CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model MLS-3020, Sanyo, Japan

Autopipette: Pipetman, Gilson, France

Cassette with intensifying screen: 35x43 cm, Kodak, U.S.A.

Centrifuge, refrigerated centrifuge: Model J2-21, Beckman Instrument Inc., U.S.A. and microcentrifuge: Model MC-15A, Tomy Seiko Co., Ltd., Japan,

Centrifuge tube: Nalgene, USA.

Electrophoresis unit: Mini protein, Bio-Rad, U.S.A. and submarine agarose gel electrophoresis unit, TOYOBO, Japan

Filter paper: Whatman No.1, Whatman, England

Gel document: SYNGENE, England

Gene pulser cuvettes: Gene Pulser^{R}/*E. coli* Pulser^{TM} Cuvettes, Bio-Rad, U.S.A.

Incubator, waterbath: Model M20S, Lauda, Germany; BioChiller 2000; FOTODYNE Inc.,U.S.A. and ISOTERM 210, Fisher Scientific, U.S.A.

Incubator shaker: Innova^{TM} 4080, New Brunwick Scientific, U.S.A.

Lamina flow: HT123, ISSCO, U.S.A.

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, U.S.A.

Membrane: Biodyne B, Pall, U.S.A.

Microcentrifuge tubes: 0.5 and 1.5 ml microcentrifuge tube, Axygen Hayward, U.S.A.

Microwave oven: Model TRX1500, Turbora International Co., Ltd., Korea

pH meter: Model S20-K, Mettler Toledo, Switzerland

PCR system: Mastercycle gradient PCR System, Eppendorf, Germany

Power supply: Model POWER PAC 300, Bio-Rad, U.S.A.

Sonicator: SONOPULS Ultrasonic homogenizers, BANDELIN, Germany

Spectrophotometer: Spectronic 2000, Bausch & Lomb, U.S.A. and
DU Series 530 and DU 650, Beckman, U.S.A.

Spin column: Bio-spin column, Bio-Rad, U.S.A.

Thin-wall microcentrifuge tubes 0.2 ml: Axygen Hayward, U.S.A.

UV gene linker chamber: GS Gene Linker™, Bio-Rad, U.S.A.

UV transluminator: Model 2011 Macrovue, San Gabriel California, U.S.A. and
M-26, UVP, U.S.A.

Vortex: Model K-550-GE, Scientific Industries, Inc, U.S.A.

X-ray film: Hyperfilm ECL, Amersham Pharmacia Biotech Inc., U.S.A.

2.2 Chemicals

Acrylamide: Merk, Germany

Agar: Merck, Germany

Agarose: SEKEM LE Agarose, FMC Bioproducts, U.S.A.

Ammonium persulfate: Sigma, U.S.A.

Ammonium sulfate: Carlo Erba Reagent, Italy

Ampicillin: Sigma, U.S.A.

D-Alanine: Sigma, U.S.A.

L-Alanine: Sigma, U.S.A.

Aquasorb: BML, Thailand

1,4-bis[2-(5-phenyloxazole)]benzene (POPOP), Sigma, U.S.A.

Boric acid: Merck, Germany

Bovine serum albumin (BSA): Sigma, U.S.A.

Bromphenol blue: Merck, Germany

Casein hydrolysate: Merck, Germany

Cetyl trimethylammonium bromide (CTAB): Sigma, U.S.A.

Calf thymus DNA: Sigma, U.S.A.

Chloroform: BDH, England

Chloramphenicol: Sigma, U.S.A.

Coomassie brilliant blue R-250: Sigma, U.S.A.

Copper sulfate: Merck, Germany

2,5-diphenyloxazole (POP): Sigma, U.S.A.

DNA marker: Lamda (λ) DNA digested with *Hin*dIII, BioLabs, Inc., U.S.A., 100 base pair DNA ladder, Promega Co., U.S.A.

dNTP mixture: Promega, Co., U.S.A.

[α-P^{32}] dCTP (1,000-3,000 Ci/mmole): Amersham Pharmacia Biotech Inc., U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

Ficoll type 400: Sigma, U.S.A.

Formamide: Fluka, Switzerland

GBX developer solution: Kodak, U.S.A.

GBX fixer solution: Kodak, U.S.A.

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glucose: BDH, England

Glycerol: Merck, Germany

Glycine: Sigma, U.S.A.

Hydrochloric acid: Carlo Erba Reagenti, Italy

Isoamyl alcohol: Merck, Germany

Isopropanol: Merck, Germany

Isopropylthio-β-D-galactosidase (IPTG): Sigma, U.S.A.

Kanamycin: Sigma, U.S.A.

2-Mercaptoethanol: Fluka, Switzerland

N-cyclohexyl-3-aminopropane sulfonic acid (CAPS): Sigma, U.S.A.

N,N-dimethyl-formamide: Fluka, Switzerland

N,N'-methylene-bis-acrylamide: Sigma, U.S.A.

N,N,N',N'-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagent, Italy

β-Nicotinamide adenine dinucleotide (oxidized form) (NAD^+): Sigma, U.S.A.

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

Peptone from casein pancreatically digested: Merck, Germany

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

Phenol: BDH, England

85% Phosphoric acid: Mallinckrodt, U.S.A.

Phenylmethylsulfonyl fluoride (PMSF): Sigma, U.S.A.

Polyvinyl pyrrolidone: Sigma, U.S.A.

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

QIA quick Gel Extraction Kit: QIAGEN, Germany

Random hexamers: Promega Co., U.S.A.

Sodium acetate: Merck, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate: Sigma, U.S.A.

di-Sodium ethylene diamine tetra acetic acid: M&B, England

Sodium hydroxide: Merck, Germany

Sodium formate: Fluka, Switzerland

Standard protein marker: Amersham Pharmacia Biotech Inc., U.S.A.

Tetracyclin: Sigma, U.S.A.

Tetramethylammonium hydroxide: Sigma, U.S.A.

Toluene: Carlo Erba Reagenti, Italy

Triton X-100: Merck, Germany

Tris (hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy

Yeast extract: Scharlau microbiology, European Union

2.3 Enzymes and restriction enzymes

DyNAzyme™ II DNA Polymerase: FINNZYMES, U.S.A.

Large Klenow fragment: New England BioLabs, Inc., U.S.A

Restriction enzymes: New England BioLabs, Inc., U.S.A.; *Bam*HI, *Hin*dIII, *Kpn*I and *Xho*I

RNaseA: Sigma, U.S.A.

T_4 DNA ligase: New England BioLabs, Inc., U.S.A.

TargeTron™ Gene Knockout System Kit: Sigma-Aldrich, U.S.A.

2.4 Oligonucleotide primers

Oligonucleotides: Bioservice Unit, Thailand., Pacific Science, Germany and Theera Trading, Germany.

