CHAPTER II

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study and their description characteristics were listed in Table 2.1

Procedure for Extraction of Plasmid DNA (Birnboim and Doly, 1979)

The plasmids were prepared by rapid alkaline extraction as described by Birnboim and Doly, 1979. The principle of this method is selective precipitation of high molecular weight chromosomal DNA while covalently closed circular DNA remains soluble. Plasmid containing cells were treated with lysozyme to weaken the cell wall and then lysed completely with SDS and NaOH. DNAs were denatured. When the lysate was neutralized by acidic sodium acetate, chromosomal DNA, still in a very high molecular weight form, renatured and aggregated to form an insoluble network. Simultaneously, the high concentration of sodium acetate caused precipitation of protein-SDS complex and high molecular weight RNA. Renatured DNA (and residual

Table 2.1 Bacterial strains and plasmids

Strains or Plasmids	Genotype or Phenotype	Source or Reference
Bacillus subtilis MI111 (RM125)	*arg*15, *leu*A8, r^-_M, m^-_M	Uozumi *et al.*, 1977*
Escherichia coli DH5α	*endA1 hsdR17*($r^-_k m^+_k$) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ(*lacZYA-arg F*)U169 (φ*80lacZ*Δ*M15*)	Hanahan, 1983
Eschrichia coli JM101	F'*traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15/supE thi* Δ(*lac-proAB*)	Yanisch-Perron *et al.*, 1985
pCSBC5 (7.9kb)	Am^r	สุรศักดิ์, 1993
pCSBC8 (9kb)	Am^r	สุรศักดิ์, 1993
pHV33 (6.8kb)	Am^r, Cm^r, Tc^r	Primrose *et al.*, 1981*

*This strain and plasmid were kindly provided by Assoc.Prof.Dr. Watanalai Panbangred, Department of Biotechnology, Mahidol University

low molecular weight RNA) remained soluble and were recovered from the supernatant by ethanol precipitation.

1. Large-scale preparations

E. coli JM101 harbouring pCSBC5 or pCSBC8, *B. subtilis* MI111(RM125) harbouring pHV33 and selected clones were grown in 100 ml of L-broth (10 g bactotryptone, 5 g yeast extract, 10 g NaCl in 1 l of water, pH7.4) containing 100 μg/ml ampicillin or 35 μg/ml chloramphenicol as appropriate. After 18 h of incubation at 37°C, bacterial cells were harvested by centrifugation at 4,000 xg and 4°C for 20 min and resuspended in 4 ml of lysozyme solution (50 mM glucose,10 mM EDTA,25 mM Tris-HCl pH 8.0 and 5 mg/ml lysozyme). After 30 min of incubation at 0°C, 8 ml of alkaline SDS solution (0.2 N NaOH, 1% SDS) was added. The mixture was gently mixed. The suspension should become almost clear and slightly viscous. Then 6 ml of high salt solution (3 M sodium acetate or potassium acetate, pH 4.8) was added. The contents of the tube were gently mixed by inversion for a few seconds during which time a clot of DNA forms. The tube was maintained at 0°C for 60 min to allow most of the protein, high moleccular weight RNA and chromosomal DNA to precipitate. Centrifugation at 4,000 xg for 20 min yielded an almost clear supernatant liquid. Equal volume of 1:1 phenol [saturated with TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0)] chloroform mixture was added and mixed by inversion several times. An aqueous phase was separated by centrifugation. An equal volume

of ethanol was added. The tube was held at-20°C overnight. The precipitate was collected by centrifugation for 10 min at 5,000 xg. The pellet was dissolved in 500 μl TE buffer and transferred to a 1.5 ml Eppendorf tube.

2. Minipreparations

A single bacterial colony was grown in 1.5 ml of L-broth containing appropriate antibiotics. After 18 h of incubation at 37°C, bacterial cells were harvested by centrifugation at 4,000 xg for 10 min and resuspended in 200 μl of lysozyme solution. After 30 min of incubation at 0°C, 400 μl of alkaline SDS solution was added. The mixture was gently mixed. The suspension should become almost clear and slightly viscous. The 300 μl of high salt solution was added. The contents of the tube were gently mixed by inversion for a few seconds during which a clot of DNA forms. The tube was maintained at 0°C for 30 min to allow most of the protein, high molecular weight RNA and chromosomal DNA to precipitate. Centrifugation at 4,000 xg for 10 min yielded an almost clear supernatant liquid. Equal volume of 1:1 phenol chloroform mixture was added and mixed by inversion several times. An aqueous phase was separated by centrifugation. An equal volume of ethanol was added. The tube was held at -20°C overnight. The precipitate was collected by centrifugation for 10 min at 5,000 xg. The pellet was dissolved in 30 μl TE buffer.

