# CHAPTER TWO

# MATERIALS AND METHODS

## Materials and Equipments

### Bacterial strains and plasmids

*Escherichia coli* XL1-Blue was purchased from Stratagene. *Streptomyces lividans*TK24 and *S. narbonensis* ATCC 19790 and plasmid pWHM3 were obtained from Dr. Suchart Chanama. Plasmid, pET-17b and pMC-picK were acquired from Asst. Dr. Manee Chanama.

### Chemicals, Enzymes and culture media

Chemicals for protoplast isolation and transformation, and for genetic manipulation, including ammonium molybdate tetrahydrate, calcium chloride, copper (II) sulphate, iron (III) chloride, magnesium chloride hexahydrate, glucose, L – proline, manganese chloride tetrahydrate, polyethelene glycol (PEG), sodium borate decahydrate spermidine, spermine, zinc chloride, X-gal and IPTG were purchased from Sigma-Aldrich, U.S.A and all general and molecular biology reagents were purchased from BDH, England, Merck, Germany and Amersham Pharmacia Biotech Inc., U.S.A.

Bacto-agar, yeast extracts, Bacto – peptone, malt extracts and casamino acid were purchased from Difco, Becton Dickinson, U.S.A.

Antibiotics, including thiostrepton, tetracycline and ampicillin were purchased from Fluka, Switzerland; Merck, Germany, and Sigma, U.S.A., respectively.

Restriction endonucleases, modifying enzymes and DNA marker such as GeneRuler$^{TM}$ 1kb DNA ladder and λ/*Hin*dIII, T4 DNA ligase were obtained from New England BioLabs, Amersham Pharmacia and MBI Fermentas. Molecular weight standard

marker of protein and QIA quick Gel Extraction Kit were purchased from Amersham Pharmacia Biotech Inc., U.S.A. and QIAGEN, Germany.

**Equipments**

Biohazard laminar air flow, model V5 (LAB Service, Ltd., Thailand), Electrophoresis unit, MINI SUB™ DNA CELL (Bio-Rad, U.S.A.) , Incubator , Model UM700 (Memmert, Germany), Microcentrifuge, Model Mikro 12 – 24 (Hettick, Germany), Mini protein and Sub – Cell, Model 192 (Bio-Rad, U.S.A), Orbital shaker, Model Innova ™2100 (New Brunswick scientific Co. Inc, U.S.A.), Pipetman, P2, P20, P100, P200 and P1000 (Gilson, France), Ultracentrifuge, model L8-70 (Beckman Instrument, Inc., U.S.A.), Water bath shaker, model G-76D (New Brunswick Scientific Co., Inc., U.S.A.)

## Methods

### Preparation of plasmid pWHM3

Plasmid pWHM3 obtained from Dr. Suchart Chanama, Department of Biochemistry, Faculty of Science, Chulalongkorn University was transformed to *Escherichia coli* strain XL1-Blue for amplification. Then, the plasmid was isolated and purified by method of PEG – plasmid preparation. *Escherichia coli* XL1-Blue containing pWHM3 was grown in Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin at 37 °C, 250 rpm. The fifty milliliters of overnight growth culture was centrifuged 4 °C at 2,000 xg for 15 min. Cell pellet was suspended in 4 ml of solution I (25 mM Tris – HCl, 50 mM EDTA, pH 8.0, 1% glucose). After cell pellet was incubated for 5 min on ice, 8 ml of solution II (1% SDS, 0.2 M NaOH) was added and the solution was incubated for 5 min on ice. The cell mixture was added with 6 ml of solution III (1.667 M potassium acetate and 3.333 M acetic acid) centrifuged for 10 min at 12,000 xg. The supernatant was added with 20 ml isopropanol, and then incubated on ice for 10 min. After centrifugation 12,000 xg, supernatant was discarded. Pellet was washed twice with 70% ethanol. Dried pellet was suspended in 4 ml of TE buffer containing 25 μg/ml RNaseA and incubated for 30 min at 37 °C. The mixture was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) and aqueous phase was collected. Aqueous phase was added with 0.5 volume of 7.5M ammonium acetate and 2 volumes of absolute ethanol. The crude DNA was pelleted and washed with 70% ethanol. Washed pellet was dissolved in 2 ml of TE buffer. Addition of 0.5 volume of PEG solution (30 % PEG 6000 in 1.8 M NaCl) to the DNA suspension, the suspension was incubated at 4 °C overnight. DNA was precipitated by centrifugation. DNA pellet was resuspended in 200 μl of sterile distilled water and sodium acetate was added to 100 mM of final concentration. DNA in solution was precipitated with 2 volumes of absolute ethanol.

