รายงานวิจัยฉบับสมบูรณ์

โครงการ

ผลิตงานวิจัยและส่งเสริมกลุ่มวิจัยด้านอณูชีววิทยา

โดย

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19 ธันวาคม 2542

สัญญาเลขที่ RTA / 01 / 2539

รายงานวิจัยฉบับสมบูรณ์

โครงการผลิตงานวิจัยและส่งเสริมกลุ่มวิจัยด้านอณูชีววิทยา

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

ชุดโครงการเมธีวิจัยอาวุโส สกว. สกล พันธุ์ยิ้ม

สารบัญ

บทคัดย่อ

บทคัดย่อ

อณูชีววิทยาเป็นวิทยาการผสมผสานความรู้และหลักการทางเคมี ฟิสิกส์ และชีววิทยา เพื่อให้เกิดความรู้ความเข้าใจปรากฏการณ์ที่ระดับอณูของสิ่งมีชีวิต อันเนื่องมาจากโครงสร้างของสารพันธุกรรม องค์ความรู้ทางอณูชีววิทยาเป็นรากฐานสำคัญของเทคโนโลยีชีวภาพ ซึ่งส่งผลในแนวกว้างทางด้านการแพทย์ เกษตรกรรม อุตสาหกรรมและสิ่งแวดล้อม การวิจัยและพัฒนาวิชาการด้านอณูชีววิทยาในประเทศไทยยังไม่ก้าวหน้าเท่าที่ควรเพราะการขาดแคลน ผู้เชี่ยวชาญ และบุคลากร จึงจำเป็นต้องดำเนินการเร่งการพัฒนางานวิจัย สร้างกลุ่มวิจัย และผลิตบุคลากรวิจัยด้านอณูชีววิทยาขึ้นภายในประเทศ

โครงการผลิตงานวิจัยและส่งเสริมกลุ่มวิจัยด้านอณูชีววิทยา ประกอบด้วยคณะผู้ร่วมวิจัย 35 คน ได้ดำเนินการเป็นระยะเวลา 3 ปี โดยสนับสนุนและฝึกนักวิจัยด้านอณูชีววิทยาให้มีความเชี่ยวชาญ 11 คน ส่งผลให้มีการผลิตผลงานวิจัยเป็นที่ยอมรับในระดับสากลโดยการตีพิมพ์ในวารสารวิชาการระดับนานาชาติ จำนวน 16 เรื่อง และได้ผลิตนักศึกษาระดับปริญญาโท-เอก ซึ่งจะเป็นนักวิจัยในอนาคต จนจบการศึกษา จำนวน 12 คน และกำลังศึกษาอยู่ 6 คน ได้นำเสนอผลงานวิจัยในการประชุมวิชาการนานาชาติ 10 ครั้ง ในการประชุมวิชาการภายในประเทศ 12 ครั้ง และจัดประชุมวิชาการอณูชีววิทยาประจำปี 3 ครั้ง

ผลงานวิจัยโดยสังเขปได้องค์ความรู้ใหม่ด้านกลไกการฆ่าลูกน้ำยุงของโปรตีนCry4B ทราบโครงสร้าง αhelix ใน pore-forming domain มี 7 สาย โดยสาย helix 4 และ helix 5 เป็นสายที่ทะลุผ่านผนังเซลล์ทำให้ลูกน้ำยุงตาย และได้ทำการสร้างแบคทีเรียสายพันธุ์ที่พบในกระเพาะลูกน้ำยุงให้ฆ่าลูกน้ำยุงได้ดี ได้ศึกษาDNAควบคุมการแสดงออกของยีน(promoter)ในcyanobacteria พบpromoter หลายชุด และ 1 ชุดมีลำดับเบสเป็น t.RNA^{pro} ได้แยกพร้อมศึกษาการเรียงลำดับเบสของยีน flagellin ใน *B.pseudomallei* และสามารถพัฒนาการจำแนก *B.pseudomallei* และ *B.cepacia* ที่พบในคนไข้โดยวิธี PCR-RFLP ได้พัฒนาเทคนิคPCR-RFLPในการจำแนก *M.tuberculosis* complex ออกจาก mycobacteria และ pathoganic bacteria 24 ชนิด ได้แยกยีน catalase ของ lactobacillus และเปลี่ยน lactobacillus ที่พบในแหนมให้ผลิต catalase ซึ่งสลาย H_2O_2 ได้ดี ได้ศึกษาเชื้อไวรัสจุดวงแหวนมะละกอ (PRV) จนทราบลำดับเบสของ coat protein ซึ่งมีความแตกต่างกันใน PRV จากราชบุรี ชุมพร ขอนแก่น และจากฮาวาย ไต้หวัน ออสเตรเลีย และได้สร้างมะละกอข้ามสายพันธุ์ป้องกันการติดเชื้อPRV ได้ศึกษายีนที่แสดงออกในเซลล์กล้ามเนื้อและเม็ดเลือดกุ้งกุลาดำ และแยกยีนฮอร์โมนจากเซลล์ก้านตากุ้งซึ่งอาจควบคุมการลอกคราบหรือระดับน้ำตาลหรือการพัฒนาเซลล์สืบพันธุ์

เนื้อหางานวิจัย

เนื้อหางานวิจัย

SINGLE PROLINE SUBSTITUTIONS OF SELECTED HELICES OF THE *BACILLUS THURINGIENSIS* CRY4B TOXIN AFFECT INCLUSION SOLUBILITY AND LARVICIDAL ACTIVITY

ABSTRACT

PCR-based mutagenesis was employed to investigate the role in toxicity of putative transmembrane helices of the 130-kDa Cry4B mosquito-larvicidal delta-endotoxin produced by *Bacillus thuringiensis* subsp. *israelensis*. Mutant toxins with a single proline substitution in the central region of α5, α6 and α7 were constructed and expressed in *Escherichia coli* as cytoplasmic inclusion bodies with a yield similar to that of the wild-type toxin. Unlike inclusions of the wild-type and that of the previous mutants for proline replacements in α3 or α4, all proline-substituted inclusions were insoluble in carbonate buffer, pH 9.0, indicating that the bend introduced in these three helices possibly interferes with the protein inclusion packing as shown by the insolubility. Similar to the previous substitution in α4, an almost complete loss of toxicity against *Aedes aegypti* mosquito-larvae was demonstrated for *E. coli* cells expressing mutant toxins in which residues Val-181, Ala-182 or Leu-186 in α5 were changed to proline. In addition, a dramatic decrease in larvicidal activity was observed for the substitution at Thr-254 in α7 while the mutation Q215P in α6 did not affect the biological activity. These results suggest that the central helix (α5) and conceivably α7, but not α6, are important determinants of toxin function. Our data therefore further support the notion that the putative pore forming helical hairpin α4-α5 together with α7 plays a crucial role in Cry toxin activity.

INTRODUCTION

Bacillus thuringiensis (*Bt*) is a Gram-positive entomopathogenic bacterium that has been used successfully as an alternative insecticide for biological control of disease vectors and other pests. During sporulation, different *Bt* strains produce larvicidal proteins (classified as Cry and Cyt delta-endotoxins) in large quantities as cytoplasmic crystalline inclusions that are specifically toxic to a variety of dipteran, lepidopteran and coleopteran insect larvae [1,2]. When ingested by susceptible larvae, the inclusions are solubilised by the alkaline pH of the larval midgut and the protoxins are activated by gut proteases. It is believed that the

activated toxins then bind to midget epithelial cells *via* specific receptors, and insert into the microvillar membrane to form ion channels or leakage pores that cause cell swelling and eventually death by colloid-osmotic lysis (see [3] for reviews). However, the insecticidal mechanism at the molecular level of these *Bt* toxins is still not entirely established.

The X-ray crystal structure of two different Cry toxins, the coleopteran-specific Cry3A toxin [4] and the lepidopteran-specific Cry1Aa toxin [5], reveals Cry proteins consisting of three distinct domains, and it is believed that each domain has a defined function including pore formation and receptor recognition [4,5]. Domain I is a group of seven α-helices in which the central helix (α5) is relatively hydrophobic and encircled by six other amphipathic helices. Domain II is the most variable part of the Cry toxin family and is composed of three anti-parallel β-sheets, each terminating in a surface-exposed loop. Domain III is a tightly packed β-sandwich of two anti-parallel sheets. It has been proposed that other members of this family will have the same overall tertiary structure since the core of the molecule including all the domain interfaces is built up from five amino acid sequence segments that are highly conserved throughout the entire Cry toxin family [1,4].

Structurally, it is immediately apparent that domain I is likely to be the transmembrane pore-forming apparatus. This domain contains five amphipathic helices (α3, α4, α5, α6 and α7) that are theoretically long enough to span the bilayer lipid membrane and form a lytic pore [4,5]. The possibility that this α-helical bundle in domain I is essential for pore formation is supported by the feature that it is highly conserved in all Cry toxins [1], and by analogy with the helical bundle pore-forming structures of two other well-characterized bacterial toxins, colicin A and diphtheria toxin, although they bear no sequence homology [6]. This notion is further supported by several studies with truncated proteins corresponding to domain I of Cry1Ac [7] and Cry3B2 [8] and with synthetic peptides of selected helices from Cry1Ac [9] or Cry3A [10] that demonstrated pore-forming activity either in phospholipid membrane vesicles or within planar lipid bilayers. A number of experiments *via* site-directed mutagenesis suggested that α4 and/or α5 of Cry1Aa [11,12] and of Cry1Ac [13] and the loop between the bottoms of α4 and α5 of Cry1Ab [14] are involved in pore formation. Recently, we have employed single proline substitutions and demonstrated that α4, but not α3, of the dipteran-specific Cry4B toxin plays a crucial role in larvicidal activity, possibly in the pore forming step rather than in receptor binding [15]. In this report, we have applied the same mutagenesis strategy to further investigate a possible involvement in toxicity of three other putative-transmembrane helices (α5, α6 and α7) of

this mosquito-active toxin, and found that these three helices are essentially involved in inclusion solubility. Our results also indicate that α5 and α7, but not α6, play a role in larvicidal activity of the Cry4B toxin.

MATERIALS AND METHODS

Construction and Expression of Mutant Toxins

Single proline substitutions *via in vitro* site-directed mutagenesis were performed using a Quickchange PCR-based mutagenesis kit (Stratagene) following the manufacturer's instructions. The plasmid pMU388, constructed by cloning the full-length *cry4B* toxin gene from *Bt* subsp. *israelensis* in a pUC12 vector [16], was used as a template. Mutagenic primers were purchased from Bio-synthesis Inc. (USA) as shown in Table I. All mutations were verified by DNA sequencing using an ABI prism 377 sequencer.

Partial Purification and Solubilisation of Protoxin Inclusions

The wild type and mutant Cry4B toxin genes were expressed in *E.coli* strain JM109 under control of the *lacZ* promoter. Cells were grown in LB medium plus 100 µg/ml of ampicillin until OD_{600} reached 0.4-0.5 and incubation was continued for another 4 hours after addition of isopropyl-β-D-thiogalacto pyranoside (IPTG) to a final concentration of 0.1 mM. *E. coli* cultures expressing each mutant as inclusion bodies were harvested by centrifugation, resuspended in 1 ml of distilled water and then disrupted in a French Pressure Cell at 16,000 psi. The crude lysates were centrifuged at 8,000 *g* for 5 minutes and pellets obtained were washed 3 times in distilled water. Protein concentrations were determined by using a protein microassay (Bio-Rad), with bovine serum albumin fraction V (Sigma) as a standard.

Protoxin inclusions (1 mg/ml) were solubilised in 50 mM Na_2CO_3, pH 9.0 and incubated at 37 °C for 60 minutes as described previously [15]. After centrifugation for 10 minutes, the supernatants were analysed by SDS-15% (w/v) polyacrylamide gel electrophoresis (PAGE) in comparison with the inclusion suspension.

Larvicidal Activity Assays

Bioassays were performed as described previously [15] with some modification using 2-day old *Aedes aegypti* mosquito-larvae reared in a container (22x30x10 cm deep) with approximately 3 litres of distilled water supplemented with a small amount of rat diet pellets. Both rearing and bioassays were done at room temperature (25 °C). The assays were carried out in 1 ml of *E. coli* suspension (10^8 cells suspended in distilled water) in a 48-well

microtitre plate (11.3 mm well diameter), with 10 larvae per well and a total of 100 larvae for each type of *E. coli* samples. Mortality was recorded after 24-hour incubation period.

RESULTS AND DISCUSSION

Recently, data on molecular determinants of membrane insertion and pore formation for *Bt* Cry toxins has increased substantially. Where studied, the helical domain of different Cry toxins has now been putatively demonstrated to be involved in membrane integration and toxin pore formation [7-15]. In the present study, we have made use of PCR-based mutagenesis to construct several more mutants in the putative pore-forming domain to determine which of the three other helices (α5, α6 or α7) would be responsible for toxicity of the mosquito-active Cry4B toxin. Proline substituted mutants were designed based on a 3D Cry4B model which was previously constructed by homology modelling using Cry3A as a template [17]. The designed mutants have single proline substitutions in the central region of the selected helices of Cry4B including α5 at residues Val-181, Ala-182 and Leu-186, α6 at Gln-215 and α7 at Thr-254 (Fig.1). Each mutant gene could be over-expressed in *E. coli* under inducible control of the *lacZ* promoter by addition of IPTG to mid-exponential phase cultures and the mutant toxins were predominantly produced as sedimentable inclusion bodies which were then partially purified. In addition, the level of protein expression of all mutant proteins was approximately the same as that of the wild type, and the expressed mutant derivatives still cross-reacted with Cry4B antibodies (data not shown).

Experiments were carried out to assess the solubility of mutant protein inclusions in carbonate buffer, pH 9.0 in comparison with that of the wild-type inclusion. The amounts of the 130-kDa soluble proteins in the supernatant were compared with those of the proteins initially used in order to determine the percentage of protein solubilisation. A complete loss of solubility at alkaline pH was observed for the inclusions of the mutants substituted in α5, α6 or α7, whilst the inclusions of the two previously constructed mutants, V118P and Q149P, exhibited the same solubility characteristics as that of the wild-type (Fig. 2). The reason for the difference in solubility between those two sets of point mutations is not clear. However, this may be explained by the fact that all the five mutated residues, *i.e.* Val-181, Ala-182 and Leu-186 in α5, Gln-215 in α6 and Thr-254 in α7 are buried in the protein core, unlike Val-118 in α3 and Gln-149 in α4 that are exposed at the protein surface (see Fig. 1C). Thus, the bend created by the proline substitution in these relatively conserved helices, *i.e.* α5, α6 or α7 possibly disturbs the structural characteristics either locally or globally that might consequently affect inclusion formation as demonstrated by a drastically

perturbed dissolvability. On the other hand, the proline mutations in α3 or α4 appear to affect only the individual helices, without significantly influencing the other helices or the overall structure, as earlier demonstrated for an α-lactalbumin folding intermediate [28].

To determine whether a single praline replacement in these three additional helices also affects the larvicidal activity of Cry4B, *E. coli* cells expressing each type of the mutant toxins were bioassayed using *Aedes aegypti* mosquito-larvae (Fig. 3). The mortality data recorded after a 24-hour incubation reveals that the α6 mutant (Q215P) still exhibited full larvicidal activity (98.7±0.7%) similar to the α3 mutant (V118P), whereas the T254P mutant produced merely 23.7±13.9%. However, mortality of all the α5 mutants, V181P, A182P and L186P, was almost totally abolished as approximately comparable to that observed with the previously described α4 mutant (Q149P). These results suggest that the integrity of α5 and α7 may indeed be important for toxin activity like that of α4.

Although toxin inclusion insolubility and larvicidal activity are seemingly correlated for all the three α5 mutants and the α7 mutant, the insolubility *in vitro* may not always necessarily reflect toxin activity *in vivo* as observed for the α6 mutant which was insoluble at pH 9.0 but still bioactive. It has been previously demonstrated that the difference detected in solubilisation *in vitro* for Cry4A inclusions which were purified from two different *Bt* recipient strains, is not a factor in larval toxicity [19]. Presumably, larval midgut proteases *in vivo* might facilitate the dissolution of the ingested toxin inclusions which would negate the differences between the observed toxicities of the α3 and α6 mutants. It was shown that incubation of the Cry2A toxin with gut proteases enhanced the solubility of the toxin inclusion, obviating the requirement for reducing agents at pH10.5 [20].

In conclusion, this report additionally demonstrates that the central helix (α5), α6 and α7, but not α3 or α4, are conceivably involved in protein packing for inclusion formation, thus destabilising these three helices individually could gives rise tc toxin insolubility *in vitro*. Moreover, our study also provides further support for a crucial role of the pore-forming helical hairpin α4-α5 as well as α7, which has been suggested to be a domain binding sensor [10], in larvicidal activity of the Cry4B toxin.

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A)

α3 α4

Cry3A 123 DYAKNKALAELQGLQNNVEDYVSALSSWQKNPVSSRNPHSQGRIRELFSQAESHFRNSMPSFA-ISGY

Cry4B 106 AYVRTDANAKMT**V**VKDYLDQYTTKFNTWKREPN---NQSYRTAVIT**Q**FNLTSAKLRETAVYFSNLVGY

118 149

α5 α6

Cry3A 190 EVLFLTTYAQAANTHLFLLKDAQIYGEEWGYEKEDIAEFYKRQLKLTQEYTDHCVKWYNVGLDKLRGS

Cry4B 171 ELLLLPIYAQ**VA**NFN**L**LLIRDGLINAQEWSLARSAGDQLYNTMV**Q**YTKEYIAHSITWYNKGLDVLRNK

181 186 215

182

α7

Cry3A 258 SYESWVNFNRYRREMTLTVLDLIALFPLYDVRL

Cry4B 239 SNGQWITFNDYKREM**T**IQVLDILALFASYDPRR

254

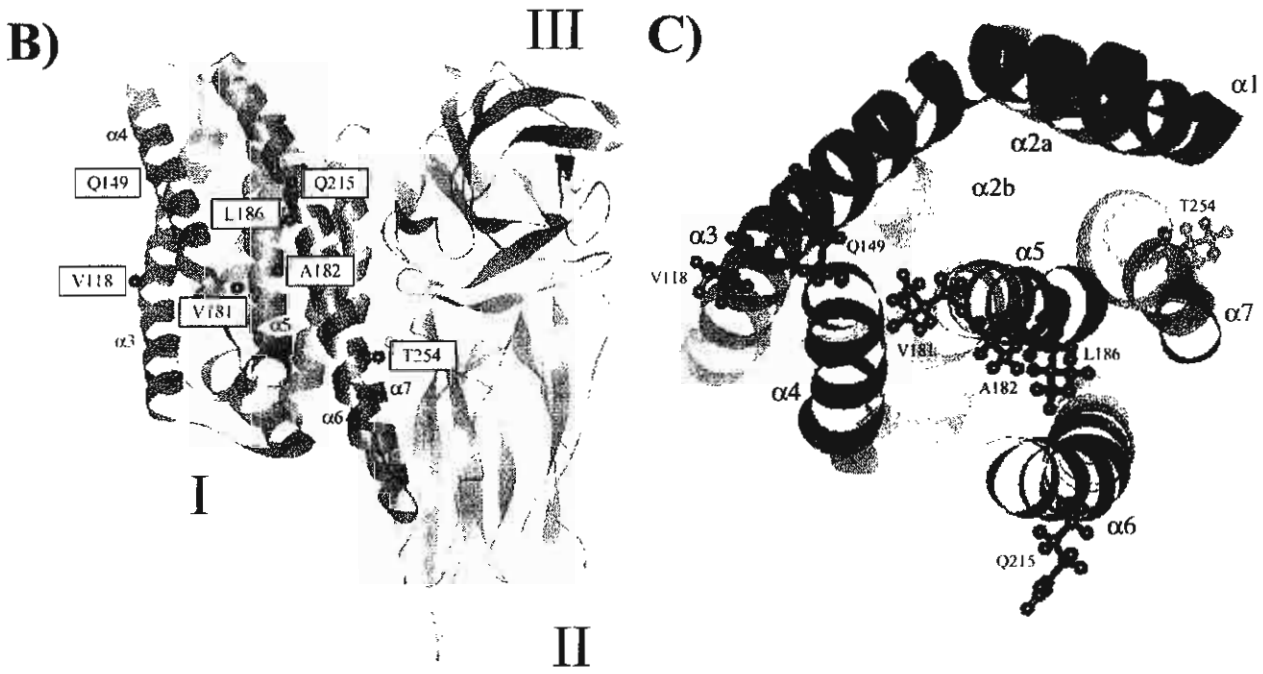

FIGURE 1 **A)** Amino acid sequence alignment of Cry4B with the known secondary structure elements of Cry3A. Single proline substitutions at each indicated position are underlined and emboldened. The corresponding helices are shown above the sequences. **B)** A ribbon representation (generated by Weblab viewer, Molecular Simulation Inc.) of the Cry4B model built by homology modelling [17], illustrating the three-domain organisation (I-III). Black beads indicate β-carbon atoms of the mutated residues. **C)** Top view of the helical bundle in domain I of Cry4B. The mutated residues are shown as ball and stick: α3 at Val-118, α4 at Gln-149, α5 at Val-181, Ala-182, and Leu-186, α6 at Gln-215 and α7 at Thr-254.