Plasmid were maintained in nuclease free solutions. There were two methods for the maintenance of plasmids:

a Short-term (about 2 months)

Plasmid DNA was dissolved in TE buffer and stored at 4°C.

b Long-term (more than 2 months)

Maintain plasmid DNA in 70% ethanol at -20°C. The plasmid DNA was collected by centrifugation and dissolved in TE buffer prior to use.

<u>Procedure for Extraction of Chromosomal DNA</u> (modified from Rodriguez *et al.*, 1983)

Bacterial cells were grown in 100 ml of L-broth. After 18 h of incubation at 37°C, the bacterial cells were harvested by centrifugation at 4,000 xg and 4°C for 20 min. The cell pellet was frozen at -70°C for 10 min and then immediately immerged in 65°C water bath. The cell pellet was resuspended in 2 ml of SET buffer (20% sucrose, 50 mM Tris-HCl pH 7.6, 50 mM EDTA). 0.2 ml of lysozyme solution [5 mg/ml of lysozyme in TEN buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 10 mM NaCl)] and 0.1 ml of RNase A solution [10 mg/ml of Pancreatic Ribonuclease A in RNase buffer (0.1 M sodium acetate pH 7.4 0.3 mM EDTA)] were added, and the mixture was incubated for 30 min at 37°C in a shaking water bath before adding 0.05 ml of 25% SDS. Incubation was continued for 3-6 h at 37°C in a shaking water bath. 0.3 ml of pronase solution (2 mg/ml of pronase in TEN buffer) was

added and mixed. 3 ml of 24:1 chloroform isoamyl alcohol mixture was added and incubated for 10-16 h at 37°C in a shaking water bath. Then, 1 ml of steriled distilled water and 10 ml of 24:1 chloroform isoamyl mixture was added. The contents of the tube were gently mixed by inversion for 5 min. An aqueous phase was separated by centrifugation at 1,000 xg for 20 min. The aqueous upper phase was transferred to a new tube by using a wide-bore pipet. An equal volume of 24:1 chloroform isoamyl alcohol was added and the extraction procedure was repeated twice. After the aqueous phase from the last extraction was transferred to a new tube, 1/100 volume of 7.5 M ammonium acetate pH 7.5 was added followed by two volumes of ethanol. The tube was held at -20°C for at least 10 min. The precipitate was collected by centrifugation for 10 min at 5,000 xg. The pellet was dissolved in 2 ml of TEN buffer.

<u>Agarose Gel Electrophoresis</u> (Maniatis, 1982)

A submarine 0.7-1% agarose gel was set up in TBE buffer (89 mM Tris-HCl,89 mM boric acid, 2.5 mM EDTA pH 8.3). 20 μl of each sample DNAs were mixed with 4 μl of tracking dye (1% bromphenol blue, 1% xylene cyanole FF, 50% glycerol) and loaded into the wells. The gel was run at 80 volts for 3 h or until bromphenol blue tracking dye reached the end of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (4 μg/ml of ethidium bromide in distilled water) for 5 to 10 min. The gel was destained for about

10 min with water. The stained gel could be photographed under UV transilluminator through a red filter onto Kodak Tri-X Pan 400 film.

Restriction Enzymes Digestion (Rodriguez et al.,1983)

About 1 μg of plasmid DNA (pCSBC5, pCSBC8) or chromosomal DNA was completely digested with 5 U of restriction endonuclease in 20 μl reaction mixture. Examples of restriction endonuclease were listed in Table 2.2. The reaction mixture was incubated overnight at 37°C. The samples were subjected to agarose gel electrophoresis to separate the DNA fragment of interest. The reaction mixture may also be extracted with 1:1 phenol-chloroform twice. The aqueous upper phase was collected. The DNA was precipitated with absolute ethanol and dissolved in TE buffer.

