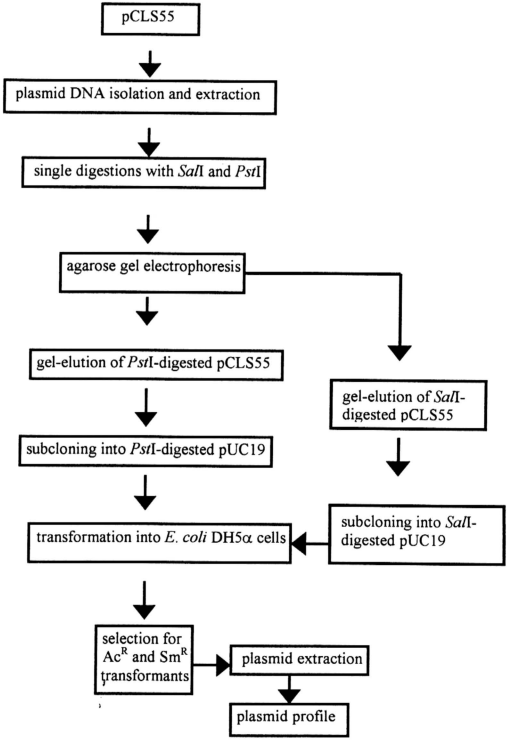
2.21.3 Analysis of nucleotide sequences

The Cy5™ 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 ALFwin 1.10 software. The sequence data generated was compiled using GeneJockey programmes until a continuous sequence had been obtained. Both strands were completely sequenced and, as a consequence of the sequencing method, each base was sequenced on average four times.

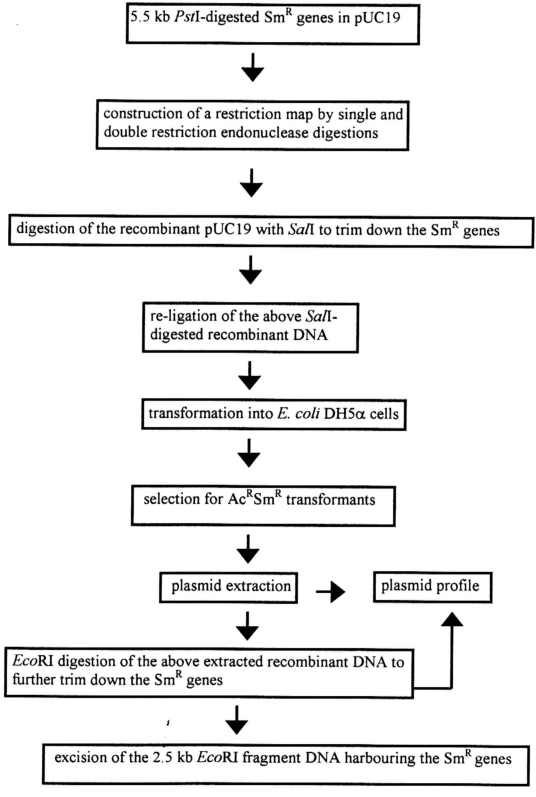
The nucleotide sequences obtained from automated DNA sequencing were analysed by using appropriate software and compared with sequences from the EMBL- or Genbank-databases by using BLAST similarity searches. The nucleotide sequences reported in this work are pending accession numbers from the GenBank database.

2.22 Flowcharts

2.22.1 Flowchart of experiments to subclone the Sm^R genes into pUC19



2.22.2 Flowchart of experiments to construct a restriction map of the 5.5 kb insert and to locate the Sm^R genes on a smaller fragment.



2.22.3 Flowchart of experiments to subclone the Sm^R genes into M13mp18 vector, sequence the Sm^R genes, and analyse the nucleotide sequences.