Alr For	5' CGGGATCCATGCAAGCGGCAACTGTTTTG 3'
Alr Rev	5' CCGGAATTCTTAATCCACGTATTTCATCGCGAC 3'
Alr IBS	5' AAAAAAGCTTATAATTATCCTTACGTCCCGGGATCGTGCG CCCAGATAGGGTG 3'
Alr EBS1d	5' CAGATTGTACAAATGTGGTGATAACAGATAAGTCGGGATC ATTAACTTACCTTTCTTTGT 3'
Alr EBS2	5' TGAACGCAAGTTTCTAATTTCGGTTGGACGTCGATAGAGG AAAGTGTCT 3'
Dad For	5' CGGAATTCATGACCCGTCCGATACAG 3'
Dad Rev	5' CCGCTCGAGTTACACCGTCACAACCGGG 3'
Dad IBS	5' AAAAAAGCTTATAATTATCCTTAGAACACCCTGATGTGCG CCCAGATAGGGTG 3'
Dad EBS1d	5' AAAAAAGCTTATAATTATCCTTAGAACACCCTGATGTGCG CCCAGATAGGGTG 3'
Dad EBS2	5' TGAACGCAAGTTTCTAATTTCGATTTGTTCTCGATAGAGG AAAGTGTCT 3'
T7 For	5' CCCAAGCTTAGGAAACAGACCATGAACACGATTAACGCT AAGAAC 3'
T7 Rev	5' CGGAATTCTTACGCGAACGCGAAGTCC 3'

2.5 Bacterial strains

Escherichia coli BL21(DE3), genotype: F⁻ *ompT* $hsdS_B$ (r^-_B m^-_B) *gal dem*(DE3), was used as a host to create the *alr* gene knockout mutant, *dad* gene knockout mutant and *alr* with *dadX* gene knockout mutant.

Escherichia coli BL21(DE3), containing pETAlaDH (Appendix A) was used as a source of alanine dehydrogenase.

2.6 Plasmid

pACD4K-C was used for create the single gene knockout in chromosomal DNA of *E. coli* BL21(DE3) (Appendix A).

pACD4K-C-loxP was used for create the double gene knockout in chromosomal DNA of *E. coli* BL21(DE3) (Appendix A).

p706-Cre was used as a vector for expression of Cre recombinase to remove antibiotic gene cassette, with flanked by loxP site from chromosomal DNA of *E. coli* BL21(DE3) (Appendix A).

2.7 Bacterial growth medium

Luria-Bertani (LB) broth containing 1% peptone, 0.5% NaCl and 0.5% yeast extract was prepared and adjusted pH to 7.5 with NaOH. For agar plate, the medium was supplemented with 1.5% (w/v) agar. Medium was steriled for 20 minutes at 121°C. If needed, selective antibiotic drug was then supplemented.

2.8 Crude extract preparation

Bacterial cells were grown in an appropriate condition. The cells were harvested by centrifugation at 8,000xg for 15 minutes, then washed twice with cold 0.85% NaCl and centrifuged at 8,000xg for 15 minutes. After that, the cell pellet was washed once in cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% β-mercaptoethanol and 1.0 mM EDTA) and centrifuged again. The cell pellet was stored at -80°C until the next step. For enzyme extraction, the cell pellet was resuspended in 5 ml of cold extraction buffer and then broken by discontinuous sonication on ice with 5 seconds pulse and 2 seconds stop interval for 15 minutes by sonicator. Unbroken cell and cell debris were removed by centrifugation at 12,000xg for 30 minutes. The supernatant was stored at 4°C for enzyme and protein assays.

2.9 Protein measurement

Protein concentration was determined by Lowry's method (Lowry *et al.*,1951) using bovine serum albumin (BSA) as a protein standard. The reaction mixture of 5 ml containing 20-300 μg of protein, 100 μl of solution A and 5 ml of solution B was mixed and incubated at 30°C for 10 minutes. After that, the solution mixture was incubated with 0.5 ml of solution C for 20 minutes at room temperature. Preparation of the solutions was described in Appendix B. The protein concentration was derived from the absorbance at 610 nm and calculated from the curve of standard curve of BSA.

2.10 Enzyme activity assay

2.10.1 Alanine dehydrogenease activity assay

The activity of alanine dehydrogenase for oxidative deamination of alanine was spectorphotometrically assayed. Reaction mixture 1 ml comprised 200 μmol of glycine-potassium chloride-potassium hydroxide buffer, pH 10.5, 20 μmol of L-alanine, 1 μmol of NAD^+, and enzyme. In a blank tube, L-alanine was replaced by water. Incubation was carried out at 30°C in a cuvette of 1-cm light path, The reaction was started by addition of NAD^+ and was monitored by measuring the initial change in absorbance of NADH at 340 nm.

One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH in 1 minute. Specific activity is expressed as units per milligram protein.

2.10.2 Alanine racemase activity assay (Watanabe, *et al.*, 1999)

The activity of alanine racemase for conversion of D-alanine to L-alanine was spectrophotometrically assayed. One milliliter of reaction mixture of comprised 100 mM of CAPS buffer, pH 10.5 (adjusted to pH 10.5 with tetramethylammonium hydroxide), 30 mM of D-alanine, 2.5 mM of NAD^+, 0.15 units of L-alanine dehydrogenase and about 400 μl of alanine racemase. In a blank tube, D-alanine was replaced by distilled water. Incubation was carried out at 37°C for 15 minutes in cuvette of 1-cm light path. The reaction was started by addition of alanine racemase and was monitored by measuring the initial change in absorbance of NADH at 340 nm.

One unit of enzyme is defined as the amount of enzyme that catalyzes the racemization of 1 μmol of D-alanine in 1 minute.

2.11 Partial purification of alanine dehydrogenase

L-alanine dehydrogenase was purified, from *E. coli* BL21(DE3) containing alanine dehydrogenease gene in pET-17b as described in a following method.

2.11.1 Bacterial cultivation

2.11.1.1 Starter inoculum

One colony of *E. coli* BL21(DE3) containing alanine dehydrogenase gene in pET-17b from agar plate was grown in 6 ml of LB medium supplemented with 100 μg/ml ampicilin, at 37°C with shaking overnight before 1 ml of overnight culture was inoculated in to LB medium 50 ml containing 100 μg/ml ampicilin. Cell culture was grown at 37°C with shaking overnight.

2.11.1.2 Enzyme production and crude enzyme preparation

The two percentage of starting inoculum from section 2.11.1.1 was transferred into 1,000 ml of LB medium containing 100 μg/ml ampicilin and shaked at 37°C. When turbidity of the culture at 600 nm had reached to 0.6, IPTG was added to 0.4 mM in order to induce enzyme production, and the cultivation was continued at 37°C for 4 hours before cell harvesting. The crude extracts were prepared as described in 2.8. Finally, The crude enzyme solution was collected for the determination of alanine dehydrogenase activity and protein concentration as described in section 2.10.1 and 2.9.

2.11.2 Purification procedures of enzyme

The crude enzyme from section 2.11.1.2 was purified according to Figure 2.1. All operations were done at 4°C. The buffer used in all step was 10 mM potassium phosphate buffer (KPB), pH 7.4 containing 0.01% 2-mercaptoethanol and 1 mM EDTA.

2.11.2.1 Ammonium sulfate precipitation

The precipitation of crude enzyme was done by slowly adding ammonium sulfate into enzyme solution to 20% saturation with gentle stirring by magnetic stirrer. After 30 minutes, the supernatant was removed by centrifugation at 10,000xg for 30 minutes then brought to final concentration at 40% saturation with solid ammonium sulfate and left for 30 minutes and then centrifuged at 10,000xg for 30 minutes. The precipitate was dissolved in 10 mM KPB, pH 7.4. The protein solution was dialyzed against the same buffer, at least 4 hours with 3 changes of buffer at 4°C before determination of the enzyme activity and protein concentration as described in section 2.10.1 and 2.9.