DNA was dissolved in 100 μl of TE buffer. Small of DNA solution plasmid was qualified and quantitated by agarose gel electrophoresis.

**Preparation of *picK* gene**

*Escherichia coli* DH5α containing pMC-*picK* was grown in LB broth containing 100 μg/ml ampicilln at 37 °C with agitation. The bacterium cell pellet was harvested by centrifugation. Plasmid isolation as described previously. Finally, plasmid DNA was dissolved in 100 μl of TE buffer and determined quality and quantity by agarose gel electrophoresis.

**Agarose gel electrophoresis**

One gram of agarose powder was dissolved in 100 ml of electrophoresis buffer, and then melted completely in microwave oven. The agarose was cooled down before pouring into an electrophoresis mould. After the gel was completely set, DNA sample mixed with 5X loading buffer (0.025 % bromophenol blue, 40 % ficoll 400, 0.5 % SDS) was loaded. Electrophoresis was performed at constant voltage of 10 volt/cm. The agarose gel was stained in ethidium bromide solution and destained by distilled water. DNA fragments on agarose gel were visualized under UV light and documented electronically by CCD-camera or photographed through a red filter using Kodak Tri X pan 400 film. Quantitative analysis of DNA was carried out by comparison to the intensity of the standard DNA marker.

**Cloning of *picK* in pWHM3**

The vector pWHM3 was linearized by *Eco*RI and *Xba*I. The digestion mixture containing of pWHM3, 1X digestion buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT and 50 mM NaCl), 30 unit of *Eco*RI and 30 unit of *Xba*I. Then the mixture was incubated at 37 °C for 18 hours and heat inactivated at 65 °C. The linearized pWHM3 was separated by agarose gel electrophoresis and extracted from agarose gel using QIA Quick gel extraction kit (QIAGEN, Germany).

Similarly, the pMC-*picK* digested by *Eco*RI and *Xba*I as follows: The digestion mixture was composed of plasmid, 1 x digestion buffer, 10 unit/ml of *Eco*RI and 10 unit/ml of *Xba*I. The digested *picK* fragment (size 1.3 kb) was separated on agarose electrophoresis and isolated from agarose gel as mentioned above.

The *picK* as an insert fragment was ligated to pWHM3 at a vector: insert molar ratio of 1:3. The ligation reaction contains 100 ng/mL of vector and 300 ng/mL of the insert, 1 x ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5 % (w/v) polyethylene glycol - 8000) and 10 units/mL of $T_4$ DNA ligase. The reaction was incubated at 16 °C for 16 hours, and then precipitated by cold ethanol. The pellet was washed and precipitated at 4 °C. Finally, DNA pellet was dissolved in water for further use in the next experiment.

**Transformation of pWHM3-*picK* into *E. coli* XL1-Blue**

The ligation product (pWHM3-*picK*) was transformed into *Escherichia coli* XL1-Blue competent by electroporation. Electro-competent cells were prepared by the method of Dower, 1988 (Appendix C). One to five microlitres of recombinant plasmid 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 settings. Subsequently, one milliliter of LB medium was added immediately to the cuvette.

The electroporation was carried out using the Gene Pluser apparatus setting a condition as follows: 25 μF, 2.5 KV and 200 ohm. The pulse time of electroporation is approximately 4.6 milliseconds. After electroporation cell suspension was incubated at 37 $^{o}$C for 2 hour with agitation and was spread onto the LB agar plates containing 100 μg/ml ampicillin, 1.0 mM IPTG and 20 μg/ml X-gal. White colonies were picked and their plasmids were isolated for further analysis.