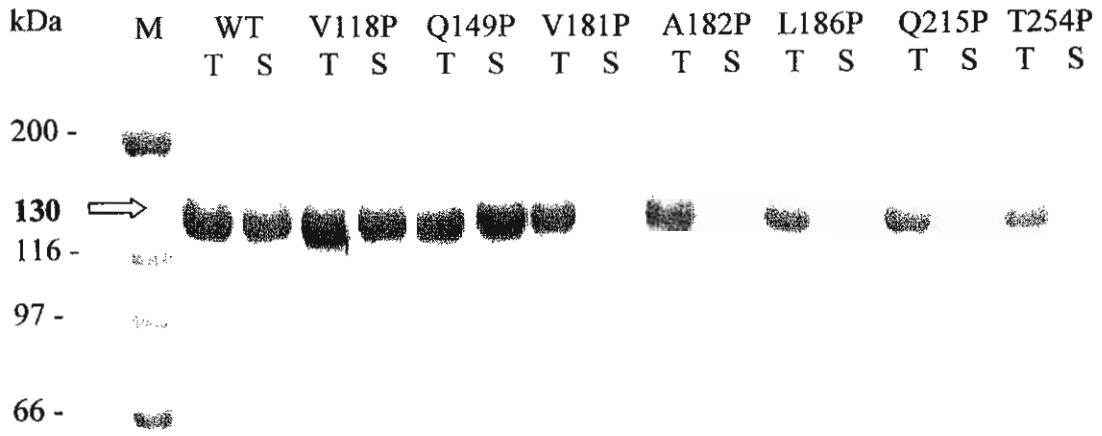

FIGURE 2 The figure shows a Coomasie brilliant blue-stained SDS-15% polyacrylamide gel of the partially purified 130-kDa protein inclusions extracted from *E. coli* expressing the wild-type (WT) and mutant Cry4B toxins and solubilised in carbonate buffer. (T) and (S) represent the total fractions and an equivalent volume of the supernatants after centrifugation, respectively. (M) represents the molecular mass standards.

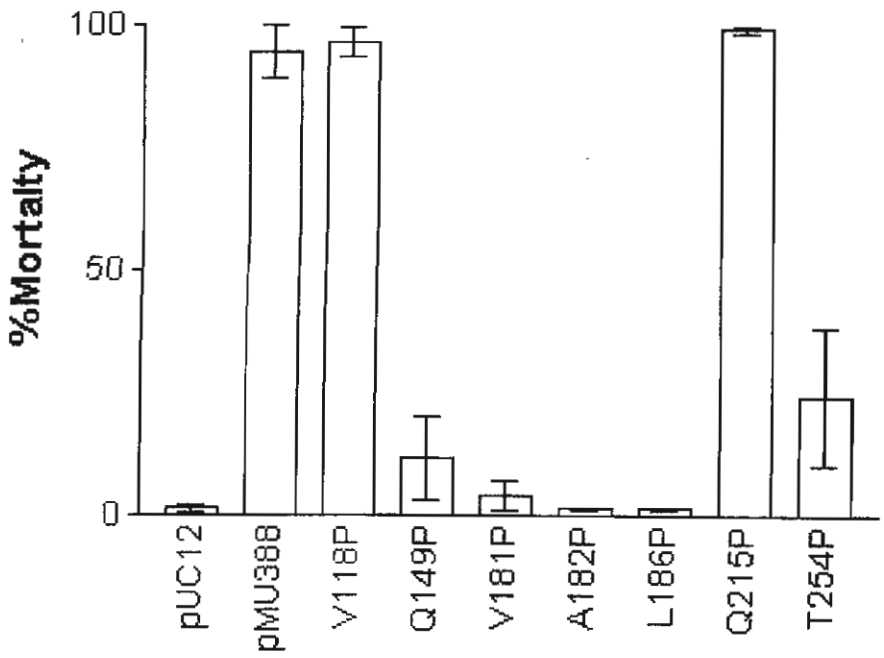

FIGURE 3 Lavicidal activities of *E. coli* cells expressing either the Cry4B wild-type toxin (pMU388) and its mutants (V118P, Q149P, V181P, A182P, L186P, Q215P and T254P) against *A. aegypti* larvae. Error bars indicate standard error of the mean from three independent experiments.

Table I Oligonucleotide primers designed to substitute a coded residue with proline

Primer	Location	Sequence[a]	Restriction Site
V181P-f	α5	P I Y A Q **P** A N F N L CCAATATACGCACAA**CCGG**CAAATTTCAATTTA	*Hpa*II
V181P-r		C GTAAATTGAAATTTG**CCGG**TTGTGCGTATATTG G	
A182P-f	α5	P I Y A Q V **P** N F N L CCAATATACGCACA**GGTCC**CAAATTTCAATTTA	*Sau*96I
A182P-r		C GTAAATTGAAATTTG**GGACC**TGTGCGTATATTG G	
L186P-f	α5	A N F N **P** L L I R D G L GCAAATTTCAAT**CC**ACTTTTAAT**CCGG**GATGGC	*Hpa*II
L186P-r		CTC GAGGCCATC**CCGG**ATTAAAAGT**GG**ATTGAAATT TGC	
Q215P-f	α6	T M V **P** Y T K E Y I CACTATGGTGCC**GTATAC**TAAAGAATATATTGC	*Acc*I
Q215P-r		GCAATATATTCTTTA**GTATAC**GGCACCATAGTG	
T254P-f	α7	D T K R E M **P** I Q V L GATTATAAAAGAGAGATGCC**GATTC**AAGTATTA	*Hinf*I
T254P-r		G CTAATACTT**GAATC**GGCATCTCTCTTTTATAATC	

[a]Introduced restriction enzyme recognition sites are underlined. Mutated nucleotide residues are shown as boldface. The deduced amino acid sequence is shown above each oligonucleotide sequence.

เนื้อหางานวิจัย

SELECTION OF STRONG PROMOTERS IN CYANOBACTERIUM *SYNECHOCOCCUS* PCC7942

ABSTRACT

Promoter-active fragments of *Synechococcus* PCC7942 were isolated by transcriptional gene fusion to the promoterless β-glucuronidase (GUS) gene of *E. coli*, which was used as a reporter gene. Several of the isolated promoter-active fragments expressed GUS activity in *Synechococcus* comparable with that of the λP_R promoter. Only 10% of the isolated promoter-active fragments also functioned in *E. coli*. The transcription initiation sites of the promoter-active fragment E3 were identified. The major transcription initiation site of E3 in *Synechococcus* was located within the nucleotides TTG which was identical to that corresponding in *E. coli*. Immediately upstream of the E3 transcription initiation sites was $tRNA^{pro}$ (GGG) gene, which contained two regions exhibiting strong homology to the major promoter elements in eukaryotic tRNA genes but did not contain the *E. coli* promoter element. Thus, the $tRNA^{pro}$ gene can act as a promoter. In addition, the promoter-active fragments E10 and E14, which contain the light-intensity responsive promoter and distant enhancer respectively, are under investigation.

INTRODUCTION

Cyanobacteria are the simplest organisms capable of performing oxygenic photosynthesis, with a thylakoid apparatus remarkably similar to that of higher plant chloroplasts [11]. They are model organisms for the cloning of genes involved in photosynthesis. Cyanobacteria have been used as hosts to express several heterologous genes. For example, attemps have been made to express the mosquitocidal protein genes of *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* in order to provide an alternative biological insecticide for control of mosquito populations [2, 4, 23]. They have also been used in the expression of salmon growth hormone gene in order to produce a feed additive for fish [12], or the expression of ethylene-forming enzyme in order to exploit atmospheric CO_2 as a substrate [8]. However, the level of heterologous gene expression in cyanobacteria is very low when compared with that in *E. coli*. A possible way to improve the gene expression is to use an endogenous strong promoter. However, current knowledge of the structure and function of promoters recognized within cyanobacteria is still limited. Although cyanobacteria are classified as eubacteria [10], their RNA polymerase holoenzyme

is unique in that it contains a subunit γ, in addition to the $\alpha_2\beta\beta'\sigma$ structure common to the RNA polymerase of other eubacteria [19]. The genetics of cyanobacteria is highly heterogeneous, the GC content of their DNA ranges from 35% to 71% which is as varied as the whole kingdom of bacteria [17].

In order to isolate cyanobacterial promoters for structure and functional studies, Chungjatupornchai et al. [6] used the unicellular *Synechococcus* PCC7942 (previously referred to as *Anacystis nidulans* R2, Pasteur Culture Collection no. 7942) as a model system. Promoter-active fragments of *Synechococcus* PCC7942 were isolated by transcriptional gene fusion to the promoterless β-glucuronidase (GUS) gene of *E. coli*, which was used as a reporter gene. From a total of 640 transformants screened, 15 clones (2.3%) and 25 clones (3.9%) of the transformants expressed high and low GUS activity respectively. Out of these 40 clones, only 4 clones (10%) could express in both *Synechococcus* and *E. coli*. The majority of the isolated promoters could not function in *E. coli*, thus it indicated that their functional sequences were different from those of *E. coli*.

In this study, the three different isolated strong promoter-active fragments, E3, E10 and E14, were characterized.

MATERIALS AND METHODS

Organisms and culture conditions

Synechococcus PCC7942 strain R2-SPc, cured of the small plasmid pUH24 [13], was grown in liquid or on solid (1% Difco Bacto Agar) BG-11 medium as described previously [24]. *E. coli* strain MC1061 [3] was grown in LB broth or agar as previously described [18].

Construction of plasmids

A promoter-probe shuttle vector pKG, capable of replication in *Synechococcus* PCC7942 and in *E. coli*, was generated previously [6]. The pKG plasmid harbored the BamHI site upstream of the promoterless-GUS gene. Plasmid pKG-E3, pKG-E10 and pKG-E14 were the pKG plasmid harboring the chromosomal DNA of *Synechococcus* at the BamHI site.

β-glucuronidase and protein assay

For the GUS assay of *Synechococcus* and *E. coli*, the harvested cells cultures were suspended in buffer (50 mM $NaPO_4$ pH7.0, 10mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, 10 mM β-mercaptoethanol). Cells were lysed using a hand-held motor homogenizer in the

presence of fine silica. The cell debris and fine silica were removed by centrifugation. GUS activity in the cell lysates was assayed using 4-methylumbelliferone-β-D-glucuronide as a substrate following the method of Scott *et al.* [20]. 4-methylumbelliferone fluorescence was measured with a TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, USA.). The values obtained were converted into specific activity using a calibration curve with known amounts of 4-methylumbelliferone product. Protein concentration of the cell lysates were determined using the Bio-Rad Detergent Compatible Protein Assay Kit (Bio-Rad Laboratories, USA).

Nucleotide sequence analysis

DNA sequences of the promoter-active fragments and recombinant plasmids from 5'RACE-PCR were determined by an automated sequence analyzer (Perkin Elmer, ABI, Model 377). Sequence comparisons and analysis were carried out using the Wisconsin Package Version 9.1 solfware, Genetics Computer Group (GCG, Madison, WI, USA) and the BLAST program [1] with the GenBank database and the genome database for *Synechocystis* PCC6803 [15].

5'Rapid Amplification of cDNA Ends (RACE) -PCR

Total RNA was extracted from *E. coli* or cyanobacterial cells using Trizol Reagent (GIBCO BRL, USA). The absence of DNA contamination in the total RNA sample was verified using PCR detection. 5'RACE-PCR method, used to identify the transcription initiation sites, were carried out following the manufacturer's manual (5'/3' RACE kit, Boehringer Mannheim, Germany) as described [6].

RESULTS AND DISCUSSION

Promoter strength of the isolated DNA fragments in *Synechococcus* PCC7942 and *E.coli*

The promoter strength of the isolated DNA fragments was determined by measuring GUS activity. In comparison with the *CaMV35S* and λP_R promoters, the GUS activity of the 7 isolated promoter-active fragments is shown in Table 1. Promoter-active fragments E3, E4, D13 and D21 were active in both *Synechococcus* and *E. coli*. E8, E10 and E14 were active only in *Synechococcus* but not active in *E. coli*. In *Synechococcus*, E3 was stronger than the λP_R promoter, whereas E10 and E14 were of similar strength. These 7 isolated promoters were not inducible by heat at 39°C. We also observed that light intensity affected expression of E10. GUS activity of E10 in *Synechococcus* grown at 1,500 Lux was 65 (±7) nmole/ min/ mg protein. This activity was approximately 4 times lower than that detected

when the *Synechococcus* were grown at 5,000 Lux, (see Table 1). GUS activity of the remaining 6 promoters was not significantly different when they were grown at 1,500 or 5,000 Lux. GUS activity of E3, E4, D13 and D21 in *Synechococcus* was higher than the corresponding activity in *E. coli*. The region immediately upstream of the initiation codon of the GUS gene did not contain a canonical Shine-Dalgarno sequence (GGAGG) similar to that found in *E. coli* (Fig. 1). This may be one of the factors that caused lower levels of GUS activity in *E. coli* than in *Synechococcus* (Table 1). Although it has been shown that in *E. coli*, the presence of canonical SD sequence increases expression of the reporter gene, SD-independent translation initiation system also exists [7]. No concensus sequence of a cyanobacterial counterpart to the SD sequence in *E. coli* has been previously reported.

None of the 7 isolated promoters were as strong as the λP_R promoter in *E. coli*. Several promoters from this study were stronger or as strong as the λP_R promoter which has been previously shown to be a strong promoter in *Synechococcus* [5, 14] and thus they are useful for high expression of heterologous genes in *Synechococcus*. Further characterization of the isolated promoters will reveal more detail of cyanobacterial promoter structures.

Nucleotide sequence analysis of promoter-active fragment E3

The insert fragment E3 exhibited the strongest promoter activity in *Synechococcus* (Table 1), and therefore further characterized. The nucleotide sequence at the 3'end of the 1.5 kb of E3 fragment, immediately upstream of the promoterless-GUS gene, was determined. Nucleotide sequence analysis revealed an ORF of 107 amino acids (Fig. 1) which was highly homologous to the hypothetical proteins slr1846 from *Synechocystis* PCC6803 and ORF107 from *Porphyra purpurea* with 76.6% and 53.3% amino acids identity respectively (Fig. 2). The 74 nucleotides following the ORF107 (Fig. 1) were 100% identical to the $tRNA^{pro}$ (anticodon GGG) gene of *Synechocystis* PCC6803 (accession no. D90900). Upstream of the 5'end $tRNA^{pro}$ gene, no conformed *E. coli* promoter element was detected (Fig. 1). At the 3'end of this gene there was an 15 bp inverted repeat (Fig. 1). Immediately downstream at the end of the $tRNA^{pro}$ gene of *Synechocystis* PCC6803, an 10 bp inverted repeat (TTCAGCTGAA) is observed (accession no. D90900). There is no Sau3A1 site within ORF107 and $tRNA^{pro}$ gene, thus the organizations of the gene could not be artifacts from cloning.

The tRNA^{pro} gene can act as promoter

Transcription initiation sites of E3-GUS were determined using 5'RACE-PCR. Sequencing analysis of 5'RACE-PCR independent clones revealed that transcription initiation sites in *E. coli* were located at TTG (6 out of 10 clones) and at G (4 clones) indicated as (a) and (b) respectively. Those in *Synechococcus* were located at TTG (11 out of 13 clones) and at A (2 clones) indicated as (c) and (d) respectively (Fig. 1). Thus, there are one major and one minor transcription initiation sites of E3-GUS. The major transcription initiation site in *E. coli* was located within nucleotides TTG identical to that in *Synechococcus*. Thus in both organisms, the GUS transcripts were probably controlled by the same functional promoter sequence. Immediately upstream of the transcription initiation sites was the tRNA^{pro} gene, and therefore the tRNA^{pro} gene can act as a strong promoter. However, it did not contain any -10 and -35 regions which conformed to those of *E. coli*σ^{70} promoter and may be one of the factors that caused low promoter activity in *E. coil* (Table 1). The tRNA^{pro} gene contained two regions, block A and B (Fig. 1), exhibiting strong homology respectively to block A (TGGCNNAGTGG) and B (GGTTCGANNCC) of the eukaryotic tRNA gene [9]. In the tRNA^{pro} gene, two additional nucleotides at nt.16 and 17 interrupt the block A (Fig. 1). It has been shown that blocks A and B are split promoter sequences of the tRNA^{Leu} gene of *Xenopus laevis* and recognized by RNA polymerase III [9]. Yeast tRNA gene containing the two conserved blocks can act as promoter in yeast [16]. Whether these two conserved blocks are the promoter elements that control the GUS transcripts in *E. coli* and in *Synechococcus* remain to be investigated. Further deletion analysis and site directed mutagenesis of the potential region in the tRNA^{pro} gene could elucidate the characteristics of a strong promoter in *Synechococcus*.

Nucleotide sequence analysis of promoter-active fragment E10

The nucleotide sequence at the 3'end of the 1.75 kb of E10 fragment, immediately upstream of the promoterless-GUS gene, was determined. Nucleotide sequence analysis revealed the 233 nucleotides (Fig. 3) whose deduced amino acid sequence was highly homologous to the *htpG* heat shock protein gene of *Synechocystis* PCC6803 (Fig. 4). However, only 3'end of the *htpG* gene was obtained. Since there was a Sau3A1 site upstream of the *htpG* gene, the missing of 5'end of the gene may be due to Sau3A1 partial digestion of the DNA used in the cloning procedure. No significant similarity to downstream sequence of the htpG gene of E10 was detected in the database.

Deletion analysis of promoter-active fragment E14

The effects of different length of E14 deletions on GUS expression are shown in Fig. 5. The GUS activity of *Synechococcus* harboring pKG-E14D2 was similar to that of cells harboring pKG-E14. Preliminary results of 5'RACE-PCR revealed that the transcription initiation sites of E14D2-GUS mRNA were located within 150 bp upstream of the PvuII restriction endonuclease site (data not shown). Thus, it was expected that the promoter region of E14D2 might locate within the HaeIII site (Fig. 5). However, the GUS activity of cells harboring further deleted plasmid pKG-E14D3 was reduced to background level. The GUS activity of cells harboring pKG-E14D4 was similar to that of cells harboring pKG-E14D2. These results indicated that the region between HincII and HaeIII sites was important for E14D2 promoter activity (Fig. 5). Whether the HincII/HaeIII region is distant enhancer remained to be investigated.

Multiple sigma factor genes have been identified in *Synechococcus* PCC7942. Their encoded products have conserved domains characteristic of principal sigma factors of the σ^{70} class [21, 22]. However, our results show that the majority of isolated *Synechococcus* promoters did not function in *E. coli*. The $tRNA^{pro}$ gene, which functions as a promoter and lacks the *E. coli* promoter element, was not recognized by sigma factors of the σ^{70} class. The use of these isolated promoters as DNA templates to probe for new RNA polymerase activity *in vitro* will clarify whether other uncharacterized sigma factors exist in *Synechococcus* PCC7942.

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Table 1. GUS activity of promoter-GUS fusion in *Synechococcus* PCC7942 and *E. coli* MC1061.

Plasmid	GUS activity (nmole/ min/ mg protein) [a]	
	Synechococcus [b]	*E. coli* [c]
Host (without pKG)	0.11 (± 0.01)	13 (± 2)
pKG (control)	0.13 (± 0.01)	13 (± 2)
pKG-35S	6 (± 1.7)	N
pKG-P_R (non induction) [d]	27 (± 10)	N
pKG-P_R (heat induction) [d]	276 (± 30)	505 (± 8)
pKG-E3	325 (± 20)	57 (± 8)
pKG-E4	36 (± 5)	21 (± 3)
pKG-E8	22 (± 9)	N
pKG-E10	298 (± 30)	N
pKG-E14	245 (± 22)	N
pKG-D13	145 (± 20)	70 (± 3)
pKG-D21	79 (± 15)	31 (± 8)

[a]: The specific activities are the means of three independent experiments (each in duplicate), with standard deviations indicated in parantheses. N: no expression (the GUS activity is not higher than the control).

[b]: *Synechococcus* was grown on BG-11 agar at 28°C with a light intensity of approximately 5,000 Lux, for 6 days.

[c]: *E.coli* was grown in LB broth at 37°C for 14 hr.

[d]: For non induction conditions, cells were grown at 28°C. For heat induction, a 5-day culture of *Synechococcus* and a log-phase culture of *E. coli* were further grown at 39°C, 24 hr and at 42°C, 18 hr respectively.

