CHAPTER II

MATERIALS AND METHODS

2.1. Bacterial strains and plasmids

The bacterial strains used in this study and their descriptive characteristics were listed in Table 2.1.

Table 2.1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or Reference
K. pneumoniae M5a1	*nif*$^+$	Dixon *et al.* (1976)
K. oxytoca NG13	*nif*$^+$	Kazuo Komagata ‡
K. R15	*nif*$^+$	Poontariga Harinasut (1981)
K. R17	*nif*$^+$	Poontariga Harinasut (1981)
pAM51	Tc^r, *glnA*	Alvarez Morales *et al.* (1984)
pBR322	Am^r, Tc^r	Bolivar *et al.* (1977)
pRD1	Carb^r, Km^r, Tc^r, *nif*$^+$	Dixon *et al.* (1976)
pSA30	Tc^r, *nif*HDKTY	Cannon *et al.* (1979)

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2.2. Media and growth condition

2.2.1. Luria - Bertani (LB) medium (Maniatis et al, 1982)

Tryptone	10 g
Yeast extract	5 g
Sodium chloride, NaCl	10 g
Distilled water to	1 l
adjust pH to 7.0 with NaOH	
Agar	15 g per l (for solid medium)

2.2.2. Cultivations

All bacterial strains were grown on LB agar plate or in LB broth with shaking at 37^{0}C. For E. coli cultures, kanamycin (Km), 15 µg/ml and tetracycline (Tc), 12.5 µg/ml were used for maintaining plasmid. Long term stock cultures were maintained in 50 % LB-glycerol medium at -70°C.

2.3. Reagents and buffers

All chemicals used were laboratory grade or otherwise specified.

2.3.1. For gel electrophoresis

TEB buffer : 89 mM Tris, 2.5 mM EDTA, 89 mM boric acid, pH 8.3

Agarose gel (Type II Sigma), 0.7 % in TEB buffer

Tracking dye solution : 0.1 % bromphenol blue, 5 mM EDTA, 40% Ficoll 400

Staining solution : 2.5 µg/ml ethidium bromide

DNA markers : Lambda (λ) DNA digested with HindIII (λ/HindIII)

2.3.2. For plasmid detection and purification

Solution A : 10 % sucrose in 25 mM Tris, 10 mM EDTA, pH 8.0, 0.2 µg/ml RNase, and 5 mg/ml lysozyme

Solution B : 5% sucrose, 4 % SDS

Lysing solution : 3 % SDS, 50 mM Tris, pH 12.6

Phenol - chloroform solution, 1:1 (V/V)

PEM buffer : 5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0

TEG : 25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8.0

Lysozyme, 5 mg/ml in TEG

SDS - NaOH solution : 1 % SDS in 0.2 M NaOH

Sodium acetate, 3 M, pH 4.8

Absolute ethanol

TE buffer : 10 mM Tris, 1 mM EDTA, pH 8.0

Cesium chloride

Ethidium bromide, 10 mg/ml

Water - saturated butanol

2.3.3. For purification of chromosomal DNA

TES : 20 % sucrose, 50 mM Tris, 50 mM EDTA, pH 7.6

Lysozyme, 10 mg/ml in TE buffer

RNase, 10 mg/ml in TE buffer

SDS, 25 %

Proteinase K, 2 mg/ml

Chloroform/isoamyl alcohol solution, 24:1 (V/V)

NaCl, 5 M

Absolute ethanol

Ethanol, 80 %

TE buffer, pH 8.0

2.3.4. For digestion of DNA

Restriction endonuclease (Type II) : BamHI, BglII, EcoRI, HindIII, PstI, SalI, SmaI and XhoI (from BRL)

10 x concentrate buffer (supplied with restriction enzymes from BRL)

