RESULTS

1. Isolation and purification of plasmid DNA

The pWC3 plasmid DNA was isolated and purified by Wizard ™ minipreps DNA purification system. The concentration was estimated by measuring the optical density (OD) at 260 nm. An OD of 1 corresponds to approximately 50 µg/ml. The ratio of the reading at 260 nm and 280 nm (OD260/OD280) provides an estimate for the purity of nucleic acid. Pure preparation of DNA has OD260/OD280 of 1.8 to 2.0 (72). The concentration of the DNA isolated by this method was 400 ng/µl and the OD260/OD280 of the prepared DNA was 1.83.

2. Construction of partial restriction map

Purified DNA was digested to completion with a variety of restriction endonuclease such as *Bam*HI, *Bcl*I, *Bst*EII, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, *Stu*I, *Xba*I, and *Xho*I for both single and double digestion and electrophoresed on 1.5% agarose gel. The partial restriction map was constructed (Fig.1). No sites were found for those restriction endonucleases except *Pst*I and *Sal*I.

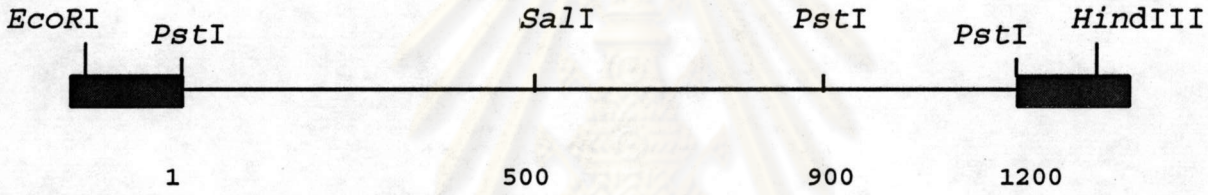

Fig. 1 Partial Restriction Map of the *Pst*I insert of pWC3. The boxes represent pUC18 and the single line represents insert DNA.

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3. Determination of DNA sequence

The double-stranded DNA was sequenced by the dideoxy chain termination method using universal primers of the pUC18 vector and several synthetic oligonucleotide primers for both strands (Table 1). The sequencing strategy was demonstrated (Fig. 2) and the example of sequence gel autoradiography was shown (Fig. 3). DNA sequence was searched for open reading frames (ORFs), ribosome binding site (RBS), promoter and termination sites by using DNASIS version 2.1. It is presented with its predicted amino acid translation (Fig. 4).

The nucleotide sequence of 1,188 bp was determined from both strands. It has *Sal*I and *Pst*I site cleavage corresponding to the partial restriction map (Fig.1). A 1,133-bp open reading frame was identified by ATG start codon at position 56 and a putative ribosome binding site (AGGG) was 7- bp upstream of the starting site. No sequences with homology to the *E. coli* concensus promoter and stop codon were present. Therefore, the ORF was capable of coding for a protein of 43.5 kDa by ending with UAA stop codon in the C-terminal of *lac* gene. The amino acid sequence deduced from the nucleotide sequence of DNA insert was determined to be 377 residues in length corresponding to a 42 kDa protein with an isoelectric point of 5.65.

The overall G+C content of this fragment was 66.6% which agrees well with the predicted genomic G+C content of *P. pseudomallei*, 67.9% (3). In addition, approximately 94.7% of the codons have a G or C in the third base position. The protein product possesses a sequence at the N terminus which resembles known secretory signal peptides (73).

A search of the public databases using blast program of National Center for Biotechnology Information (NCBI) revealed no homology to DNA or amino acid sequence of any known hemolysin in the databases.