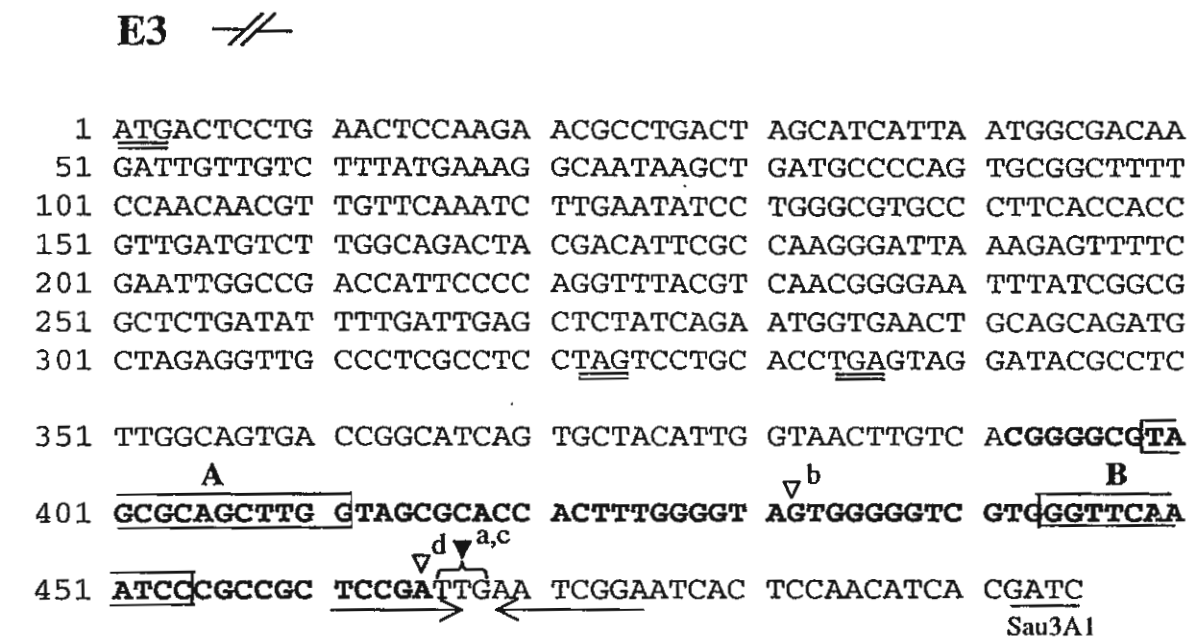

Fig. 1. Nucleotide sequence of the E3 promoter-active fragment.

The nucleotide sequence shown is the 3' end of the 1.5 kb E3 fragment immediately upstream of the promoterless-GUS gene (GenBank Database accession no. AF083392). The start and stop codons of an open reading frame of 107 amino acids (shown in Fig. 17) are marked by double lines. The 74 nucleotides (nt. 392-465) of $tRNA^{Pro}$ gene is in bold. The two boxed regions A and B conform to the two conserved sequence blocks within eukaryotic tRNA genes. The transcription initiation sites of GUS transcripts were determined by 5' RACE-PCR. In *E. coli*, the transcription initiation sites are indicated as (a) and (b). In *Synechococcus*, the transcription initiation sites are indicated as (c) and (d). Major and minor transcription initiation sites are marked by black and white triangles respectively. The inverted repeat sequence at the 5' end of the transcript is marked by arrows.

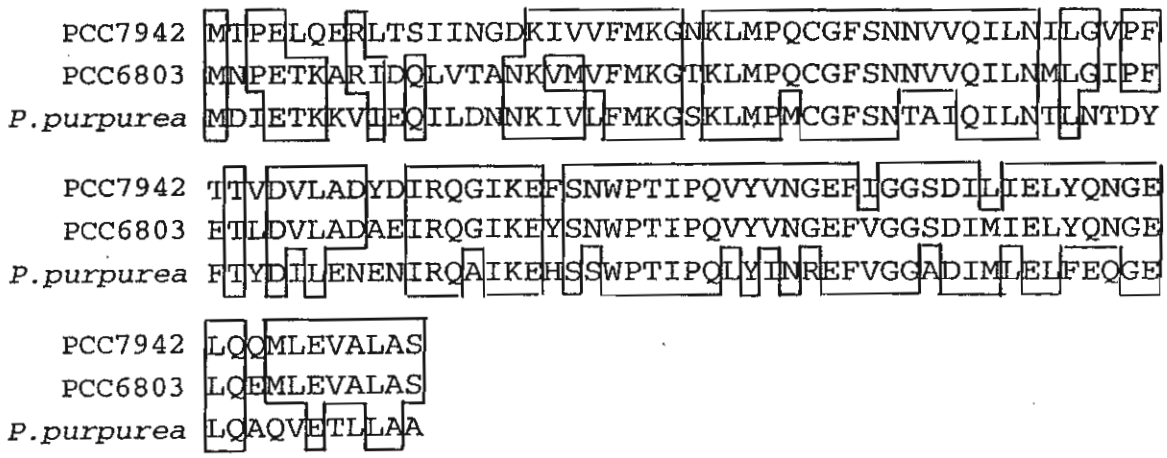

Fig. 2. Comparison of the ORF107 of E3 promoter-active fragment.

The ORF from *Synechococcus* PCC7942 (this study, accession no. AF083392), slr1846 from *Synechocystis* PCC6803 (accession no. P73056) and ORF107 from *Porphyra purpurea* (accession no. U38804). Boxed residues represent identical amino acids.

E10 //

```
  1  CCCAGTCAGT AGTCAGAGAG CAATACCGTT AAACCCTGCC CACGCGGGGT
 51  TTTTTTATGG GCGATCGCAG ACGCGTCCGT AAGATGAAGG GGTGATGTTG
                  Sau3A1
101  GCCATCGCCT CAATGCTGAT CCGGCTAATA TACCCGATGA CCATGTCCTG
                       Sau3A1
151  CTCGTGAATA CCGCGCATCC TCTTGTCCAG AACATCCTTA GTCTGCAGCA
201  GGGGGCTATC CTCAGCAGTG ATGGACACTC TCCAAGCCAG GTCCTGGCAG
251  AGCAGCTCTG TCGACACATC TATGACTTGG CCTTGATGAC CCAAAAAGGG
301  TTTGATGCTG AGGGAATGAA AGCCTTCATT GAGCGTTCTA ATGCGGTCTT
351  GACGGCGTTG ACGACTCGCC AGTGAAGGGT TTAGGGGAAC TCTAAACTTC
401  ACAGAACGTC ATCCTAGCTA TCTCGCCCTC AGACGTGAGC CTCGCTAAGC
451  TGAGGGCGAC GATCGCAGCG GAAAAATGTG ACCGGGGCGG CGCAGATC
                                                  Sau3A1
```

Fig. 3. Nucleotide sequence of the E10 promoter-active fragment.

The nucleotide sequence shown is the 3'end of the 1.75 kb E10 fragment immediately upstream of the promoter-less GUS gene. The 233 nucleotides of htpG heat shock protein gene are in bold. The stop codons of htpG are marked by double lines.

```
E10            PDDHVLXVNT AHXLVQNILS LQQGXILSSD GHSPSQVLAE
               P+ HVL  NT  H L++NILS L QG I++   G   SPSLA+
PCC6803  580 PEQHVLAINT NHPLIKNILS LSQGGIVTGS GESPSAELAK

E10          QLCRHIYDLA LMTQKGFDAE GMKAFIERSN AVLTALT
             LC+ H+YDLA LM QKGFDAE GMK FIERSN AVLT LT
PCC6803      SLCQHVYDLA LMAQKGFDAE GMKGFIERSN AVLTRLT 657
```

Fig. 4. Amino acid aligment of E10 and htpG protein of *Synechocystis* PCC6803.

Deduced amino acids of nt. 131-363 of E10 promoter-active fragment was highly homologous to the C-terminal of htpG heat shock protein of PCC6803. There are 68% (53/77) identities and 80% (62/77) positives.

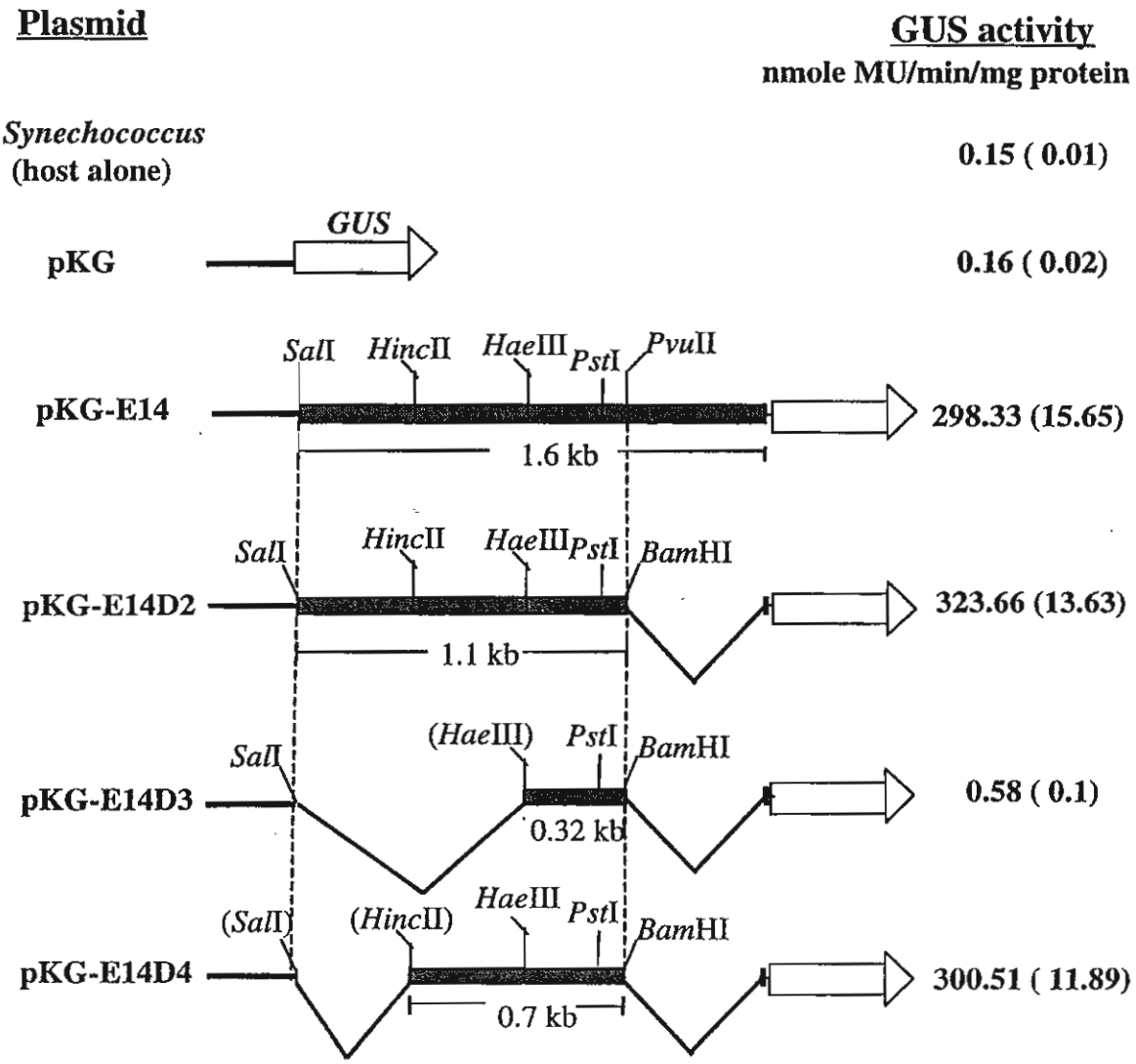

Figure 5. Effect of different lenght of E14 deletions on GUS expression

Synechococcus harbouring pKG, pKG-E14 and their derivatives were grown in BG-11 broth at 28 C with a light intensity of approximately 5,000 Lux, for 9 days (OD_{730} ~ 5). The GUS specific activities are means of three independent experiments (each in duplicate), with standard error mean indicated in parentheses. The figure is not drawn to scale. The restriction sites in parentheses are lost after ligation.

เนื้อหางานวิจัย

CHARACTERIZATION OF EXPRESSED SEQUENCE TAGS FROM BLACK TIGER SHRIMP 'S HEMOCYTES

ABSTRACT

Two lambda ZAPII-based cDNA libraries was prepared from poly(A)+ RNA of hemocytes from Black Tiger Shrimp (*Penaeus monodon* Fabricius). Random clones were isolated and their 5' end partial nucleotide sequence determined by automated procedure. A segment of each DNA sequence was used to search online nucleotide and protein sequence databases by two BLAST programs. From the first cDNA library with a lower phage titer, 53 DNA sequences were obtained. Among these, 12 sequences (22%) did not match existing DNA sequence in online databases by either BLASTN or BLASTX searches, while 12 sequences (23%) matched known DNA sequences for known protein identities and 29 sequences (55%) matched 16S ribosomal rRNA sequences. Among those matching protein coding sequences, 3 sequences (6%) matched those of unique genes, 5 sequences (9 %) matched those of ribosomal protein genes, 4 sequences (7%) matched those of mitochondrial genes. A second cDNA library constructed from higher amount of poly(A)+ RNA provided higher phage titer. Analysis of random cDNA sequences from the second cDNA library was in progress. The study of partial cDNA sequences should allow rapid characterization of the DNA sequences, some of which may be relevant to immune system of the shrimp.

INTRODUCTION

Shrimps in the subphylum Crustaceae are a group of highly diversed organisms of over 38,000 known species (1). Human consume a number of marine shrimps species, yet those in the Family Penaeidae constitute the bulk of the catches (2). With the adoption of certain fast growning *Penaeus* species for aquaculture around the World, its commercial impact is even higher. In South and Southeast Asia, *Penaeus mondon* is the choice species. For Thailand alone, the World 's number one exporter of frozen shrimps, a yearly export value for about 250,000 tonnes of frozen shrimps amounted to US$ 2 billions.

Despite its economic importance to the World, little attention has been paid to study the shrimps in Molecular Biology aspects. Online DNA sequence databases possessed only a hundred plus records, as of early 1999. In recently years' a number of viruses infecting

useful genetic marker for studying diversity and significance in pathogenesis[13] as well as variations within populations of closely related bacteria.[14]

The flagellin gene of *B. pseudomallei* has been studied and its sequence reported.[15] Ithas been shown to be a typical flagellin in that there are two conserved regions at the N- and C-terminal ends and a variable part at the middle region of the gene. Commonly, the conserved regions are most highly conserved among the same genus.[16] Thevariable region shows significant differences between species and even between strains in the same species.[17,18] In this study, the characteristics of the flagellin gene are used to isolate and compare the flagellin gene are used to isolate and compare the flagellin sequences between *B. pseudomallei* and *B. cepacia* from different clinical isolates. We also report here a simple method for identification of both organisms based on the flagellin sequence by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis.

MATERIALS AND METHODS

Bacterial species, plasmid and growth conditions

Clinical isolated *B. pseudomallei* and *B. cepacia* used in this study were obtained from the National Institute of Health, Thailand and are listed in Table 1. All isolated were grown at 37°C on nutrient agar and broth. *Escherichia coli*K12 JM109,19 which was used as the recipient for recombinant plasmids was grown at 37°C on Luria-Bertani (LB) agar and broth without or with antibiotic (100 μg ml^{-1} ampicillin) for plasmid selection. PUC19[19] was used as a vector for cloning and sequencing analysis of the flagellin genes.

Genomic DNA purification and PCR amplification of flagellin gene

Genomic DNA purification was performed by QIAGEN Genomic-tip according to QIAGEN protocol. The purified genomic DNA was directly used as a template for PCR amplification reaction. The PCR reaction was carried out in a 50 μl mixture containing 1 μl of DNA template (about 200 ng), reaction buffer (10 mM Tris-HCl pH 8-8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 200 μM of each dNTP, 0-4 μM of each primer (Forward primer; 5′ ATG CTC GGA ATC AAC AGE AAC AT 3′ and Reverse primer ; 5′ CAG GAG CTT CAG CAC TTG CTG 3′). All reactions were overlaid with two drops of light minerol oil (Sigma) and heated at 100°C for 10 min before adding 1 unit of *Vent* DNA polymerase (New England Biolabs). The synthesis was performed for 30 cycles in an automated DNA Thermal Cycler (480 Perkin-Clmer Cetus). The PCR condition of all isolates was carred out with the debaturation temperature at 94°C for 1 min, the annealing temperature at 65°C for 1 min, and followed by the synthesis temperature at 72°C for 1 min.

Cloning and DNA sequencing

The PCR-amplified products were excised from the agarose gel and purified with Gene Clean II kit (Bio 101, Inc.). Cloning in plasimid pUC19 vector was performed by blunt-end ligation at the *Sma*I cloing site following the standard protocol described proviously.[20] DNA sequencing fo both strands of pUC19-based clones and subclones was carried out by the dideoxy-chain termination method.[21] An automated DNA sequencer (Applied Biosystem, model 377) was utilized to determine the nucleotide sequences of both strands of the entire fragment. Computer analysis of the sequences was performed by using PC/Gene software package (Intelligenetics), the CLUSTAL W[22] for multiple sequence alignment, and the NCBI BLAST e-mail server.[23]

PCR-restriction endonuclease analysis

The PCR-amplified products (5 µl) were subsequently used for restriction endonuclease analysis either by *Pst*I or *Xho*I digestion. The reaction was carried out in a total volume of 20 µl using the condition srecommended by the sepplier (Ner England Biolabs).

RESULTS

PCR-amplification of *B. pseudomallei* and *B. cepacia* flagellin genes

Specific primers were designed based on the 5′-and 3′-end conserved sequences from the flagellin gene of *B. pseudomallei* 1026b, and the flagellin gene was seccussfully amplified from different clinical isolates of both *B. pseudomallei* and *B. cepacia*. As expected, a single PCR product of apporximately 1-1 kb in size was obtained from all *B. pseudomallei* isolated and a PCR product of the same size was also found from two clinical isolates of *B. cepacia*, as shown in Fig.1

Cloning and DNA sequencing

All of the PCR-amplified products were subsequently cloned into the pUC19 vector and were consuently analysed for their DNA sequences using primers for both orientations. The results can be accessed from the GenBank database under the accession numbers AF078151; AF078152; AF080259; and AF080260 for flagellin sequences of *B. pseudomallei* NF 10/38, NF 47/38, NF 105/37; *B. pseudomallei* NF 154/37; *B. cepacia* DMS 2555; and *B. cepacia* DMS 3027, respectively.

The deduced amino acid sequences were examined for similarity to sequences previously reported.[15] In *B. pseudomallei*, the sequences showed 99% homology among

themselves and with the sequences previously reported, whereas the sequences of *B. cepacia* showed 100% homology in our two isolates, and 97% homology with the *B. cepacia* flagellin type II sequences recently reported,[24] as shown in Fig. 2a and 2b, respectively. Comparison of the amino acid sequences between these two species in Fig.3 reveals the general features of the flagellin sequence in that there are the N- and C-terminal conserved regions and the central part is diverse, resulting in less homology (84%).

PCR-restriction endonuclease analysis

Figure 4 represents a restriction enzyme mapping of the flagellin sequences from both apecies. According to the maps, the restriction sites for endouncleases *Psi*I and *Xho*I are apparently distinct between the two species. Using either *Psi*I and *Xho*I digestion of the PCR products, the obtained restriction endonuclease patterns can be used to identify the two species, as shown in Fig. 5a and 5b, respectively, In the PCR-restricted *Psi*I patternm, three fragments of 676, 264 and 216 bp were obtained from *B. pseudomallei*. The PCR procuct form *B. cepacia* contains five restriction sites for *Psi*I and should yield fragments of 417, 264, 165, 164, 70 and 69 bp. Three bands can be visualized in the gel in Fig. 5a, the 417, 264 and the combined 165 and 164 bp fragments, In the PCR-restricted *Xho*I pattern, two fragments of about 584 and 567 bp were detected from *B. pseudomallei*, whereas the recognition site for *Xho*I in absent in *B. cepacia*.

DISCUSSION

PCR-based isolation of the flagellin gene from the bacterial species *Pseudomonas*[16-18] has been achieved, making use of the highly conserved 5′ and 3′ regions of the gene. Since, the specific primers designed from the first codon of the N-terminal and the third codon from the C-terminal sequences were not able the amplify flagellin gne of bacterial species in the genus *Burkholderia*, the conservation of these regions has been shown to be restrictive and more diverse across the genus.[16] In this study, we therefore designed specific primers based on the 5′ and 3′ conserved flagellin sequence from *B. pseudomallei* 1026b,15 and they wete secessfully employed to isolate the flagellin gene from four clinical isolates of *B. pseudomallei*. In an attempt to extend this method for isolation of the flagellin gene in other spceies in the same genus *Burkholderia* using the same pair of primers, two chinical isolates of *B. cepacia* were also successfully amplifiied. The PCR-amplified flagellin products from both species were of a similar size of 1-1 kb, which is the experted size of the flagellin gene of *B. pseudomallei*. In the case of *B. cepacia* there have been recently reported at least two types of flagellin genes.[24] Type I is 1-4 kb in size and type II is 1-1kb.