2.3.5. For recovery of DNA fragments from low-melting temperature agarose gel electrophoresis

Low-melting temperature agarose (Type VII Sigma) : 1 % in TEB buffer

Phenol solution : saturated phenol in TE, pH 8.0 with 0.1 % 8-hydroxy-quinoline

Chloroform/isoamyl alcohol solution

Water-saturated ether

2.3.6. For labelling of DNA by nick translation reaction

NEK 004, nick translation system (from Biotechnology Systems, NEN Research Products of Dupont):

3,000 Ci/mmol, 10 µCi/µl [α-^{32}P] dCTP in 10mM tricine, pH 7.6

Cold dATP, dGTP and dTTP mixture, each at a concentration of 100 µM in 50 mM Tris, pH 7.5

Nick translation buffer, 50 mM Tris, 10 mM $MgCl_2$, 50 µg/ml bovine serum albumin (BSA), 1 mM dithiothrietol (DTT), pH 7.4

DNA polymerase I, 0.6 units/µl

DNase I, 0.2 units/ml

Nick translation stop buffer, 10 mM EDTA, 0.1 % SDS, 100 µg/ml salmon sperm DNA

Trichloroacetic acid (TCA), 10 % and 5 %

Ethanol, 95 %

Bray's solution : 6 % naphthalene, 0.4 % PPO, 0.02 % POPOP, 10 % methanol and 2 % ethylene glycol in dioxane

Sodium - magnesium acetate : 1 M sodium acetate, 20 mM magnesium acetate, pH 7.7

Absolute ethanol

2.3.7. For Southern blot transfer

Hydrochloric acid (HCl), 0.25 M

Denaturation solution : 0.5 M NaOH, 1.5 M NaCl

Neutralization solution : 0.5 M Tris, 1.5 M NaCl, pH 7.5

20 x SSC : 3 M NaCl, 0.3 M tri-sodium citrate, pH 7.0

1 X SSC : 0.15 M NaCl, 15 mM tri-sodium citrate, pH 7.0

2.3.8. For Southern hybridization and washing

5 x SSC : 0.75 M NaCl, 75 mM tri-sodium citrate, pH 7.0

5 x Denhardt's solution : 0.1 % Ficoll 400, 0.1 % polyvinyl pyrrolidone, 0.1 % BSA fraction V

Tris, 5 mM pH 8.0

Formamide, 50%

Calf thymus DNA, 100 µg/ml

3 x SSC : 0.45 M NaCl, 45 mM tri-sodium citrate, pH 7.0

3 x SSC, 1 mM EDTA and 0.1 % SDS solution

0.1 x SSC, 1 mM EDTA and 0.1 % SDS solution

2.4. Detection and preparation of plasmid DNA

Three conventional methods were used. Two rapid procedures were described for detecting plasmid in bacterial strains within 3 to 4 h. The last one was used for large scale preparation of plasmid DNA.

2.4.1. Modified Eckhardt lysate electrophoresis (Eckhardt, 1978)

One single colony of bacterial culture from LB agar plate was suspended in 25 µl of solution A. After this lysozyme treatment, 25 µl of spheroplast suspension was pipetted into the front slot of 0.7 % agarose gel plate (100 x 80 x 5 mm in size) containing two slots for each array. Then, 50 µl of solution B was added into the concomitant back slot. Lambda DNA digested with HindIII was used as molecular weight markers. The electrophoresis buffer (TEB) was poured into the chamber until the level of buffer reached the gel edge. The samples were electrophoresed at 15 volts for 30 min, then 80 volts for 3 h. After electrophoresis, the gel was stained by submerging in staining solution for 20 min and destained with distilled water. The DNA bands were visualized and photographed under UV light on a Kodak tri-X film.

