CHAPTER II

MATERIALS AND METHODS

2.1. Bacterial strains , plasmid DNA and standard DNA

The bacterial strains and plasmid DNA used in this study and their descriptive characteristics are listed in Table 2.1.

Standard DNA used as molecular weight markers in this study is lambda-DNA (Sigma) digested with *Hind*III showing 6 DNA fragments: 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 2.1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Source or Reference
Azospirillum brasilense	associative with rice	
Sp7	and other Gramineae	NifTAL
Bradyrhizobium japonicum		
THA5	symbiont with *Glycine max*	NifTAL
THA7	symbiont with *Glycine max*	NifTAL
Escherichia coli		
HB101	F^- *hsd* S20($r_B^- m_B^-$) *rec*A13 *leu*B6 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20(str^r)*xyl*-5 *mlt*$^{-1}$ *sup*E44 $^-$	Bolivar and Backman (1979)
JM101	F^- *tra*D36lacI^q Δ(*lac*Z) M15 *pro*AB/*sup*E thiΔ (*lac-pro*AB)	Messing *et al.* (1981)
Klebsiella oxytoca		
NG13	associative with rice	Kazuo Komagata*
R15	associative with rice	Boonjawat *et al.* (1986)
R17	associative with rice	Boonjawat *et al.* (1986)
Klebsiella pneumoniae		
M5a1	free-living soil bacteria	Dixon *et al.* (1976)

Table 2.1. (continued)

Bacterial strain or plasmid	Relevant characteristics	Source or Reference
Rhizobium leguminosarum		
TAL1402	symbiont with *Pisum*	NifTAL
Rhizobium meliloti		
TAL380	symbiont with *Medicago sativa*	NifTAL
TAL1372	symbiont with *Medicago sativa*	NifTAL
pE39	Am^r, *nod*D1	Egelhoff and Long (1985)
pRmSL42	Am^r, *nod*ABC	Egelhoff and Long (1985)
pSA30	Tc^r, *nif*HDKTY	Cannon *et al.* (1979)
pUC18	Am^r, *lac*Z'	Messing (1983); Yanisch-Perrom *et al.* (1985)

*National Institute of Genetics, Japan.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2.2. Media for bacterial culture

2.2.1. Luria-Bertani (LB) medium for *Klebsiella* spp. (Luria *et al.*, 1960)

Tryptone	10 g
Yeast extract	5 g
Sodium chloride, NaCl	10 g
Distilled water to	1 l
adjust pH to 7.0 with 1.0 N NaOH	
Agar	15 g per l (for solid medium)

2.2.2. Medium for *A. brasilense* Sp7 (Döbereiner, 1977)

Potassium dihydrogen phosphate, KH_2PO_4	0.4	g
Magnesium sulphate, $MgSO_4.7H_2O$	0.2	g
Sodium chloride, NaCl	0.1	g
Calcium chloride, $CaCl_2.2H_2O$	0.02	g
Ferric chloride, $FeCl_3.6H_2O$	0.01	g
Sodium molybdate, $Na_2MoO_4.2H_2O$	0.002	g
Potassium hydroxide, KOH	4.0	g
Yeast extract	0.2	g
Malic acid	5.0	g
Distilled water to	1	l
adjust pH to 6.8 with 1.0 N NaOH		
Agar	15 g per l (for solid medium)	

2.2.3. Yeast-Mannital (YM) Medium for *Bradyrhizobium* spp., *Rhizobium* spp. (Vincent, 1970)

D-mannital	10.0	g
Yeast-extract	0.4	g
Dipotassium hydrogen phosphate, K_2HPO_4	0.5	g
Magnesium sulphate, $MgSO_4.7H_2O$	0.2	g
Distilled water to	1	l

adjust pH to 6.8 with 1.0 N HCl

Agar 15 g per l (for solid medium)

2.3. Cultivations of bacterial strains and plasmid

Klebsiella strains were grown on LB agar plate or in LB broth with shaking at 37 °C., *Bradyrhizobium* and *Rhizobium* were grown on YM plate or in YM broth with shaking at 28 °C., and *Azospirillum* was grown on medium agar plate or in medium broth for *A. brasilense* Sp7 with shaking at 37 °C. For *Escherichia coli* HB101 cultures containing plasmids, ampicillin (Ap) 50 μg/ml (for pE39 and pRmSL42) and tetracyclin (Tc) 12.5 μg/ml (for pSA30) were used for maintaining plasmids. Long term stock cultures were maintained by adding equal volume of sterile glycerol in medium culture and kept in a deep-freezer at -70 °C for 1-2 years.