Thus, our isolate should contain the type II lfagellin gene. As the majority of *B. cepacia* clinical isolates have been found to have typeII flagellin,[24] it is similar to the size of flagellin gene found in *B. pseudomallei* clinical isolates. This finding also indicates a close relationship of the two organisms.

In order to be sure that the PCR-amplified products botained fromthese two species were not artifacts of contamination in the clinical samples or in the PCE reaction, DNA sequencing analysis was applied. The results demonstrated that the 1-1 kb PCR-amplified products from the two species have differents flagellin sequecnes. Moreover, the DNA sequences found in the four clinical isolates and a published sequence of *B. pseudomallei*[15] are highly conserved throughout the gene, especially at the amino acid level (more than 99% homology). This was also found in the two clinical isolates and a published sequence of a type II flagellin gene of *B. cepacia*24 (97% homology). In contrast, the amino acid sequence comparison between the two species in more divergent (84% homology). The variations are commonly located in the middle part of the gene which is the characteristic[25-28] of a flagellin gene. Interestionly, the variable region of the *B. cepacia* type II lfalgellin sequence has a four amino acid deletion a position 248-251 **(-PSFO-)** compared to the *B. pseudomallei* flagellin sequence. This finding suggests that a nested PCR could be developed for the bacteriological classification by designing specific primers in this region.

To simply identify these two species based on the PCR-amplified flagellin products, *Psl*I and *Xho*I restriction endonuslease reactions were performedI Both restriction endonucleases are also found and localize in the same regions of a type II *B. cepacia* flagellin sequence.[24] Either the *Psl*I and *Xho*I digestion is able to distinguish the two species, therefore the flagellin sequence is a useful marker for identification of the closely related species. Although PCR-RFLP analysis enabled flagellin type classification within population of *B. cepacia*,[24] it was not applicable for studying variation among species. Our inverstigation is mainly to study variation between species, *B. pseudomallei* and *B. cepacia*, parricularly from clinical isolates. Previously, 165 rRNA sequences have been applied for identification of the species.[8] the flagellin sequence is an interesting target because a correlation may be established with the serological and pathogenesis of the diseases, particularly from clinical isolates. A study of larfer numbers of clinical isolated from both species are required to berrer assess these connections as well as to study diversity of the organisms.

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Tabele 1. *B. pseudomallei* and *B. cepacia* from clinical isolates used in this study

Species and corresponding isolates number	Source of isolation
B. pseudomallei NF[a] 10/38	Blood
B. pseudomallei NF 47/38	Blood
B. pseudomallei NF 105/37	Pus
B. pseudomallei NF 154/37	Pus
B. cepacia DMS[b] 2555	Pus
B. cepacia DMS 3027	Blood

NF[a] is Nofermentation

DMS[b] is Department of Medical Sciences, Ministry of Public Health, Thailand

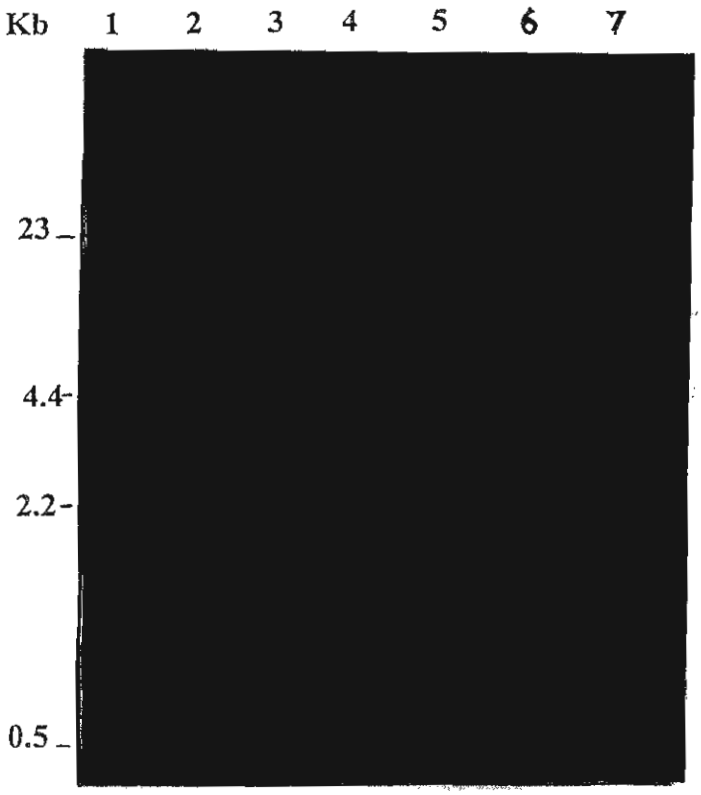

Figure1 Ethidium bromide staining of an 1% agarose gel electrophoresis of PCR-amplified flagellin gene from clinical isolates of B.pseudomallei NF 10/38 (lane 2), NF 47/38 (lane 3), NF 105/37 (lane 4), NF 154/37 (lane 5) and B.cepacia DMS 2555 (lane 6), DMS 3027 (lane 7), respcectively. Lane 1 is a standard size marker.

```
NF10/38 MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQINGL   60
NF47/38 MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQINGL
NF105/37MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQINGL
NF154/37MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQINGL
Bps      MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQINGL
         ************************************************************

NF10/38  NQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQIS  120
NF47/38  NQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQIS
NF105/37NQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQIS
NF154/37NQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQIS
Bps      NQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQIS
         ************************************************************

NF10/38  EVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQTL  180
NF47/38  EVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQTL
NF105/37EVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQTL
NF154/37EVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQTL
Bps      EVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQTL
         ************************************************************

NF10/38  GTIKVAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGSSTA  240
NF47/38  GTIKVAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGSSTA
NF105/37GTIKVAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGSSTA
NF154/37GTIKVAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGSSTA
Bps      GTIKVAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGSSTA
         ************************************************************

NF10/38  GTGTAASPSFQTLALSTSATSALSATDQANATAMVAQINAVNKPQTVSNLDISTQTGAYQ  300
NF47/38  GTGTAASPSFQTLALSTSATSALSATDQANATAMVAQINAVNKPQTVSNLDISTQTGAYQ
NF105/37GTGTAASPSFQTLALSTSATSALSATDQANATAMVAQINAVNKPQTVSNLDISTQTGAYQ
NF154/37GTGTAASPSFQTLALSTSATSALSATDQANATAMVAQINAVNKPQTVSNLDISTQTGAYQ
Bps      GTGTAASPSFQTLALSTSATSALSATDQANATAMVAQINAVNKPQTVSNLDISTQTGAYQ
         ************************************************************

NF10/38  AMVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLS  360
NF47/38  AMVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLS
NF105/37AMVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLS
NF154/37AMVSIDNALATVNNLQAMLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLS
Bps      AMVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETANLS
         ********** ***************************************************

NF10/38  RAQVLQQAGISVLAQANSLPQQVLKLL-     388
NF47/38  RAQVLQQAGISVLAQANSLPQQVLKLL-
NF105/37RAQVLQQAGISVLAQANSLPQQVLKLL-
NF154/37RAQVLQQAGISVLAQANSLPQQVLKLL-
Bps      RAQVLQQAGISVLAQANSLPQQVLKLLQ
         ***************************
```

Figure 2(A). Alignmet of the amino acid sequences for flagellin gene from clinical isolates NF10/38, NF47/38, NF105/37, NF154/37 and a published sequence[15], Bps, of *B. pseudomallei*. Identical amino acids are indicated by an asterisk.

```
DMS3027  MLGINSNINSLVAQQNLNGSQNALSQAITRLSSGKRINSAADDAAGLAISTRMQTQINGL   60
DMS0704  MLGINSNINSLVAQQNLNGSQNALSQAITRLSSGKRINSAADDAAGLAISTRMQTQINGL
BcII     MLGINSNINSLVAQQNLNGSQNALSQAITRLSSGKRTNSAADDAAGLAISTRMQTQINGL
         ************************************************************

DMS3027  NQGVSNANDGVSMIQTASSALSSLTNSLQRIRQLAVQASTGTMSTTDQAALQQEVSQQIQ  120
DMS0704  NQGVSNANDGVSMIQTASSALSSLTNSLQRIRQLAVQASTGTMSTTDQAALQQEVSQQIQ
BcII     NQGVSNANDGVSMIQTASSALSSLTNSLQRIRQLAVQASTGTMSSTDQAALQQEVAQQIQ
         ******************************************.***********.****

DMS3027  EVNRIASQTTYNGTNILDGSAGIVSFQVGANVGQTISLDLSQSMSAAKIGGGLVQKGQTV  180
DMS0704  EVNRIASQTTYNGTNILDGSAGIVSFQVGANVGQTISLDLSQSMSAAKIGGGLVQKGQTV
BcII     EVNRIASQTTYNGTNILDGSAGIVSFQVGANVGQTMSLDLSQSMSAAKIGGGLVQKGQTV
         ***********************************.************************

DMS3027  GTVTGLSLDNAGAYTSSGAAITAINVLSDGKGGYTFTDQNGGAISQTVAQSVFGANATTG  240
DMS0704  GTVTGLSLDNAGAYTSSGAAITAINVLSDGKGGYTFTDQNGGAISQTVAQSVFGANATTG
BcII     GTVTGLSLDNAGAYVSSGATITAINVISDGQGGYTFTDQNGQSISSGAATAVFGSNATTG
         ************** ****.******.***.************.**    * .*********

DMS3027  TGTAVGNLTLQTGATGTGTSAAQQTAITNAIAQINAVNKPATVSNLDISTVSGANVAMVS  300
DMS0704  TGTAVGNLTLQTGATGTGTSAAQQTAITNAIAQINAVNKPATVSNLDISTVSGANVAMVS
BcII     SGTAVGALSLQPSATGANTTAAQLTAITNAIAQINAVNKPVTVSGLDISTVSGANVAMVS
         .***** *.**..***. *.*** ****************.*** ***************

DMS3027  IDNALQTVNNVQAALGAAQNRFTAIATSQQAESTDLSSAQSQITDANFAQETANMSKNQV  360
DMS0704  IDNALQTVNNVQAALGAAQNRFTAIATSQQAESTDLSSAQSQITDANFAQETANMSKNQV
BcII     IDNALQTVNNLQAALGAAQNRFTAIATAQQAESTDLSSAQSQITDANFAQETANMSKNQV
         **********.****************.**********************************

DMS3027  LQQAGISVLAQANSLPQQVLKLL-      384
DMS0704  LQQAGISVLAQANSLPQQVLKLL-
BcII     LQQAGISVLAQANSLPQQVLKLLQ
         ***********************
```

