CHAPTER 2

MATERIALS AND METHODS

2.1 Equipment

- Autoclave: Model HA-3D (Hirayama Manufacturing Corporation, Japan)
- Automatic micropipette P20, P100, P200, P1000 (Gilson Medical Electrics S. A., France)
- Refrigerated centrifuge, Beckman: Model J2-21C (Beckman, U.S.A.)
- Gene Pulser™ (Bio-RAD, U.S.A.)
- GS GENE LINKER™ UV CHAMBER: Model GS Gene Linker® UV Chamber (Bio-RAD, U.S.A.)
- Gyrotory water bath sharker: Model G76D (New Brunswick Scientific Co., Inc.U.S.A.)
- High speed microcentrifuge: Model Kubota 1300 (Kubota Corporation, Japan)
- Horizontal gel electrophoresis apparatus for 8.5 x 12.5 cm gel former
- Incubator: Model BM600 (Memert GmbH, W., Germany)
- pH/ ION meter: model PHM 95 (Radiometer, Copenhagen)
- Plateform sharker: Model inniva™ 2100 (New Brunswick Scientific Co., Inc. U.S.A.)
- Power supply: Model Power PAG 300 (Bio-RAD, U.S.A.)
- Spectrophotometer, Beckman: Model DU 650 (Beckman, U.S.A.)
- UV transilluminator: Model 2011 (Macrovue, San Gabrial, U.S.A.)
- Vortex: Model K-550-GE (Scientific Industrial, USA)
- Vaccum blotter: Model 785 (Bio-RAD, U.S.A.)

2.2 Inventory supplies

- Filter paper wathman 3 MM (Wathman Internation Ltd., England)
- X-ray film, X-Omat XK-1 (Eastman Kodak company Rochester, U.S.A.)
- MagnaCharge, Nylon Transfer Membrane (Micron Separations Inc., MA)

2.3 Chemicals

- Absolute ethanol (Merck, Germany)
- Acetic acid (Merck, Germany)
- Agarose (Seakem LE, FMC Bioproducts, USA)
- Bacto agar (DIFCO Laboratories, U.S.A.)
- Bacto tryptone (DIFCO Laboratories, U.S.A.)
- Bacto yeast extract (DIFCO Laboratories, U.S.A.)
- Boric acid (BDH, England)
- Chloroform (BDH, England)
- Developer (Eastman Kodak Company, Rochester, U.S.A.)
- DIG High Prime Labelling and Detection Starter Kit II (Boehringer Mannheim, U.S.A.).
- dNTPs (dATP, dGTP, dCTP and dTTP) (Promega Corporation Medison, U.S.A.)
- Dikalcium hydrogenphosphate (Merck, Germany)
- Disodium ethylenediamine tetraacetic acid (Fluka, Switzerland)
- Ethidium bromide (Sigma Chemical Co., U.S.A.)
- Ferrous sulfates heptahydrate (Fluka, Switzerland)
- Fixer (Eastman Kodak Company, Rochester, U.S.A.)
- Hexaadecyl trimethyl ammonium sulfate (Sigma Chemical Co., U.S.A.)
- Isoamylalcohol (Merck, Germany)
- Isopropyl alcohol (Merck, Germany)
- Kaliumhydrogenphosphate (Merck, Germany)

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- Lamda phage DNA (New England Biolabs Inc., U.S.A)
- L-glutamic acid sodium salt (BDH, England)
- Malelic acid disodium salt (Sigma Chemical Co., U.S.A.)
- N-lauroyl sarcosine sodium salt (Sigma Chemical Co., U.S.A.)
- Phenol crystal (Carlo Erba France)
- Polyethyleneglycol 8000 (Fluka, Switzerland)
- Protenase K (Gibco-BRL Technology, Inc., U.S.A.)
- QIAEX II Agarose Gel Extraction Kit (QIAGEN GmbH, Germany)
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chamical Co., U.S.A)
- Sodium hydroxide (Merck, Germany)
- Sodium nitrate (BDH, England)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Tween 20 ((Fluka, Switzerland))

2.4 Bacterial strains and plasmids

Sphingomonas chlorophenolica strain ATCC 39723 (kindly provided by R. L.Crawford, University of Idaho, USA). *S. chlorophenolica* was cultured in mineral salts (MS) medium (0.82 g K_2HPO_4 $3H_2O$, 0.19 g KH_2PO_4, 0.5 g $NaNO_3$, 0.1 g $MgSO_4$ $7H_2O$, 4 g of L- sodium glutamate, 20μM of $FeSO_4$ per one liter) with shaking at 220 rpm, 28 ^{o}C.