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Table I. List of primers used in sequencing for both strands

Primers	Sequence (17 mer)	Position
Universal reverse	5' CAGGAAACAGCTATGAC 3'	
B-1	5' CTTCACGACCTCCGACC 3'	136-152
B-2	5' CTGTACGCGGAGAAGCT 3'	413-429
B-3	5' GTCCACCCGCTCGTCGA 3'	671-687
B-4	5' GGAAGGCCAGGCGAAGT 3'	910-926
Universal forward	5' GTTTTCCCAGTCACGAC 3'	-40
F-1	5' GCACCGAGTCGATCACG 3'	931-947
F-2	5' CCCGAATCCTGGCCCGT 3'	803-819
F-3	5' GCCGTCGTCCATCGCCT 3'	489-505
F-4	5' ATCTGCGTGGCGAGGCA 3'	248-264

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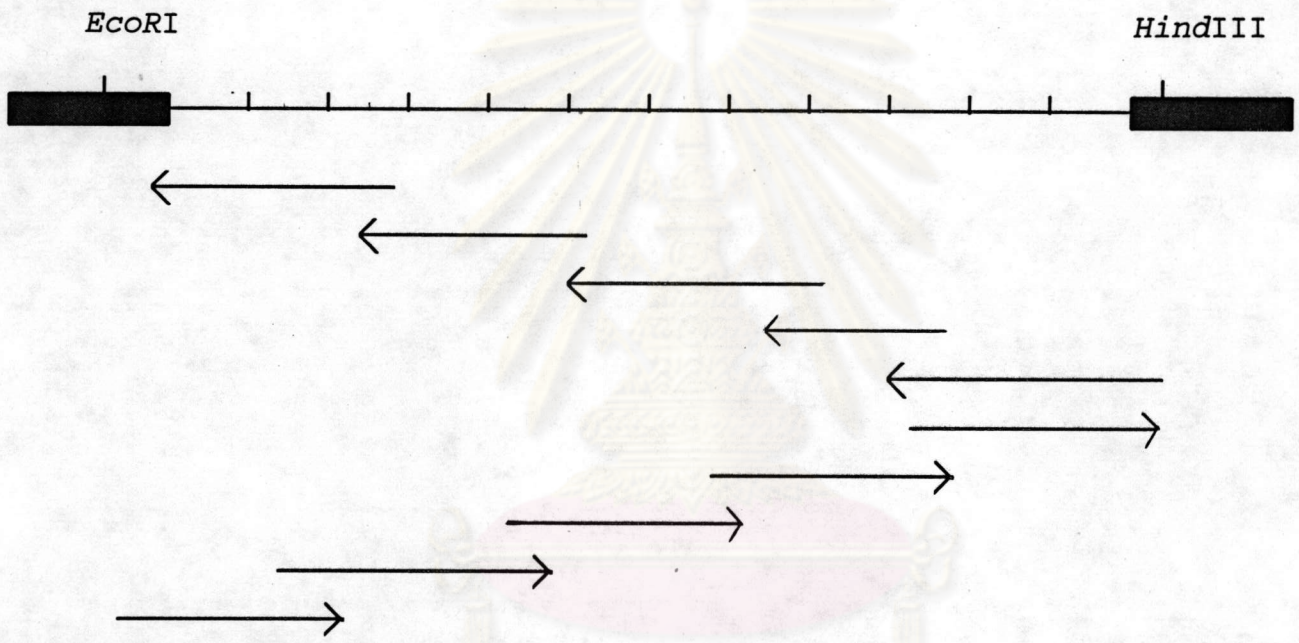

Fig. 2 Sequencing strategy of pWC3. The scaled line represents *Pst*I insert and the broad line represents pUC18. Arrows indicate the length and direction of sequencing for each fragment.