2.4. Reagents

All chemicals used for preparation of reagents were laboratory grade or otherwise specified.

2.5. Amplification, isolation and purification of plasmid DNA (Mandel and Higa, 1970, Cohen, 1972, and Birnboim and Doly, 1979)

Purified plasmid pE39 and pRmSL42 were obtained from Dr. Ann M. Hirsch, Department of Biology, UCLA Los Angeles, California. Plasmid pSA30 was kindly provided by Dr. Chistina Kenedy.

E. coli strain HB101 was used in this study. One loop of cells was inoculated into 1 ml of LB broth which was incubated at 37 °C for 16-18 hr, and then 100 µl of this culture was inoculated into 10 ml of LB broth in a 50 ml flask and incubated at 37 °C with shaking about 2.5-3.0 hr or until the cells were in the mid-log stage ($A_{600\ nm} \approx 0.6$). The cells were chilled on ice for 5 min before centrifuging at 1000 xg for 5 min at 4 °C and then resuspended in 5 ml of cold 0.01 M $CaCl_2$. The solution was centrifuged again and pelleted cells were resuspended in 0.5 ml of cold 0.1 M $CaCl_2$ and chilled on ice at least 60 min. The transformation tube contained 100 µl of $CaCl_2$ treated cells (competent cells) and 500 ng of each purified plasmid DNA (pE39, pRmSL42 and pSA30). The tube was incubated on ice for 30 min prior to heat-shock at 42 °C for 5 min and 100 µl LB broth was added into the tube, and incubated at 37 °C for 1 hr. The cell suspension was then spreaded on LB agar plate containing 50 µg/ml of ampicillin for pE39 and pRmSL42 or 12.5 µg/ml of tetracyclin for pSA30 and incubated at 37 °C for 16-18 hr.

Next day, pick a single colony which can grow on LB agar plate containing ampicillin or tetracyclin and inoculated into 100 ml of LB broth in a 250 ml flask. Ampicillin was added into the medium to a concentration of 50 μg/ml for pE39 and pRmSL42 or tetracyclin 12.5 μg/ml in order to reselect only the cells containing plasmid. The cells were grown to log phase ($A_{600nm} \simeq 0.6$). The cells were harvested by centrifugation at 5,000 xg, 4 °C for 20 min and washed once with TE buffer (10 mM Tris-HCl, 50 mM Na_2EDTA, pH 8.0). The packed cells were resuspended in 2 ml of freshly prepared lysozyme solution (50 mM glucose, 25 mM Tris-HCL, pH 8.0, 10 mM Na_2EDTA, and 5 mg/ml lysozyme) and left on ice for 30 min in order to weaken the cell walls prior to complete lysis on ice for 15 min with 4 ml of freshly prepared lysis solution (0.2 M NaOH, 1% SDS). The solution was then neutralized by addition 3 ml of 3 M sodium acetate, pH 4.8, mixed by gently inversion for several times and left on ice for 1 hr. The high molecular weight host chromosomal DNA, at first denatured by alkali was then renatured to form a insoluble network while the plasmid DNA was still in an soluble intact form. Furthermore, the high concentration of sodium acetate would cause precipitation of protein-SDS complexes and of high molecular weight RNA. In this way, most of the three major contaminating macromolecules were co-precipitated and removed by single centrifugation at 20,000 xg, 4 °C for 20 min. The plasmid DNA was then precipitated by adding 2 Vol of cold absolute ethanol and stored at -20 °C overnight. The DNA pellet was obtained by centrifugation at 12,000 xg, 4 °C for 10 min and 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 tube. 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 hr. After centrifugation, the tube was punctured with a 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 Vol of 3 M sodium acetate plus 2 Vol of cold absolute ethanol and stored -20 °C overnight. After centrifugation, the DNA pellet was redissolved in TE buffer, pH 8.0 and quantified by measuring absorbance at 260 nm (1 $A_{260\ nm}$ = 50 μg/ml of nucleic acid), and checked for purity by the ratio $A_{260\ nm}/A_{260\ nm} > 1.8$.