2.4.2. Rapid procedure (Kado and Liu, 1981)

Two colonies were picked up from culture plate and suspended in 50 µl of lysing solution. The lysate was subsequently heated at 55 or 65°C for 0, 30 and 60 min in a water bath, followed by extraction with an equal volume of phenol-chloroform solution. The solution was emulsified by shaking briefly and the emulsion was broken by centrifugation at 2,000 x g, 4°C for 5 min. Then, 20 µl of aqueous phase was mixed with 5 µl of tracking dye solution and loaded on a 0.7 % agarose gel for electrophoresis in TEB buffer at 80 volts for 2-3 h.

2.4.3. Large scale preparation of plasmid by rapid alkaline extraction (Birnboim and Doly, 1979)

Cells from log-phase culture (250 ml) were harvested by

centrifugation at 5,000 x g, 4°C for 20 min and washed once with PEM buffer, pH 8.0. The packed cells were resuspended in 5 ml of solution containing 5 mg/ml lysozyme in TEG, pH 8.0 and left on ice for 30 min. Then, 10 ml of freshly prepared 1 % SDS in 0.2 M NaOH was added into the lysate on ice for 5 min. The mixture was neutralized by adding 7.5 ml of 3 M sodium acetate, pH 4.8, mixed by gently inversion for several times and left on ice for one hour. After centrifugation at 20,000 x g, 4°C for 20 min, the plasmid DNA was fractionated by adding 2 volumes of cold absolute ethanol and stored at -20°C overnight. The DNA pellet was obtained by centrifugation at 12,000 x g, 4°C for 10 min and then redissolved in TE buffer, pH 8.0.

For further purification, 9 g of cesium chloride and 9 ml of plasmid DNA solution were mixed in each 13.5 ml polyallomer tubes. When cesium chloride was completely dissolved, 100 µl of 10 mg/ml ethidium bromide was added and mixed thoroughly. The tubes were capped and put into a rotor (Beckman type 70.1 Ti). Isopycnic centrifugation was performed at 45,000 rpm, 20°C for 24 h. After centrifugation, the tube was punctured with 18 G needle and the band of plasmid was drawn into a 5-ml syringe under UV light. Ethidium bromide was removed from the DNA solution by water-saturated butanol extraction. To remove cesium chloride, the solution was dialysed against TE buffer, pH 8.0. The plasmid DNA was recovered by adding 1/10 volume of 3 M sodium acetate plus 2 volumes of absolute ethanol and stored at -20°C overnight. After centrifugation, the DNA pellet was redissolved in TE buffer and quantified by measuring absorbance at 260 nm (1 A_{260nm}=50 µg/ml of nucleic acids), and checked for purity by the ratio A_{260nm}/A_{280nm} > 2.0.

2.5. Isolation and purification of chromosomal DNA (modified from Rodriguez and Tsit, 1983)

Cells were harvested from culture (100 ml) at early stationary phase by centrifugation at 5,000 x g, 4°C for 20 min and washed once with PEM, pH 8.0. The packed cells were frozen at -70°C for 10 min and thawed in warm water before resuspending in 2 ml of TES, pH 7.6 and put on ice. Cell lysis was performed by adding 200 µl of 10 mg/ml lysozyme and 100 µl of 10 mg/ml RNase in TE, pH 8.0. The mixture was incubated on ice for 15 min. The cell lysate was poured into a 25-ml screw-cap tube and 50 µl of 25 % SDS was added. After mixing by inversion, the mixture was incubated at 37°C with gently shaking for 3-6 h. After the addition of 300 µl of 2 mg/ml proteinase K and 1.5 ml of chloroform/isoamyl alcohol solution, the mixture was further incubated at 37°C with gently shaking for overnight. Then, 1 ml of sterile distilled water and 2 volumes of chloroform/isoamyl alcohol solution were added into the mixture. After mixing by gently inversion, the mixture was centrifuge at 1,000 x g for 20 min. The aqueous (upper) phase was removed into a new tube and the chloroform/isoamyl alcohol extraction was repeated twice. The chromosomal DNA in aqueous phase was precipitated by adding 1/25 volume of 5 M NaCl plus 2 volumes of ethanol. After gently inversion, the content was placed at -20^0C for 10 min. Fibrous strands of DNA were spooled out with a glass rod and dipped in 80% alcohol to remove excess salt. The DNA was allowed to air-dry and redissolved in TE buffer, pH 8.0. The amount and purity of DNA were checked by measuring A_{260nm}/A_{280nm}.