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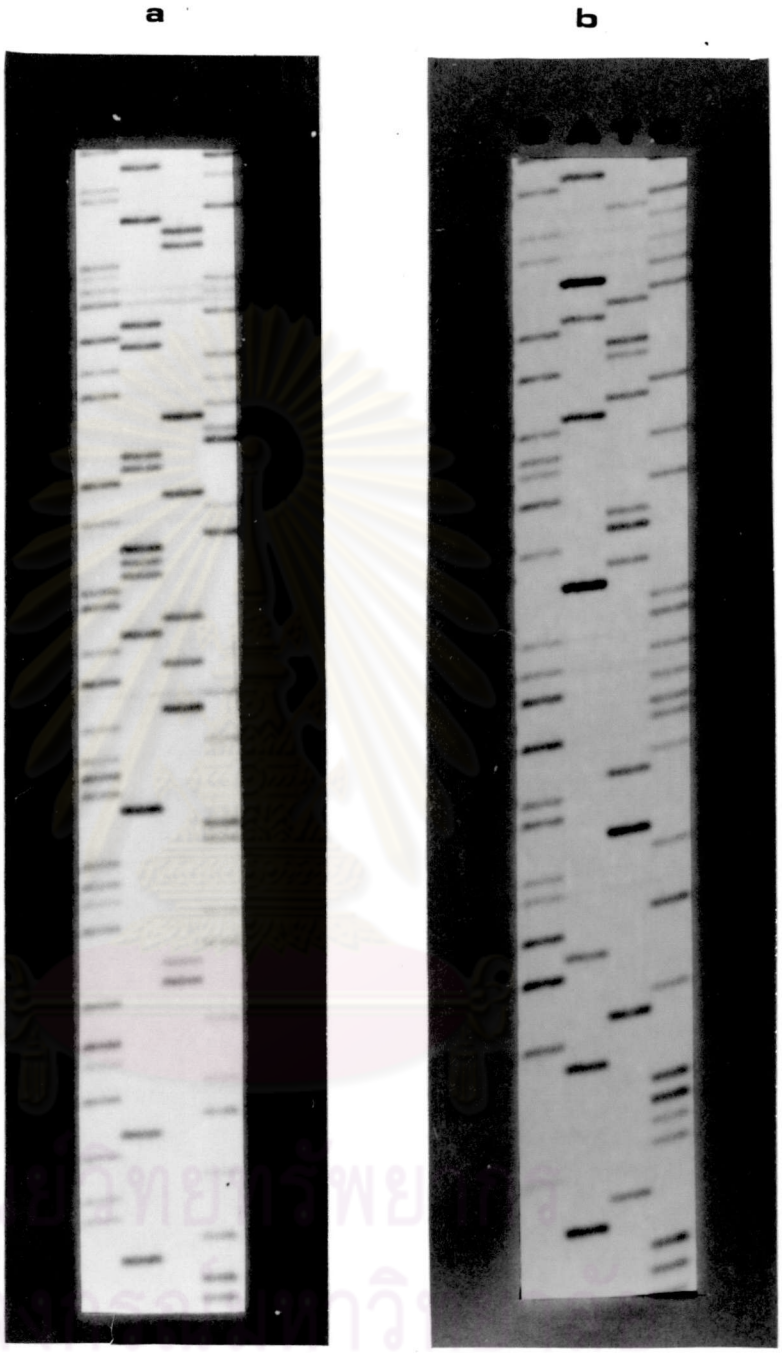

Fig 3. Example of sequence gel autoradiography of *Pst*I insert. Reactions were performed with universal reverse primer (a) and F-2 primer (b).

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v          v          v          v          v     ____ v
 GGTGCTGCCCGTGCAGATCCACGGCGACGCGGCGTTCGCGGGCCAGGGCGTCGTG 55

    v          v          v          v          v          v
ATGGAAACGCTGAACCTCGCGCAGACGCGCGGTTACGGCACGCACGGCACGCTGCA 111
M  E  T  L  N  L  A  Q  T  R  G  Y  G  T  H  G  T  L  H   19

         v          v          v          v          v
CATCGTCATCAACAACCAGATCGGCTTCACGACCTCCGACCCGCGCGACGCGCGCT 167
 I  V  I  N  N  Q  I  G  F  T  T  S  D  P  R  D  A  R  S   38

  v          v          v          v          v          v
CGACGCTGTACTGCACGGACGTCGTCAAGATGATCGAGGCACCGGTGCTGCACGTG 223
  T  L  Y  C  T  D  V  V  K  M  I  E  A  P  V  L  H  V   56

       v          v          v          v          v
AACGGCGACGATCCGGAAGCCGTGGTCCTCGCCACGCAGATCGCGATCGACTACCG 279
N  G  D  D  P  E  A  V  V  L  A  T  Q  I  A  I  D  Y  R   75