Figure 2(B). Alignment of the amino acid sequences for flagellin gene from clinical isolates DMS 2555, DMS 3027 and a published sequence24, BelI, of type II *B. cepacia.* Identical amino acide are indicated by an asterisk and similar amino acids are marked by a dot.

```
NF10/38  MLGINSNINSLVAQQNLNGSQGALSQAITRLSSGKRINSAADDAAGLAIATRMQTQINGL   60
DMS3027  MLGINSNINSLVAQQNLNGSQNALSQAITRLSSGKRINSAADDAAGLAISTRMQTQINGL   60
         ********************* ************************************

NF10/38  NQGVSNANDGVSILQTASSGLTSLTNSLQRIRQLAVQASNGPLSASDASALQQEVAQQIS  120
DMS3027  NQGVSNANDGVSMIQTASSALSSLTNSLQRIRQLAVQASTGTMSTTDQAALQQEVSQQIQ  120
         ************..***** *.***************** * .*..* .**********

NF10/38  EVNRIASQTNYNGKNILDGSAGTLSFQVGANVGQTVSVDLTQSMSAAKIGGGMVQTGQTL  180
DMS3027  EVNRIASQTTYNGTNILDGSAGIVSFQVGANVGQTISLDLSQSMSAAKIGGGLVQKGQTV  180
         *********.*** ******** .***********.*.**.***********.** ***.

NF10/38  GTIKVAIDSSGAAWSSGSTGQETTQINVVSDGKGGFTFTDQNNQALSSTAVTAVFGS-ST  240
DMS3027  GTVTGLSLDNAGAYTSS--GAAITAINVLSDGKGGYTFTDQNGGAISQTVAQSVFGANAT  240
         **.         * .*   *   * ***.******.******. **** *  .***. .*

NF10/38  AGTGTAASPSFQTLALSTSATSA-LSATDQANATAMVAQINAVNKPQTVSNLDISTQTGA  300
DMS3027  TGTGTAVG----NLTLQTGATGTGTSAAQQTAITNAIAQINAVNKPATVSNLDISTVSGA  300
         .*****       *.* * ** .  **. *.  *  .********* ********* .**

NF10/38  YQAMVSIDNALATVNNLQATLGAAQNRFTAIATTQQAGSNNLAQAQSQIQSADFAQETAN  360
DMS3027  NVAMVSIDNALQTVNNVQAALGAAQNRFTAIATSQQAESTDLSSAQSQITDANFAQETAN  360
           ********* ****.**.*************.*** *. *. *****  * *******

NF10/38  LSRAQVLQQAGISVLAQANSLPQQVLKLL-     388
DMS3027  MSKNQVLQQAGISVLAQANSLPQQVLKLL-     384
         .*. ************************
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Figure 3. Alignment of the amino acid sequences of *B. pseydimallei* flagellin (NF10/38) and *B. cepacia* flagellin (DMS 3027). Identical amino acide are indicated by an asterisk and aimilar amino acids are marked by a dot.

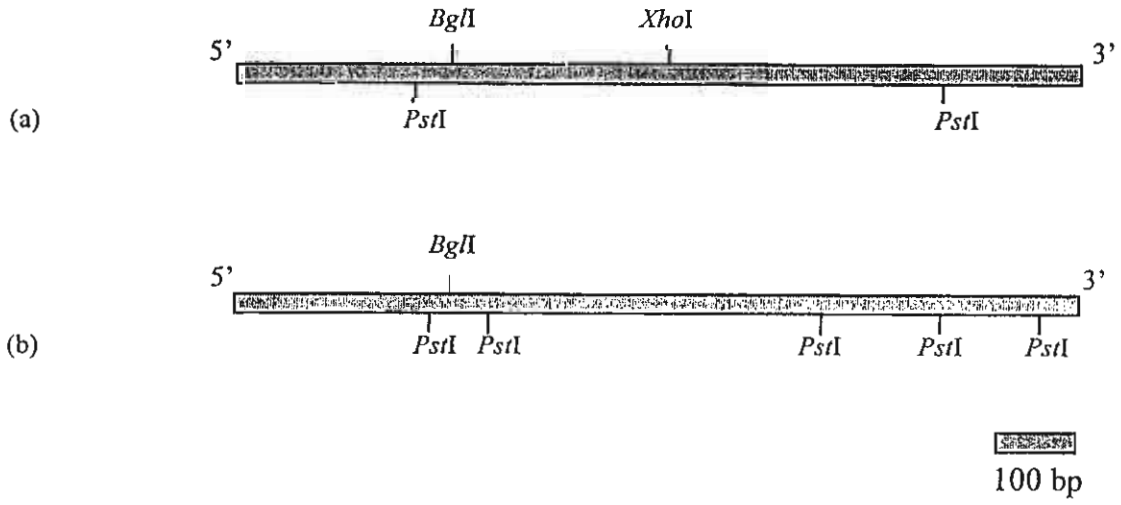

Figure 4. Partial restriction enzymes map of the flagellin genes of *B. pseydimallei* (a) and of *B. cepacia* (b).

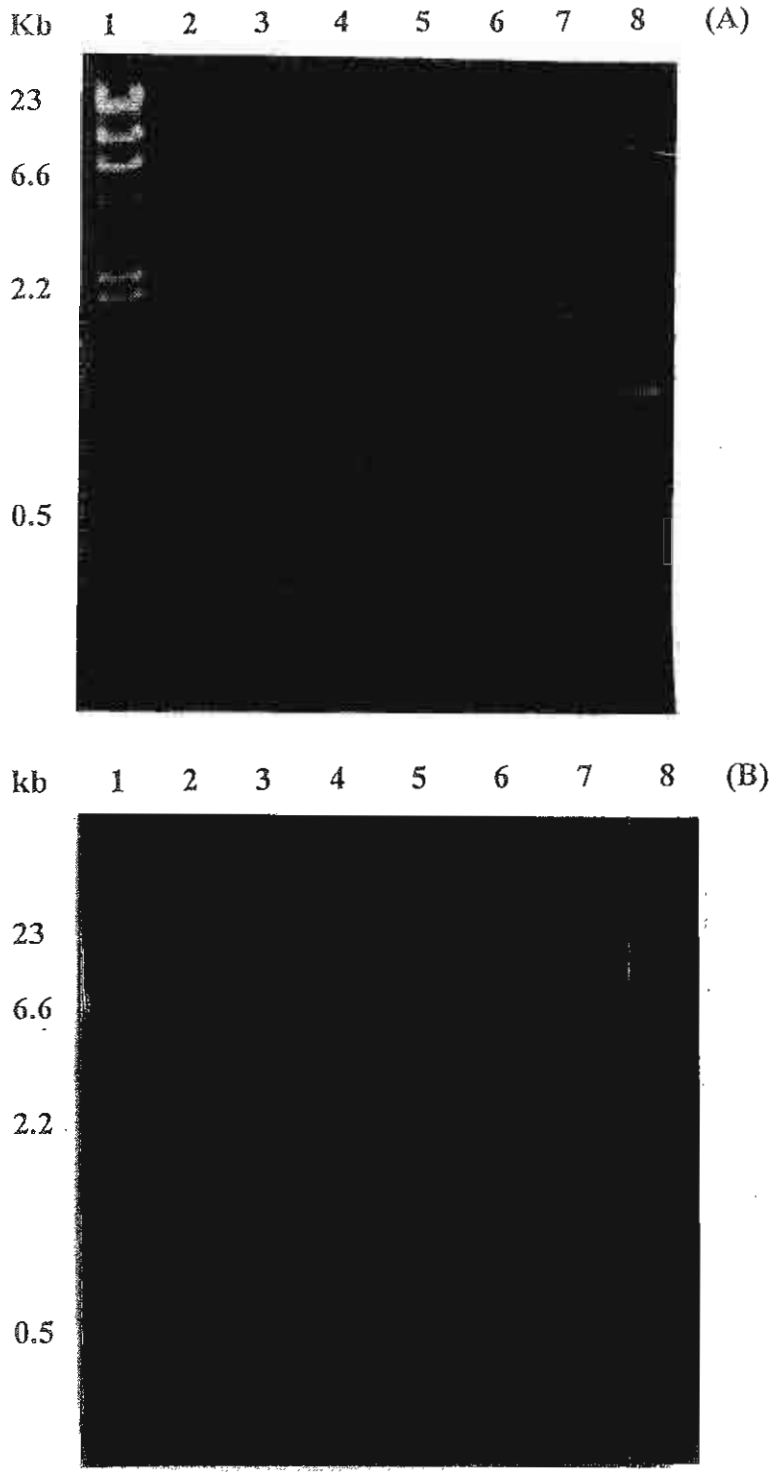

Figure 5. Ethidium bromide staining of an 1% agarose gel electrophoresis of the PCR-restricted PstI pattern (A) and PCR-restricted XhoI pattern (B) of *B. pseudomallei* NF 10/38 (lane 2), NF 47/38 (lane 3), NF 105/37 (lane 4), NF 154/37 (lane 5) and *B.cepacia* DMS 2555 (lane 6), DMS 3027 (lane 7) and undigested PCR-amplified product (lane 8). Lane 1 is a standard size marker.

เนื้อหางานวิจัย

DIFFERENTIAL ANALYSIS OF THE *RPO*B GENE OF MYCOBACTERIA AND PATHOGENIC BACTERIA BY PCR-RFLP TECHNIQUE

ABSTRACT

We have analysed the *rpo*B gene of *M.tuberculosis* and compared to other 13 mycobacteria and 11 pathogenic bacteria using PCR-RFLP technique. Results demonstrated that the 5'portions (region A, nucleotides 36-1,073 and region B, nucleotides 1,024-1,666) and the 3'portion (region F, nucleotides 2,856-3,645) of the *rpo*B gene were conserved among the *M.tuberculosis* complex (*M.tuberculosis, M.bovis* BCG and *M.africanum*). Due to the absence of these fragments in many bacteria, they could be used to distinquish the *M.tuberculosis* complex from other mycobacteria (*M.avium, M.chelonae, M.duvalli, M.gordonae, M.kansasii, M.phlei, M.scrofulaceum, M. smegmatis, M.vaccae and M.intracellulare)* and respiratory bacteria (*S.aureus, S.epidermidis, S.pyogenes, S.viridans, H.influenzae, Ps.aeruginosa, Ps.pseudomallei, K.pneumoniae, C.diptheriae, N.meningitidis and E.coli). M.microti*, one of the *M.tuberculosis* complex, differed from the group by giving PCR products only from regions B and F. PCR-RFLP patterns of region C (nucleotides 1,615-2611) using *Sal*I and region E (nucleotides 2,335-2962) using *Alu*I confirmed the specificity of the *rpo*B gene of *M.tuberculosis* complex. Two regions, D (nucleotides 2,335-2,492) and H (nucleotides 3,935-4,357) were highly conserverd in all mycobacteria and respiratory bacteria tested as they yielded similar sizes of PCR products. However, *Bsa*AI digestion pattern of the amplified region D could be used to differentiate the *M.tuberculosis* complex from *H.influenzae, E.coli* and other mycobacteria while *Hae*III digestion could be used to separate *M.tuberculosis* complex from *M.avium, M.gordonae, M.kansasii, M.scrofulaceum* and *M.intracellulare*. As a whole, we suggested that the PCR-RFLP pattern of regions D and E were useful for identification of the *rpo*B gene of the *M.tuberculosis* complex, hence assuring the detection of rifampicin resistance genotype in unpurified specimens.

INTRODUCTION

Tuberculosis (TB) is a serious health problem in Thailand. The rising incidence rate is believed to accompany the acquired immunodeficiency syndrome (AIDS) epidemic. Moreover, the high occurrence of drug resistant *M. tuberculosis*, the causal agent,

contributes considerably to an ineffective treatment of TB and therefore compromises the Tuberculosis Control Programme. Rifampicin is one of the first-line drugs used in TB chemotherapy. Its mechanism of action is the inhibition of bacterial ribonucleic acid (RNA) polymerase by forming a complex with the holoenzyme, thus blocking the transcription step[1-3]. Resistance to rifampicin is one major cause of ineffective treatment. Alterations in the hot spot region covering 25 amino acids in the *rpoB* gene (encoding for the β subunit of RNA polymerase) are responsible for the rifampicin resistant phenotype[3-8]. Several laboratories have developed rapid techniques for detection of points mutations lying in this region including heminested PCR[9], PCR-single stranded conformation polymorphism[6], dideoxy fingerprinting[10] specific probe hybridization (the line probe assay kit)[11] and DNA sequencing[12,13]. However, these techniques require a separate bacterial species identification procedure. Current identification of *Mycobacterium tuberculosis* complex utilizes at least two target genes for example; genes encoding 65kDa, 19kDa, MPB 64 and the MPB 70 proteins[14].

In this study, we have examined the specificity of each DNA fragment in the *rpoB* gene of *M. tuberculosis* comparing to other mycobacteria and respiratory bacteria using PCR-RFLP technique. The analysis was also extended to compare the identity of the *rpo*B gene sequence among 10 different isolates of *M. tuberculosis*. Specific RFLP pattern obtained from this study could be used to differentiate the *M. tubeculosis* complex from 13 other mycobacteria and 11 pathogenic bacteria therefore were useful for *rpo*B gene mutation analysis especially when the samples were unsure of the purity.

MATERIALS AND METHODS

Bacterial strains

The standard strain *M. tuberculosis* H37Rv and 13 other mycobacteria were generously obtained from Dr. Prasit Palittapongarnpim Department of Microbiology, Faculty of Science, and Dr. Angkana Chaiprasert Department of Microbiology, Faculty of Medicine, Mahidol University. Eleven other bacteria (Table 1) were obtained kindly from Dr. Roongnapa Prachaktam and Dr. Poonpilas Hongmanee, Department of Medicine, Ramathibodi Hospital.

Table 1 Mycobacteria and bacteria used in this work.

Mycobacteria species	Bacteria species
Mycobacterium tuberculosis H37Rv	*Corynebacterium diptheriae*
Mycobacterium africanum	*Escherichia coli*
Mycobacterium avium	*Haemophilus influenzae*
Mycobacterium bovis BCG	*Krebsiella pneumoniae*
Mycobacterium chelonae	*Neisseria meningitidis*
Mycobacterium duvalii	*Pseudomonas aeruginosa*
Mycobacterium gordonae	*Pseudomonas pseudomallei*
Mycobacterium intracellulare	*Staphylococcus aureus*
Mycobacterium kansasii	*Staphylococcus epidermidis*
Mycobacterium microti	*Streptococcus pyogenes*
Mycobacterium phlei	*Streptococcus viridans*
Mycobacterium scrofulaceum	
Mycobacterium smegmatis	
Mycobacterium vaccae	

DNA extraction

The chromosomal DNA was prepared by the enzymatic method. Briefly, a loopful of mycobacteria grown on Lowenstein-Jensen (LJ) medium was transferred to a 1.5 ml microcentrifuge tube containing 200 µl of 10 mM Tris HCl pH8.0, 1 mM EDTA (1XTE) buffer and heated at 80°C for 30 min. The solution was incubated at 37°C overnight with 200 µl of 10 mg/ml lysozyme. Then 75 µl 10% SDS/10 mg/ml proteinase K solution was added and the mixture was incubated at 65°C for 10 min. 50 µl of 5 M NaCl was then mixed and followed by 50 µl of cetylpyridinium chloride. The solution was further incubated at 65°C for 10 min. The DNA was extracted by chloroform/isoamyl alcohol and precipitated with 0.7 volume isopropanol. The DNA pellet was washed with 70% ethanol before air-drying and resuspending in 20 µl 1XTE buffer.

PCR-RFLP analysis

Nine regions covering the entire *rpoB* gene of *M. tuberculosis* were amplified by using primers designed from the *rpoB* gene sequence of *M. tuberculosis* H37Rv strain as shown in Table 2. Nucleotide sequences of the *rpo*B gene were based on Miller's report in 1994[8]. Regions D and E were amplified using different downstream primers (DD, for region

D and ED for region E) but the same upstream primer (DU). Generally, the amplification was carried out for 30 cycles in a total volume of 50 µl consisting of 50 ng genomic DNA, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmoles of each primer and 1 unit of *Taq* polymerase. The cycles of reaction were denaturated at 94°C, 1 min, annealing at the appropriate temperature for 1 min, and extension at 72°C for 1 min (10 min in the last cycle). The amplified PCR products were analyzed by electrophoresis in 1.5% agarose gel.

Table 2. List of primers, oligonucleotide sequences and their melting temperatures.

Primer	Length (base)	Oligonucleotide sequence (5'→3')	Melting temperature (T_m, °c)	Nucleotides of amplified regions in the *rpo*B gene (region)
AU	19	CGATGAACTCAACGACTTG	56	36-1,073 (A)
AD	18	TTCATGCGACCAACGAGA	54	
BU	20	GTGGGCACCGCTCCTCTAAG	66	1,024-1,666 (B)
BD	20	GCTGTGCAGCGTCTTGTCGG	66	
CU	20	GGGTGTACTTCGACGAGACC	64	1,615-2,611 (C)
CD	19	TACGGCGTTTCGATGAACC	58	
DU	21	TGGTCGCCGCGATCAAGGAGT	68	2,335-2,492 (D)
DD	20	TGCACGTCGCGGACCTCCAG	68	
ED	20	CGCAGCTCCATCCCGGTGCC	72	2,335-2,962 (E)
FU	20	GAGCACGACGACGCCAACCG	68	2,856-3,645 (F)
FD	20	GCAACTCGTCCTCGTCCTGG	66	
GU	20	CCTGCTGGTGGCCATCATGC	66	3,216-4,028 (G)
GD	20	CAACAGGCCCTGCAGCTCGG	68	
HU	20	GCTGCCCGACGAACTGCTCG	66	3,935-4,357 (H)
HD	20	CGGCCGACGGTGTCATCGGA	68	
IU	20	GGTTCGGGGAGATGGAGTGC	68	4,261-4,652 (I)
ID	20	GACGTCGAGCACGTAACTCC	68	

Enzyme digestion

The amplified PCR products were digested with various kinds of restriction endonucleases (Table 3). The digestion reaction was performed in a total volume of 20 µl consisting of 2 µg DNA, 1X reaction buffer, and 10 units restriction endonuclease. The mixtures were incubated at the appropriate manufacturer-recommended temperature for 2 h before being electrophoresed on 1.5% agarose gel. The positive control for the *Bsa*AI

digestion was the amplified PCR product from a part of the *rpoB* gene containing the *Bsa*AI cutting site while for the *Alu* I digestion, pUC18 was used.

Table 3. Amplified regions and the restriction endonucleases used for species differentiation.

Region	Nucleotide regions in the *rpo*B gene	Size of PCR product (bp)	Restriction endonuclease
A	36 - 1,073	1,028	*Alu* I, *Eco*R V, *Mbo* I, *Msc* I, *Sac* II, *Sf* I, *Sph* I, *Stu* I, *Taq* I
B	1,024 - 1,666	644	*Alu* I, *Hae* III, *Msp* I, *Sal* I, *Taq* I
C	1,615 - 2,611	997	*Bcl* I, *Msc* I, *Sal* I
D	2,335 - 2,492	160	*Apa* I, *Bsa*A I
E	2,335 - 2,962	628	*Alu* I, *Cla* I, *Hae* III, *Msp* I, *Pvu* II
F	2,856 - 3,645	789	*Hae* III, *Msc* I, *Rsa* I
G	3,216 - 4,028	812	*Cla* I, *Sph* I, *Xho* I
H	3,935 - 4,357	423	*Bcl* I, *Bgl* II, *Msc* I, *Rsa* I, *Sal* I
I	4,261 - 4,652	392	*Alu* I, *Msp* I, *Pst* I

RESULTS AND DISCUSSION

We have used the PCR-RFLP technique to analyse the *M.tuberculosis rpo*B gene. The patterns and product sizes generated after restriction enzyme digestion of *rpo*B gene fragments could help distinquish different species of mycobacteria and bacteria, although many of them were similar.

Our data showed that region A (nucleotides 36-1,073), region B (nucleotides 1,024-1,666) and region F(nucleotides 2,856-3,645) were conserved to the *M. tuberculosis* complex due to the presence of expected DNA fragments in *M. tuberculosis, M. bovis* BCG *and M. africanum* (Tables 4 and 5). Regions A, B and F could not be amplified from other mycobacteria except for *M.microti* which gave PCR products only from regions B and F. To examine if the PCR product obtained from regions B was belonging to the *M.tuberculosis* complex, we further digested this fragment (644 bp) with *Sal*I and compared the pattern of digestion with the control *M.tuberculosis*. *Sal*I digestion of region B from *M.tuberculosis* complex and *M.microti* gave rise to specific products of 247 bp and 397 bp fragments. However, this enzyme could not be used to separate the *M.tuberculosis* complex from *M. microti*.

Region D of the *rpo*B gene covering the hot spot of mutations is always the target for analysis of rifampicin resistant genotype. This region exhibited high homology in DNA sequences among mycobacteria and other bacteria whereas the 5' and 3' regions of the *rpo*B gene seemed to be conserved only among mycobacteria, especially those belonging to *M. tuberculosis* complex. Unfortunately, PCR-RFLP pattern of region D investigated so far could not discriminate the *M.tuberculosis* complex from most pathogenic bacteria. Therefore, the combination of results from other regions of the *rpo*B gene is necessary. In this work, nested PCR of regions D and E was performed in order to eliminate 11 respiratory bacteria in the amplified DNA. Patterns of digestion of region D products excluded other mycobacteria, *H.influenzae* and *E.coli* from the *M. tuberculosis* complex. PCR-RFLP patterns of region E generated by *Hae*III should separate *M. tuberculosis* complex from *M.avium, M.gordonae, M.kansasii, M.scrofulaceum* and *M.intracellulare* (Figures 1and 2). To avoid false negative results, two additional control experiments were performed. In the first experiment, DNA extract of *M.tuberculosis* was added to the tested DNA and reamplified with the same set of primers. This experiment should confirm whether the PCR condition was optimal or not. The second experiment was conducted to test the quality of DNA templates by reamplifying with different set of primers. In both cases, specific PCR product were detected indicating the appropriate PCR condition (data not shown).

Regions D, E, H and I were more conserved among mycobacteria whereas regions D and H were conserved in both mycobacteria and bacteria. The results of PCR amplification were summarized in Tables 4 and 5.

Although we could not distinguish *M. tuberculosis* from the *M. tuberculosis* complex due to a very high homology in gene sequences among them, tuberculosis occured in human are mainly caused by *M. tuberculosis.* RFLP patterns tested by *Alu*I, *Mbo*I *Taq*I for region A, *Sal*I, *Hae*III *Taq*I for region B, *Cla*I, *Msp*I, *Pvu*II for region E, *Hae*III *Rsa*I for region F, and *Bcl*I, *Bgl*II, *Msc*I, *Rsa*I, *Sal*I for region H did not show any variations among 10 isolates of *M.tuberculosis.*

In conclusion, the PCR-RFLP patterns of the *rpoB* gene obtained in this study could rapidly differentiate the *M.tuberculosis* complex from 13 other mycobacteria and 11 pathogenic bacteria. This data should therefore be useful as a confirmation that DNA fragments amplified from the *rpo*B gene belongs to the *M. tuberculosis* complex prior to mutation analysis.

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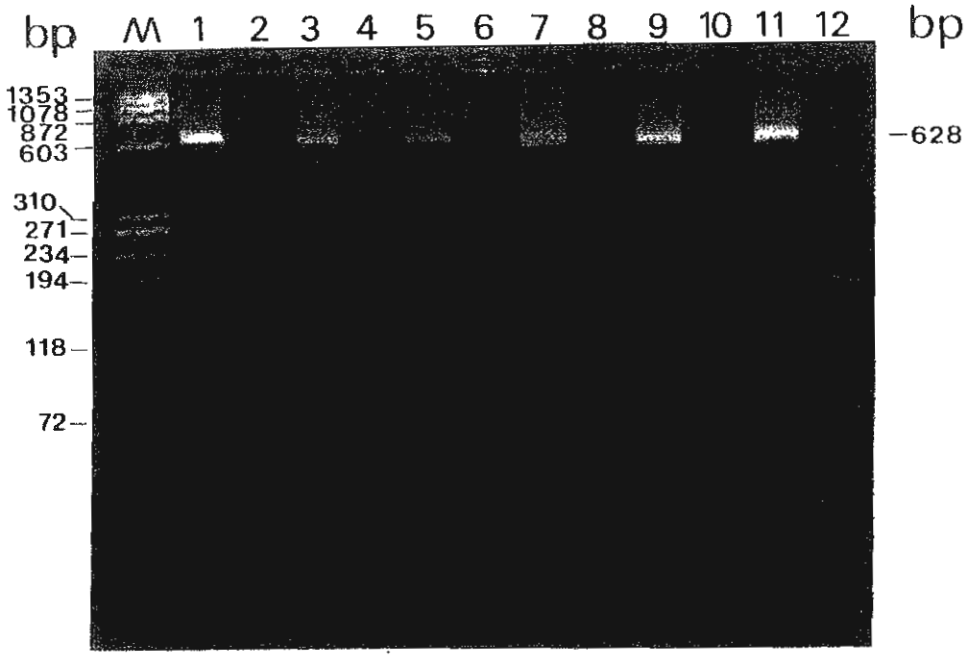

Figure 1. Digestion of region E of the *rpo*B gene with *Hae*III. Even and odd numbers indicate uncut and cut PCR products respectively. Amplified products of 628 bp from *M.tuberculosis* (Lanes1,2), *M.avium* (Lanes 3,4), *M.gordonae* (Lanes 5,6), *M. intracellulare* (Lanes 7,8), *M.kansasii* (Lanes 9,10) *and M.scrofulaceum* (Lanes11,12) generated distinct patterns of different sizes after cutting with *Hae*III.

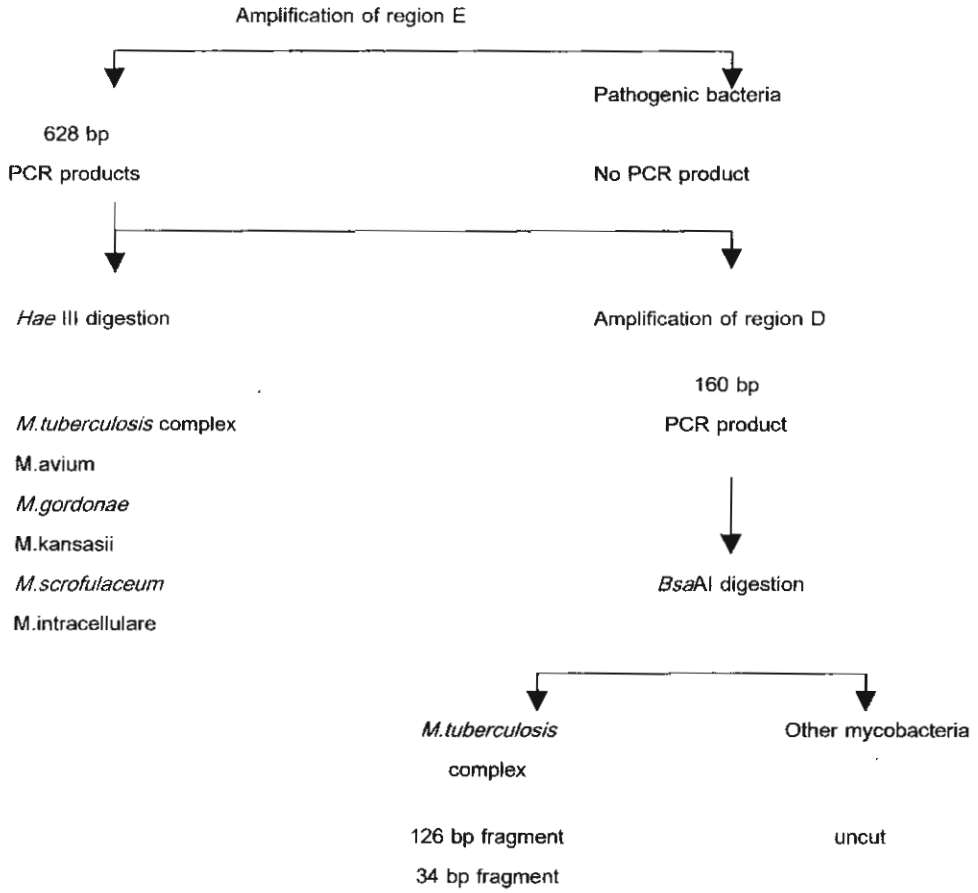

Figure 2. Summary of differentiation of the *rpo*B gene of *M.tuberculosis* complex from other mycobacteria and pathogenic bacteria using a combination of PCR-RFLP pattern of regions E and D.

Table 4 Summary of PCR amplification of 9 *rpoB* gene fragments in bacteria.

	A	B	C	D	E	F	G	H	I
	36-1073	1024-1666	1615-2611	2335-2492	2335-2962	2856-3645	3216-4028	3935-4357	4261-4652
1. *C. diptheriae*	-	-	-	+	-	-	-	+	-
2. *E. coli*	-	-	-	+	-	-	-	+	-
3. *H. influenza*	-	-	-	+	-	-	-	+	-
4. *K. pneumoniae*	-	-	-	+	-	-	-	+	-
5. *N. meningitidis*	-	-	-	+	-	-	-	+	-
6. *Ps. aeruginosa*	-	-	-	+	-	-	-	+	-
7. *Ps. pseudomallei*	-	-	-	+	-	-	-	+	-
8. *S. aureus*	-	-	-	+	-	-	-	+	-
9. *S. epidermidis*	-	-	-	+	-	-	-	+	-
10. *S. pyogenes*	-	-	-	+	-	-	-	+	-
11. *S. viridans*	-	-	-	+	-	-	-	+	-

+ = amplified; - = not amplified

Table 5 Summary of PCR amplification of 9 *rpoB* gene fragments in mycobacteria.

	A	B	C	D	E	F	G	H	I
	36-1073	1024-1666	1615-2611	2335-2492	2335-2962	2856-3645	3216-4028	3935-4357	4261-4652
1. *M. tuberculosis*	+	+	+	+	+	+	+	+	+
2. *M. africanum*	+	+	+	+	+	+	+	+	+
3. *M. bovis* BCG	+	+	+	+	+	+	+	+	+
4. *M. microti*	-	+	+	+	+	+	-	+	+
5. *M. avium*	-	-	+	+	+	-	+	+	+
6. *M. chelonae*	-	-	-	+	-	-	-	+	-
7 *M. duvalii*	-	-	ND	+	ND	-	-	+	ND
8 *M. gordonae*	-	-	-	+	+	-	+	+	+
9. *M. kansasii*	-	-	-	+	+	-	+	+	-
10. *M. phlei*	-	-	ND	+	ND	-	-	+	ND
11. *M. scrofulaceum*	-	-	+	+	+	-	-	+	-
12. *M. smegmatis*	-	-	ND	+	ND	-	-	+	ND
13. *M. vaccae*	-	-	ND	+	ND	-	-	+	ND
14. *M. intracellulare*	-	-	+	+	+	-	+	+	+

+ = amplified; - = not amplified; ND = not determined

เนื้อหางานวิจัย

CATALASE-GENETICALLY MODIFIED *LACTOBACILLUS PLANTARUM* AS A STARTER CULTURE IN FERMENTED MEAT PRODUCT

ABSTRACT

The property of catalase is very rare in lactobacilli but desirable for starter cultures as it prevents food product defects caused by hydrogen peroxide. *Lactobacillus sake* SR911 is a local strain which produces a heme dependent catalase. The catalase gene of *Lactobacillus sake* SR911 was amplified by polymerase chain reaction and cloned in pMEx8 vector and introduced into E.coli UM2. The catalase gene was subcloned into *E.coli-lactococcus* shuttle vector pGKV210 and introduced into *Lactobacillus plantarum* a catalase negative strain isolated from Thai fermented sausage(NHAM). The transformed lactobacilli were shown to decompose hydrogen peroxide and the molecular size of the recombinant protein was 65,000 Da on SDS-PAGE. The DNA sequence contains 1637 bp encoding 478 amino acids. The level of expression of catalase gene was increased when expressed under strong lactococcus promoter p59 in vector pIL1020. The transformed strain showed stable catalase activity and the recombinant plasmid was retained in more than 95% of the cell after 20 generations. The stability of recombinant plasmid was also investigated when using as starter culture in the process of meat fermentation. This catalase-genetically modified strain may prove to be useful as a starter culture in fermented meat product.

INTRODUCTION