2.6. DNA manipulation by restriction enzymes (Maniatis et al., 1982 and Blakesley, 1983)

The restriction endonucleases used in this study are shown in Table 2.2, with their recognition sequences.

Table 2.2 Recognition sequences of type II restriction endonucleases

Enzyme	Recognition sequence
BamHI	G/GATCC
BglII	A/GATCT
EcoRI	G/AATTC
HindIII	A/AGCTT
PstI	CTCGA/G
SalI	G/TCGAC
SmaI	CCC/GGG
XhoI	C/TCGAG

2.6.1. Chromosomal DNA digestion

The digestion of chromosomal DNA was performed in 20-100 µl reaction mixture containing 1-3 µg of DNA, 10-30 units of restriction enzymes (from BRL), appropriate buffer (as recommended by the suppliers) and H_2O to adjust the volume. The reaction mixture was incubated at 37^0C overnight. After digestion, the cohesive end of DNA was denatured at 65^0C for 2 min and analysed by agarose gel electrophoresis.

2.6.2. Plasmid DNA digestion

To assure that the plasmids used in this study are pAM51 and pSA30, the purified plasmid were characterized by the sizes of DNA fragments after restricted digestion. The experiment was performed by digesting 1 µg of the plasmid DNA with 5 units of restriction enzymes (BamHI, EcoRI and HindIII) in 20 µl reaction mixture containing the appropriate buffer at 37°C for 2 h. After digestion, the sizes of DNA fragments were determined by electrophoretic mobility on 0.7% agarose gel in TEB, pH 8.3.

2.7. Recovery of DNA fragments from low-melting temperature agarose gel electrophoresis (modified from Maniatis et al., 1982)

After digestion with proper restriction enzymes, 20 µg of plasmid DNA was loaded on 1% low-melting temperature agarose (Type VII Sigma) and electrophoresis was carried out at 80 volts for 3 h. The region of gel containing the wanted DNA fragments estimated from DNA markers were carefully cut from the whole gel plate and put into 5 volumes of TE buffer. After heating at 65°C for 20 min, the melted gel was extracted with an equal volume of phenol solution. The aqueous phase was recovered by centrifugation and reextracted with chloroform/isoamyl alcohol solution followed by extraction with water-saturated ether. After ethanol precipitation in the presence of 0.2 M NaCl, the recovered DNA was dissolved in TE buffer.

2.8. Labelling of DNA probes with [α-^{32}P] dCTP by nick translation reaction (Rigby, 1977 and Maniatis et al., 1982)

The 30 µl reaction mixture contained : 10 µl of 3,000 Ci/mmol, 10 µCi/µl [α-^{32}P] dCTP ; 5 µl of nick translation buffer ; 4 µl of 100 µM dATP, dGTP and dTTP mixture in 50 mM Tris, pH 7.4 ; 500 ng of plasmid

DNA (volumes was adjusted up to 7 µl with distilled water) ; 2 µl of 600 units/ml DNA polymerase I and 2 µl of 0.2 units/ml of DNase I to start reaction. All additions were carried out on ice and then incubated at 12-14°C in a thermobath until maximum incorporation of the radioactive ^{32}P into the DNA was obtained.