v          v          v          v          v          v
GATGCAGTTCCACAAGGATGTCGTGATCGACATCGTCTGCTTCCGCAAGCTCGGCC 335
 M  Q  F  H  K  D  V  V  I  D  I  V  C  F  R  K  L  G  H   94

    v          v          v          v          v          v
ACAACGAACAGGACACGCCGGCCGTCACGCAGCCGCTGATGTACAAGAAGATCGCG 391
  N  E  Q  D  T  P  A  V  T  Q  P  L  M  Y  K  K  I  A  112

         v          v          v          v          v
CAGCACCCGGGCACCCGTGCGCTGTACGCGGAGAAGCTCGTCCAGCAGGGCGTGAT 447
Q  H  P  G  T  R  A  L  Y  A  E  K  L  V  Q  Q  G  V  I  131

  v          v          v          v          v          v
CAGCGCCGACGACGCGGACGGCTTCGTGAAGGCGTACCGCAAGGCGATGGACGACG 503
 S  A  D  D  A  D  G  F  V  K  A  Y  R  K  A  M  D  D  G  150

       v          v          v          v          v    ↓SalI
GCCACCACACGGTCGATCCGGTGCTGTCGAACTACAAGAGCAAGTACGCGGTCGAC 559
  H  H  T  V  D  P  V  L  S  N  Y  K  S  K  Y  A  V  D  168

v          v          v          v          v          v
TGGATGCCGTTCCTGAACCGCAAGTGGACGGACGCCGCCGACACCGCGGTGCCGCT 615
W  M  P  F  L  N  R  K  W  T  D  A  A  D  T  A  V  P  L  187
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Fig. 4 Nucleotide and deduced amino acid sequence of *PstI* insert. The putative ribosome binding site is overlined. The predicted signal peptide is underlined. The sequence of *lac* gene is boxed. Restriction endonuclease sites corresponding to those in Fig. 1 are indicated.