2.6. Isolation and purification of chromosomal DNA (Modified from Rodriquez and Tsit, 1983)

Rhizobia strains were obtained from NifTAL and/or BNF Resource Center, Division of Soil Science, Department of Agriculture, Bangkok, Thailand.

K. oxytoca R15, R17, NG13 and *K. pneumoniae* M5a1 were grown in LB broth with shaking at 37 °C, *B. japonicum* THA5, THA7, *R. meliloti*

TAL380, TAL1372 and *R. leguminosarum* TAL1402 were grown on YM broth with shaking at 28 °C and *A. brasilense* Sp7 was grown in medium broth for *A. brasilense* Sp7 with shaking at 37 °C. Cells were harvested from culture (100 ml) at early stationary phase ($A_{600\ nm}$ ≈ 0.7-0.8) by centrifugation at 5,000 x g, 4 °C for 20 min and washed once with SET buffer (20 % sucrose, 50 mM Tris-HCl, 50 mM Na_2EDTA, pH 7.6). The packed cells were frozen at -70 °C for 10 min and thawed in warm water (65 °C) before resuspending in 2 ml of SET buffer, pH 7.6 and put on ice. Cell lysis was performed by adding 200 µl of 5 mg/ml lysozyme in TEN buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, 10 mM NaCl, pH 7.6) and 100 µl of 10 mg/ml RNase A in RNase buffer (0.1 M sodium acetate, 0.3 mM Na_2EDTA, pH 7.4). The mixture was incubated on ice for 15 min (for *Klebsiella*) or incubated at 37 °C for 30 min (for *Azospirillum*, *Bradyrhizobium* and *Rhizobium*). 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 hr. After the addition of 300 µl of 2 mg/ml pronase in TEN buffer, pH 7.6 and 1.5 ml of chloroform/isoamyl alcohol solution 24:1 (V/V), the mixture was further incubated at 37 °C with gently shaking for overnight. Then, 1 ml of sterile distilled water and 2 Vol of chloroform/isoamyl alcohol solution were added into the mixture. After mixing by gently inversion, the mixture was centrifuged at 1,000 xg 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 Vol of 5 M NaCl plus 2 Vol of cold absolute ethanol. After gently inversion, the content was placed at -20 °C for 10 min. Fibrous strands of DNA were spooled out with a glass rod and dipped in 70 % ethanol to remove excess salt. The DNA was allowed to air-dry and redissolved in TEN buffer, pH 7.6. The amount and purity of DNA were checked by measuring $A_{260\ nm}/A_{280\ nm}$.

2.7. Restriction endonucleases digestion (Maniatis *et al.*, 1982)

Table 2.2. shows the restriction endonucleases type II used in this study and their recognition sequence. They were purchased from Bethesda Research Laboratories (BRL) Co. Ltd., U.S.A., Biolabs Co. Ltd., U.S.A. and Sigma Chemical Company Co. Ltd., U.S.A.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

I1690669X

Table 2.2. Recognition sequences of type II restriction endonucleases

Enzyme	Recognition sequence
*Bam*HI	G/GATCC
*Bgl*II	A/GATCT
*Eco*RI	G/AATTC
*Hind*III	A/AGCTT
*Pst*I	CTGCA/G
*Sal*I	G/TCGAC
*Sma*I	CCC/GGG
*Xho*I	C/TCGAG

2.7.1. Plasmid DNA digestion

The experiment was performed by digesting 1 μg of the plasmid DNA with 5 units of a restriction enzyme, (pE39 digested with *Bam*HI, pRmSL42 digested with *Bam*HI, *Eco*RI and *Hind*III and pSA30 digested with *Eco*RI) to separate inserted genes from vector, in 20 μl reaction mixture containing the appropriate buffer at 37 °C overnight. The reaction was stopped by denaturation at 65 °C for 20 min and addition of tracking dye (50% glycerol, 0.1% bromphenol blue, 0.1% Xylene cyanol FF) 1:5 (V/V) of the reaction mixture.