DNA Recovery after Agarose Gel Electrophoresis

1. Freeze-Squeeze method (Tautz and Renz, 1983)

After agarose gel electrophoresis of the DNA samples was complete, the gel was stained with ethidium bromide, and the DNA bands were visualized under UV light. DNA bands of interest were excised as agarose strips. The agarose strip was minced as fine as possible with a razor blade and placed into a 0.5 ml microfuge tube which was punctured at the bottom and plugged with siliconized glass wool, 0.12 ml of DNA elution buffer (0.3 M sodium acetate pH 7.0, 1 mM EDTA)

Table 2.2 List of restriction endonucleases used

Restriction Endonuclease	Recognition Sequence	Nature of ends generated by cut
Acc I	5'-GT/(AorC)(GorT)AC-3'	cohesive ends
*Bam*H I	5'-G/GATCC-3'	cohesive ends
Cla I	5'-AT/CGAT-3'	cohesive ends
*Eco*R I	5'-G/AATTC-3'	cohesive ends
*Hin*c II	5'-GTPy/PuAC-3'	blunt ends
*Hin*d III	5'-A/AGCTT-3'	cohesive ends
Hpa I	5'-GTT/AAC-3'	blunt ends
Kpn I	5'-GGTAC/C-3'	cohesive ends
Pst I	5'-CTGCA/G-3'	cohesive ends
Pvu II	5'-CAG/CTG-3'	blunt ends
Sal I	5'-G/TCGAC-3'	cohesive ends

per 20 mm^3 of gel volume was added. The mixture was incubated at room temperature for 30 min and placed in the liquid nitrogen or dry ice for 30 min. Then the 0.5 ml tube was placed into a 1.5 ml microfuge tube and rapidly centrifuged for 10 min at 5,000 xg, 4°C. 1/100 volume of 10% acetic acid - 1 M $MgCl_2$ was added to the collected DNA solution followed by 2.5 volume of cold ethanol. The mixture was then placed at -20°C for at least 30 min. The DNA precipitate was collected by centrifugation for 5 min at 4,000 xg. The supernatant liquid was discarded. The DNA precipitate was dissolved with TE buffe

2. The Geneclean II Kit

The Geneclean II Kit (BIO 101 Inc.) contains a specially formulated silica matrix, called GLASSMILK, that binds single and double stranded DNA but not the DNA contaminants. The protocol of DNA recovery is as following:

DNA band of interest from ethidium bromide stained agarose gel was excised with razor blade and then transferred to a plastic tube. The volume of the gel slice was estimated (1 g equals approximately 1 ml). 3 volumes of NaI stock solution was added. The tube was incubated at a 45°C to 55°C with occasional mixing. After five minutes of incubation, the agarose gel should be completely dissociated. The insoluble silica matrix stock was mixed by vortexing until all contents were in suspension, and 5 μl of this GLASSMILK suspension was added to the solution containing 5 μg or less of DNA. The solution was mixed and placed on ice for 5 min to allow binding of

the DNA to the silica matrix. The silica matrix with the bound DNA pellet was separated in a microcentrifuge for approximately 5 seconds at 5,000 xg. 250 μl of ice cold NEW WASH was added to the pellet. The pellet was resuspended in the wash by pipeting back and forth while digging into the pellet with the pipet tip. The pellet was spun down for 5 seconds in the centrifuge, and the supernatant liquid was decanted. The wash procedure was repeated twice. After the supernatant liquid from the third wash had been removed, the tube was quickly spun for a few seconds, and the last bit of liquid was removed with a fine tipped pipet. The washed white pellet was resuspended with 10 μl of TE buffer, incubated at 45°C to 55°C for 2 or 3 minutes, and then centrifuged for about 30 seconds at 5,000 xg. The supernatant containing the eluted DNA was carefully removed and placed in a new tube.

<u>Ligation</u> (Rodriguez et al, 1983)

DNA insert (CGTase gene containing inserts from plasmid, pCSBC5,pCSBC8) and plasmid vector (pHV33 or pUC18) that had been digested with appropriate restriction enzymes were mixed in a 3:1 molar ratio. The mixture was incubated at 50°C for 10 min. The 8 μl of T_4 ligation 5X buffer (with ATP), 0.5 μl (200 NEB unit) of T_4 DNA ligase and steriled distilled water were added to make 40 μl of reaction mixture. The solution was mixed and incubated at 12-15°C for 15 h.

