MATERIALS AND METHODS

1. Bacterial strains and culture conditions

E. coli JM109 harbored pWC3, called WC3 (23), was used in this study. This transformant was cloned from *P. pseudomallei* strain K1/88 and found to posses hemolytic activity of 2 hemolytic unit (HU)/ ml. It carries recombinant plasmid pWC3 (3.9 kb) of which the insert was approximately 1.2 kb with two *Pst*I fragments of about 300 and 900 bp in pUC18 at *Pst*I site. The culture was grown overnight with shaking (180 rpm) at 37oc in 3 ml of Luria-Betani broth (LB broth) containing 50 µg/ml of ampicillin.

2. Isolation and purification of plasmid DNA

Plasmid DNA was isolated and purified by WizardTM minipreps DNA purification system (Promega,Madison, WI,USA) as the following procedures. Cells were centrifuged at 12,000xg for 1 min and the pellets were suspended in 200 µl of cell resuspension solution (50 mM Tris-Cl [pH 7.5], 10 mM EDTA, 100 mg/ml RNase A). Then 200 µl of cell lysis solution (0.25 M NaOH,1% SDS) was added and mixed by gently inverting the tube until the suspension was clear. Next, 200 µl of neutralization solution (1.32 M potassium acetate, pH 4.8) was added and the lysates were centrifuged at 12,000xg for 5 min. The supernatant was collected and added with 1 ml of the WizardTM minipreps DNA purification resin. The mixture were passed through

the minicolumn by syringe and washed with 2 ml of column wash solution (200 mM NaCl, 20 mM Tris-Cl [pH 7.5], 5 mM EDTA and diluted with 1.4 volumes of 95% ethanol). The minicolumn was dried by centrifugation at top speed in microcentrifuge tube for 2 min and the plasmid was eluted with 50 µl of distilled water by spinning at top speed for 20 sec. To estimate concentration and check purity, the plasmid DNA was measured the optical density at wavelengths of 260 nm and 280 nm. The purified plasmid DNA was used for sequencing, partial restriction mapping, and characterization of gene products.

3. Partial restriction mapping

The plasmid DNA (1.2 µg) was digested to completion in the total volume of 50 µl with various restriction endonucleases. The one unit of enzyme was used with 1 µg of DNA at 37 °c for 24 h. The cleavage sites were determined after 1.5 % agarose gel electrophoresis in Tris-acetate buffer (TAE), using lambda/*Hin*dIII and ϕ x174/*Hae*III as molecular weight standard. In this manner, the partial restriction map was constructed.

4. DNA sequencing

DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al*. (66) using doubled-stranded DNA. Both strands were sequenced by utilizing the universal primers of pUC18 vector and by synthesizing oligonucleotide primers for the remaining region of the fragments. This study used Sequenase™ version 2.0 DNA sequencing

kit, USB, Cleaveland, OH, USA and the regions of compression in sequencing gels were resolved by replacing dGTP with 7-deaza-dGTP with the use of 7-deaza-dGTP Sequenase™ version 2.0 DNA sequencing Kit. The protocols for sequencing, using [α-35S] dATP as a label, were those suggested by the manufacturer as follows.

4.1 Preparation of sequencing reaction

4.1.1 Denaturation of double stranded template.

[alkaline-denaturation method (67)]

Plasmid DNA (3-5 µg) was denatured by adding 0.1 volumes of 2 M NaOH, 2 mM EDTA and incubating for 30 min at 37oc. The mixture was neutralized by adding 0.1 volumes of 3 M sodium acetate pH 5 and the DNA was precipitated with 2 to 4 volumes of absolute ethanol for 15 min at -70oc. After centrifugation at 12,000xg for 10 min the DNA pellet was washed with 70% ethanol and dissoloved in 7 µl of distilled water.

4.1.2 Annealing reaction

The 2 ul of sequenase reaction buffer(200 mM Tris-Cl [pH 7.5], 100 mM $MgCl_2$, 250 mM NaCl) and 1 µl of primer (0.5 pmol/ µl) was added to the denaturated DNA. The mixture was heat at 65oc for 2 min,cool slowly to less than 35oc over 15-30 min and chilled on ice.