2.7.2. Chromosomal DNA digestion

The digestion of chromosomal DNA was performed in 20-100 µl reaction mixture containing 1-4 µg DNA, 10-20 units of restriction enzyme in appropriate buffer (as recommended by the suppliers) and distilled water to adjust the volume. The reaction mixture was incubated at 37 °C (except *Sma*I at 30 °C) overnight. After digestion, the cohesive end of DNA was denatured at 65 °C for 20 min, tracking dye 4µl/20µl reaction mixture was added and analysed by agarose gel electrophoresis.

2.8. Agarose gel electrophoresis (Modified from Maniatis *et al.*, 1982)

The sizes of DNA fragments were determined in a submarine horizontal gel electrophoresis apparatus using agarose gel (Type II Sigma) 0.7 % in TEB buffer, pH 8.3 (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM Na_2EDTA, pH 8.3). The size of gel was 100 x 90 x 6 mm and electrophoresis was carried out at 80 volts for 3 hr in TEB buffer, pH 8.3. DNA bands were stained in 0.5 µg/ml ethidium bromide 30 min and destained in distilled water 60 min before examination by ultraviolet light in a UV transilluminator and photographed of the restriction fragment length polymorphism (RFLP).

2.9. 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 (pE39, pRmSL42, and pSA30) was loaded on 1% low-melting temperature agarose (Type VII Sigma) and electrophoresis was carried out at 80 volts for 3 hrs. The region of gel containing the wanted DNA fragments (genes and vector) estimated from DNA markers were carefully cut from the whole gel plate and put into 1 Vol of TE buffer, pH 8.0. After heating at 65 °C for 20 min, the melted gel was extracted with an equal volume of phenol solution. The aqueous phase was recoverd 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, pH 8.0.

2.10. Dot hybridization technique (Modified from Ishii, 1992)

Prepared dilution series of genes (*nod*D1, *nod*AB, *nod*C and *nif*HDK) and chromosomal DNA of 10 strains of N_2-fixing bacteria (*K. oxytoca* R15, R17, NG13, *K. pneumoniae* M5a1, *A. brasilense* Sp7, *R. meliloti* TAL380, TAL1372, *R. leguminosarum* TAL1402, *B. japonicum* THA5, THA7 in suitable amounts. Denatured these DNA in dot denaturing solution (10:1) for 10 min at room temperature. Marked a nylon membrane (Boehringer Mannheim; Biochemica) lightly with a pencil to identify each dilution before spotting. Dispensed each dilution onto the membrane, fixed the DNA to the membrane by UV

crosslinking with a UV transilluminator for 3 min. The membrane was kept in a sealed-plastic bag at 4 °C until prehybridization and hybridization step.

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

After electrophoresis on agarose gel (100 x 90 x 6 mm in size), the gel was stained with 2.5 µg/ml of ethidium bromide and photographed. The DNA in the gel was depurinated (partial hydrolysis) by soaking in a plastic box containing 100 ml of 0.25 M HCl for 15 min. This process induces cleavage, so facilitating the transfer of large DNA fragments. The gel was rinsed with sterile water immediately and then transferred to a box containing a 200 ml of denaturation solution (0.5 M NaOH, 1.5 M NaCl) twice, with constant shaking for 25 min each. Then the gel was transferred to a box containing a 200 ml of transfer solution (0.25 M NaOH, 1.5 M NaCl) for 15 min. The gel was now ready for blot transfer. The gel was finally laid on top of a transfer solution, taking care to remove all air bubbles trapped between the gel and the paper wick. A piece of nylon membrane, cut about 1 mm larger than the gel on all sides, was carefully placed on top of the gel, again avoiding trapped air bubbles. A 3 cm stack of 3 MM Whatman paper, cut about 1 mm smaller than the nylon membrane on all sides, was placed over the nylon membrane. A polyethylene sheet (Glad Wrap) was used to cover the blotting box to prevent evaporation of the solution. The weight 700-