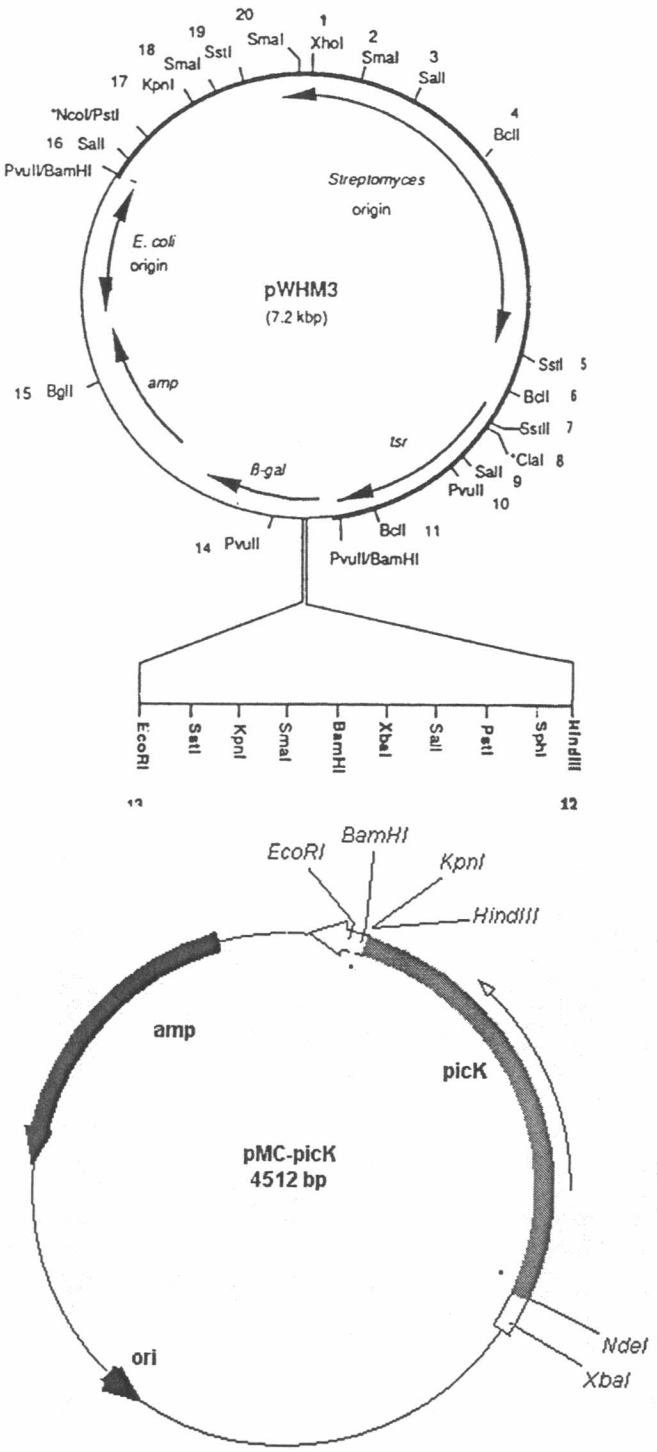


**Figure 2.1** Plasmid maps of pWHM3 and pMC-*picK*.

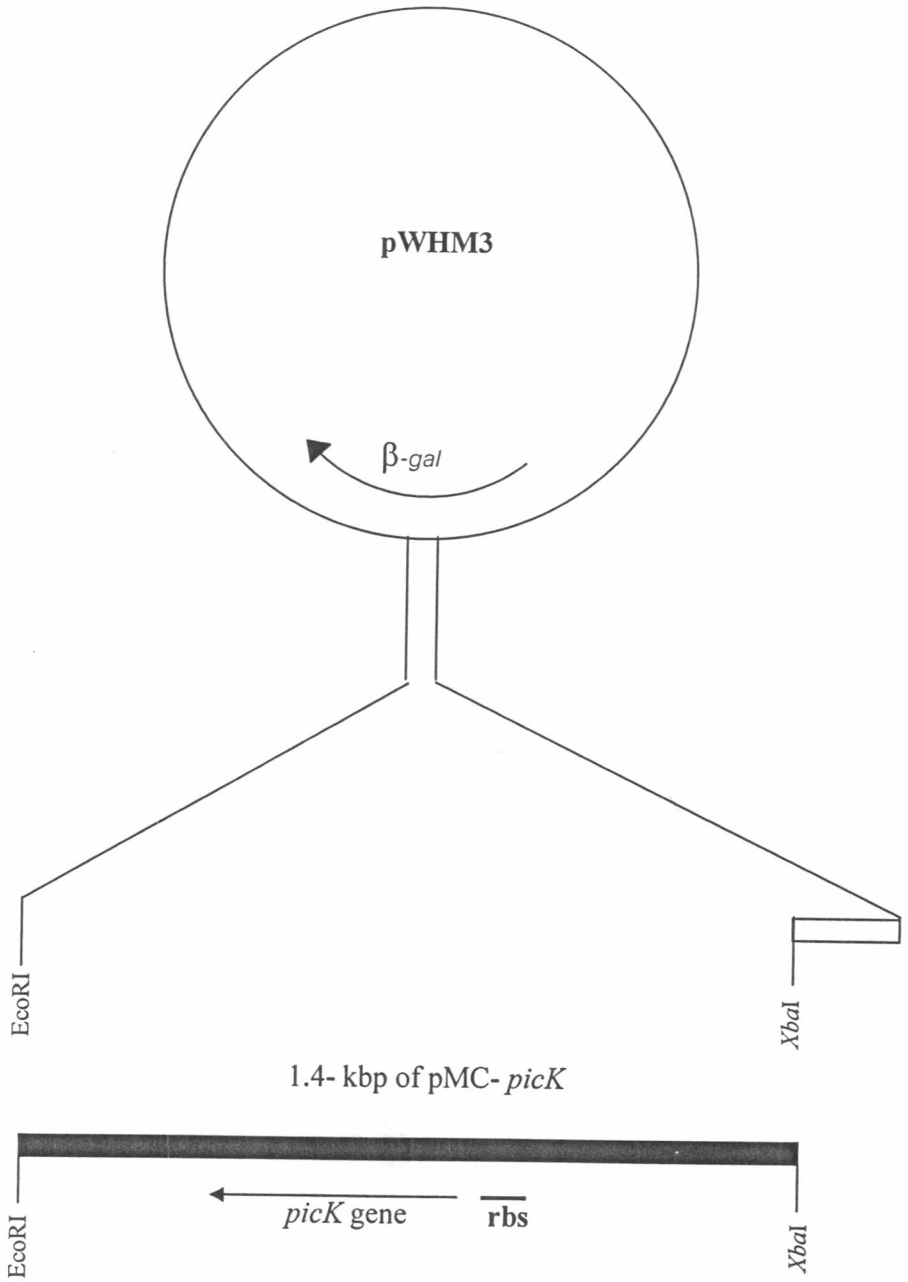


**Figure 2.2** Construction of pWHM3-*picK*. The 1.4-kpb *Eco*RI - *Xba*I fragment of pMC-*picK* was cloned into the *Eco*RI - *Xba*I site of pWHM3.

**Screening of recombinant *E. coli* clone carrying pWHM3-*picK***

The white colonies selected on LB agar containing 100 μg/ml ampicillin were inoculated into LB broth containing 100 μg/ml ampicillin. The overnight cell culture was harvested in 1.5 ml microcentrifuge tube. Then, 100 μl of ice-cold TE buffer, pH 8.0 was added to suspend cells 200 μl of NaOH/SDS solution (0.2 N Sodium hydroxide and 1 % SDS) was added and placed on ice for 10 minutes. The 3 M sodium acetate, pH 4.8 was added and the tube was placed on ice for 10 minutes. The mixture was centrifuged at 10,000xg for 10 minutes and then the supernatant was transferred to a new tube. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added, mixed and centrifuged at 12,000 rpm for 10 minutes. The upper phased liquid was transferred to a new tube. The plasmid DNA was precipitated with absolute ethanol and washed with 70 % ethanol. After drying, the pellet was finally dissolved in an appropriate volume of TE buffer, pH 8.0 containing 20 μg/ml DNase-free pancreatic RNaseA. The recombinant DNA was sized and proved by restriction endonuclease digestion (Ausuble *et al.* 1995).