Lactobacilli are important microorganisms and are widely used in food and agricultural industries. Their main function is to produce acid which lowers the pH thus prevents the growth of spoilage bacteria in the fermented product. They are also involved in the development of flavor and texture in the final product (Mckay et.al., 1990). Their significant roles in food conversion and preservation have provided the opportunity to develop new strains with specialized role. In the fermentation process the presence of oxygen may occur and lactic acid bacteria may produce hydrogen peroxides as their common metabolites (Whittenbury et.al., 1964). Hydrogen peroxide is a very strong oxidizing agent and if accumulated may affect the sensory quality of the product as a result of rancidity or color defects (Niven et.al., 1957). Lactobacilli are commonly considered as

lacking of catalase until recently whereby many studies have shown that lactic acid bacteria contain at least 2 groups of catalase (Wolf et.al., 1991; Engesser et.al., 1994). The two groups of catalase include true catalase or heme catalase and the non-heme catalase or pseudocatalase or manganese catalase. The first group of enzyme can be detected in many species of lactic acid bacteria if heme or hematin is present with different activities. In the second group, the enzyme is active without addition of heme and is found in very few species (Engesser et.al., 1994). The *katA* gene encoding the true catalase from *L. sake* LTH677 and *L. sake* SR911 has been cloned and characterized (Knauf et.al., 1992; Noonpakdee et.al., 1996). Recently the pseudocatalase gene from *Lactcoccus plantarum* has been also cloned and characterized (Igarashi et. al., 1996).

Lactobacillus sp. TISTR891 and *Lactobacillus plantarum* TISTR850 are isolated from local fermented meat products(NHAM) and are catalase negative. *Lactobacillus sake* SR911 on the contrary contains high heme dependent catalase. Since catalase is one of the desired properties for starter cultures used in food fermentation as it can prevent flavor and color defects in the products due to the effect of hydrogen peroxide (Lucke et.al., 1985), the transfer of the catalase gene into these lactobacilli can give them the ability to produce catalase and simplify starter preparations (Hammes et.al., 1994). Moreover the catalase gene may be used as food grade marker since detection of oxygen producing colonies is very simple. In this study, we report the cloning and transferring of the catalase gene from *L. sake* SR911 into selected lactic acid bacteria that lack this enzyme. The work presented in this report demonstrates a successful cloning and expression of the catalase gene into *Lactobacillus sp.* TISTR891 and *L. plantarum* TISTR 850 using an *E.coli-lactococcal* shuttle vector pGKV210 and expression vector pIL1020. The amount of catalase activity obtained from these strains were compared. Study on the properties of transformed *L.plantarum* TISTR 850 as starter culture in food was also investigated.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

Media and Growth Conditions

The *E. coli* and lactobacilli strains were grown in LB medium (Davies et.al., 1980) and MRS medium (DeMan et.al., 1960) as previously described (Tanasupawat *et.* al., 1993). To detect catalase activity in lactobacilli, cells were grown in MRS media in the presence of hematin at a final concentration of 30 μM as previously described (Wolf et.al.,

1991). Selective media containing ampicillin at 100 µg/ml or erythromycin at 200 µg/ml were used for selection of *E. coli* and 20 µg /ml erythromycin was used for selection of lactobacilli.

DNA Preparation

Chromosomal DNA from *L. sake* was isolated according to the method previously described (Anderson et.al., 1983). Proteinase K was used at the final concentration of 20 µg/ml before extraction with phenol/chloroform/isoamyl alcohol. Plasmid DNA of *E. coli* was isolated by standard alkaline lysis method (Sambrook et.al., 1989). Plasmid DNA of lactobacilli were isolated according to the method described by Anderson and McKay (Anderson et.al., 1983).

Enzymes

DNA-modifying enzymes supplied with appropriate buffers were obtained from Boehringer Mannheim. *Hind* III cut λ DNA was used as molecular weight marker.

Cloning Procedure

The catalase gene including its promoter region was amplified from chromosomal DNA of *L. sake* SR911 by polymerase chain reaction. PCR amplification was carried out in 50 µl mixture containing 5 µl of 10x PCR buffer (10 mM KCl, 10 mM ammonium sulfate, 20 mM Tris-HCl (pH 8.8 at 25 °C), 2 mM Mg_2SO_4 and 0.1% Tritonx-100), 1 unit of Vent^R DNA Polymerase (New England Biolabs, Beverly, Massachusetts, U.S.A) and 200 µM each of the deoxynucleoside triphosphate. The amplification was performed on a DNA thermal cycler (Perkins Elmer Cetus Model TCI) using the following programme; denaturation (1 min at 90 °c) ; annealing (1 min at 55° C) and extension (2 min at 72° C) for 29 cycles. The final cycle consisted of the following steps, denaturation (1 min at 90 ° C), annealing (1 min at 55° C) and extension (5 min at 72 °C). PCR primers were designed from –35 region of the gene encoding catalase of *L. sake* LTH677 as follow

oligo1, 5'primer 5' CGC GGA TCC GAG CAA GCT CTT CAT TGACG 3'

- 3 5

oligo2, 3'primer 5' AAA ACT GCA GAG ATA ACG AAT AAA AAA TAG GTG 3'

The amplified product was gel purified and ligated into pUC19 which had been digested with *Sma* I. The resulting recombinant plasmid referred as pUCK was subcloned

into the *E.coli-lactococcus* shuttle vector pGKV210 yielding pGKV8E. The amplified product was also ligated to pIL1020 vector which had been digested with *Eco* RV to yield the plasmid pILK with two possible orientations of the catalase gene under the control of p59 lactococcal promoter.

Transformation of *E. coli* UM2 and lactobacilli.

Competent *E. coli* cells prepared by $CaCl_2$ method (Sambrook et.al., 1989) were transformed and transformants were screened by plating on LB medium with 100 µg/ml of ampicillin or 200µg/ml erythromycin. All antibiotic resistant transformants were further analyzed for expression of the catalase. The presence of plasmids with the catalase gene casette were analysed by restriction digestion. Transformation of *Lactobacilus sp.* TISTR891 and *L. plantarum* TISTR850 were carried out by electroporation according to the protocol previously described using Biorad Gene Pulser in a 0.2 cm cuvette with the field strength of 1.25 kV/cm (Bringell et.al., 1990; Thompson et.al.,1996).

Preparation of cell-free extracts from lactobacilli

To determine the catalase activity in crude cell extracts, aliquots of overnight culture (30ml) were harvested by centrifugation at $4^{\circ}C$ and washed with ice-cold phosphate buffer (50mM, pH 7). The washed cells were suspended in the same buffer with 0.5 mM phenylmethylsulfonyl fluoride and sonicated for three 1-min periods in an ice bath. After centrifugation, the crude cell extracts were assayed immediately. The protein concentrations were determined by Bio-Rad protein assay.

Catalase activity test

Catalase positive clones were detected by flooding colonies on agar plate with hydrogen peroxide solution (0.87 M) and the bubble producing colonies were selected for further analysis. The quantitative assay for the presence of catalase in bacterial cells was performed according to the method previously described (Sinha, 1972; Noonpakdee et.al., 1996). Total cellular catalase activity was expressed as µmole of hydrogen peroxide decomposed per minute per $3x10^8$ CFU. The specific activity of catalase in crude cell extract was expressed as µmole of hydrogen peroxide decomposed per minute per mg protein.

Protein pattern analysis

Total soluble proteins from *E. coli* UM2 and lactobacilli were extracted and resolved by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Standard protein markers from Bio-Rad were used as molecular weight markers.

In-situ activity staining of the catalase.

Total soluble proteins were isolated as described above and separated on a 7.5 % nondenaturing polyacrylamide gel. The gel was stained using ferricyanide according to the method previously described (Wayne et.al., 1986).

DNA sequencing

DNA sequencing was achieved by using automated sequencing analyzer (Perkin Elmer ABI No377) with ABI Prism Dye Terminater Cycle Sequencing Ready kit. Reactions were performed as described by the instructions of the manufacturer. Subclones were constructed in pUC19, and the insert was sequenced from both sides by using universal primer and or reverse oligonucleotides primer. DNA sequence and amino acid sequence were analyzed with PCgene software Release Version 6.8.

Preparation of fermented meat product(NHAM)

To prepare fermented meat product(NHAM) , Culture of *L. plantarum* TISTR 850 or L. plantarum TISTR 850 harboring the recombinant plasmid was inoculuated into mixture of ground pork, minced skin pork, rice and garlic at concentration of 10^8 CFU/g , then incubated at 30 C for 36-48 h. As a control, no culture was inocuated.

Lipid extraction method

Lipid extraction of fermented meat product was extracted according to Bligh-Dyer method (Bligh and Dyer,1959).

Iodine number value determination

Iodine number of the fermented meat product was determined according to Wijs method (AOAC:official method,1995)

RESULTS

Cloning of the catalase gene from *L. sake* SR911 in pGKV210 and pIL1020

The catalase gene of *L. sake* SR911 including its promoter region was amplified using the primers derived from the nucleotide sequence of *L. sake* LTH677 (Knauf et.al., 1992). The catalase gene from *L. sake* SR911 was cloned as a 1.6 kb amplified fragment into *Sma* I cut pUC19. The resulting plasmid was designated pUCK 233 and then

introduced into *E. coli* UM2 which is catalase negative as a result of mutation of *KatG and KatE*. Three transformants were selected on LB agar plate containing ampicillin and expression of catalase was revealed by flooding the colonies with hydrogen peroxide. All transformants were found to contain a 1.6 kb size of catalase gene in plasmid DNA and exhibited the same level of catalase activity as measured by quantitative catalase assay. One of the recombinant plasmid was subcloned into *E.coli-lactococcus* shuttle vector pGKV210 resulting in plasmid pGKV8E and later introduced into *Lactobacillus sp.* TISTR891 by electroporation. The catalase activity produced by transformed lactobacilli must be due to expression of the catalase gene under the control of its own promoter since *Lactobacillus sp.* TISTR891 is catalase negative. Plasmid pGKV210 is a general cloning vector which contained the origin of replication pWV01. All transformants grown in erythromycin plate were found to be catalase positive. In order to increase the catalase activity, we also cloned this amplified 1.6 kb DNA fragment into *EcoR* V cut pIL1020, an expression vector which contained the same origin of replication of pWV01 and the strong lactococal promoter p59 (Van der Vossen et.al., 1985). The ligation mixture was used to transform *E. coli* UM2 as intermediate host. More than 300 erythromycin resistant transformants were screened for catalase activity. Three clones were found to be catalase positive. After restriction analysis of the purified plasmid DNA, we found that these three recombinant plasmids contained the same size 1.6 kb insert in either of the two possible orientations. Plasmid from one transformant designated pILK01 had the catalase gene cloned downstream from the p59 promoter. Two recombinant plasmid designated pILK02 and pILK03 had the catalase gene cloned in the opposite direction from p59 promoter (figure1). All 3 recombinant plasmids were then used to transform *Lactobacillus sp.* TISTR891 and pILK01 was also used to transform *Lactobacillus plantarum* TISTR850 by electroporation.

Expression of the catalase gene of *Lactobacillus sake* SR911 from pGKV8E, pILK01, pILK02 and pILK03 in *E. coli* UM2

All transformed *E. coli* UM2 harboring plasmid pGKV8E, pILK01, pILK02, and pILK03 exhibited catalase activities by quantitative assay as compared to control *E. coli* UM2 host strain and *E. coli* UM2 with shuttle vectors pGKV210, pIL1020. The expression of catalase in transformed *E. coli* UM2 did not depend on exogeneous hematin unlike in *L. sake* SR911 which we had to supply hematin in the growth medium before catalase assay

suggesting that *E. coli* strain can synthesize porphyrin group (heme) unlike most lactic acid bacteria (Whittenbury et.al., 1964).

The protein expressed from the 1.6 kb amplified DNA insert in *E. coli* UM2 using pGKV8E, pILK01, pILK02 and pILK03 were analysed on denaturing 7.5 % SDS polyacrylamide gel (SDS-PAGE) and the result is shown in Figure 2. In *E. coli* UM2 bearing the recombinant plasmids, the protein patterns showed the presence of an additional protein band with the molecular size of approximately 62,000 Da (lane 3, 5, 6 and 7). This protein band was not present in *E. coli* UM2 with vector pGKV210 or expression vector pIL1020 (lane 2, 4). The protein of the same molecular size was also observed in the protein pattern of wild type catalase strain *L. sake* SR911 (lane 1). This apparent recombinant protein is most likely to be encoded by the gene carried on the plasmids pGKV8E, pILK01, pILK02 and pILK03 since *E. coli* UM2 host is catalase negative. The intensity of protein band from transformed *E. coli* harboring pILK01 (lane 5) is approximately 2 fold (as determined by scanning with a densitometer) as compared with that of transformed *E. coli* cells harboring pGKV8E, pILK02 and pILK03 (lane 3, 6, 7).

Expression of the catalase gene of *L. sake* SR911 from pGKV8E, pILK01, pILK02 and pILK03 in *Lactobacillus sp.* TISTR891 and *Lactobacillus plantarum* TISTR850

The 1.6 kb insert of the catalase gene in the recombinant plasmid pGKV8E, pILK02 and pILK03 were expressed under its own promoter. The pILK01 however, had the catalase gene expressed under the transcriptional control of the lactococcal promoter p59. The expression of these different constructs was evaluated in the transformed lactobacilli by quantitative catalase assay and by in-situ activity staining in polyacrylamide gel electrophoresis. The data in table 2 showed that all transformed lactobacilli with recombinant plasmids exhibited heme dependent catalase activity. However, *Lactobacillus sp.* TISTR891 and *L. plantarum* TISTR850 host strains with and without the plasmids showed no activity at al. The specific catalase activity from transformed *Lactobacillus sp.* TISTR891 harboring pILK01 was approximately 2 times higher than those from *Lactobacillus sp.* TISTR891 harboring pGKV8E, pILK02 and pILK03. Therefore, the expression of the catalase gene under the trascriptional control of lactococcus promoter p59 was markedly increased compared to the expression under the control of its own promoter. Plasmid pILK01 was then used to transform another catalase negative strain, *Lactobacillus plantarum* TISTR850. The specific catalase activity of transformed *L. plantarum* harboring pILK01 increased almost 3 times higher than that of *Lactobacillus sp.*

TISTR891 harbouring plasmid pILK01 and about 6 times higher than that of *Lactobacillus sp.* TISTR891 harbouring pILK02 and pILK03. The catalase activity in transformed *L. plantarum* TISTR850 was about 3 times higher than that of wild type *L. sake* SR911.

The result of catalase in-situ activity staining on a non-denaturing polyacrylamide gel was shown in Figure 3. The pattern of catalase protein bands from transformed lactobacilli with recombinant plasmids (lane 3, 5, 6, 8), was similar to that of wild type *L. sake* SR911 (lane 1). The intensity of the recombinant protein from transformed *Lactobacillus sp.* TISTR891 harboring pILK01 was higher than that of transformed lactobacilli cell harbouring pGKV8E or pILK02 in the same strain. The negative control harbouring only the cloning vector pGKV210 or pIL1020 showed no band with catalase activity (lane 2, 4, 7). Transformed *L. plantarum* TISTR850 harboring pILK01 (lane 8) showed the same pattern and intensity of catalase bands as compared to wild type and transformed *Lactobacillus sp.* TISTR891 with pILK01 (lane 1, 5). The amount of protein from transformed *L. plantarum* (lane 8) is only one third in amount as compared to the rest. Hence, the activity of transformed *L. plantarum* by in-situ activity gel assay correlates with the result obtained by quantitative catalase assay from Table 2.

Nucleotide sequence of the catalase gene

Figure 4. shows the nucleotide sequence of the 1.6 kb insert in pKP01 which encodes the catalase of *L. sake* SR911. The sequence contained 1436 bp from the structural gene of *L. sake* SR911 encoding 478 amino acids and the 17 amino acids from pMEx8 vector. The additional amino acids from cloning vector did not affect the expression of the gene. The base composition of the catalase gene has 55.41 % AT content. The nucleotide sequence shows 93.75 % homology to that of the catalase gene of *L. sake* LTH 677 (data not shown). The difference of DNA and amino acid residues between 2 strains are also indicated.

Characteristics of recombinant strain *L. plantarum* TISTR 850 in culture

Recombinant strain *L. plantarum* TISTR 850 was grown under aerobic and anaerobic conditions, and the catalase activities of intact cells and crude extracts were determined quantitatively. Catalase activity is at the highest level at stationary phase when grown under aerobic condition(data not shown). As shown in Table 3, there was no difference in the catalase activity of the transformed cells in anaerobic and aerobic

condition. The stability of the plasmid pILK01 in *L. plantarum* was stable and the expression of catalase was retained after 20 generations.

L. plantarum TISTR 850 in fermented meat product

The stability of plasmid under condition of meat fermentation (ie. No shaking, no adding antibiotics) was investigated. The plasmid was retained in more than 70% of the cells after 2 days of fermentation. The physical properties of fermented products using *L. plantarum* as a starter culture were similar as compared to control (ie pH, color, texture) (figure 5)

Iodine number in fermented meat product

Rancidity of the fermented meat product was determined by measuring iodine number from lipid in the products made from L.plantarum and recombinant L.plantarum. The result in Table 4 showed no difference in iodine value in lipid extracted from fermented meat product kept at 4 C for one month or at room temperature for 2, 5 and 8 days.

DISCUSSION

Catalase activity in lactobacilli is a very rare property. It can prevent flavor and food color defects in fermented foods. Certain number of strains isolated from Thai fermented products were found to exhibit catalase activity if exogeneous hematin was added. *Lactobacillus sake* SR911 is one of the strains that exhibits strong catalase activity and was selected for further manipulation. The main reason to transfer this gene to other catalase negative lactic acid bacteria is to improve desired characteristic of starter organisms in food fermentation to yield a more desirable product. In this study, we have successfully cloned and expressed the catalase gene of *L.sake* SR911 in *E.coli* UM2. This gene was then transferred to *Lactobacillus sp.* TISTR891 and *L. plantarum* TISTR850 which are naturally deficient in catalase.

From previous report (Noonpakdee et.al., 1996), we expressed the structural catalase gene of *L. sake* SR911 under the control of the *tac* promoter in *E. coli* UM2. However, following the subcloning of this gene into a *E. coli-lactococcus* shuttle vector pGKV210 and transforming the lactobacilli, no expression could be detected. This means that the transcription and translation signals from the strong *E. coli* promoter was probably not recognized by the lactic acid bacteria even though the so-called –35 (TTGACA) and –10 (TATAAT) boxes of