4.1.3 Labeling reaction

The ice-cold annealed DNA mixture (10 µl) was labeled by adding 1 µl of 0.1 M dithiothreitol solution (DTT), 2 µl of 1:5 diluted labeling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP),

[α-35S] dATP (1,000 Ci/mmol at 10 μCi/μl, Amersham Corp.,UK), 2 μl of Sequenase Polymerase (13 units/μl) with dilution 1:8 in ice-cold enzyme dilution buffer (10 mM Tris-Cl [pH 7.5], 5 mM DTT, 0.5 mg/ml BSA) and incubated 2-5 min at room temperature.

4.1.4 Termination reaction

The 3.5 μl of labeling reaction was transferred to 2.5 μl of each termination mixture : ddGTP, ddATP, ddTTP, ddCTP, and incubated at 37oc for 5 min. The reaction was terminated by adding 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and heating the sample to 75oc for 2 min immediately before loading onto sequencing gel.

4.2 Preparation of denaturing polyacrylamide gel

The 6% polyacrylamide sequencing gel (see Appendix III) was prepared 2-20 hr prior to perform using the 20x60 cm gel of Model SA Sequencing Gel Electrophoresis System (BRL,Life Technologies, Inc.) with sharktooth comb (0.4 mm in thickness) for DNA separation.

4.3 Electrophoresis

Pre-electrophoresis was performed for 15 to 45 min at 2,000 volts using 1x Tris-borate buffer (1xTBE), pH 8.3. Then electrophoresis started after loading 2-3 μl of sample per well in the same voltage. The progress of electrophoresis was determined by migration of a marker dye front. After running, the gel was soaked in 5% acetic

acid and 15% methanol for 15 min to remove the urea, subsequently dried at 80°c for 90 min using the gel dyer and the DNA bands were visualized by autoradiography using an exposure to X-ray film.

4.4 Analysis of DNA sequence.

DNA sequence was analysed by using DNASIS version 2.1 (Hitachi software) and the blast (basic local alignment search tool) program of National Center for Biotechnology Information,NCBI was used for searching the homology to other DNA or amino acid sequence in the public databases such as GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank of Japan (DDBJ), Swissprot and Genpept.

5. Characterization of gene products

In vitro transcription/translation reaction was performed on purified plasmid DNA to identify the polypeptide product encoded by recominant plasmid pWC3. *E. coli* S30 Extract system for circular DNA (Promega, Madison, WI, USA) based on the method of Zubay (68,69) was performed according to the protocol of the manufacturer as follows.

5.1 *In vitro* transcription/translation

DNA template (3 µg) was added with 5 µl of 1 mM amino acid mixture minus methionine, 20 µl of S30 Premix which contained all other required component, including NTPs, tRNAs, ATP regenerating system, IPTG and appropriate salts, 1 µl of L-[^{35}S] methionine (1,000

Ci/mmol at 15 µCi/µl,Du Pont,NEN, Boston, MA), 15 µl of S30 Extract, and nuclease-free water to a final volume of 50 µl. The reaction was incubated at 25°c for 210 min. The tube was then placed in ice bath for 5 min to stop reaction.

5.2 Sample preparation (an acetone precipitation)

The 5 µl of the completed S30 Extract reaction was removed, added to 20 µl of acetone in a microcentrifuge tube, and placed on ice for 15 min. This step was required to remove polyethylene glycol (PEG) from the extract to prevent background staining. The unused portion of the reaction was stored at -20°c. The acetone-precipitated S30 sample was centrifuged at 12,000xg for 5 min. The pellet was kept and dried for 15 min under vacuum and then suspended with 20 µl of SDS sample buffer (2%SDS, 5% β-mercaptoethanol, 10% glycerol, 0.0005% bromophenol blue, 0.0625 M Tris-Cl, pH 6.8) before heating to 100°c for 2-5 min. The sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

5.3 Analysis of the product

The product of *in vitro* transcription/translation was analyzed by SDS-PAGE. The separating gel was 12% polyacrylamide (see Appendix III) prepared in a glass 16x18 cm slab gel system (model DASG-250, C.B.S. Scientific CO.). The stacking gel was 4% polyacrylamide (see Appendix III). The 20 µl of sample was applied

into gel and the electrophoresis was performed in 1XSDS electrophoresis buffer (see Appendix III), with the constant current at 25 mA until the marker dye had moved to the end of the gel. The gel was stained with coomassies brillient blue R 250 solution (0.05% coomassie brillient blue R 250, 50% methanol, 10% acetic acid) overnight, subsequently destained with a destaining solution (5% methanol, 7% acetic acid) until the background was clear. The labeled protein bands were visualized by autoradiography using an overnight exposure to X-ray film after drying at 60°c for 1 h.