2.11.2.2 DEAE-Toyopearl column chromatography

DEAE-Toyopearl was activated by washing with 0.5 N sodium hydroxide for 2-3 times before rewashing by deionized water until the pH reaced to 8.0. The activated DEAE-Toyopearl was resuspended in the buffer and packed into 2.2 x 20 cm column followed by equilibrating with the same buffer for 5-10 column at flow rate 1 ml/min. The dialyzed protein solution from section 2.11.2.1 was applied to the DEAE-Toyopearl column. The unbound proteins were

Crude enzyme

↓

20-40% saturated ammonium sulfate precipitation

↓

DEAE-Toyopearl column

(Linear gradient eluted with 0-1 M KCl solution)

↓

Blue Sepharose high performance column

(cibacron blue 3G-A affinity column)

(Linear gradient eluted with 0-2 M KCl solution)

↓

Partial purification enzyme solution

(No contaminate with alanine racemase)

Figure 2.1 Flow chart of purification process of alanine dehydrogenase

eluted from the column with the buffer. Normally, keep washing until the absorbance at 280 nm of eluent decreased to a low value. After the column was washed thoroughly with the buffer, the bounded proteins were eluted from column with linear salt gradient of 0 to 1 M potassium chloride in the same buffer. The fractions of 5 ml were continuously collected using fraction collector. The elution profile was monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was determined as described in section 2.10.1. The potassium chloride concentration was investigated by measuring the conductivity. The active fractions were pooled for further purification step and then dialyzed against the buffer at least 1 hour for 3 times. The enzyme activity and protein concentration were measured as described in section 2.10.1 and 2.9.

2.11.2.3 Blue Sepharose column chromatography

Blue Sepharose column was used to separate the pyridine nucleotide-dependent enzyme. It contains cibacon blue 3G-A which is specific for $NAD(P)^+$-dependent enzyme. The other proteins such a albumin are bound to the column with weaker force, hydrophobic interaction and/or electrostatic force, than $NAD(P)^+$-dependent dehydrogenase so separation of $NAD(P)^+$-dehydrogenase from other proteins was occurred by eluted. In this experiment, the Blue Sepharose column with void volume 5 ml was used. The column was equilibrated with 10 mM KPB, pH 7.4 for 5 column volume (25 ml) at flow rate 1 ml/min. The dialyzed enzyme from 2.11.2.2. was filtered through a 0.45 μm filter and then applied into Blue Sepharose column by pumping with flow rate 1 ml/min. The 10 mM KPB, pH 7.4 were used to eluted the unbound proteins, fractions of 2 ml were continuously

collected and measured for the proteins at absorbance 280 nm until the absorbance decreased to low value. The bound proteins were eluted with 0-2 M potassium chloride linear salt gradient elution. Fractions of 1.0 ml were collected. The determination of the enzyme activity, protein and conductivity of potassium chloride were similar to section 2.11.2.2. The active fractions were pooled before concentrating with aquasorb, and then dialyzed against 10 mM KPB, pH 7.4 for 4 hours, at 4°C with 3 times changes. The protein concentration and enzyme activity were determined as described in section 2.10.1 and 2.9.

2.12 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

2.12.1 Pouring the separating gel (10% acrylamide)

The gel sandwich was assembled according to the manufacturer's instruction. For 2 slab gels, the 3.3 ml of solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide) and 2.5 ml of solution B (1.5 M Tris-HCl, pH 8.8, 4% SDS) were mixed with 4.2 ml of distilled water. Then 50 µl of 10% ammonium persulfate and 5 µl of TEMED were added and mixed rapidly by swirling. The solution was carefully introduced into gel sandwich using a Pasteur pipette. After the appropriate amount of separating gel solution was added, water was gently layered about 0.5 cm height on top of the separating gel solution. The gel was allowed to polymerize, distinguished by clear interface between the separating gel and the water. The water had been poured off before the stacking gel was set.

2.12.2 Pouring the stacking gel (5% acrylamide)

The 0.67 ml of solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide) was mixed with 1.0 ml of solution C (0.5 M Tris, pH 6.8, 4% SDS) and 2.3 ml of distilled water. Subsequently, 30 µl of 10% ammonium persulfate and 5 µl of TEMED were added and mixed rapidly. This stacking gel solution was loaded onto separating gel until solution reached the top of short plate. Then, the comb was carefully inserted into gel sandwich. After stacking gel was polymerized, the comb was removed carefully. Then, the gel was placed into electrophoresis chamber. The electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.8) was added into the inner and outer reservoir. The air bubbles, which were occurred in the well, were removed.

2.12.3 Sample preparation

The protein sample was mixed with 5x sample buffer (0.3 mM Tris-HCl, 50% glycerol, 20% SDS, 5% 2-mercaptoethanol and 0.05% bromophenol blue), boiled for 10 minutes at 95°C and cooled to room temperature. After that, the sample solution was introduced into well.

2.12.4 Gel running

An electrode plugs was attached to proper electrodes. Current flowed towards the anode at constant current (20 mA). The electrophoresis was continued until the dye front reached the bottom of the gel. Then, the gel was removed from glass plates and transferred to a small container.

2.12.5 Staining Procedure

Protein staining

The gel from section 2.12.4 was transferred to Coomassie staining solution (1% Coomassie Blue R-250, 45% methanol, and 10% glacial acetic acid). The gel was agitated for 10 - 20 minutes on a rocking shaker. The stain solution was poured out and the Coomassie destaining solution (10% methanol and 10% glacial acetic acid) was added. The gel was shaked slowly. To complete destaining, the destaining solution was changed many times and agitated until the blue-clear bands of protein were occurred (Appendix F).

2.13 Agarose gel electrophoresis

The 0.7 g of agarose powder was added to 100 ml electrophoresis buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) in an Erlenmeyer flask and heated until complete solubilization in a microwave oven. The agarose solution was then left at room temperature to 50°C before pouring into an electrophoresis mould. After the gel was completely set, the comb and seal of the mould was carefully removed. When ready, the DNA samples were mixed with one-fifth volume of the gel-loading buffer (0.025% bromphenol blue, 40% ficoll 400 and 0.5% SDS) and loaded the mixture into an agarose gel. Electrophoresis had been performed at constant voltage of 10 volt/cm until dye migrated to approximately distance through the gel. The gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide in distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration or molecular weight of DNA sample

was determined by comparison of the intensity or relative mobility with those of the standard DNA fragment.

2.14 Extraction of DNA fragment from agarose gel

Extraction of DNA fragment from agarose gel was performed using QIAquick gel extraction kit protocol. DNA fragment was excised from an agarose gel and transferred to an eppendorf tube. Three volumes of QG buffer was then added and incubated for 10 minutes at 50°C. After the gel slice had been dissolved completely, the sample was applied to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded. QG Buffer was added and centrifuged for 1 minute. The column was washed twice with PE buffer and centrifuged for 1 minute. Finally, the elution buffer was added to elute the DNA. Subsequently, the column was stand for 1 minute, and then centrifuged for 1 minute. The DNA solution was kept at 4°C.

2.15 Transformation of plasmid

2.15.1 Competent cell preparation

A fresh colony of *E. coli* host cell was cultured as a starter in 10 ml of LB-broth and incubated overnight with shaking at 37°C. The starter was inoculated to 1 liter of LB-broth and was then incubated at 37°C with shaking until the optical density at 600 nm reached 0.6. After that, the culture was chilled on ice for 15 to 30 minutes and the cells were harvested by centrifugation at 6,000 xg for 15 minutes at 4°C. The cell pellet was washed twice with 1 volume and 0.5 volume of cold sterile water, respectively. After that, the cell pellet was washed with 10 ml of 10% (v/v) ice cold sterile glycerol and finally resuspended in a final volume of 2 - 3 ml of 10% ice

cold sterile glycerol. The cell suspension was divided into 50 µl aliquots and stored at -80°C until used.