E. coli promoter resembles that of lactobacilli. Similar phenomenon was also described by Roy *et al.* They failed to express the manganese superoxide dismutase gene of *E coli* in lactobacilli

(Roy et.al., 1993)

The 1.6 kb PCR amplified catalase gene which includes the promoter region was cloned into the vector pGKV210, and pIL1020 and expressed in both *E. coli* UM2 and lactobacilli. Both shuttle vectors pGKV210 and pIL1020 contain the same origin of replication of pWV01 derived from the cryptic plasmid of *Lactococcus lactis* subsp. *cremoris* plasmid. Plasmid pGKV210 is commonly used as a promoter screening vector but pIL1020 is an expression vector with a strong lactococcal promoter p59. The level of expression of the catalase gene under the control of its own promoter in recombinant plasmids pGKV8E and pILK02 is similar. The expression under the strong promoter p59 in recombinant plasmid pILK01, however, is approximately 2 fold higher. This is indicated by the catalase activity in transformed lactobacilli (Table 2) and by the intensity of the protein band in the region of 62,000 Da isolated from recombinant *E. coli*. The increase in the expression of catalase protein should be due to the increase in the rate of mRNA synthesis from under the strong p59 promoter or under its own promoter since transcriptional signal of p59 does not contain a Shine Dalgarno sequence (Vossen et.al., 1987). Brurberg *et al.* also observed higher chitinase activity under p59 control using pGKV259 to express a chitinase gene from *Serratia marcescens* in *Lactococcus lactis* and *Lactobacillus plantarum* (Brurberg et.al., 1994).

Interestingly, the catalase activity expressed under the control of p59 in transformed *L. plantarum* TISTR850 is increased approximately 3 fold as compared to that of *Lactococcus sp* TISTR891 and the natural host *L. sake* SR911. This explains the fact that the expression level of catalase gene under p59 in pILK01 varies among different lactobacilli species or strains.

The subunit size of the *L. sake* SR911 catalase as previously reported (Noonpakdee et.al., 1996) and in this study is approximately 62,000 Da which corresponds to the sizes of catalase isolated from animals, plants and microorganisms (Nadler et.al., 1986; Ossowski et.al., 1991). The catalase *in situ* activity staining in non-denaturing polyacrylamide gel shows 2 bands of proteins. A prediction as to the quaternary structure of the catalase from lactic acid bacteria may indicated that it is a hexameric and similar to catalase-1 from *B. subtilis* (Loewen et.al., 1987) and catalase HPII from *E. coli* (Ossowski et.al., 1991). However, the conditions in the gel may cause a partial breakdown of the

catalase fusion protein product to a trimeric form which may be the smallest active form of the enzyme. This phenomenon was also observed by Knauf *et al.*

It has been shown in this study that the catalase gene from *L. sake* can be transferred to negative catalase strains such as *Lactobacillus sp.* TISTR891 and *L. plantarum* TISTR850 to express catalase with the elevated amount of activity compared to the wild type. The manipulated strains may be useful in fermentation process.

L .plantarum harboring pILK01 has been studied in culture. Catalase activity is at the highest level at early stationary phase. There is no difference of the catalase activity when growing cells under aerobic or anaerobic condition. This may be because p59 promoter is not regulated by oxygen. The recombinant plasmid in L. plantarum is stable when used as a starter culture in meat fermentation. Iodine number of the fermented meat products from recombinant *L.plantarum* shows no differennce to that of control.

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Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant properties	Source/reference
Bacterial strains		
E. coli UM2	*katE2 katG15*	12
Lactobacillus sake SR911	wild type, heme catalase	11
Lactobacillus sake LTH677	wild type, heme catalase	6
Lactobacillus sp. TISTR891	catalase negative	11
Lactobacillus plantarum TISTR850	catalase negative	11
Plasmids		
pUC19	Ap^r, lacZ, 2.7kb	13
pUCK	Ap^r, lacZ, 3.3kb,pUC19 carrying the catalase gene *KatA*	This study
pGKV210	Em^r, Cm^r, 4.5 kb, pW01 replicon	14
pIL1020	Em^r, Cm^r, 5.1 kb, pW01 replicon	INRA
pGKV8E	Em^r, Cm^r, 6.1 kb, pGKV210 carrying the catalase gene *Kat* A	This study
pILK01	Em^r, Cm^r, 6.7 kb,pIL1020 carrying the catalase gene *KatA*	This study
pILK02	Em^r, Cm^r, 6.7 kb,pIL1020 carrying the catalase gene in opposite direction	This study
pILK03	same as pILK02	This study

Abbreviations: TISTR, Thailand Institute of Scientific and Technological Research; INRA, Institut National de la Researche Agronomique Research Center, Jouy-en-Josas, France; Em^r ,erythromycin resistance; Cm^r, chloramphenical resistance; Ap^r, ampicillin resistance

Table 2. Catalase activity expressed by transformed lactobacilli harboring the gene encoding the catalase of *L. sake* SR911

Strains	Catalase activities μmoles of H_2O_2 decompose x min^{-1} x mg of $protein^{-1}$
L.sake SR 911(wild type)	154
Lactobacillus sp. TISTR891	0
Lactobacillus sp.(pGKV210)	0
Lactobacillus sp.(pGKV8E)	67
Lactobacillus sp.(pIL1020)	0
Lactobacillus sp.(pILK01)	167
Lactobacillus sp. (pILK02)	74
Lactobacillus sp.(pILK03)	82
L. plantarum TISTR850	0
L. plantarum (pILK01)	492

[a] Data represent average number of 3 experiments, each performed in duplicate.

Table 3. Catalase activity of *L.plantarum* and *L.plantarum* harbouring pILK01 under aerobic and anaerobic condition

Strain	Growth condition	Catalase activities (μmoles H2O2 decomposed/min.)	
		$/OD_{600}$	/mg protein
L.plantarum	aerobic	0	0
	anaerobic	0	0
L.plantarum	aerobic	70.3	233
(pILK01)	anaerobic	87.2	223

Table 4. Iodine number of extracted lipid from Nham prepared from *L.plantarum* TISTR850 *L.plantarum* TISTR850 harboring pILK01

Starter	Iodine number					
		at room temperature			at 4 °C	
	0 day	2 days	5 days	8 days	1 month	2 months
Control	81.51	-	-	-	87.98	-
L.plantarum TISTR850	83.17±3.38 (n=10)	85.70±4.43 (n=2)	83.47±1.87 (n=3)	81.97±2.54 (n=3)	86.16±2.46 (n=5)	-
L.plantarum TISTR850 harboring pILK01	84.30±3.41 (n=9)	87.94±0.23 (n=3)	82.69±5.39 (n=4)	78.49±1.57 (n=4)	83.94±1.97 (n=7)	-

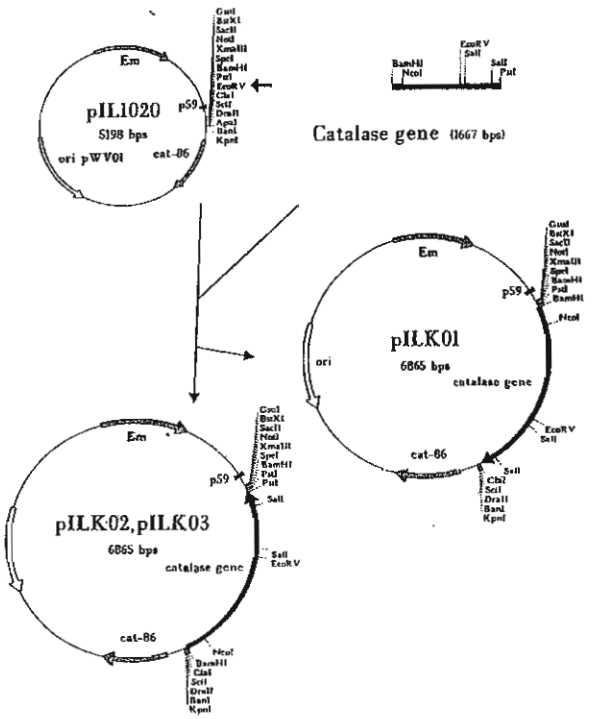

Figure 1. Schematic representation of the construction of a plasmid pILK01, pILK02, and pILK03 using *E. coli-lactococcus* expression vector pIL1020. The linear fragment represents the fragment of catalase gene including its promoter region. P59 is lactococcal promoter region. Arrows marked ori denote DNA regions required for replication and the transcriptional direction of the major open reading frames in the region. EmR and CmRindicate resistance to erythromycin and chloramphenical respectively.

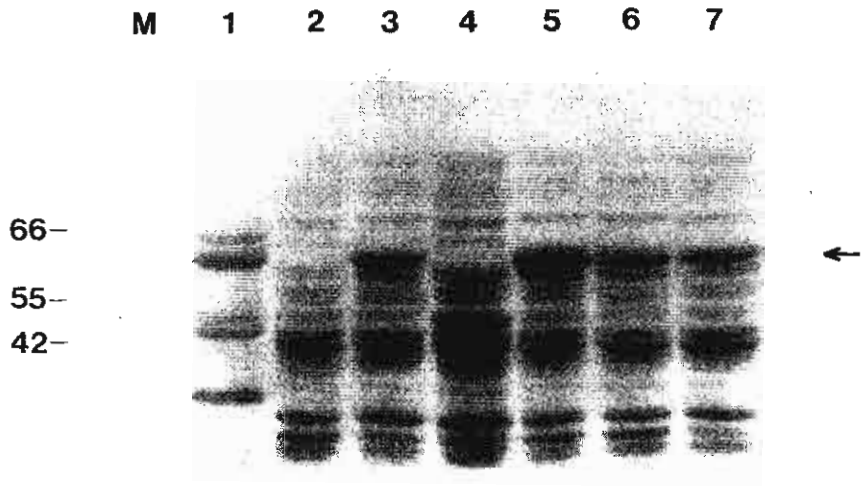

Figure 2. SDS-PAGE of total soluble proteins of transformed *E. coli* UM2 harboring the gene encoding the catalase gene of *L. sake* SR911. Lanes: M, molecular weight marker: 1, *L. sake* SR911; 2, *E. coli* UM2(pGKV210); 3, *E. coli* UM2 (pGKV8E); 4, *E. coli* UM2 (pIL1020); 5, *E. coli* UM2 (pILK01); 6, *E. coli* UM2 (pILK02); 7, *E. coli* UM2 (pILK03) .

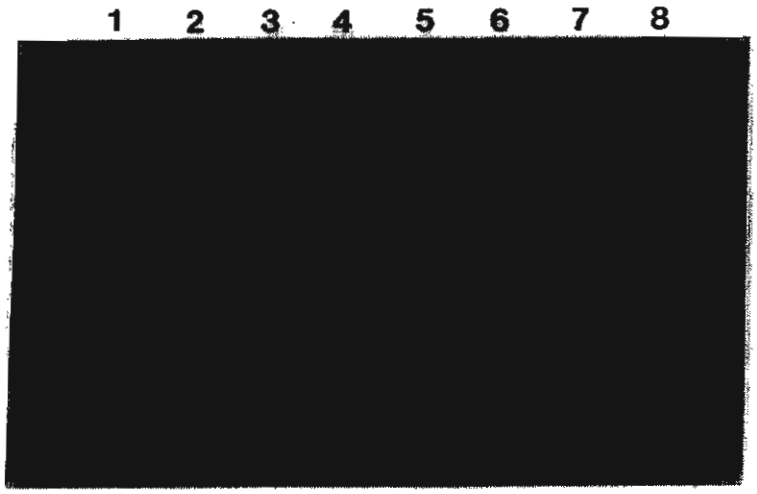

Figure 3. Catalase activity staining in PAGE of soluble proteins of strains harboring the gene encoding the catalase gene of *L. sake* SR911. Lanes: 1, *L. sake* SR911; 2, *Lactobacillus sp.*TISTR891(pGKV210); 3, *Lactobacillus sp.* TISTR891(pGKV8E); 4, *Lactobacillus sp.*TISTR891(pIL1020); 5, *Lactobacillus sp.*TISTR891(pILK01); 6, *Lactobacillus sp.*TISTR891 (pILK02); 7, *L. plantarum* TISTR850(pIL1020); 8, *L. plantarum* TISTR850(pILK01).

-35 -10 *

1 **CGC GGA TCC GAG CAA GCT CTT CAT TGA** CGG TTG CCT GTT GAA GGT CCT ATA GTG ACT AGG TAA GTT 66

67 GCG CAC AAT TAT TTT CTA GTG TGT GTT CCT AGT TTT TAA ACT AAT CTT AGG AGG TCA AAT ATT ATG 132
M

133 ACA AAT CAA CTA ACG ACT AAC GAA GGG CAA CCA TGG GCG GAC AAT CAA CAT TCA CAA ACT GCC GGC 198
T N Q L T T N E G Q P W A D N Q H S Q T A G

199 CAA CGC GGT CCC GTC TTA ATC CAA GAT TAT CAA TTA CTC GAA AAA CTC GCC CAT TTT AAC CGC GAA 264
Q R G P V L I Q D Y Q L L E K L A H F N R E

265 CGC ATT CCT GAA CGA GTG GTT CAC GCC AAA GGT GCT GGC GCT AAA GGT TAT TTC AAG GTC ACA AAA 330
R I P E R V V H A K G A G A K G Y F K V T K

331 GAC ATG AGC GCC TAC ACC AAA GCT GCT GTC TTC AGT GGC GTC GGC AAA AAA ACA CCG CTT ATC ACT 396
D M S A Y T K A A V F S G V G K K T P L I T

397 CGT TTT TCT CAA GTC GCT GGT GAA GCT GGC TAT CCC GAT ACA TAC CGC GAT GTT CGT GGT TTC GCC 462
R F S Q V A G E A G Y P D T Y R D V R G F A

463 GTT AAA TTC TAC ACG GAA GAA GGC AAT TAC GAT ATT GTT GGC AAT AAT ACG CCG GTC TTC TTC GTC 528
V K F Y T E E G N Y D I V G N N T P V F F V

529 AAT GAT CCA CTA AAA TTC CCC GAT TTC ATT CAT TCA CAA AAA CGT GAT CCT CGG ACA CAT GCC CGT 594
N D P L K F P D F I H S Q K R D P R T H A R

595 AGC CAA GAT ATG CAA TGG GAT TTC TGG TCC CTT TCA CCC GAA TCT GTC CAT CAA GTT ACG ATT CTC 660
S Q D M Q W D F W S L S P E S V H Q V T I L

661 ATG AGT GAC CGC GGG ATT CCT GCC AGT TAC CGA ATG ATG CAC GGC TTT GGT AGC CAC ACC TTC AAA 726
M S D R G I P A S Y R M M H G F G S H T F K

727 TGG GTC AAC GCA CAA GGT GAA CAA TTC TGG GTT AAA TAT CAT TTC AAA ACG AAC CAA GGC GTT CAT 792
W V N A Q G E Q F W V K Y H F K T N Q G V H

793 AAT CTC AGC AAC GAA CTC GCC GAT GAA CTC GCT GGT AAG GAT ACT GAT TAC CTT CAA AAC GAT TTA 858
N L S N E L A D E L A G K D T D Y L Q N D L

859 TTC GAC GCA ATT GAA ACG GGC GAT TAT CCC AGT TGG ACT GTC GCC GTC CAA CTC GTC CCT TAT GAA 924
F D A I E T G D Y P S W T V A V Q L V P Y E

925 GAT GGT TTG AAT TAT CCC CAA GAT ATT TTT GAT GTT ACC AAG GTT ATT TCA CAA AAG GAT TAT CCA 990
D G L N Y P Q D I F D V T K V I S Q K D Y P

991 TTA ATC GAA ATC GGT CAA ATG GTC CTC GAT GAA AAT CCA ACG AAT AAC TTC GAA GAT ATC GAA GAA 1056
L I E I G Q M V L D E N P T N N F E D I E E

1057 TTG GCC TTC TCA CCA GCT AAC TTA GTC CCT GGA ATT GAA GCA TCA CCG GAC AAA TTA CTC CAA GGT 1122
L A F S P A N L V P G I E A S P D K L L Q G

1123 CGA CTA TTT GGT TAT AAG GAT GCG GAA CGT TAC CGA CTT GGT GCC AAC TAC GAA CAA CTG CCA ATC 1188
R L F G Y K D A E R Y R L G A N Y E Q L P I

1189 AAC CGA CCA AAA GTC CCT GTT CAT AAT TAC GAA CGT GAC GGT GTC ATG GCC CAA AAC CAA GAA ACG 1254
N R P K V P V H N Y E R D G V M A Q N Q E T

1255 GGC GTT AAC TAC GAA CCA AAT AGT CAA GAT GGA CCC ACT GAA GTC CCA GCA GCT AAG ATT CAT AGT 1320
G V N Y E P N S Q D G P T E V P A A K I H S

1321 GAT CAA CTC TCT GGT ACA ACT GGC AAC TTC TCT ACC GAT CCC GAT TAT TAC TCA GCA GCT GGC AAA 1386
D Q L S G T T G N F S T D P D Y Y S A A G K

1387 CTT TAT CGG CTA CTA TCC GCT GAT GAA CAA ACC CGT TTA ATC GAA AAT ATT CGC ATG AAT CTT GGT 1452
L Y R L L S A D E Q T R L I E N I R M N L G

1453 CAA GTA ACT AAA CCA GAA ATT CAA ATT CGC GAA GTT AAA CAA TTT TAC CAA GCT GAT CCA GAA TAC 1518
Q V T K P E I Q I R E V K Q F Y Q A D P E Y

1519 GGT CGA CGC GCC GCA ACC GCA TTA AAT TTA GAT TTA GCA CAG TTT GAA TAA CGC CGA GCA CAA AAA 1584
G R R A A T A L N L D L A Q F E

1585 AAT AGG TGG TCC CAT TGA GGA CCA CCT ATT TTT TAT TCG TTA TCT TTA TTA TC 1637

Figure 4. Nucleotide sequence of the catalase gene and deduced amino acid.

เนื้อหางานวิจัย

TRANSFORMATION OF THAI PAPAYA CULTIVAR BY PARTICLE GUN BOMBARDMENT

ABSTRACT

Embryogenic calli and somatic embryos of Thai papaya cultivar Khakdum were transformed with binary vector containing kanamycin as a selectable maker. The tissues were bombarded by M10 tungsten and 1.0 μm gold particles at helium pressure 1300 psi. After bombardment transgenic calli were selected on the media containing 100 mg/l kanamycin. One transgenic callus was obtained from 810 zygotic embryos of embryogenic calli bombarded with M10 tungsten particles (0.12%) and non transgenic callus was obtained from 1590 embryogenic calli bombarded with and 1.0 μm gold particles. Somatic embryos obtained from the culture of papapa embryos grew on the media containing 10 mg/l 2,4-D for 2-3 months are more susceptible for the transformation than the embryogenic calli. Seven transgenic calli were obtained from 1200 clumps of somatic embryos bombarded with M10 tungsten particles (0.58%) and four transgenic calli from 780 clumps of somatic embryos or about 0.51% were obtained from somatic embryos. Transgenic papaya plants cultivar Khakdum were sucessfully regenerated and transferred to soil.

INTRODUCTION

Papaya (*Carica papaya* L.) is a fruit crop grown in tropical and subtropical countries. In Thailand, papaya is one of the staple food. The green fruits are used as salad and the ripened fruits are consumed as fresh dessert. Papaya trees are commercially grown in plantations and on a small scale in home gardens. Since 1975, the papaya production in Thailand has been limited by papaya ringspot virus (PRSV). The major cultivars of papaya grown in Thailand, Khak Dum and Khak Nuan are very susceptible for this virus. This virus causes seriously damage to papaya plantation. The infected area increases each year and it is now endemic throughout Thailand.

The use of genetic engineering to produce transgenic papaya by *Agrobacterium* and microprojectile bombardment had been reported (Fitch et al, 1990; Fitch et al, 1992, Cabrera-Ponce et al, 1995; Yang et al, 1996, Cheng et al, 1996). In these experiments the transgenic Hawaiian papaya cultivar containing the coat protein gene of PRSV mild strain isolated from Hawaii (HA 5-1) were generated. The transgenic papaya showed resistance to the severe strain of PRSV isolated from Hawaii (HA). However this transgenic plant was not

resistance to PRSV isolated from Thailand and other countries (Tennant *et al*,1994). Further the papaya cultivar. "Sunset" and "Kapoho" used in these experiments are not widely consumed in Thailand. In this research we report the DNA transfer of binary vector into Thai papaya cultivar Khakdum using microprojectile bombardment.

MATERIALS AND METHODS

Plasmid

The binary vector pSA1006 is based on pGV941 (Deblaere *et al.*, 1987) and contains the *nos* promoter- *nptII* gene-3'*nos* as a selectable marker and the CaMV35S promoter-*uidA* gene-3'*nos* from pBI 121 (Jefferson, 1987) as a reporter gene.

Plant material and culture

Immature zygotic embryos were dissected from seeds of immature fruits of papaya var. Khakdum. Zygotic embryos were cultured on the induction medium (M1 medium) which consisted of: half strength MS salts medium (Murashige and Skoog's, 1962) plus 50 mg/l myo-inositol, full strength MS vitamins, 400 mg/l glutamine, 10 mg/l 2,4-D, 6% sucrose, 8 g/l Difco Bactoagar, pH 5.8. After the zygotic embryos were maintained in the dark at 26°C for 3-4 weeks, the embryogenic calli which produced one or more somatic embryos on the apex were developed. These embryogenic calli were used in this transformation experiment. The second type of papaya culture used in this experiment is the somatic embryos. A small clump of somatic embryos was developed on the embryogenic callus when the callus was maintained in the induction medium for two to three months with monthly subcultured. Both types of tissues were transferred to the fresh M1 medium in group of 30 calli per petri dish one week before the bombardment

Plant transformation

Embryogenic calli and somatic embryos were bombarded with M10 tungsten and 1.0 μm gold particles coated with the binary vector using the PDS 1000/helium-driven biolistic device (Bio-Rad, Hercules, Calif.). The gap distance between the rupture disk and the macrocarrier is 1.2 cm and the petri dish was placed at the target level 2 (6.0 cm). The chamber is evacuated to 26 in Hg and the helium gas pressure is set at 1300 psi. After bombardment the embryogenic calli and somatic embryos were cultured in the M1 medium for 2 days and transferred to the callus propagation medium (M1 medium supplemented with 75 mg/l kanamycin and 500 mg/l cefotaxime). After 14 days the tissues were transferred to the selective media which is the callus propagation medium containing 100 mg/l kanamycin.

The tissues were subcultured monthly to fresh selective media until resistant embryogenic calli developed.

Plant regeneration

The resistant embryogenic calli were transferred to glass vessels with plastic lids containing the regeneration medium (MS medium supplemented with 0.2 mg/l BAP, 0.1 mg/l kinetin, 3% sucrose, 8 g/l Difco Bacto agar, 100 mg/l kanamycin, pH5.7). The tissues were cultured under 12 hour photoperiod using cool white Sylvania fluorescent lamps until the embryos regenerated and formed shoots. Shoots with 3-4 leaves and about 1.5 cm tall were sliced and transferred to the rooting medium. Two formulas of rooting media were used in this experiment. The R1 medium is a hormone free medium containing half strength MS salts, half strength MS vitamins, 1.5% sucrose, 8 g/l Difco Bactoagar at pH 5.8. The R2 medium contains full strength MS salts, full strength MS vitamins, 100 mg/l myo-inositol, 1 mg/l indol butyric acid, 3% sucrose, 8 g/l Difco Bactoagar at pH 5.8. Rooting plants were acclimatized by replaced the plastic lids of the culture vessels with a sheet of double layer sterile tissue paper and covered with saran wrap for 2 weeks before the plant transferred to 1:1 mixture of soil and vermiculite.

GUS histochemical staining

The kanamycin resistance callus was analyzed for β-glucuronidase activity by histochemical assay (Jefferson, 1987). About 50 mg of callus was placed in a 1.5 ml microcentrifuge tube and incubated in 100 ml of 1 mM X-gluc in 50 mM phosphate buffer for 1-16 hr at 37°C.

RESULTS AND DISCUSSIOIN

Transformation of Thai papaya cutivar Khakdum

Two type of papaya cultures were used for DNA transformation by particle gun bombardment, the zygotic embryos and the clump of somatic embryos. After bombardment the cultures were transferred to the selectable medium containing 100 mg/l kanamycin and transferred monthly to fresh selective medium until transgenic calli were selected which took about 4-5 months. Transgenic calli were identified as light-yellow somatic embryo clusters among the brown tissues of the non-transformed calli. Zygotic embryos of Thai papaya cultivar Khakdum are not susceptible for particle gun bombardment when compared with somatic embryos. After the tissues were cultured on selective medium for 5 months only one transgenic callus was obtained from 810 zygotic embryos bombarded with M10 tungsten particles (0.12%) and non of the transgenic callus was obtained from 1590 zygotic embryos

bombarded with 1.0 μm gold particles. In the experiment using somatic embryos for bombardment seven transgenic calli were obtained from 1200 clumps of somatic embryos bombarded with M10 tungsten particles (0.58%) and four transgenic calli were obtained from 780 clumps of somatic embryos bombarded with 1.0 μm gold particles (0.51%). The efficiency of transformation of papaya cultivar. Khakdum using somatic embryos is comparable to the previously reported in particle gun bombardment of zygotic embryos of papaya cultivars "Sunset" (Fitch *et al*, 1990) and " Maradol" (Cabrera-Ponce *et al*, 1995). However the papaya cutivar Khakdum took about 1 month longer to regenerate. The genotype of the papaya, the type and age of tissue used in the particle gun bombardment are the important factors that effect the transformation efficiency by particle gun bombardment .

Plant regeneration