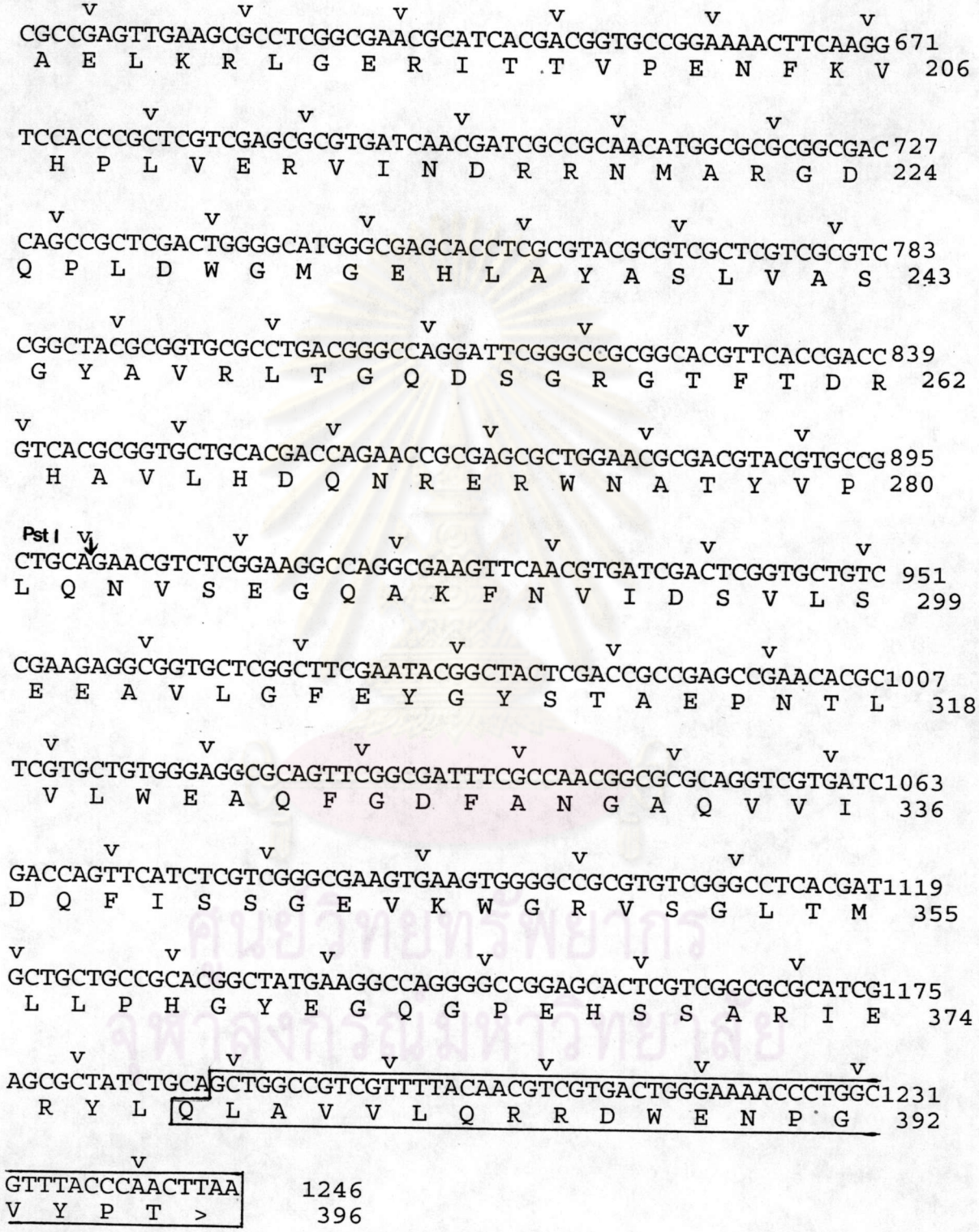
CGCCGAGTTGAAGCGCCTCGGCGAACGCATCACGACGGTGCCGGAAAACTTCAAGG 671
A E L K R L G E R I T T V P E N F K V 206
TCCACCCGCTCGTCGAGCGCGTGATCAACGATCGCCGCAACATGGCGCGCGGCGAC 727
H P L V E R V I N D R R N M A R G D 224
CAGCCGCTCGACTGGGGCATGGGCGAGCACCTCGCGTACGCGTCGCTCGTCGCGTC 783
Q P L D W G M G E H L A Y A S L V A S 243
CGGCTACGCGGTGCGCCTGACGGGCCAGGATTCGGGCCGCGGCACGTTCACCGACC 839
G Y A V R L T G Q D S G R G T F T D R 262
GTCACGCGGTGCTGCACGACCAGAACCGCGAGCGCTGGAACGCGACGTACGTGCCG 895
H A V L H D Q N R E R W N A T Y V P 280
Pst I
CTGCAGAACGTCTCGGAAGGCCAGGCGAAGTTCAACGTGATCGACTCGGTGCTGTC 951
L Q N V S E G Q A K F N V I D S V L S 299
CGAAGAGGCGGTGCTCGGCTTCGAATACGGCTACTCGACCGCCGAGCCGAACACGC 1007
E E A V L G F E Y G Y S T A E P N T L 318
TCGTGCTGTGGGAGGCGCAGTTCGGCGATTTCGCCAACGGCGCGCAGGTCGTGATC 1063
V L W E A Q F G D F A N G A Q V V I 336
GACCAGTTCATCTCGTCGGGCGAAGTGAAGTGGGGCCGCGTGTCGGGCCTCACGAT 1119
D Q F I S S G E V K W G R V S G L T M 355
GCTGCTGCCGCACGGCTATGAAGGCCAGGGGCCGGAGCACTCGTCGGCGCGCATCG 1175
L L P H G Y E G Q G P E H S S A R I E 374
AGCGCTATCTGCAGCTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGC 1231
R Y L Q L A V V L Q R R D W E N P G 392
GTTTACCCAACTTAA 1246
V Y P T > 396

Fig. 4 (cont.)