**DNA sequencing of *pic*K in pWHM3-*pic*K recombinant**

The following sequencing primers were used in nucleotide sequencing of the *pic*K gene in plasmid pWHM3-*pic*K: M13 primers forward (5'GTT TTC CCA GTC ACG AC 3'), M13 reverse primers (5'GTT GTG TGG AAT TGT G 3') and 5' CAA GCG CGG GCA GGA TGG C 3'. The DNA sequencing carried out Bioservice Unit, NSTDA.

## Expression of pWHM3-*picK* in *E. coli* XL-Blue

Plasmid pWHM3-*picK* was electrotransformed into *Escherichia coli* XL1-Blue. The recombinant clone was selected to do induction experiment.

### Induction of *picK* in *E. coli* XL1-blue

The recombinant clone was grown in LB-broth containing 100 μg/ml ampicillin at 37 °C until optical density at 600 nm of 1.0. IPTG was added to a final concentration of 1 mM and the culture was shaker incubated at 37 °C. The sample was collected at post induction time of 0, 1, 2, 3, 4 and 5 hr. Protein in prepared from collected samples were analyzed by SDS-PAGE.

### Protein analysis by SDS-PAGE

The protein sample was mixed with 5 x sample buffer (0.3 mM Tris-HCl, 50 % glycerol, 20% SDS, 5% 2 – mercaptoethanol and 0.05 % bromophenol blue) in a microcentrifuge tube. The sample mixture was boiled for 10 min. The sample was clarified by centrifugation. Supernantant was collected for loading into a well by using syringe. SDS-PAGE was set from twelve percentage of separating gel and four percentage stacking gel. The system was run at constant current of 40 mA. The SDS-PAGE gel was removed and stained in Coomassie solution (1 % Coomassie Blue R-250 overnight. The gel was shaking slowly. To complete destain, the destaining solution was changed. The background was destained by destaining solution.

### Carbon monoxide difference Spectra Analysis

Bacterial cell suspension in buffer (50 mM potassium phosphate, pH 7.4) was added with sodium dithionite. A baseline of the mixture was spectrophotometrically determined using UV/Vis spectrophotometer (Model DU Series 650 Beckman, U.S.A.) at wavelength between 400 and 500 nm. The mixture was bubbled with carbon monoxide gas for 1 min, and then the carbon monoxide difference spectra were recorded and determined using an extinction coefficient of 91 $nM^{-1}cm^{-1}$ (Omura and Sato, 1964a). The microsome was used to be positive control.

## Expression of pWHM3-*picK* in *Streptomyces* species

### Preparation of *Streptomyces lividans* TK24 and *S. narbonensis* protoplasts

*Streptomyces* spores from either *S. lividans* TK24 or *S. narbonensis* ATCC 19790 were inoculated in 25 ml YEME medium (0.3% yeast extract, 0.5% Bacto-peptone, 0.3% malt extract, 1% glucose, 34% sucrose and 5mM $MgCl_2$-$6H_2O$ was added after autoclaving) in Erlenmeyer baffled flasks (Becton, U.S.A.) and the culture was incubated at room temperature for 40 hr. The culture broth was poured into a 50 ml screw cap bottle and centrifuged (10,000xg, 4 °C, 30 min). Supernatant was discarded and pellet was washed twice 15 ml of 10.3% sucrose. Pellet containing mycelium was resuspended in 4 ml of lysozyme solution (2 mg/ml lysozyme in L buffer) and incubated for 45 min at 30 °C. Mycelium suspension was mixed by syringe and mixed gently. Protoplasts were collected by centrifugation and were resuspended in 1 ml P buffer. The fifty microlitres of protoplast suspend overnight before use. The protoplasts suspension was counted in a haemocytometer and distributed samples of c. 4 x $10^9$ protoplasts (amount used for one transformation) 0.5 ml microcentrifuge tube for immediate used.