2.15.2 Electroporation

The cuvettes and sliding cuvette holder were chilled on ice. The Gene Pulser apparatus was set to 25 µF capacitor, 2.50 kV and the pulse controller unit was set to 200 Ω. Competent cells were gently thawed on ice. One to two microliter of DNA solution was mixed with 40 µl of the competent cells and then placed on ice for 1 minute. This mixture was transferred to a cold cuvette. The cuvette was applied one pulse at the above setting. After that, one milliliter of SOC medium was added immediately to the cuvette. The cells were quickly resuspended with a pasteur pipette. The cell suspension was transferred to new tube and incubated at 37°C for 1 hour with shaking. Finally, this suspension was spread onto the LB agar plate containing selective antibiotic drug for the selection of recombinant plasmid.

2.16 Southern Blot Analysis

2.16.1 Chromosomal DNA extraction (Frederick *et al.*, 1995)

A single colony was inoculated into 6 ml of LB medium and incubated at 37°C for 24 hours with shaking. Then each 1.5 ml of cell culture was centrifuged in microcentrifuge tube at 8,000xg for 2 minutes. The pellet was resuspended in 550 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by repeated pipetting. The cell solution was then treated with 30 µl of 10% SDS followed by the addition of 3 µl of 20 mg/ml proteinase K and incubated for 1 hour at 37°C. After incubation,

100 µl of 5 M NaCl and 50 µl of CTAB-NaCl solution (10% CTAB and 0.7 M NaCl) were added and incubated for 10 minutes at 65°C. The DNA was extracted with an addition of an equal volume of chloroform-isoamyl alcohol (24: 1 V/V), mixed gently, and centrifuged at 10,000xg for 10 minutes. A viscous fluid formed at the aqueous layers was carefully transferred to a new microcentrifuge tube and extracted with equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1 V/V) to ensure the complete extraction of DNA. DNA was precipitated by the addition of 2 volume of absolute ethanol to the aqueous phase and collected by centrifugation at 10,000xg for 10 minutes. The DNA was washed with 70% ethanol. After drying, the pellet was dissolved in an appropiate volume of TE buffer. Finally, DNA was digested with each of appropriate restriction enzyme.

2.16.2 Southern blotting

Southern blot analysis, the general outline by Sambrook *et al.* (1989) is followed. The digested DNA is run on a 1.2% agarose gel in TAE buffer (2M Tris-acetate, pH 7.0 and 100mM Na_2EDTA) and then blotted onto a biodyne B membrane by capillary blotting. After electrophoresis, the gel to be blotted was removed from the electrophoresis chamber and soaked in 400 ml of depurination solution (250 mM HCl). Treatment should be stopped immediately after the bromophenol blue dye had turned completely yellow. Depurination solution was discarded the gel was rinsed with distilled water. After that, the gel was covered with 400 ml of denatureation solution (1.5 M NaCl and 0.5 M NaOH) and agitated for 25 minutes after the bromophenol blue dye had returned to its blue colour. The solution was then discarded and the gel was rinsed with distilled water, then the gel was covered with

400 ml of neutralizing solution (1.5 M NaCl ans Tris-HCl, pH 7.0). A tray was filled with 20xSSC transfer buffer (0.3 N Na-citrate and 3 M NaCl, pH 7.0) and a supporting platform was made. The filter paper was cut into three pieces in the same size of the gel and one as long to be used as a wick. The plateform was covered with a wick so that the edges was submerged in the transfer buffer. The filter paper was placed on the top of wick, same size of the gel. The gel was laid face down on the filter paper, any bubbles was smoothed out. The membrane was laid over the gel. Strips of saran wrap were cut and laid on each edge of the membrane and draped over the side of the tray. The same size as the gel of the filter paper was placed. The paper towels with the same size as the membrane were cut and stacked on top of filter paper to a height of about 10 cm. Then the weigth was placed on the top of everything. When transfer had been completed, the nylon membrane was carefully removed from the gel by flat-tipped forceps. The blotted membrane was immobilized by UV cross-linking in Bio-Rad GS Gene Linker™ UV Chamber and stored in desicator at room temperature.

2.16.3 Probe labeling

The nine microliter of probe two-hundred nanogram DNA was mixed with 1 µl of 60 ng/µl of random hexanucleotide in a final volume of 10 µl. The mixture was incubated in boiling water for 3-5 minutes. Then, the denatured probe DNA was chilled on ice for 30 seconds, and any condensation was collected by a 2-second spin in a microcentrifuge. The tube was held behind a plexiglass shield. Then 2 µl of 1x klenow buffer, 5 µl of [α-^{32}P]dCTP (specific activity 3,000 Ci/mMole), 2 µl of H_2O and 1 µl of 5 -6 units of klenow fragment were added into the tube. The mixture was

incubated for 60 minutes at room temperature. Then the reaction was stopped with 5 µl of 0.5 M Na_2EDTA. After that, the reaction volume was adjusted to 100 µl with 100 µl with H_2O. The solution was passed through a spin column to remove the unincorporated [α-^{32}P]dCTP. For the Bio-Rad spin columns, the excess liquid was removed and the column was packed by spinning for 2 minutes at 2,500 rpm. Then the sample was load into the center of the packed resin and the excluded fraction were collected by spinning for 4 minutes at 2,500 rpm.

The labeled DNA can be stored at -20°C for up to 24 hours before using, but it is probably best if used immediately. To prepare for hybridization, the DNA solution was incubated in boiling water at least 5 minutes, then immediately added to the hybridization mixture.

2.16.4 Southern blot hybridization and autoradiography

The membrane was rehydrated in 500 ml of boiling 20 mM Tris/HCl, pH 8.0. The solution was allowed to cool to room temperature for 15-20 minutes. During this time, the prehybridization solution containing 50%(v/v) deionized formamide, 5x SSC (pH 7.0), and 20mM $Na_2EDTA.2H_2O$, 1x Denhardt's solution (100x Denhardt's solution consisted of BSA, Ficoll, and polyvinylpyrrolidone(PVP) at 2%[w/v] each), 0.2% (w/v) sodium dodecyl sulfate (SDS) and 100 µg/ml denatured DNA stock (calf thymus DNA and TE buffer, pH 8.0) was prepared and warmed to the desired temperature. The prehybridization solution was added to the blotted membrane in a plastic bag. The blotted membrane was incubated in a hybridization oven at 42°C, overnight. When prehybridization was complete, the hybridization solution was prepared (same as prehybridized solution, but without denatured DNA

stock) and warmed to the desired temperature. The prehybridization solution was discarded and replaced with the hybridization solution, then prepared denature ^{32}P-oligolabeled DNA probes was added. After moving bubbles, the bag was sealed by a heat-sealer and incubated the blotted filter for at least 16 hours in a hybridization oven at 42°C. When hybridization was completed, the blotted membrane filter was removed and immediately washed in 2x SSC, 0.1% SDS twice at the room temperature for 20 minutes and once with 1x SSC, 0.1% SDS at room temperature. If the general level of cpm is too high, washing were repeated in 1x SSC, 0.1% SDS at a room temperature. After that the blotted membrane was wrapped in plastic wrap and placed in an x-ray cassette with an Hyperfilm ECL. The film was exposed overnight at -80°C. When the blotted membrane contained barely enough cpm, exposures of 4 to 7 days were necessary in some cases. For Detection, X-ray film was washed in developer solution, water, fixer solution, and water, respectively for 1 minute in every step and air-dried.

Preparation of the southern blot analysis solution was described in Appendix G.