4. Characterization of gene products

In vitro transcription/translation

Plasmid pWC3 and pUC18 DNA used were added to *E. coli* S30 Extract System containing L-[35 S] methionine. The resulting translation products were analysed by SDS-PAGE and autoradiography (Fig. 5). Two of the products with relative molecular weights of 33 kDa and 45 kDa were detected (Fig. 6). A 33 kDa-protein is β-lactamase corresponding to the product of pUC18 (Fig. 5, lane 1). Thus, pWC3 with *Pst*I insert produced a protein migrating at 45 kDa (Fig 5, lane 2) which is in close agreement with the size predicted from the nucleotide sequence.

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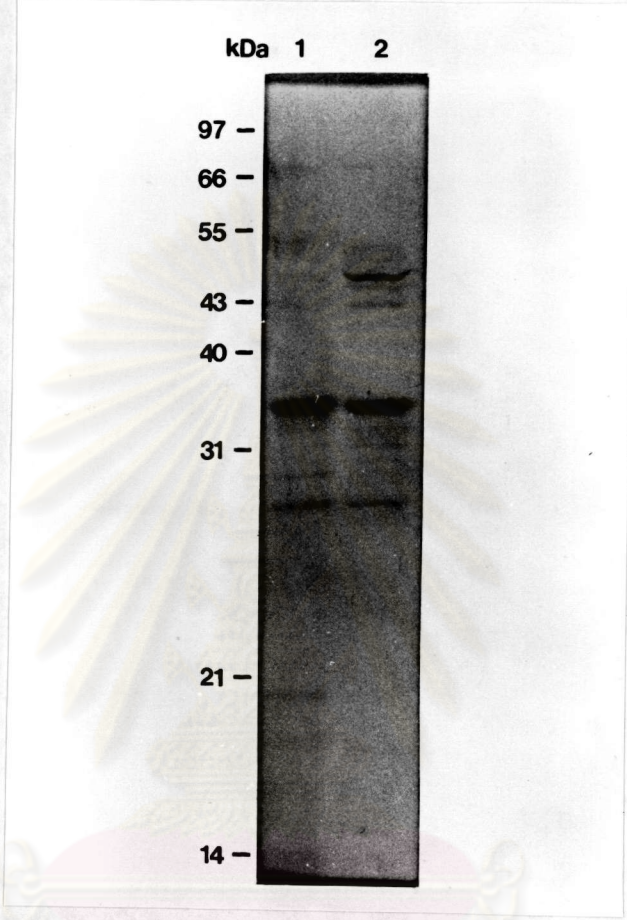

Fig. 5 Autoradiography of SDS-PAGE of L-^{35}S-methionine-labeled *in vitro* transcription/translation polypeptide products. Lanes: 1, control DNA (pUC18); 2, pWC3. Position of molecular weight markers (in kilodaltons) are shown on the left.