1,000 g was placed on top of the stack of 3 MM Whatman paper so that a flow of liquid from the reservoir through the gel and the nylon membrane could be set up. DNA fragments were therefore eluted from the gel and deposited onto the nylon membrane as shown in Figure 2.1. The transfer of DNA fragments was allowed to proceed for 12-24 hr. The rate of transfer of DNA depended on the size of DNA fragments and porosity of the gel. Smaller fragments of DNA (<1 kb) transferred from 0.7% agarose gel within one or two hours while larger fragments of DNA (>10 kb) took 15 hours or more. After blotting, the 3 MM Whatman paper sheets were removed from the nylon membrane, The orientation of the nylon membrane was indicated with a soft pencil before it was peeled off from the dried gel which was then stained with ethidium bromide to check whether the transfer was complete or not. The nylon membrane was soaked in 5 x SSC buffer (750 mM NaCl, 75 mM sodium acetate, pH 7.0) for 1 min. The membrane was blotted between 3 MM Whatman paper and dry at room temperature. The DNA fragments were immobilized on the nylon membrane by UV crosslinking with a UV transilluminator for 3 min. The membrane was kept in a sealed-plastic bag at 4 °C until prehybridization and hybridization step.

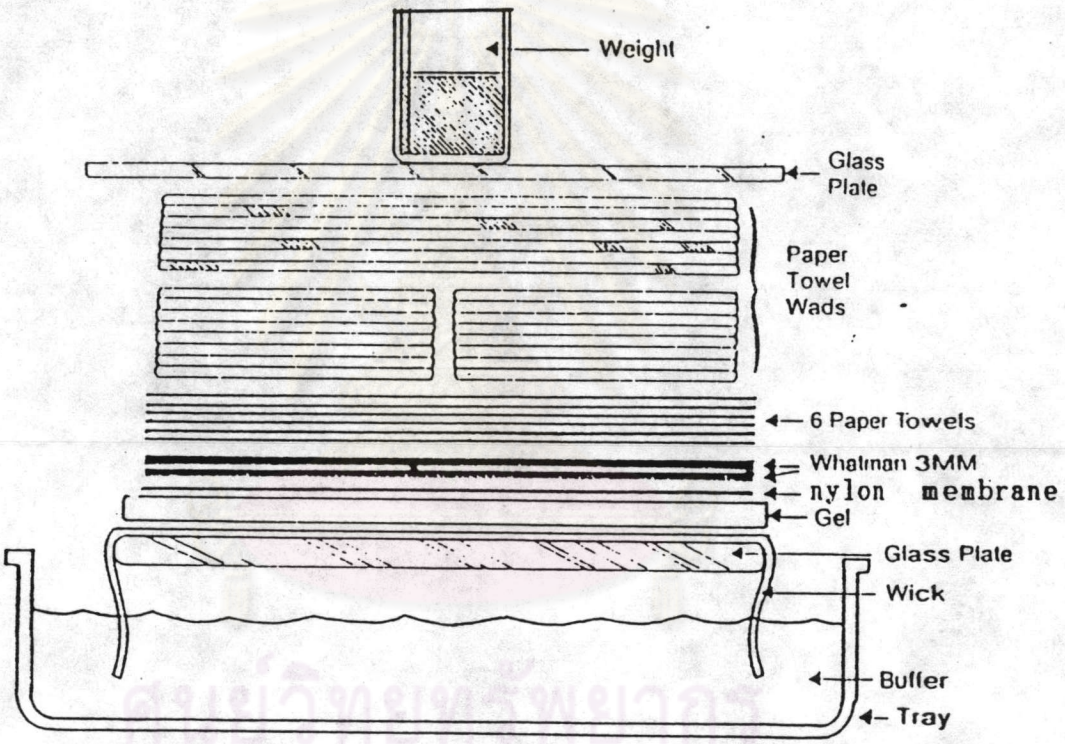

Figure 2.1. Diagram of Southern blot transfer (Modified from Southern, 1975)