Escherichia coli strain XL1-BLUE (obtained from Stratagene, U.S.A.) genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F *pro*AB *lacI*^{q}ZΔ*M15* Tn10 (Tet^{r})]^{c} . *E. coli* strain XL 1-BLUE was grown in Luria-Bertani (LB) medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1% NaCl) with shaking at 250 rpm, 37°C.

pVR46 (V. Rimphanitchayakit, Chulalongkorn University) was used as a source of kanamycin resistance gene.

pUC19 was used as a vector for construction of targeting vector.

2.5 Enzymes

The following restriction endonucleases and modifying enzymes were purchased from New England Biolabs Inc., U.S.A. and Gibco-BRL Technologies, Inc., U.S.A. : *Apa* I, *Bam*H I, *Cla* I, *Eco*R I, *Pst* I, DNA Polymerase I, Large (Klenow) Fragment, T_4 DNA Ligase T_4 DNA Polymerase, and Vent® DNA Polymerase

2.6 DNA isolation

2.6.1 Genomic DNA extraction

Sphingomonas chlorophenolica was grown to saturation in 100 ml mineral salt medium. Cells were centrifuged for 10 min at 4,500xg. Pellet was resuspended in 9.5 ml TE buffer (10 mM Tris-Cl, 1mM EDTA pH 8.0). Five hundred-µl of 10% SDS and 50 µl of 20 mg/ml proteinease K were added, mixed, and incubated at 37°C for 1 h. Addition of 1.8 ml of 5 M NaCl and mixed thoroughly, 1.5 ml of CTAB/NaCl solution (10% hexadecyltrimethyl ammonium sulfate, 0.7 M NaCl), mixed, and

incubated at 65°C for 20 min. An eqaul volume of chloroform/isoamyl alcohol (24:1) was added, extracted, and centrifuged for 10 min at 7,500xg. Supernatant was transferred with wide-bore tip to a new microcentrifuge tube. An eqaul volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, extracted, and centrifuged 10 min at 7,500xg. The supernatant was transferred with wide-bore tip to new tube. Isopropanol (0.6 volume) was added and mixed gently to precipitate DNA. The precipitate was transferred to a new tube containing 70% ethanol with a sealed pasture pipette, then washed and centrifuged for 15 min at 9,500xg. Supernatant was discarded. DNA pellet was resuspended in 0.5 ml TE buffer (10mM Tris-HCl, pH 8.0 and 1mM EDTA).

2.6.2 Alkaline extraction of plasmid DNA

A single isolated colony of *E. coli* strain XL-1-BLUE containing the desired plasmid was grown in 100 ml of LB broth containing appropriate antibiotic with shaking at 250 rpm, 37°C overnight (18 hr). The overnight culture was transferred to a fresh tube and bacterial cells were harvested by centrifugation at 4,500xg for 10 min. The pellet was resuspened in 2 ml of solution I (25mM Tris-HCl ,pH 8.0, 10mM EDTA, 50mM glucose) and the mixture was incubated at room temperature for 5 min. Four-ml of fresh solution II (10N NaOH and 20% SDS) was added, mixed gently and placed on ice for 10 min. Added 3 ml solution III (3M NaOAc, pH4.8) to a mixture, then mixed and placed on ice for 15 min. The mixture was centrifuged at 7,500xg for 10 min and supernatant was transferred to a new tube and extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) mixture. DNA was precipitated by addition of 2.5 volumes of ice-cold absolute ethanol and incubated at -20°C overnight. DNA was pelleted by centrifugation at 10,000xg for 15 min, then washed by two volumes of ice-cold 70% ethanol. The DNA pellet was air dried and

dissolved in 1 ml of TE (10mM Tris-HCl, pH 8.0 and 1mM EDTA). DNA solution was kept at -20°C until use.