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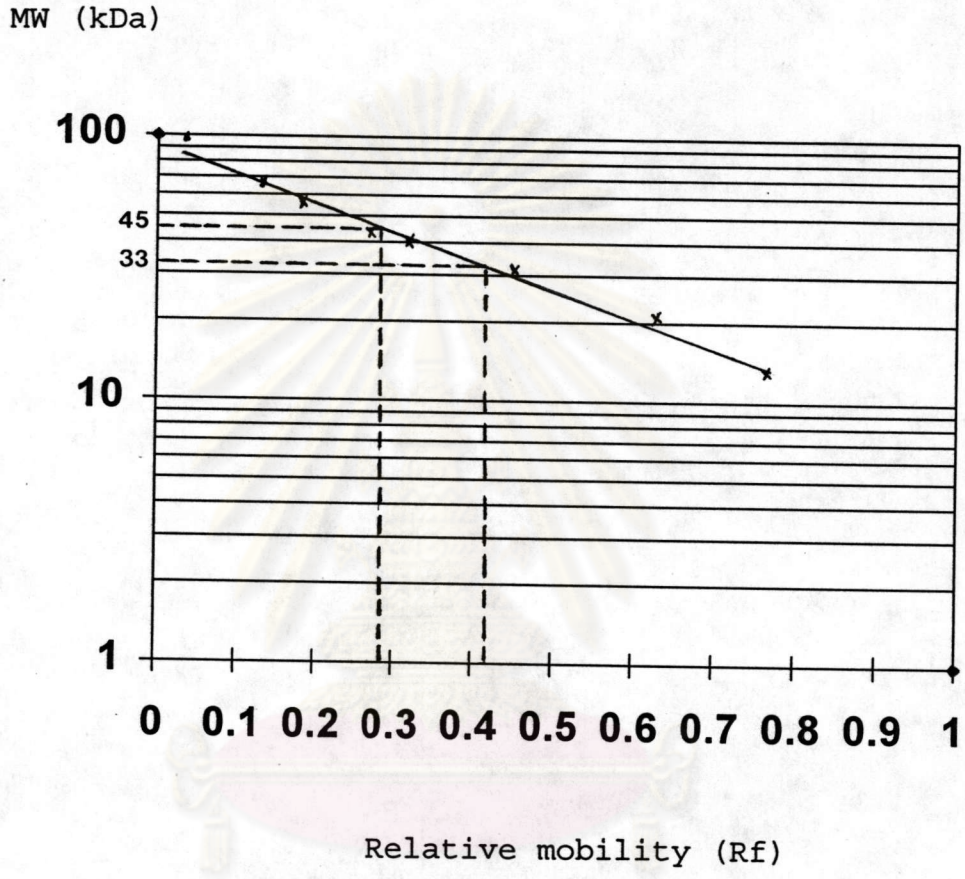

Fig. 6 Estimation of the molecular weight of pWC3-coded protein by 12% SDS polyacrylamide gel. The molecular weight of 33 kDa and 45 kDa are β-lactamase and insert-encoded protein, respectively.

5. Confirmation of the fragment orientation

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

In order to flip cloned gene in the reverse orientation, the *Hind*III-*Eco*RI fragment from pWC3 was ligated into pUC19 and transformed into *E.coli* JM109. The desired clone was selected and designated as *E. coli* JM109 (pWC3R), WC3R. Plasmid pWC3R was isolated and purified by Wizard™ minipreps DNA purification system given the concentration of 200 ng/ml.

5.2 Determination of the protein product of pWC3R

To determine the product of pWC3R , *in vitro* transcription/translation was performed and analysed by SDS-PAGE and autoradiography (Fig. 7). There was only a 33 kDa protein detected in production of pWC3R (Fig 7, lane 1). It migrated identically to a protein encoded by pUC19 which is β-lactamase product. This indicated that the *Pst*I inserted DNA was controlled under *lac* promoter of pUC vector, since it had no expression of 45 kDa protein which was expected to be the product of this gene.

5.3 Determination of hemolytic activity

The hemolytic activity of WC3R compared with *E. coli* JM109 (pUC19) as negative control were determined by using cellophane plate technique. The result showed no hemolytic activity detected in WC3R that was correlated to the result of *in vitro* transcription/ translation.

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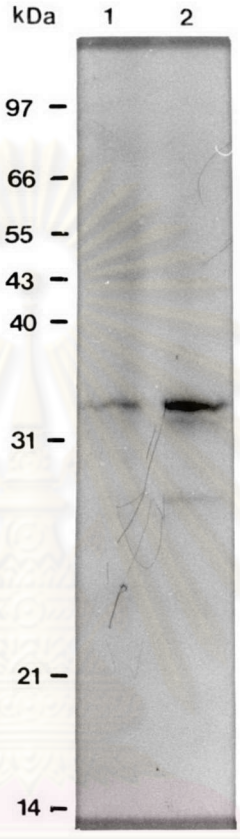

Fig. 7 Autoradiography of SDS-PAGE of L-^{35}S-methionine-labeled *in vitro* transcription/translation polypeptide products. Lanes: 1, control DNA (pUC19); 2, pWC3R. Position of molecular weight markers (in kilodaltons) are shown on the left.

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