2.17 Construction of *alr* gene knockout mutant

2.17.1 Construction of pACD4K-C-Alr

To target the intron insertion to *alr* gene in *E. coli* BL21(DE3), the intron sequence was modified base on the sequences of *alr* gene by using the InGex Intron Prediction Program (www.Sigma-Aldrich.com/Targetronaccess) (Figure 2.2). The Alr IBS, Alr EBS1d and Alr EBS2 primers were designed. The PCR reaction was performed to construct the 350 bp retargeting PCR product by primer-mediated

mutation. The 50 µl of PCR reaction contained 25 µl of JumpStart REDTaq ReadyMix, 10 pmole of Alr IBS primer and Alr EBS1d primer, 2 pmol of Alr EBS2 primer and EBS universal primer and 1 µl of intron PCR template. The PCR condition was predenaturation at 94°C for 30 seconds, and 30 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds following by final extension at 72°C for 2 minutes.

The amplified 350 bp fragment was extracted from agarose gel by using method from section 2.14 and digested with *Hin*dIII and *Bsr*GI and ligated into the pACD4K-C fragment, which was digested with the same enzymes.

2.17.2 Creation of a *alr* gene knockout with the pACD4K-C-Alr

The ligation product from section 2.17.1 was transformed into *E. coli* BL21(DE3) by electroporation (section 2.15.2) and transformants were grown in SOC medium (Appendix H) at 37°C for 1 hour. An aliquot of 100 µl of the culture was then inoculated into 3 ml of LB medium containing 25 µg/ml chloramphenical, 1% glucose and incubated overnight at 37°C with shaking. Fourty microliter of overnight culture was added to 2 ml of LB medium containing 25 µg/ml chloramphenical, 1% glucose and grown at 37°C until OD_{600} reach 0.2. Then IPTG was add to 0.5 mM and cells were induced for 30 minutes at 30°C. The cells were then pelleted, resuspended in 1 ml of LB medium containing 1% glucose and incubated at 30°C for 1 hour with shaking. The recombinant clones were screened on LB agar plates containing 25 µg/ml kanamycin and incubated at 30°C for 2-3 days.

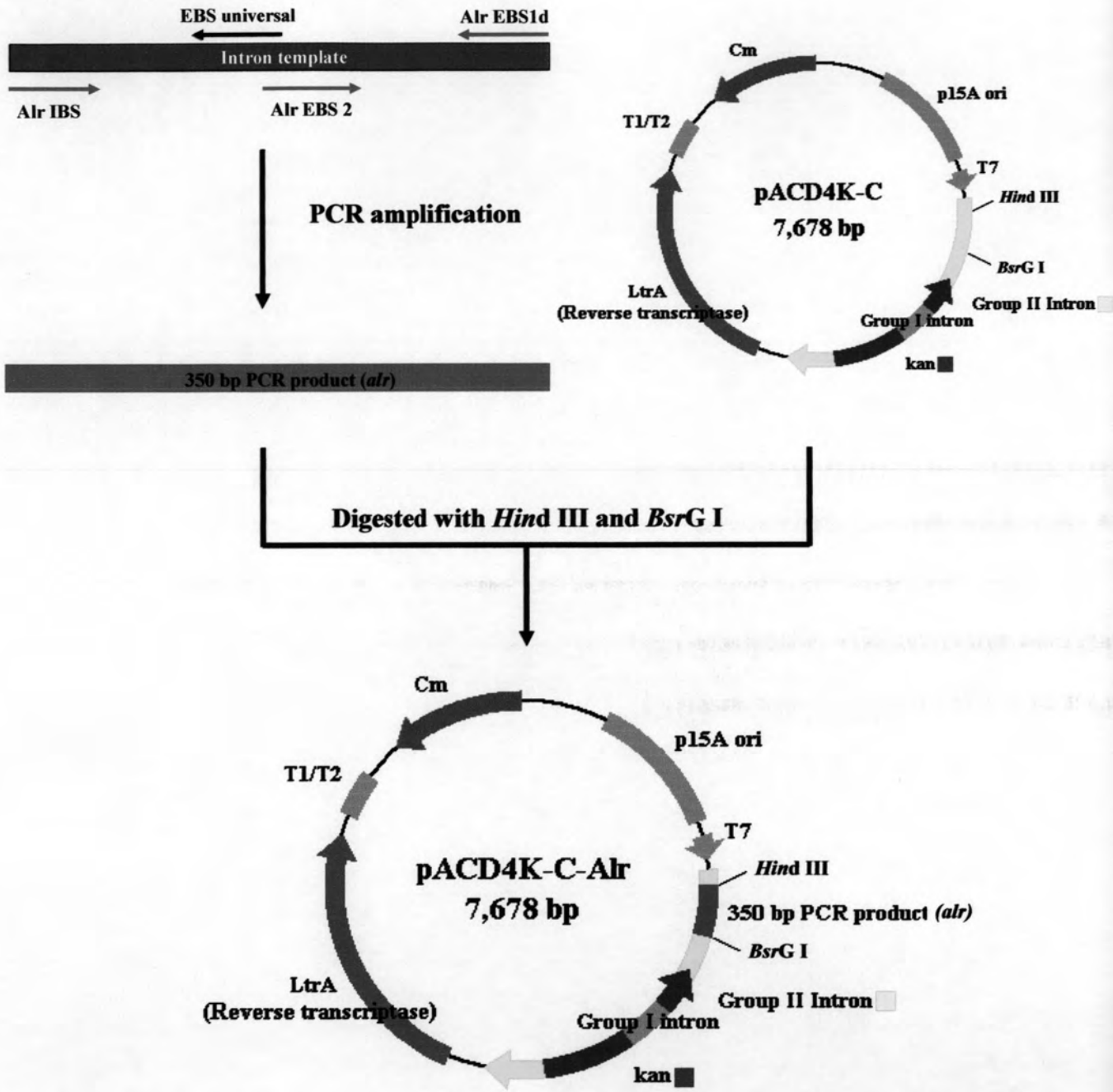

Figure 2.2 Construction of pACD4K-C-Alr

2.17.3 Confirmation of *alr* gene inactivation

2.17.3.1 PCR analysis

Kanamycin-resistant colonies from section 2.17.2 were screened for insertion of the intron into *alr* gene by colony PCR using specific *alr* gene primers, Alr For and Alr Rev and specific *T7* gene primers, T7 For and T7 Rev. The fifty microliter of PCR reaction contained of 0.7 unit of DyNAzyme™ II DNA Polymerase, 10 pmole of each specific *alr* gene and *T7* gene primer, 2.5 mM each dNTPs, 1x PCR buffer, 1.5 mM $MgCl_2$ and bacterial cells. The colony PCR condition was predenaturation at 94°C for 10 minutes, and 30 cycles of denaturation at 94°C for 1 minutes, annealing at 60°C for 2 minutes, extension at 72°C for 3 minutes following by final extension at 72°C for 7 minutes.

PCR product was estimated by agarose gel electrophoresis compared with known size of λ/*Hin*dIII marker.

2.17.3.2 Southern blot analysis

The southern blot analysis was followed method from section 2.16. The chromosomal DNA of *alr* gene knockout mutant was digested with each restriction enzyme, *Bam*HI, *Hin*dIII and *Xho*I.