Transformation

1. Transformation of competent *E. coli* (modified from Mandel and Higa, 1970)

A single colony of *E. coli* strain DH5α , or JM101 was cultured in 1 ml of L-broth medium and incubated at 37°C for an overnight. The starter was diluted to 1% with 10 ml of L-broth medium and cultured at 37°C for 2-3 h or until the culture was in the mid log-phase stage. The cells were then chilled on ice for 5 min, spun down by centrifugation at 4,000 xg for 10 min. The pellet was washed with 5 ml of cold 10 mM $CaCl_2$, spun down, resuspended in 500 μl of cold 100 mM $CaCl_2$ and left on ice for at least 1 h before used.

100 μl of the competent cells were mixed with 10-20 μl of ligation mixture and then placed on ice for 30 min. The mixture was heat-shocked at 42°C for 5 min. 500 μl of L-broth medium was added and incubated at 37°C for 1 h. 100 μl of the cells were spread onto an L-agar plate containing appropriate antibiotics. The plate was incubated overnight at 37°C, and the transformant colonies were counted and selected.

2. Transformation of *Bacillus subtilis* MI111 (RM125) protoplast

2.1 Prepaeation of protoplast of *B. subtilis* MI111 (RM125) (Chang and Cohen, 1979) Mid log-phase cell cultures freshly grown in Penassay broth (PAB, see Appendix I for medium composition) at

37°C to 1-2 x 10^8 cfu/ml were harvested and resuspended in 1/10 volume of SMMP solution (see Appendix I for medium composition). Lysozyme was added to 2 mg/ml final concentration, and the suspension was incubated at 37°C with gentle shaking. Although protoplasts were formed within 30 min, incubation usually was carried out for 2 h to ensure complete formation. Cells were pelleted by centrifugation at 2,000 xg for 15 min and washed once by resuspending them gently in SMMP. After centrifugation, the protoplasts were suspended in 1/10-1/15 volume of the starting culture by the addition of SMMP. The resulting preparation is stable at room temperature for at least 5 h.

2.2 The protoplast transformation procedure 1 pg to 5 μg of recombinant plasmid DNA in 50 μl or less of TE buffer was mixed with equal volume of 2x SMM solution in a sterile culture tube. 0.5 ml protoplast suspension was added, followed immediately by the addition of 1.5 ml of 40% (w/v) PEG solution. The contents of the tube were gently mixed. After two minutes exposure to PEG, 5 ml of SMMP medium was added to the mixture to dilute the PEG, and protoplasts were recovered by centrifugation for 10 min at 2,000 xg. The treated protoplasts were resuspended in 1 ml SMMP and incubated for 1.5 h at 30°C in a gentle shaking water bath to enable phenotypic expression of genetic determinants carried by the plasmid. The protoplast were then plated onto antibiotic DM3 medium for direct selection of transformants.

Selection of CGTase Producing Transformant

1. Indicator test (Park *et al.*, 1989)

A single transformant colony was inoculated on PM-agar (see Appendix I for medium composition) containing appropriate antibiotics and incubated at 37°C for 72 h. CGTase producing transformant showed orange hollow area around the colony.

Alternatively a single transformant colony was inoculated in PM-broth containing appropriate antibiotics and incubated at 37°C for 72h. CGTase producing transformant could change the color of the culture from red to orange.

2. Dextrinizing activity test (I_2 Method) (Fuwa,1954)

The transformants were further tested for dextrinizing activity. A single transformant colony was inoculated on L-agar containing 1% soluble starch and appropriate antibiotics, and incubated at 37°C for 72 h. The culture plates were sprayed with I_2 solution (0.02% w/v I_2 in 0.2% KI). CGTase producing transformant showed clear zone around the colony.

Alternatively a single transformant colony was inoculated in L-broth containing 1% soluble starch and appropriate antibiotics, and incubated at 37°C for 72 h. The supernatant was separated by centrifugation at 4,000 xg for 20 min. Then 100 μl of supernatant was added to 0.3 ml of starch solution (0.2% w/v potato soluble starch in 0.2 M phosphate buffer, pH 6.0) and placed in 40°C water bath.

After 10 min, 4 ml of 0.2 M HCl was added to stop reaction and then 0.5 ml of I_2 solution (0.02% I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water, and its absorbance at 600 nm was measured. For a control tube, the supernatant sample was added after adding HCl. One unit of enzyme was defined as the amount of enzyme which produced 10 % reduction in the intensity of the blue color of the starch iodine complex per min under the described conditions.