5.4 Estimation of Molecular Weight (MW)

Molecular weight of a protein was estimated by the relative mobility according to the method described by Laemmli (70). SDS-PAGE marker proteins (Pharmacia,Sweden) was applied at the same time as unknown protein. The relative mobility (Rf) of the marker proteins: phosphorylase b, bovine serum albumin (BSA), glutamate dehydrogenase, ovalbumin, aldolase, carbonic anhydrase, soy bean trypsin inhibitor and lysozyme whose MWs were 97,000, 66,000, 55,000, 43,000, 40,000, 31,000, 21,000, 14,000 daltons, respectively, was determined from the following formular

$$Rf = \frac{\text{distance of protein migrated from origin.}}{\text{distance from origin to marker dye.}}$$

The Rf values were plotted against MWs on a semi-logarithmic paper as a standard MW curve , calculated and estimated for the MW from this standard curve.

6. Confirmation of the fragment orientation

6.1 Cloning of *Pst*I-inserted DNA into pUC19

6.1.1 Digestion and elution

The pWC3 DNA (2 µg) was completely double-digested with *Hin*dIII and *Eco*RI in a total volume of 100 µl. The *Hin*dIII-*Eco*RI fragments were electrophoresed through 1% low melting temperature agarose gel (71). The gel containing target bands was cut out, added with TE to decrease agarose percentage to 0.4 % or less and melted at 65 °c. DNA was extracted with an equal volume of buffered phenol, mixed vigorously for 5 to 10 min, and centrifuged at 12,000xg for 10 min at room temperature. The aqueous phase was collected and reextracted with an equal volume of TE and centrifuged as described above. After ethanol precipitation, DNA was suspended in TE buffer.

6.1.2 Ligation and transformation

The 80 ng of the eluted fragment was ligated with 120 ng of dephospholyrated *Hin*dIII-*Eco*RI cut pUC19 DNA (72) with T_4 ligase (3 units) in a total volume of 20 µl at 16 °c for 24 hr. *E. coli* JM109 made competent by $CaCl_2$ treatment (72) was transformed with the ligation mixture and then plated onto LB agar containing 50 µg/ml of ampicillin and 100 µl of 2 % X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in dimethylformamide.

6.1.3 Selection of subclones

Transformants which carried recombinant plasmids formed white colonies whereas those with self-ligated pUC19 formed blue colonies. Thus, the recombinant plasmids in white colonies were isolated by an alkaline lysis procedure (72). Cells were suspended in 100 µl of GTE (0.05 M glucose, 0.025 M Tris-Cl, pH 4.8, 0.01 M EDTA), lysed with 200 µl of lysis buffer (0.2 N NaOH,1% SDS) and added with 150 µl of potassium acetate pH 4.8 at 4oc for 5 min. The supernatant was kept by centrifugation at 12,000xg for 1 min. The DNA was precipitated with ethanol and resuspended the pellet in 40 µl of TE buffer. The resuspened DNA was used for restriction cleavage with *Pst*I and analysed on 1.5% agarose gel. The purified plasmid DNA was prepared by Wizard™ minipreps.

6.2 Determination of the protein products

In vitro transcription/translation reaction was performed on purified plasmid DNA to identify the polypeptide product encoded by the recombinant pUC19, pWC3R as described in section 5.

6.3 Determination of hemolytic activity

The hemolytic actvity of the transformant containing recombinant pUC19 and pUC19 was determined by using cellophane plate technique (29) as the followings. Cells were grown overnight at 37 oc

in 5 ml of tryptone-glucose broth. The 60 µl of broth-culture was spreaded over the cellophane placed on tryptone-glucose agar. After 48-hr incubation, bacterial culture on the cellophane plate was washed off with 3 ml of normal saline and centrifuged at 2,600xg for 15 min. The supernate fluid obtained was determined for hemolytic activity by preparing 0.5 ml of supernatant in two-fold dilutions with normal saline. The 0.5 ml of 1% washed human group O red blood cells were added and incubated 37 °c for 2 hr. The reciprocal of the highest dilution showing complete hemolysis was taken as the hemolytic unit.