The probe was generated via PCR with primers, Alr EBS1d and Alr IBS by using uncutting 350 bp PCR product from section 2.17.1 as a template. The PCR reaction contained with 0.7 unit of DyNAzyme™ II DNA Polymerase, 10 ng of uncut 350 bp PCR product, 10 pmole of each Alr EBS1d and Alr IBS primer, 2.5 mM each dNTPs, 1x PCR buffer, 1.5 mM $MgCl_2$. PCR reaction were performed with the following reaction: predenaturation at 94°C for 10 minutes,

and 30 cycles of denaturation at 94°C for 1 minutes, annealing at 60°C for 2 minutes, extension at 72°C for 3 minutes following by final extension at 72°C for 7 minutes.

2.18 Construction of *dadX* gene knockout mutant

2.18.1 Construction of pACD4K-C-Dad

To target the intron to *dadX* gene in chromosome of *E. coli* BL21(DE3), the pACD4K-C-Dad was constructed by the same method from section 2.17.1. The primers in PCR reaction, Dad IBS, Dad EBS1d and Dad EBS2, were used for generation for 350 bp PCR product (Figure 2.3).

2.18.2 Creation of a *dadX* gene knockout mutant with the pACD4K-C -Dad

The ligation product from section 2.18.1 was transformed into *E. coli* BL21(DE3). The method for creation of a *dadX* gene knockout mutant was similar to the method described in section 2.17.2.

2.18.3 Confirmation of *dadX* gene inactivation

2.18.3.1 PCR analysis

Kanamycin-resistant colonies from section 2.18.2 were screened for insertion of the intron by PCR using specific *dadX* gene primers, Dad For and Dad Rev. Specific *T7* gene primers, T7 For and T7 Rev were used to amplified T7 RNA polymerase gene. The colony PCR condition was the same method as describe in section 2.17.3.1.

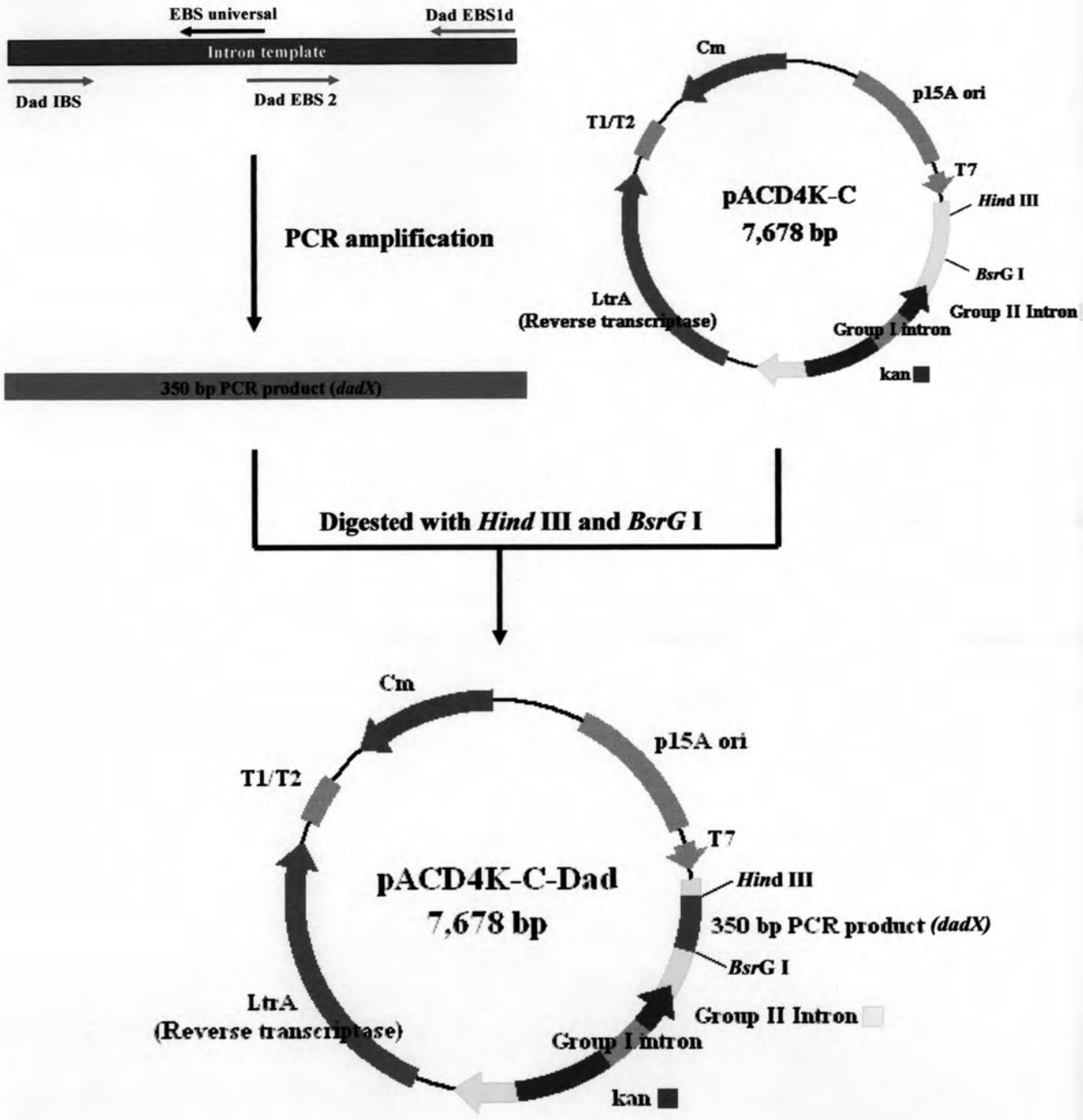

Figure 2.3 Construction of pACD4K-C-Dad

2.18.3.2 Southern blot analysis

The method from section 2.16 was used for Southern blot analysis of *dadX* gene knockout mutant. The chromosomal DNA from *dadX* gene knockout mutant was digested with each enzyme, *Bam*HI, *Hin*dIII and *Kpn*I, respectively. The uncutting 350 bp PCR product from section 2.18.1 was used as a template for generation of *dadX* gene knockout probe by using Dad IBS and Dad EBS1d primers. The PCR condition was performed for probe synthesis by using the same PCR method as section 2.17.3.2.

2.19 Construction of *dadX* and *alr* genes knockout mutant

The step of creation the *dadX* with *alr* gene knockout mutant was shown in Figure 2.4.

2.19.1 Construction of pACD4K-C-loxP-Dad

The method from section 2.18.1 were used for generation the 350 bp PCR product. The cutting 350 bp PCR product was ligated into the pACD4K-C-loxP (Figure 2.5).

2.19.2 Creation of a *dadX* gene knockout mutant with the pACD4K-C-loxP-Dad (first gene inactivation)

The ligation product from section 2.19.1 was transformed into *E. coli* BL21(DE3). The method from 2.18.2 was used for creation of a *dadX* gene knockout mutant.