2.7 Measurement of DNA concentration

The concentration of extracted DNA was spectrophotometrically measured at the optical density of 260 nm (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 50 μg/ml double-stranded DNA. Therefore, the DNA concentration is calculated by following formular:

$$[DNA]\ (\mu g/ml) = OD_{260} \times 50 \times \text{dilution factor}$$

The concentration of DNA fragments in agarose gel are estimated by comparison with a standard DNA. DNA fragments and standard DNA (500ng lamda phage DNA digested with *Hind* III) were run on 1% agarose gel dissolved in 1x TBE buffer (89mM Tris-HCl, 89mM boric acid and 2.5 mM Na_2EDTA,pH 8.3). Agarose gel electrophoresis was run at 100 Volt. After electrophoresis, the gel was stained with ethidium bromide. DNA concentration was estimated from the fluorescent band by comparing to the intensity of the red fluorescent band of the standard DNA. The red fluorescent intensity of the standard DNA bands of 23.1, 9.4, 6.6, 4.4, 2.3, 2.1 and 0.6 kb corresponds to 240, 98, 67, 45, 24, 21 and 6 ng of DNA.

2.8 Amplification of *pcp D* open reading frame (ORF) from *Sphingomonas chlorophenolica* ATCC 39723 by polymerase chain reaction (PCR)

Genomic DNA, isolated from *S. chlorophenolica*, was used as the DNA template in the PCR reaction. A hundred-μl PCR reaction contain, 100 ng of template DNA, 200 μM each of dNTP (dATP, dGTP, dTTP and dCTP), 100 pmole each of

forward primer (5′-ATG ACA AAC CCC GTT TCG AC-3′) and reverse primer (5′-GAG CTG CAG ATT CAG ATG TC-3′), 1X buffer for Vent® DNA polymerase (10mM KCl, 20mM Tris-HCl (pH 8.8 at 25°C), 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100), 1 unit of Vent® DNA polymerase. Initially, the PCR reaction mixture was preheating at 95°C for 3 min, then followed by 30 cycles of consecutive step: denaturation at 94°C for 1 min, annealing at 60°C for 30 sec and extension at 75 °C for 1 min. An addition extension step at 75°C was carried out for 1 min, then kept at 4°C until use.

2.9 DNA manipulation for gene cloning

2.9.1 Digestion of double stranded DNA

A digestion reaction was carried out in volume of 40 µl containing 2-3 µg of DNA, 1X digestion buffer, 10 unit of restriction endonuclease enzyme(s) and sterile distilled water. The reaction was incubated at the recommended temperature for 2-3 hours. Complete digestion of DNA was monitored by agarose gel electrophoresis.

2.9.2 Elution of DNA fragments

DNA fragments were resolved and recovered from an agarose gel by QIAEX II Gel Extraction Kit. After electrophoresis, DNA band of interest was excised from agarose gel with a clean sharp blade and put into a 1.5 ml-microcentrifuge tube. The gel slice was weighted, then added with 3 volumes of buffer QX I. Ten-µl of QIAEX II was added into a mixture, incubated at 50°C for 10 min mixed every 2 min. The mixture was centrifuged for 30 sec at 12,000xg and supernatant was removed by pipetting. Spin washing the pellet with 500 µl of QX I buffer at 12,000xg for 30 sec. Then, washing twice with 500 µl of QX II buffer. The pellet was air dried. DNA was

eluted by addition of 20 µl of 10 mM Tris-Cl, pH 8.5, incubated at room temperature for 5 min and centrifuged at 12,000xg for 30 sec. Pipetted the supernatant into new tube and kept this elute DNA at -20°C until use.

2.9.3 DNA ligation

An amount of vector and insert in ligation reaction mixture is calculate as follows:

insert (ng) = vector (ng) × (size of insert (kb)/ size of vector (kb))

x (molar ratio of insert to vector)

Ligation reaction was conducted in a volume of 15 µl, which contain 50 ng of vector, an amount of insert could calculate from above equation (molar ratio of insert : to vector = 3:1), 1X T4 DNA ligase buffer (50mM Tris-HCl (pH 7.5), 10 mM dithiothreitol, 1mM ATP, 25 µg/ml bovine serum albumin), 200 units of T4 DNA ligase and sterile distilled water. The reaction mixture was incubated at 16°C for 16 hours.