2.12. Labeling of DNA probes with DIG-dUTP by random primer (Boehringer Mannheim; Biochemica, 1993)

In vitro labeling of purified DNA by DIG-dUTP is used in this study. The random primer reaction for labeling of DNA probes (pE39:*nod*D1, pRmSL42:*nod*AB, *nod*C and pSA 30:*nif*HDK) was from Boehringer Mannheim; Biochemica, 1993.

The genes (*nod*D1, *nod*AB, *nod*C and *nif*HDK) were denaturated by heating in a boiling waterbath (100 °C) for 10 min and chilling quickly on ice/NaCl. The reagents were added in to a 1.5 ml microfuge tube on ice .

Reagent	Volume	Final concentration
Freshly denatured DNA	10 ng-3 µg	0.5-150 ng/ml
Hexanucleotide mixture	2 µl	1x
dNTP labeling mixture	2 µl	1x
Sterile distilled water	to 19 µl	-
Klenow fragment of DpI	1 µl	100 units/ml
Total volume	20 µl	

Centrifuge briefly and incubate for at least 60 min at 37 °C. Longer incubation (up to 20 hr) can increase the amount of labeled DNA.

Table 2.3. The amount of synthesized DIG labeled DNA depends on the amount of template DNA in the labeling reaction and on the length of the incubation time at 37 °C.

Amount of template DNA per labeling reaction	10 ng	30 ng	100 ng	300 ng	1000 ng	3000 ng
Amount of synthesized DIG-labeled DNA						
after 1 hr	15 ng	30 ng	60 ng	120 ng	260 ng	530 ng
after 20 hr	50 ng	120 ng	260 ng	500 ng	780 ng	890 ng

Stop the reaction by adding 2 µl of 0.2 mol/l EDTA pH 8.0. Precipitate the labeled DNA with 2.5 µl of 4 mol/l LiCl , and 75 µl prechilled (-20 °C) ethanol. Mix well and leave for at least 30 min at -70 °C or 2 hr at -20 °C. After centrifugation, the DNA pellet was washed with 70% cold ethanol, dried and dissolved in 50 µl TE buffer, pH 8.0. Then stored at -20 °C.

2.13. Hybridization and washing (Boehringer Mannheim; Biochemica, 1993)

2.13.1. Prehybridization step

The blotted nylon membrane was placed in a plastic bag containing at least 20 ml of standard prehybridization solution (5 x SSC, 1.0% casein as Blocking reagent, 0.1% N-lauroylsarcosine, Na-salt, 0.02% SDS) per 100 cm^2 of membrane. Air bubbles in the bag were removed. The bag was then sealed with a plastic sealer and incubated at 65-68 °C for at least 2 hrs. During prehybridization, denature the labeled DNA by heating for 10 min at 95 °C and chilling quickly on ice/ethanol.

2.13.2. Hybridization step

After prehybridization, one edge of bag was cut off and the prehybridization solution was replaced with 2.5 ml per 100 cm^2 membrane of hybridization solution containing freshly denatured labeled DNA. Air bubbles in the bag were removed. The bag was then sealed and incubated overnight (or at least 6 hrs) at 65-68 °C.

2.13.3. Washing non-hybridization probe from nylon membrane

The membrane was recovered from the hybridization bag (the hybridization solution containing the probe could be reused) and washed in a plastic box containing 2 x wash solution (2 x SSC containing 0.1% SDS) and shaken at room temperature for 5 min. This was done twice. The membrane was then shaken with a non-stringent

0.5 x wash solution (0.5 x SSC containing 0.1% SDS) for 2 times at 65 °C, 15 min each time. The washed membrane was used directly for detection of hybridized DNA or stored air-dried for later detection.