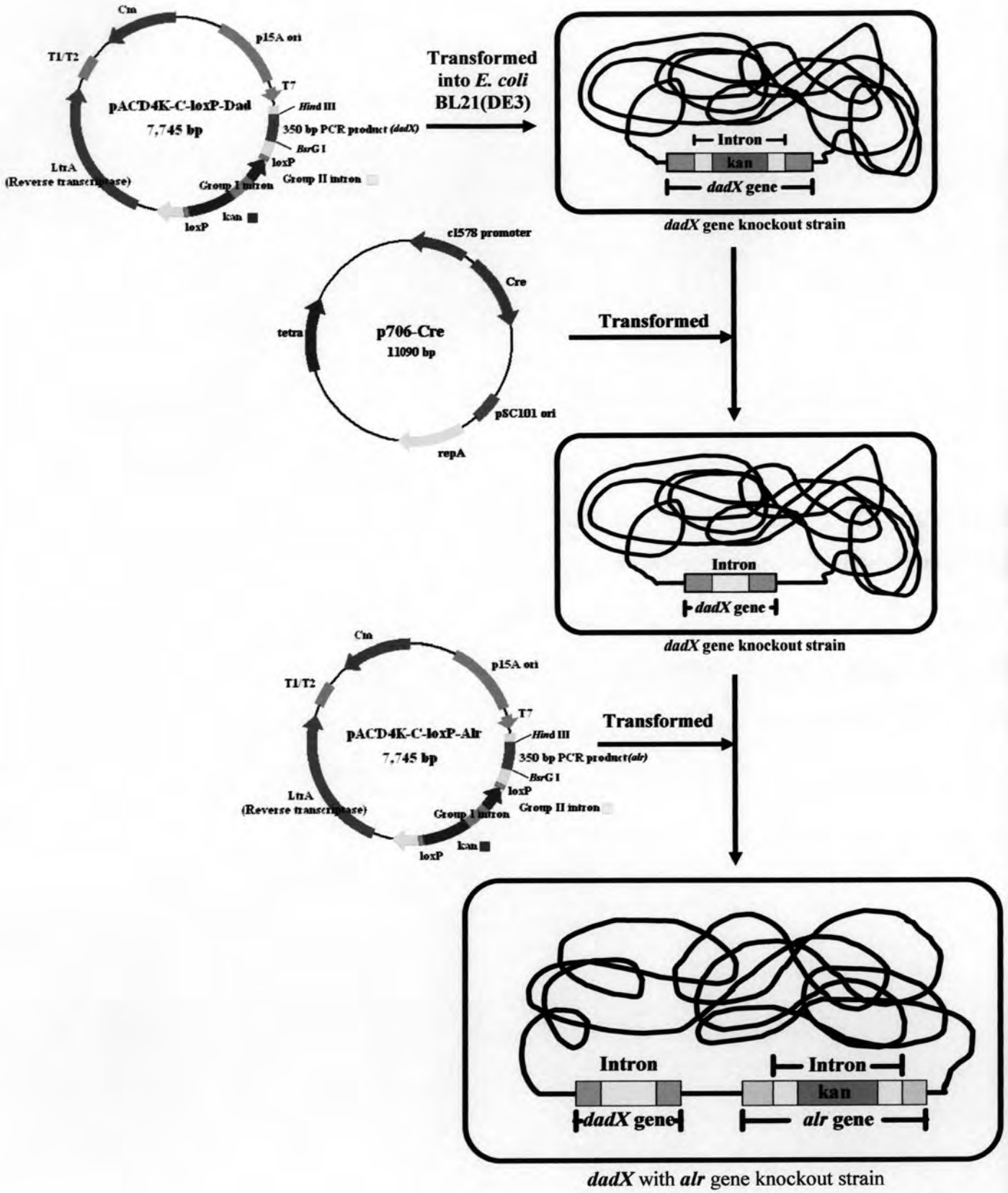

Figure 2.4 The step of creation the *dadX* and *alr* genes knockout mutant

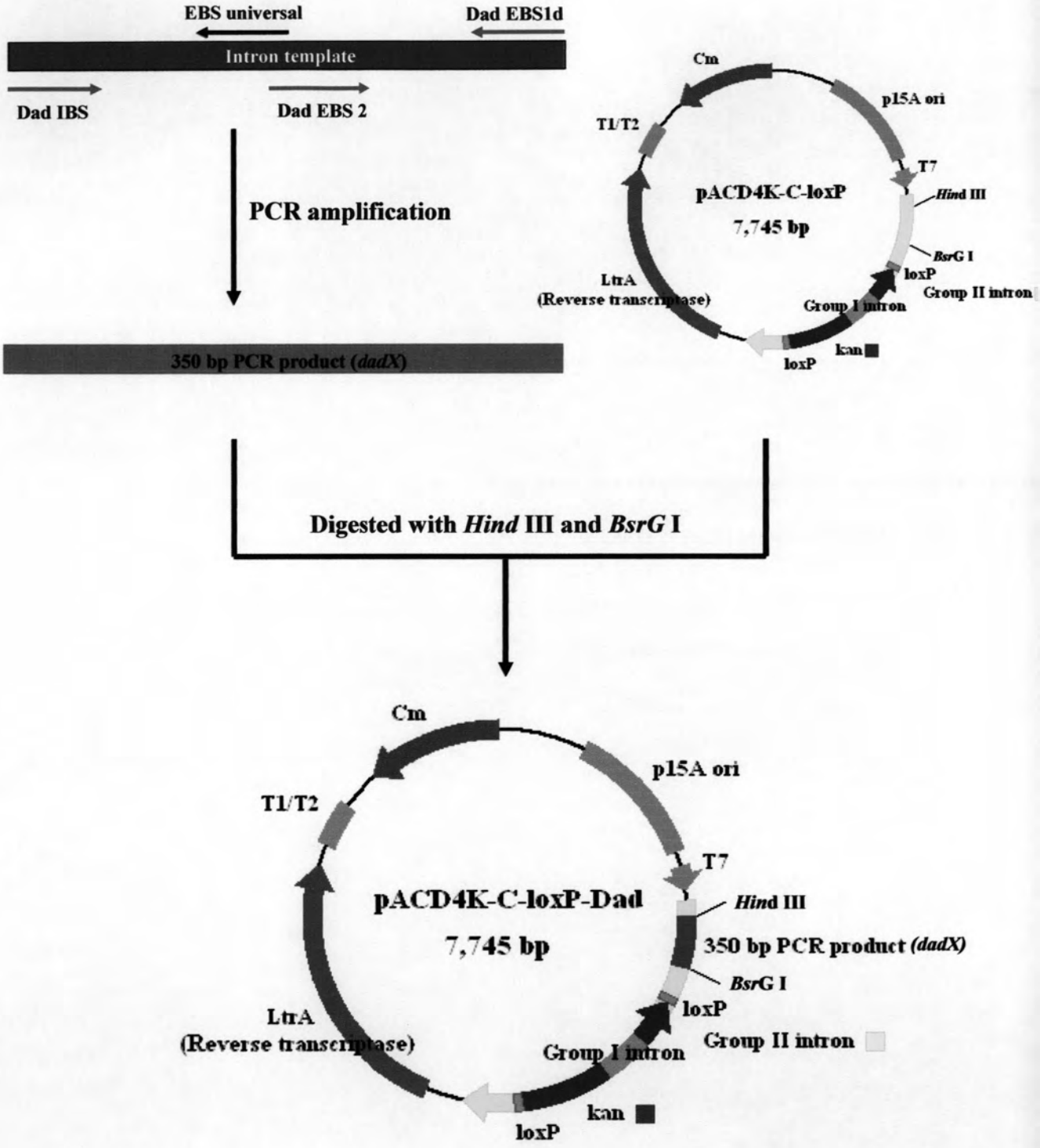

Figure 2.5 Construction of pACD4K-C-loxP-Dad

2.19.3 Confirmation of a *dadX* gene knockout mutant

The PCR analysis and southern blot analysis of *dadX* gene knockout mutant which created from pACD4K-C-loxP-Dad was performed using the same method from section 2.18.3.1 and 2.18.3.2, respectively.