2.9.4 Generation of blunt end

3′ protruding end

The 3′ protruding end was blunted by flushing the 3′ overhang with T_4 DNA polymerase. The reaction to generate blunt ends was carried out in 20 µl reaction which consisted of 2-3µg DNA , 100 µM of each dNTP (dATP, dTTP, dGTP and dCTP), 1X buffer (500mM Tris-HCl pH 8.0, 100mM $MgCl_2$, 500mM NaCl), 3 units of T_4 DNA polymerase and sterile distilled water, incubated at 12°C for 20 min and heat inactivated the enzyme at 75°C for 10 min.

5′ protruding end

The 5′ protruding end was blunted by filling in the 3′ recessed end with DNA Polymerase I, Large (Klenow) Fragment. The reaction was carried out in 20 µl reaction which consisted of 2-3 µg DNA, 25 µM of each dNTP (dATP, dTTP, dGTP and dCTP), 1X buffer (500mM Tris-HCl pH 8.0, 100mM $MgCl_2$, 500mM NaCl), 3 units of klenow fragment and sterile distilled water, incubated at 30°C for 20 min and heat inactivated the enzyme at 75°C for 10 min.

2.10 Gene cloning (Construction of targeting vector)

Plamid pUC 19 and *pcp D* fragment from PCR were digested with *Bam*H I and *Pst* I. DNA fragments of 2.6 kb from pUC 19 and 0.8 kb from *pcpD* were separated by 1% agarose gel electrophoresis and eluted from gel by QIAEX II Agarose Gel Extraction Kit and subjected to ligation reaction. Ligation mixture was transformed to *E. coli* by electroporation, and desired clones were screened on LB agar plate containing 4 µg of X-gal/ ml, 5 µg of IPTG/ ml and 100 µg of ampicillin/ml.

Plasmid carrying *pcpD* was called as pPA. pPA was digested with *Apa*I to interrupt the ORF of *pcpD*, and 3′-overhang ends of linearized pPA were converted to blunt ends with T_4 DNA Polymerase. This fragment was then ligated to the DNA Polymerase I, Large (Klenow) Fragment filled-in, kanamycin resistance (kan^r) gene containing 1 kb *Bam*H I fragment from pVR 46. Ligation product was electroporated into *E. coli* XL 1-BLUE and the clones were selected on LB agar medium containing 15 µg of kanamycin/ml.

2.11 Transformation of plasmid DNA into bacterial cells by electroporation

2.11.1 Preparation of bacterial competent cells.

E. coli strain (XL 1-BLUE)

E. coli (XL 1-BLUE) was grown in LB broth with shaking at 250 rpm, 37°C for overnight (18 hours.). Inoculation of 5 ml of overnight seed culture into a 500 ml L-broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 0.5% NaCl). Then the bacterial cells were grown to an OD_{600} of approximately 0.5. Cells were chilld on ice and harvested by centrifuging at 4°C, 4,500×g for 10 min. The cell pellet was resuspended in 250 ml of cold sterile water, centrifuged as mentioned previously. Bacterial cells were washed twice with 150 ml cold sterile water, once with 100 ml of cold sterile 10% glycerol and resuspended in 1.0-1.5 ml of cold 10% glycerol. Finally, 40 µl of competent cells were aliquoted into each microcentrifuge tube and stored at -70°C for later use.

Sphingomonas chlorophenolica ATCC 39723

A single colonies of *S. chlorophenolica* was inoculated into mineral salts medium (0.82 g of K_2HPO_4 $3H_2O$, 0.19 g of KH_2PO_4, 0.5 g $NaNO_3$, 0.1 g $MgSO_4$ $7H_2O$, 4 g of L- sodium glutamate, 20µM of $FeSO_4$ per one liter) and grown at 28°C with shaking at 200 rpm. The culture was transferred into 500 ml mineral salts medium and the growth was continued at 28°C with shaking at 200 rpm until an OD_{600} of 0.5-1.0 was obtained. Cells were harvested by centrifugation at 4,500×g for 15 min at 4°C. The cell pellet was washed three times with sterile deionized water and once with strile 10% glycerol, as previously described in *E. coli*. Finally the cell pellet was resuspended in 1.0-1.5 ml of cold 10% glycerol and 30% polyethylene glycol 8000. Competent cells were aliquoted into 50 µl portions and stored at -70°c for later use.