2.14. Chemiluminescent detection with Lumigen™ PPD (Boehringer Mannheim; Biochemica, 1993)

The membrane was washed briefly in Genius buffer 1 (150 mM NaCl, 100 mM Tris-HCl, pH 7.5). The membrane was then incubated for 60 min with 100 ml Genius buffer 2 (1.0% Blocking reagent in Genius buffer 1) followed by 30 min with about 20 ml of diluted antibody-conjugate alkaline phosphatase solution (150 mU/ml in Genius buffer 2) per 100 cm^2 of membrane. Unbound antibody-conjugate was removed by washing the membrane twice for 15 min each in 100 ml of Genius buffer 1. Equilibrated the membrane for 2 min with 20 ml of Genius buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). The membrane was placed between two sheets of plastic bag, gently lift the top sheet of plastic, and added approximately 0.5 ml per 100 cm^2 of membrane of diluted Lumigen™ PPD (1:100 in buffer 3) with a sterile micropipette to the top surface of the membrane, scattering the drops of the substrate over the surface of membrane. Air bubbles in the bag were removed. The bag was then sealed and the membrane was incubated for 5 min at room temperature followed by 30 min at 37 °C. The blotted nylon membrane in plastic bag was exposed to X-ray film, between two intensifying screens in a cassette. The cassette was incubated at room temperature for 15 min-2 days.

2.15. Stipping and reprobing chemiluminescence-detected membrane (Boehringer Mannheim; Biochemica, 1993)

The membrane could be re-hybridization with a new probe by washing the membrane in sterile distilled water for 1 min. Incubated the membrane twice for 10 min in 0.2 N NaOH, 0.1% SDS solution at 37 °C. This incubation removed the DIG-labeled probe. The membrane was rinsed throughly in 2 x SSC and hybridization can be carried out without the prehybridization step.

2.16. Cloning (Modified from Maniatis *et al.*, 1982)

The chromosomal DNA from *K. oxytoca* strain NG13 purified according to Method 2.6. was digested with *Bam*HI (1 μg DNA/ 10 units *Bam*HI) and recovered 4.0-4.9 kb from low-melting temperature agarose gel electrophoresis (see in Method 2.9.). Plasmid pUC18 used as the vector DNA, was prepared by using Method 2.5. and 1 μg of the plasmid DNA was digested with 5 units of *Bam*HI. Then, the linearized plasmid DNA was dephosphorylated by adding 0.06 unit of Calf intestinal phosphatase (CIP), 1 unit of alkaline phosphatase. After that, the mixture was then incubated at 37 °C for a half to one hour. The plasmid DNA was recovered by adding 1/10 Vol of 3 M sodium acetate plus 2 Vol of cold absolute ethanol. The pellet was redissolved in TE buffer and the concentration determined.

DNA fragment of *K. oxytoca* NG13, after digested with *Bam*HI were mixed with dephosphorylated plasmid pUC18 DNA in a 5 : 1 (W/W) ratio in 20 μl of reaction mixture (500 ng of DNA fragment and 100 ng

of dephosphorylated plasmid DNA). The mixture was incubated at 50 °C for 10 min, then 5x ligation buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 20 μg/ml nuclease-free BSA (Biolabs) and 5 units of T_4 DNA ligase were added. The solution were mixed and frozen for 5 min, then incubated at 15 °C for 16-18 hr. The control was the same except the chromosomal DNA was not added. After phenol /chroloform extraction, ethanol precipitation was performed. Finally the DNA pellet was dissolved in a suitable volume of TE buffer. This recombinant plasmid DNA solution was now ready for transformation.

Transform ligation between plasmid and chromosomal DNA into *E. coli* strain JM101 (competent cells prepared as described in Method 2.5.). The cell resuspension was then spreaded on LB agar plate containing 50 μg/ml of ampicillin, 20 μg/ml of 5-bromo-4-chloro-indolyl-β-D-galactoside (x-gal) and 25 μg/ml of Isopropylthio-β-galacto-side (IPTG) and incubated at 37 °C for 16-18 hr in order to select only white colonies that contained recombinant *nod*D1-pUC18, for further plasmid purification and checked for inserted DNA of fragment size 4.0-4.9 kb.