3. Cyclodextrin - trichloroethylene (CD-TCE) complex precipitation methods (Nomoto *et al.*, 1986)

The CGTase activity was determined by the formation of cyclodextrin-trichloroethylene (CD-TCE) complex precipitation. The sample supernatant was successively diluted with 0.2 M phosphate buffer pH 6.0 to obtain a series of dilution of $1:2^n$. One ml of each diluted solution was mixed with 5 ml of a 2% soluble starch solution prepared in the same buffer, and the mixture was incubated at 40°C for 48 h. 1 ml of TCE was added, mixed vigorously and left at room temperature overnight. The CD-forming activity of CGTase is expressed by the dilution limit ($1:2^n$) that is capable of producing a CD-TCE complex precipitation.

DNA-DNA Hybridization

In principle, DNA was fixed onto the membrane and then hybridized with the labeled probe. After washing out the non-specific binding of labeled probe, the hybridization of fixed-DNA and the labeled probe was detected. In this study DNA was labeled using DNA labeling kit from Boehringer Mannheim GmbH, Biochemica.

1. DNA labeling and detection with the DIG system

DIG DNA labeling kit from Boehringer Mannheim GmbH, Biochemica was used in the experiment. The DIG System uses digoxigenin, a steroid hapten, to label DNA for hybridization and subsequent detection. DNA probes are labeled with DIG-11-dUTP via random primed labeling. The DIG-labeled probes are hybridized to membrane-bound nucleic acid on a Southern blot. The hybridized probes are immuno-detected with an alkaline phosphate-conjugated anti-digoxigenin antibody and then visualized with the chemiluminescent substrate Lumigen™ PPD. The DIG system for nucleic acid analysis can be used for single copy gene detection on Southern blot.

1 μg DNA template was heat-denatured in a boiling water bath for 10 min and immediately chilled on ice bath for 1 min. 2 μl of hexanucleotide mixture (10x), 2 μl of dUTP labeling mixture and 1 μl of Klenow enzyme (2U/μl) were added. The volume was made up to 20 μl by adding steriled distilled water. The reaction was mixed and incubated at 37°C for 20 h. 2 μl of 200 mM EDTA, pH 8.0 was added to

terminate the reaction. The labeled DNA was precipitated by adding 0.1 volume of 4 M LiCl and 2.5-3.0 volumes chilled 95% ehtanol, mixing well, incubating at -20°C overnight and spinning at 5,000 xg for 10 min. The DNA pellet was washed with 100 μl of chilled 70% ethanol, dried and resuspended in 50 μl of TE buffer. DNA labeled probe suspension was stored at -20°C.

The yield of DIG-labeled probe was estimated by dot-blot hybridization. The intensities of signal from dots using DIG-labeled probe were compared with those of labeled control DNA (5 ng of digoxigenin labeled pBR322), at 5000, 1000, 100, 1 and 0.1 pg/μl respectively.

Two techniques of DNA-DNA hybridization were performed in this study, "dot blot hybridization" and "Southern blot hybridization". These techniques consisted of the steps described below.

2. Fixing DNA onto the membrane

2.1 Dot-blot 1 μg of chromosomal DNA *Bacillus* sp. A11 was denatured by incubating with 0.1 volume of denature solution (4 M NaOH, 100 mM Na_2EDTA) at room temperature for 10 min and was spotted on a positively charged nylon membrane.

DNA could be efficiently bound to the nylon membrane by irradiating with UV light for 3-5 min, or baking in an oven at 80°C for 2 h.

The membrane could be used immediately for prehybridization, or could be stored dry at room temperature for future use.

2.2 Southern-blot (modified from Maniatis, 1982 ; Carter, 1991) 4 μg of *Bacillus* sp. A11 DNA was completely digested with restriction endonucleases *Bam*HI, *Hin*dIII, *Eco*RI, *Pst*I and partially digested with restriction endonuclease *Sau*3AI in 60 μl reaction mixture at 37°C. The digests were subjected to 0.7% agarose gel electrophoresis. The gel was soaked with 250 mM HCl for 10 min, rinsed with distilled water, submerged in the denaturing solution (0.5 M NaOH, 1.5 M NaCl) with gentle shaking for 45 min and rinsed with distilled water. Then, The gel was soaked with transfer solution (0.25 M NaOH, 1.5 M NaCl) for 15 min. The blotting apparatus was set up as shown in Fig.2.1. The reservoir was filled with transfer solution. A Whatman 3 MM filter paper was prewetted wjth transfer solution, and placed over a glassplate with the ends hanging down into the transfer solution as a wick. All air bubbles were pressed out. The gel, DNA side down, was then placed on the prewetted filter paper. The nylon menbrane, soaked with the transfer buffer for 1 min, was placed over the gel. Make sure that all air bubbles were removed. The membrane was covered with 3-4 sheets of the transfer solution wetted Whatman 3 MM filter paper and then dry absorbent papers. The blotting apparatus was then covered with a plastic wrap. Finally, about 0.75-1.0 kg of weights were put on top to allow the capillary transfer process to proceed for 12-16 h.