2.11.2 Electroporation

E. coli strain XL 1-BLUE

For *E. coli*, 1 µl of plasmid DNA (100-200ng) was added into 40 µl of *E. coli* competent cells for electroporation in a 0.2 cm electrode gap cuvette using a Bio-RAD Gene Pulser with the following setting condition: 2.5 kV, 25 µF and 200 Ohms. One ml of SOC medium (0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 20mM $MgSO_4$ and 20mM glucose) was added to the cuvette immediately after electric pulse. Electroporated cells were transferred to a sterile culture tube and incubated for 1 hour with shaking at 225 rpm, 37°C. Cell suspension was plated on LB plates containing appropriate antibiotics.

Sphingomonas chlorophenolica ATCC 39723

For *S. chlorophenolica*, 4-5 µl of plasmid DNA (5 µg) was added into 50 µl of *S. chlorophenolica* competent cells for electroporation in a 0.2 cm electrode gap cuvette using a Bio-Rad Gene Pulser with the following setting condition: 2.5 kV, 25 µF and 600 Ohms. One-ml of SOC medium (0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$ and 20 mM glucose) was added to the cuvette immediately after electric pulse. Electroporated cells were transferred to a sterile culture tube and incubated for 16 hours with shaking at 150 rpm, 28°C. Cell suspension was plated on mineral salts agar plates containing kanamycin 15 µg/ml.

2.12 Southern-blot hybridization analysis

2.12.1 Southern blotting

Two micrograms of chromosomal DNA of *S. chlorophenolica* ATCC 39723 were digested with *Eco*R I. Digested DNA was electrophoresis to give good distribution on agarose gel. The agarose gel containing digested DNA was subjected to depurination with 250 mM HCl, for 10 min, at room temperature. The gel, then, was denatured in the solution containing 0.5 N NaOH, 1.5 M NaCl for 15 min at room temperature, twice. Finally, gel was neutralized with neutralization solution (0.5 M Tris-HCl, pH 7.5; 3 M NaCl) for 15 min at room temperature. The DNA fragments were readily transferred to a nylon membrane by the vacuum blotting, setting at 5 mm of Hg. The transferred DNA was crosslinked to the membrane by UV setting at 150mJoune. The membrane was rinsed briefly with water and allow to air dry.

2.12.2 Southern blot hybridization

The Southern analysis was performed using non-isotopic DNA detection technique according to the manufactureing protocol (DIG High Prime DNA Labeling and Detection Starter Kit II, Boehringer Mannheim, U.S.A.).

DNA labeling

DNA template was diluted with sterile water to final concentration of 1 μg in 16 μl of total volume. DNA solution was denatured by heating in a boiling water bath for 10 min, then quickly chilling in an ice. Addition of 4 μl DIG-High Prime and mixed briefly. The mixture was incubated at 37°C for 16 hours. The labeling reaction was stopped by heating to 65°C for 10 min and kept at -20°C until use.

Southern Hybridization

A DNA-linked nylon membrane was preincubated with prewarmed standard hybridization buffer at 68°C for 30 min before addition of 5-25ng/ml of heat denature DIG-labeled probe. DNA probe diluted in the same hybridization solution. Incubated the nylon membrane with DNA probe at 68°C for 16 hours with gentle agitation. After hybridization, the membrane was washed twice with 2X SSC, 0.1% SDS at room temperature for 15 min, then washed twice with 0.5X SSC, 0.1% SDS at 68°C for 5 min under constant agitation. Washed membrane was rinsed briefly (1-5 min) in washing buffer and incubated for 30 min in 100 ml blocking solution. The filter membrane was subsequently incubated in 20 ml of anti-DIG-Alkaline Phosphatease conjugate (1:10000) in blocking solution for 30 min. After incubation, the membrane was washed twice with 100 ml of washing buffer for 15 min and equilibrated in 20 ml detection buffer for 3 min. The membrane was then placed on a plastic bag and ready-to-use CSPD substrate solution was applied approximately 20 drops on the surface of nylon membrane. The membrane was incubated at room temperature for 5 min, then followed by incubation at 37°C for 5-15 min. Hybridization signal was detected by exposure of membrane to X-ray film at room temperature for suitable period of time. The solutions and buffers for Southern blot hybridization are in appendix F.