### Transformation of pWHM3-*picK* in *S. lividans* TK24 and *S. narbonensis*

One aliquot of protoplasts was centrifuged in bench – top centrifuge at 3000xg for 7 min at room temperature. The protoplasts was resuspended in 50 µl of P buffer left after pouring off supernatant, by tapping the tube (not by vortex mixing). The recombinant DNA was adjusted up to 20 µl by TE buffer added. Immediately added 0.5 ml T buffer containing 25% PEG 1000 (polyethylene glycol MW 1000, tissue culture grade, Sigma, U.S.A) and mixed gently. As soon as possible (no longer than 3 minutes after adding the PEG 1000), added 5 ml P buffer and spun in the bench – top centrifuge. The transformant protoplasts were resuspended 1 ml P buffer and then 0.2 ml spreaded on R2YE plates by made serial dilution was $10^{-2}$, $10^{-4}$, $10^{-6}$ and $10^{-8}$ diluents. After 18 hours incubated plates at 30 °C overlaid for selection (soft nutrient agar medium containing 12 µg/ml Thiostrepton). The resistant colonies after 3 days were scored.

**Screening of recombinant clone(s) carrying pWHM3-*picK***

One milligram of mycelium was harvested by centrifugation and suspended in total volume of 500 µl TESLR (25 mM Tris – HCl, 10 mM EDTA, pH 8.0, 50 mM glucose, 1mg/ml lysozyme, and optional 5 µg/ml pre – boiled Rnase A). Then mycelium suspension incubated at 37 °C for 30 min, and 250 µl NaOH/SDS solution (0.3 M sodium hydroxide and 2% SDS) and incubated at 70 °C for 15 min cap tubes were opened, cooled tubes to 37 °C. Extraction with 80 µl acid phenol/chloroform was added and transferred 700 µl supernatant to a new microcentrifuge tube containing 70 µl unbuffered 3 M potassium acetate and added 700 µl isopropanol. The pellet was redissolved in 50 µl TE butter and 5 µl 3M unbuffered sodium acetate and 25 µl neutral phenol/chloroform was added. Upper aqueous phase was transferred to new microcentrifuge tubes and precipitated with isopropanol. Spermine or spermidine precipitation by dissolved DNA in 500 µl TE, added 25 µl 100 mM spermine and 300 µl NaOAc/$MgCl_2$-$6H_2O$ solution (0.3

M sodium acetate and 10 mM magnesium chloride hexahydrate). The mixture was incubated 1 hour on ice and the pellets were collected by high speed centrifugation. The recombinant DNA was dissolved in 20 μl of TE buffer. The concentration or molecular weight of DNA sample was compared with the intensity and relative mobility of the standard DNA fragment.

**Expression of *picK* gene in *S. lividans* TK24 and *S. narbonensis***

The thiostrepton resistant colony was treated generate to spores on oatmeal plates (boiled oatmeal, trace salt solution and 2% Bacto - agar) containing 50 μg/ml thiostrepton, incubated 5 – 8 days at 30 °C. Spore suspension made up with 20% (v/v) glycerol (Merck, Germany) was inoculated in 25 ml YEME broth containing 50 μg/ml thiostrepton, cultured 2 days at room temperature on orbital shaker 250 rpm. After that, 1 % of the cell culture was inoculated into each 50 ml YEME broth containing 50 μg/ml thiostrepton and shake at room temperature, 250 rpm. When the turbidity of the culture at 600 nm had reached to 0.6, and then 1 mM IPTG was added to induce expression. The cultivation was continued at room temperature and collected by post-induction times at 0, 1, 2, 3, 4 and 5 hours. The cell collections were analyzed by SDS-PAGE and carbon monoxide difference spectra.