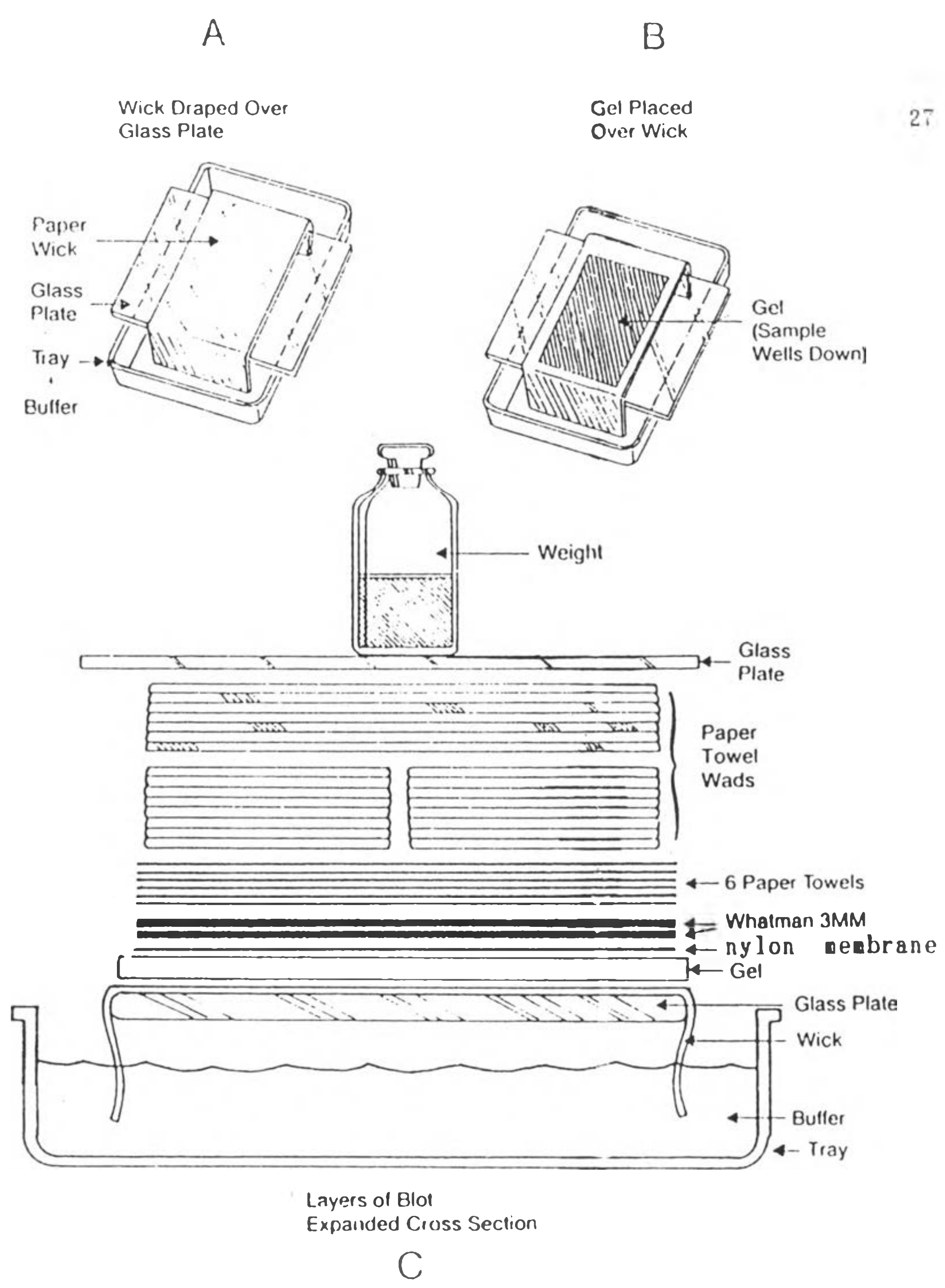

Figure 2.1 Southern transfer of DNA fragment from an agarose gel onto nylon membrane.
(A) Position of a wick over a glassplate.
(B) Gel is placed on a wick.
(C) Schematic illustration of complete Southern blotting set-up, (Devis *et al.*, 1986)