2.19.4 Excision of kanamycin resistance gene cassette by the p706-Cre

The p706-Cre was transformed into *dadX* gene knockout mutant from section 2.19.3 by electroporation (section 2.10.2) and the transformants were grown in 1 ml of SOC medium at 30°C for 1.5 hours with shaking. The cells were plate on LB agar plate containing 5 μg/ml tetracyclin and 25 μg/ml kanamycin and incubated at 30°C for more than 24 hours. A single colony had cultured in 1 ml of LB medium containing 25 μg/ml of kanamycin at 30°C for 4 hours befor the temperature was switched to 37°C. The overnight culture was plated on LB agar plate. The colonies were screened for excision of the kanamycin gene cassette by PCR using two specific *dadX* gene primers, Dad For and Dad Rev.

2.19.5 Construction of pACD4K-C-loxP-Alr

The 350 bp PCR product was amplified by method described in section 2.17.1 (Figure 2.6), then ligated into the pACD4K-C-loxP.

2.19.6 Creation of a *dadX* and *alr* genes knockout mutant with the pACD4K-C-loxP-Alr (second gene inactivation)

The ligation product from section 2.16.5 was transformed into *dadX* mutant from section 2.19.4 by electroporation. The step for creation of *dadX* and

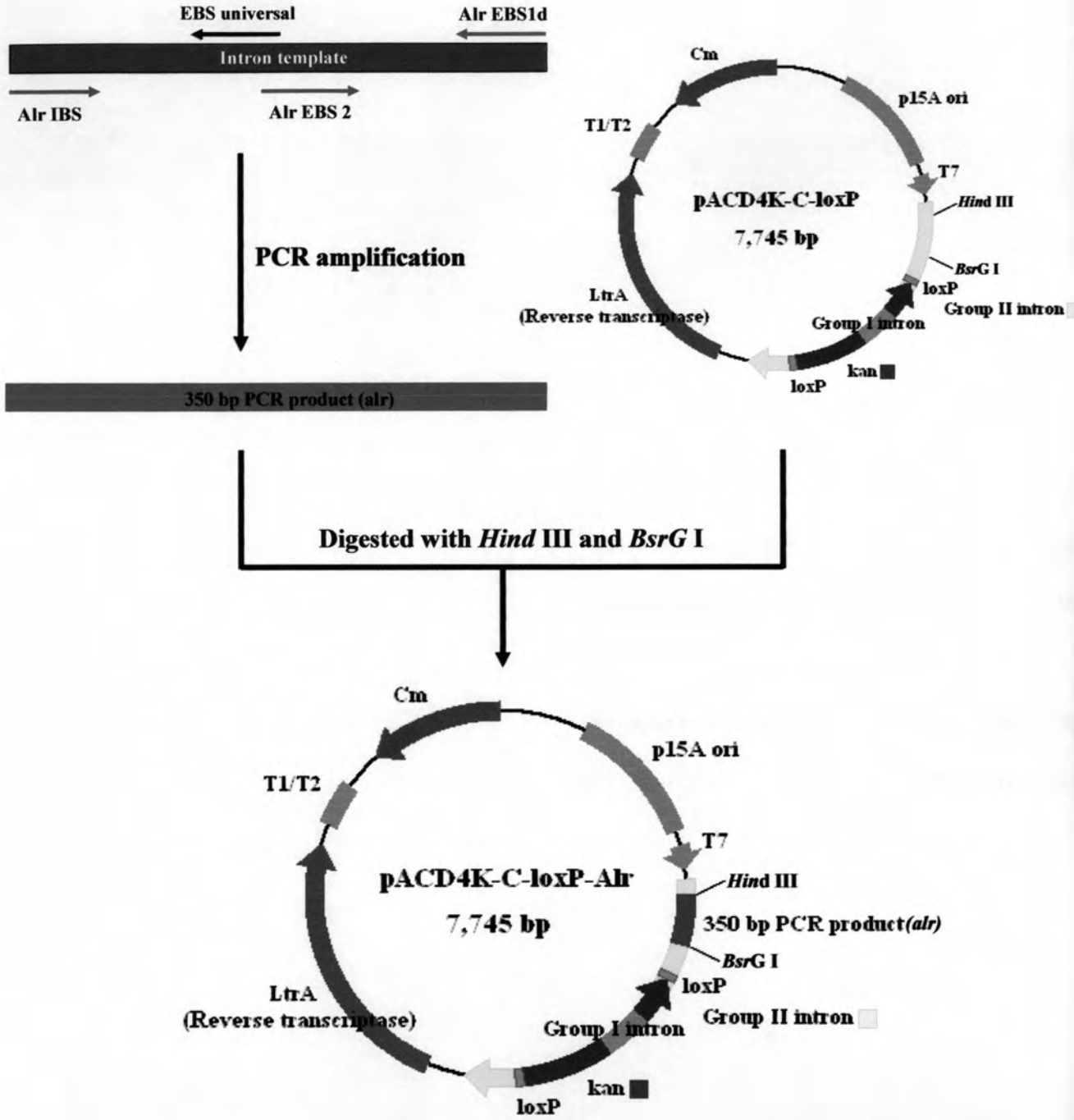

Figure 2.6 Construction of pACD4K-C-loxP-Alr

alr genes knockout mutant was done using the same method as described in section 2.17.2.

2.19.7 Confirmation of *dadX* and *alr* gene inactivation

The confirmation of *dadX* and *alr* gene inactivation was performed in the same method as section 2.17.3.1 and 2.17.3.2.

2.20 Growth curve determination (Iain, 2000)

The single colony of all mutants and wild type was inoculated into each 6 ml of LB medium. The 25 μg/ml kanamycin was added into the medium for all mutants. The cell culture was incubated at 37°C for overnight with shaking. The four milliliters of starter was inoculated into 200 ml of LB medium supplemented with an appropriate antibiotic then incubated at 37°C with shaking. The culture was monitored by measuring the turbidity at OD_{600} in every hour and every two hours after the ten hours. The graph between log OD_{600} and times was plotted. The growth rate constant was calculated from

$$k = \frac{ln2}{g}$$

which, k = Growth rate constant (h^{-1})

g = Doubling time (The time in hours taken from the population to double)

The g was calculated from the graph by chosen the OD_{600} value at the end of straight line then find the point which OD_{600} was double. The time between the two point was the doubling time.

2.21 Alanine racemase activity assay

The single colony of all mutants and wild type was inoculated into each 6 ml of LB medium. The 25 μg/ml kanamycin was added into the medium for all mutants and 10 mM D-alanine was added for *dadX* and *alr* genes knockout mutant. The cell culture was incubated at 37°C for overnight with shaking. The four milliliters of starter was inoculated into 200 ml of LB medium supplemented with an appropriate antibiotic and D-alanine (as described previously). Then incubated at 37°C with shaking until the optical density at 600 nm reached to 0.6. Then the crude enzyme was collected as described in section 2.8. The alanine racemase activity from the crude enzyme in each cultures was assayed

2.22 Stability test

The all mutants were daily subcultured by streaking on LB plate for 20 days. LB medium was supplemented with 10 mM D-alanine for *dadX* and *alr* genes knockout mutant Then the 1^{st}, 5^{th}, 10^{th}, 15^{th} and 20^{th} subcultured colonies were picked up to test of their intron insertion stability by colony PCR with each specific primer pair. The colony PCR condition was predenaturation at 94°C for 10 minutes, and 30 cycles of denaturation at 94°C for 1 minutes, annealing at 60°C for 2 minutess, extension at 72°C for 3 minutes following by final extension at 72°C for 7 minutes. Molecular weight of PCR product was estimated by agarose gel electrophresis compared with known size of λ/*Hin*dIII marker.