For kinetic studies, the progress of the reaction was followed by determining the accumulation of acid insoluble radioactivity with time. Measuring of the incorporation of [α-^{32}P] dCTP into acid insoluble product was done by sampling 1 µl aliquot at appropriate time intervals (30, 60, 90 and 120 min) and putting in prelabelled tube containing 200 µl nick translation stop buffer. A 25 µl aliquot was withdrawn from each tube and dispensed into a separate tube containing 2 ml of cold 10% TCA. The precipitated DNA was collected on 10% TCA - soaked Millipore HA filter disc by using a vacuum filtration apparatus. The filter was washed repeatedly with 20 ml of cold 5% TCA and 95% ethanol respectively. The filter was dried in a scintillation vial and counted in 5 ml of Bray's solution for incorporated radioactivity. To measure the total count of radioactivity added into the incubation mixture, another 25 µl aliquot was spotted into another vial and counted as described previously.

When the time required for maximum incorporation of [α-^{32}P] dCTP into the DNA was obtained, the entire mixture was stopped by adding 200 µl of stop buffer. The labelled DNA was separated from free dCTP by passing through a Sephadex G-50 spun column. The eluted DNA fraction was precipitated by adding 1/10 volume of 1 M sodium acetate and 20 mM magnesium acetate, pH 7.7 plus 2.5 volumes of cold ethanol and kept at -20°C overnight. The labelled DNA was resuspended in 100 µl of TE buffer and denatured by boiling for 5 min and immediately cooled on ice.

2.9. Southern blot transfer (Southern, 1975 and Meinkoth and Wahl, 1984)

After digested chromosomal DNAs were separated by electrophoresis on agarose gel (12 x 15 x 0.5 cm in size), the gel was photographed. The DNA in the gel was depurinated (partial hydrolysis) by soaking in 0.25 M HCl for 15 min. This process induces cleavage, so facilitating the transfer of large DNA fragments. The gel was rinsed with water and then transferred to denaturation solution (0.5 M NaOH, 1.5 M NaCl) twice with constant shaking for 25 min each. After neutralization for 25 min, twice, with 0.5 M Tris pH 7.5, 1.5 M NaCl, the DNA was transferred to a nitrocellulose membrane (from Bio-Rad) by the capillary action in the presence of 20 x SSC overnight at room temperature. After blotting, the membrane was removed from the agarose gel and soaked in 1 x SSC for 5 min. The membrane was blotted between Whatman 3 MM paper and then baked at 80°C for 1 hour in an vacuum oven. The membrane was kept in a sealed-plastic bag at 4°C until used. The blotted gel was stained with ethidium bromide to check whether the transfer was complete or not.

2.10. Hybridization (Maniatis et al., 1982 and Yod Yotee, 1987)

The blotted nitrocellulose membrane in a plastic bag was filled up with 16 ml of prehybridization solution (50-100 $\mu l/cm^2$) made up of 5 x SSC, 5 x Denhardt's solution, 5 mM Tris, pH 8.0, 50% formamide and 100 µg/ml denatured calf thymus DNA. Air bubbles in the bag were removed. The bag was then sealed and incubated at 37°C for at least 6 h. After prehybridization, the prehybridization solution was replaced with 16 ml fresh prehybridization mixture. The ^{32}P- labeled probe (specific activity 10^7-10^8 cpm/µg) after denaturing at 100°C for 3 min followed by rapid chilling in ice-water, was added into the bag,

resealed and incubated overnight at 37°C. Next day, the membrane was recovered from the hybridization bag and washed with 3 x SSC at room temperature in a shaking baht twice, 10 min each. The membrane was further washed with 3 x SSC, 1 mM EDTA and 0.1% SDS at 55°C twice, 15 min each. Then, the membrane was finally washed with 1 x SSC, 1 mM EDTA and 0.1% SDS at 55°C twice, 15 min each. The washed membrane was blotted dry between Whatman 3 MM paper and sealed in plastic bag.

2.11. Autoradiography (Swanstorm and Shank, 1978)

The blotted-dry nitrocellulose membrane in plastic bag was exposed to Kodak RX100-Xray film, between two intensifying screens in a cassette. The cassette was incubated at -70°C for 1 h - 2 days.