The transfer solution transferred the DNA fragments from the gel directly to the nylon membrane. After the transfer was completed, the membrane was removed, rinsed in 5xSSC buffer (750 mM NaCl, 75 mM sodium citrate, pH 7.0) for 1 min to remove residual agarose and dried at room temperature.

DNA could be efficiently bound to the nylon membrane by irradiating with UV light for 3-5 min, or baking in an oven at 80°C for 2 h.

The membrane could be used immediately for prehybridization, or could be stored dry at room temperature for future use.

3. Prehybridization and hybridization

Prehybridization is a step to prepare the membrane for probe hybridization. It blocks non-specific nucleic acid binding sites on the membrane in order to lower the background of hybridization.

The nylon membrane was submerged in at least 20 ml of standard prehybridization solution [5xSSC buffer, 1%(w/v) blocking reagent, 0.1% N-lauroylsarcosine and 0.02% SDS] per 100 cm^2 of membrane, and prehybridized at 65°C for at least 2 h before hybridization.

The probe (DIG-labeled probe) was diluted in standard hybridization solution to the final concentration of 5-10 ng/ml, heated in a boiling water bath for 10 min to denature the DNA and chilled immediately on ice before use. The nylon membrane was removed

from prehybridization solution, and placed in a hybridization bag. The hybridization solution containing the DIG-labeled probe was added. The required amount of the hybridization solution for a 8.5x10 cm nylon membrane was at least 3 ml. The hybridization bag was sealed after pressing out all air bubbles. Then it was hybridized overnight at 65°C.

At the end of the hybridization, the membrane was washed twice, 5 min per wash, in 2x wash solution (2xSSC containing 0.1% SDS) at room temperature. Then the membrane was washed twice, 15 min per wash, in 0.5x wash solution (0.5xSSC containing 0.1% SDS) at 65°C.

The hybridization solution containing unannealed DIG-labeled probe was poured from the bag into a capped tube that could stand freezing and boiling. The entire solution could be reused in future hybridization experiments. The tube was stored at -20°C. DIG-labeled probes stored in this manner were stable for at least 1 year. For reuse, the solution was thawed and denatured by heating.

4. Detection (Boehringer,1993)

Detection of DIG-labeled nucleotides could be accomplished with a chemiluminescent or colorimetric reaction. In this experiment, a three-steps chemiluminescent detection process was used. In the first step, the membrane was treated with Blocking Reagent to prevent non-specific attraction of antibody to the membrane. Then, the membrane was incubated with dilute solution of Anti-digoxigenin Fab fragment conjugated to alkaline phosphate. In the third step, the

membrane carrying the hybridized probe and bound antibody conjugate was reacted with Lumigen™ PPD and exposed to X-ray film to record the chemiluminescent signal.

After hybridization and post-hybridization wash, the membrane was equilibrated in Genius I buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 1 min. The membrane was blocked by gently agitating in Genius II buffer [1%(w/v) Blocking Reagent for nucleic acid hybridization dissolved in Genius I buffer] for 60 min. At the end of the blocking period, the antibody solution was prepared by diluting the anti-DIG-alkaline phosphate 1:20,000 in Genius II buffer. The Genius II buffer was poured off and the membrane was incubated for 30 min in the antibody solution. This incubation was performed in plastic tray that was agitated gently. The membrane should always be covered with the antibody solution. Then, the antibody solution was discarded, and the membrane was gently washed twice, 15 min per wash, in Genius I buffer. At the end of the washing, the membrane was equilibrated in Genius III buffer (100 mM Tris-HCl, pH 9.5,100 mM NaCl, 50 mM $MgCl_2$) for 2 min and soaked in Lumigen™ PPD diluted 1:200 in Genius III buffer. The membrane was placed in a plastic bag, sealed, and incubated in the dark at 37°C for 1 h. X-ray film exposure was done in a cassette with two intensifying screens for 30-60 min at room temperature. The signal intensity could be adjusted by decreasing or increasing the exposure time. The results were visualized after developing the film with developer and fixer solutions.