## DISCUSSION

An *E. coli* clone carrying recombinant plasmid harbored *P. pseudomallei* DNA that expresses a potential hemolysin has been isolated. In this study, DNA sequencing and *in vitro* gene expression were performed to determine the DNA sequence and gene product of the insert DNA fragment (1.2 kb) of this clone.

Wizard ™ miniprep DNA purification system was used in purification of plasmid DNA instead of CsCl gradient purification which is laborious, time-consuming and expensive. In contrast, the method performed in this study is simple, rapid, and effective for DNA sequencing and *in vitro* transcription/translation. Double-stranded DNA is directly used as template in the dideoxy chain termination method as the sequencing of phospholipase C (*plc*) gene of *P. aeruginosa* (63). In addition, the universal primers that anneal to vector sequence are also provided for double-stranded sequencing of DNA cloned into pUC vector. Moreover, progressive oligonucleotide strategy (74) was developed by using each sequence obtained to design next synthetic oligonucleotide primers along the DNA in both strands. Sequencing of two *Pst*I subclones was also performed and the results corresponded with those obtained from the intact clone (data not shown).

Because of the high G+C content of *P. pseudomallei* gene, the compression occured in some regions due to the formation of secondary structure. In general, it was resolved by using a nucleotide analog

such as dITP or 7-deaza dGTP and separating in 40% formamide sequencing gel (71). In this study, the compression could be resolved only by using 7-deaza dGTP substituted for dGTP to reduce the formation of secondary structure, whereas others could not. Furthermore, the sequencing in the complementary strand and the use of primers designed close to the compression region were carried out to solve the problem.

*In vitro* transcription/translation was used for identifying the encoded polypeptide. *E. coli* S30 extract is deficient in endo-protinase and protease activity so the expressed protein is stable. Nevertheless, the protein obtained by *in vitro* expression had no hemolytic activity, because some processes such as signal cleavage to process the protein into active form may be needed, or extremely small amount of protein was produced, as has been reported in the study of *Actinobacillus pleuropneumoniae* hemolysin (33).

For the analysis of DNA sequence, there is only one frame given the largest open reading frame which was not fused in-frame with *lac* gene. The open reading frame (ORF) showed only N-terminal sequence, due to the ATG start codon, and it had no stop codon shown. The predicted protein of 43.5 kDa, which was the fusion product with the C terminus of β-galactosidase (α-fragment), corresponded to the *in vitro* expressed protein of 45 kDa. No apparent promoter with significant homology to the *E. coli* concensus promoter was found. This finding suggests that its promoter was not cloned. In addition, the expression of this gene should be controlled under *lac* promoter

since hemolytic activity was increased when IPTG was added in the medium (data not shown). At this point, the cloned gene was flipped by cloning into pUC19 which has polylinker in opposite direction to that of pUC18. No protein product was detected in recombinant pUC19 and no hemolytic activity was detected by cellophane plate technique. Therefore, this cloned gene was not transcribed from its own promoter in *E. coli*, but it was expressed under the control of *lac* promoter. It was noticed that the MWs of both β-lactamase and the insert-encoded protein identified by *in vitro* expression (33 kDa, 45 kDa, respectively) were higher than the MWs of β-lactamase (31.5 kDa [75] ) and that of which estimated from the predicted amino acid sequence (43.5 kDa). It may be due to the nature of the proteins expressed in *in vitro* transcription/translation. The weak bands resulted from the incomplete translational products and other proteins in the reactions.

According to earlier study (23) , WC3 possesses low hemolytic activity (2 HU/ml) which may be due to the weak promoter of pUC18, the truncated gene cloned, or the codon usage bias in *E. coli* given in low expression. In this study, the result revealed that WC3 contains *P. pseudomallei* gene which codes for only an N-terminal truncated protein of 42 kDa and no upstream transcriptional control region. In addition, the codon usage of *P. pseudomallei* gene was different from that seen for *E. coli* genes (76) with a bias towards a G or C in the third codon position. In order to clone the entire gene, the cloned gene from WC3 may be used as probe to detect a larger fragment generated by restriction endonucleases that have no site in WC3 DNA insert such as *Hin*dIII, *Bam*HI, *Xho*I and *Xba*I. In addition, either *in*

*vivo* expression such as maxicell analysis (77) to demonstrate the mature hemolysin or preparation of purified hemolysin from the parental strain should be performed in order to compare the properties with each other. The mature hemolysin is also required for N-terminal amino acid sequencing to confirm the predicted signal peptide.