The transgenic calli started to regenerate after culturing for 3-4 months on the regeneration medium containing NAA, BAP and kanamycin and multiple shoots were formed within 6-9 months. Shoots with 3-4 leaves and about 1.5 cm tall were sliced and transferred to the rooting medium. When using media R2 which containing 1 mg/l indol butyric acid for rooting, few thick and short roots are formed at the end of the shoot. However this type of root is not healthy and not elongate when compared with the root of plant grew on the media R1 which is hormone free media. Thus the regenerated shoots are cultured in the R1 media until the thin long healthy roots formed which took about 1 month and then transferred to soil.

GUS expression in transgenic calli.

Transgenic calli resistant to 100 mg/l kanamycin were assayed for the β-glucuronidase expression by histochemical test. Five transgenic lines out of eight bombarded with M10 tungsten particles and one transgenic line out of four bombarded with gold particles showed *gus* expression in form of spot staining on the callus. Some reports sugested that transgenic plants may not express the *gus* gene product due to the deletion of the *gus* gene from the plasmid which was subjected to fragmentation during particle gun bombardment and the gene might not be linked to the kanamycin resistance gene (Fitch *et al*, 1990). Moreover, it has been also reported that the papaya callus tissue might contain detrimental factors leading to degradation of the GUS transcript and the GUS protein (Yang *et al*, 1996).

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เนื้อหางานวิจัย

CHARACTERIZATION OF EXPRESSED SEQUENCE TAGS FROM BLACK TIGER SHRIMP 'S HEMOCYTES

ABSTRACT

Two lambda ZAPII-based cDNA libraries was prepared from poly(A)+ RNA of hemocytes from Black Tiger Shrimp (*Penaeus monodon* Fabricius). Random clones were isolated and their 5' end partial nucleotide sequence determined by automated procedure. A segment of each DNA sequence was used to search online nucleotide and protein sequence databases by two BLAST programs. From the first cDNA library with a lower phage titer, 53 DNA sequences were obtained. Among these, 12 sequences (22%) did not match existing DNA sequence in online databases by either BLASTN or BLASTX searches, while 12 sequences (23%) matched known DNA sequences for known protein identities and 29 sequences (55%) matched 16S ribosomal rRNA sequences. Among those matching protein coding sequences, 3 sequences (6%) matched those of unique genes, 5 sequences (9 %) matched those of ribosomal protein genes, 4 sequences (7%) matched those of mitochondrial genes. A second cDNA library constructed from higher amount of poly(A)+ RNA provided higher phage titer. Analysis of random cDNA sequences from the second cDNA library was in progress. The study of partial cDNA sequences should allow rapid characterization of the DNA sequences, some of which may be relevant to immune system of the shrimp.

INTRODUCTION

Shrimps in the subphylum Crustaceae are a group of highly diversed organisms of over 38,000 known species (1). Human consume a number of marine shrimps species, yet those in the Family Penaeidae constitute the bulk of the catches (2). With the adoption of certain fast growning *Penaeus* species for aquaculture around the World, its commercial impact is even higher. In South and Southeast Asia, *Penaeus mondon* is the choice species. For Thailand alone, the World 's number one exporter of frozen shrimps, a yearly export value for about 250,000 tonnes of frozen shrimps amounted to US$ 2 billions.

Despite its economic importance to the World, little attention has been paid to study the shrimps in Molecular Biology aspects. Online DNA sequence databases possessed only a hundred plus records, as of early 1999. In recently years' a number of viruses infecting

Penaeid shrimps have been identitifed which caused epidemics among the farm-reared *P. monodon* shrimp of several countries (3,4,5). Outbreak of viral diseases such as white spot disease and yellow head disease for *P.monodon* in 1996 destroyed almost half of Thailand 's annual shrimp output. Sporadic infections of those viruses were still reported in many localities. It is clear that works in the DNA level on the shirmp and its viruses have become an urgent necessity.

Work on cDNA library and the clones in various organisms have allowed rapid identification of expressed sequences (6). Such partial cDNA sequences, widely known as Expressed Sequence Tags (or EST), are well characterized in human with over one million sequence records believed to cover most of the functioning human gene sequences. There are many useful application of EST information, such as serving as gene-specific tags in DNA cloning and gene characterization, as chromosomal markers for selective breeding programs and for genome mapping research, as known targets for PCR amplification in preparation of DNA probes, etc. With the severe deficiency in Peneid shrimps DNA sequence data, as well as overall deficiency for those across the wide taxa of crustacea as well, it was also very difficult for researchers to start working on any gene systems of the shrimp although some information of the particular genes might already be available from other invertebrate models such as *Drosophila* and *Caenorhabsitis*. To accumulate data fast with minimal cost, we had embarked on characterization of the shrimp 's random cDNA clones from muscular tissues to prepare ground work on the shrimp's molecular genetics (7). Result from EST work had let our group to identify and characterize full-length sequences of many cDNA from the black tiger prawn, such as enolase (8), two actin sequences (9), arginine kinase (10).

With some known cDNA sequences serving as our foundation for addressing other molecular biology questions, we then started to ask question on how much did we know about on the genes relevant to the defense and immune mechanism of the shrimp. Limited information from work conducted in a primitive crustacean, *Limulus polyphemus*, and many insect species which belong to the same phylum indicated that phagocytosis is the main defense mechanism (11). Hymocyte became the natural choice for us as starting material for collecting gene information by the same approach. In this communication, we describe our recent work on the EST from *P. monodon* 's hemocytes.

MATERIALS AND METHODS

Preparation of cDNA library and isolation of cDNA clones

Two months old *Penaeus monodon* shrimp specimens (hatched from eggs of a mother prawn catched from Andaman sea) were purchased from local farms in western Thailand. Live shrimps were briefly rinsed in sterile distilled water, then the homolymph was quickly withdrawn from the cardiac by a sterile 22 G needle and chilled in ice until sufficient volume was obtained. The first preparation of the total RNA for preparation of the first cDNA library was prepared directly from whole hemolymph by extracting by Trizol reagent. The second cDNA library was prepared from pelleted hemocytes by the acid guanidinethiocyanate method (12,13). Poly (A)+ RNA was obtained by means of either an oligo(dT) cellulose column (14) or magnetic procedure. About 0.15 ug and 2 ug of poly (A)+ RNA were used in the two library constructions, respectively. A cDNA synthesis kit of Strategene was used and the cDNA was cloned into EcoRI - XhoI site of a lambdaZAPII in *E.coli* XL1-Blue MRF' host, using Gigapack III Gold *in vitro* packaging kit. The two cDNA libraries yielded unamplified titers of around 4 x 10^4 pfu and 3 x 10^5 pfu, respectively. An aliquot from the unamplified library was taken and plated at one time to give few hundred pBluescript SK$^-$ phagemid clones using supertransfection of a f1 helper (ExAssist) phage. Random clones were picked from white colonies in LB medium containing X-gal and IPTG, and separately grown at 37 C in LB medium containing 50 ug / ml Ampicillin for 18 hr. Plasmids were extracted and partially purified by CTAB procedure (14) and cleaned up by QIAGEN. Clones whose inserts could be recovered by cutting with Eco RI and Xho I were selected for sequencing.

DNA sequence determination

Each plasmid DNA was sequenced (15) by using AmpliTaq DNA Polymerase FS enzyme and a sequencing kit from Perkin-Elmer. Reactions were performed mainly with the M13 Universal reverse sequencing primer in a thermal cycler (Perkin Elmer model 2400) set at 10 sec at 96 C, 5 sec 50 C, and 4 min 60 C for 25 cycles. The reactions were then fractionated and analyzed by Perkin Elmer ABI Prism model 377 automated DNA sequencer, using 6% acrylamide (+ 7 M Urea) running xxx volts, xxx watts, for about 10 hr.

DNA sequence search and alignment

DNA sequences from the regions of clear chromatogram (typically 400-500 bp out of a maximum sequence read from the machine) were used in the computer searches of GenBank (16), conducted via the world-wide web server of NCBI (http://www.ncbi.nlm.nih.gov).

The program used was BLASTN 1.4.9 MP and BLASTX (17, 18). The cDNA clones which showed statistically significant matching scores were then individually compared to some selected DNA sequences by ClustalW program, preferentially to *Drosophila melanogaster*, if available. Most of 5'-EST sequences reported in this paper will be deposited in the dbEST database. Only few upon which complete sequences were later determined and confirmed will be reported elsewhere.

Result and Discussion

A first *P.monodon* 's hemocyte cDNA library was constructed from a small amount of poly(A)+ RNA and was found to have a low unamplified titer of around 4 x 10^4 . The insert sizes of most clones range from 0.5 kb upto about 1 kb. Few hundred clones were isolated and verified that each possessed an insert cloned in between correct restriction sites (EcoRI and Xho I) as used in the library construction procedure before they were chosen to be excised in the form of pBluescript SK- phagemid. They were later sequenced using fluorescent dye-terminator chemistry on a Perkin-Elmer ABI Prism 377. Screening of several hundred clones revealed a small fraction, 53 clones which could be digested with the restriction enzymes and thus considered as containing legitimate inserts. Most clones gave DNA sequences, with the readable portion was about 300-500 bases for each clone When only the partial sequence from each clone was used for sequence querry via the Blast programs, 12 sequences (22% of 53 clones) did not match known gene sequences in existing databases by either BLASTN or BLASTX searches. This might that the shrimp DNA sequences were quite diverged from those of known eukaryotic species including those of *S.cerevisiae* and *C.elegans*. Only 12 sequences (23%) matched known gene sequences and 29 sequences (55%) matched 16S ribosomal rRNA sequences. Among those matching protein coding sequences, 3 sequences (6%)matched those of unique genes, 5 sequences (9 %) matched ribosomal protein gene sequences, 4 (7%) sequences matched mitochondrial genes.

Due to high level of contamination of the putative 16S rRNA sequences in the cDNA library, a decision was made to prepare a second cDNA library using much more hemolymph sample. The protocol was also slightly modified such that total RNA was extracted from pelleted hemocytes (which include phagocytic cells) and thus higher amount of poly(A)+ RNA was obtained. That resulted in much higher titer of the recombinant lambda phage of around 3 x10^5 pfu. Analysis of random cDNA sequences from a second cDNA library was still in progress.

The availablility of our DNA sequences in the online databanks should facilitate investigators working in other invertebrates in identifying their gene products of known or unknown function in the near future. The study of partial cDNA sequences allow rapid characterization of the DNA sequences some of which may be relevant to immune system of the shrimp.

Table I List of putative identity of some *P.monodon* EST from the first hemocyte cDNA library.

Clone ID	Putative Identity	Ref. organism (with accession no.)	sequence% identity
PMH08	Thymosin beta-11	(CAA21832)	38 %
PMH17	Neuronal protein NP45	Rattus norvegicus (M84725)	91%
	or Calponin homolog	Schistosoma mansoni (AAB47536)	52 %
PMH25	ADP-ribosylating factor	A. thaliana (M95166)	88 %
PMH38	Ribosomal protein S12	Sus scrofa (P46405)	79 %
PMH74	Cytochrome oxidase subunit 1	Penaeus nortialis (CAB40364)	75 %
PMH83	NADH dehydrogenase subunit 5,	Panorpa japonica (AF056494)	83 %
PMH95	Ribosomal protein S5	S.pombe (AB029515)	83 %
PMH140	Ribosomal protein P2, EL12	Artemia sp. (P02399)	39 %
PMH172	Cytochrome oxidase III	Penaeus nortialis (X84350.2)	85 %

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เนื้อหางานวิจัย

MOLECULAR CLONING OF TWO CDNAS ENCODING NEUROPEPTIDES IN CHH/MIH/GIH FAMILY FROM *PENAEUS MONODON* AND THEIR GENE STRUCTURE

ABSTRACT

Crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH) are crustacean neuropeptides that control sugar metabolism, molting process and gonad maturation, respectively. They are synthesized by the X-organ-sinus gland complex (XOSG) in the eyestalks. In this study, we used a technique of Rapid Amplification of cDNA Ends (RACE) to clone cDNAs that encode peptide hormones in CHH/MIH/GIH family from *Penaeus monodon*'s eyestalks. Two cDNA clones, *Pem-CMG1* and *Pem-CMG2*, were obtained. *Pem-CMG1* and *Pem-CMG2* coded for putative peptides of 127 and 128 amino acids, respectively. The putative mature peptides of both *Pem-CMG1* and *Pem-CMG2* comprised 74 amino acid residues. These two peptides shared 43% identity in amino acid sequences of the leading peptide region whereas 95% identity was found in their mature peptide sequences. Genomic fragments of *Pem-CMG1* and *Pem-CMG2* were obtained by PCR. The gene for *Pem-CMG1* was composed of two exons that were separated by an intron of 314 nucleotide pairs in the mature peptide coding region. *Pem-CMG2* gene harbored two introns, one was 405 nucleotides in length inserting into leading peptide coding region. The other intron was 268 nucleotides long inserting into a coding region for mature peptide. These results suggest that Pem-CMG1 and Pem-CMG2 were encoded by two related genes that have similar sequences in the coding region for their mature peptides but are structurally different.

INTRODUCTION

A number of physiological processes in decapod crustaceans are known to be regulated by diverse neuropeptides synthesized by a neurosecretory system called X-organ-sinus gland (XOSG) complex located in the optic ganglia of the eyestalks (1). In contrast to most of the peptide hormones isolated from the XOSG complex e.g. pigment dispersing hormone (PDH) and crustacean cardioactive peptide (CCAP) that have also been identified in insect, a group of related peptides constituting a novel hormone family has been described and has thus far been found only in crustaceans (2). This family includes crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad

inhibiting hormone (GIH) that are involved in blood sugar regulation, inhibition of ecdysteroid synthesis and regulation of reproduction, respectively (3, 4).

Amino acid sequences of the hormones in this CHH/MIH/GIH family have recently been determined in several crustaceans (5-8). Comparison of amino acid sequences of these hormones revealed significant degrees of similarity (2, 9) suggesting that these hormones constitute an authentic peptide family across species boundaries. In addition to several similarities in their structure, some hormones in the CHH/MIH/GIH family also exhibit overlapping biological activities (7, 10, 11). However, the certainty concerning the primary physiological functions of different peptides still needs to be clarified.

In recent years, the cDNA coding for CHH, MIH and GIH have been cloned based on the information of their available amino acid sequences (8-14). Despite this increasing number of reports on cDNA cloning and nucleotide sequence analysis of neuropeptides in the CHH/MIH/GIH family, the hormones in several crustaceans still await complete characterization, especially at molecular level.

We report here the molecular cloning of two cDNAs coding for member of CHH/MIH/GIH family, Pem-CMG1 and Pem-CMG2, from black tiger prawn, *Penaeus monodon*. Open reading frames of *Pem-CMG1* and *Pem-CMG2* cDNAs were cloned and primary structure of the genes encoding these two peptides were also analyzed.

MATERIAL AND METHODS

Animals

Black tiger prawns, *Penaeus monodon*, were obtained from Shrimp Culture Research Center, Chareon Pokphand Group and were kept in aerated seawater at room temperature until used.

Oligonucleotide primers

Abridged anchor primer (AAP) and abridged universal amplification primer (AUAP) were provided with the 5'RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL). Other oligonucleotides were purchased from Bio-Synthesis, USA. The sequences of the primers are shown in Figures 1 and 2.

RNA Isolation

A total of 100 eyestalks were collected from *Penaeus monodon* immediately after anesthetized with crushed ice. After the cuticle and non-neural tissues were removed, the dissected eyestalks were ground to fine powder in liquid nitrogen by means of mortar and pestle. Total RNA was extracted using TRIzol Reagent (GIBCO BRL). Poly $(A)^+$ RNA was

isolated using Dynabeads mRNA purification kit (DYNAL) and was quantified by measuring the absorbancy at 260 nm.

Amplification of the 3' ends of cDNA

The reaction was carried out according to the protocol of the 3' RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL). First strand cDNA was synthesized from 75 ng of poly $(A)^+$ RNA in a total volume of 20 µl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 500 nM PRT primer, 500 mM each dATP, dCTP, dGTP, dTTP and 200 units of SuperScript II Reverse Transcriptase. The reaction was incubated at 50°C for 50 minutes and then terminated at 70°C for 15 minutes. The RNA template was degraded with RNaseH before proceeding to amplification step. A 2µl aliquot of cDNA was amplified with primers PM1 and PM2 (see Figure 1A) in a reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 nM each primer, 200 mM each dATP, dCTP, dGTP, dTTP and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer). Amplification was performed in a DNA Thermal Cycler (GeneAmp System 2400, Perkin Elmer) with 35 cycles of 94°C for 1 minute, 50 °C for 1 minute and 72°C for 2 minutes following with 7 minutes incubation at 72°C as a final extension.

Amplification of the 5' ends of cDNA

The detailed protocol of the 5' RACE System for Rapid Amplification of cDNA Ends Version 2.0 (GIBCO BRL) was followed. First strand cDNA synthesis was carried out as described for the 3' RACE except that 100 nM of RM3 primer was substituted for PRT. The cDNA synthesized was then purified using a GlassMax DNA isolation spin cartridge (GIBCO BRL).

A 10 µl aliquot of purified cDNA was tailed with dCTP in 25 µl of 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.5 mM $MgCl_2$, 200 mM dCTP and 1 µl of terminal deoxynucleotidyl transferase (TdT). The reaction was incubated at 37°C for 10 minutes and TdT was heat inactivated at 65°C for 10 minutes. The PCR amplification using 5 µl of dC-tailed cDNA was performed as described for 3' RACE using 200 mM of PM4 and abridged anchor primer (AAP). The second round PCR was performed using primer PM5 and abridged universal amplification primer (AUAP) to obtain specific amplified product (Figure 1).

Amplification of an open reading frame of Pem-CMG1 peptide

Total RNA extracted from eyestalks and primer CMG-R were used to synthesize a cDNA template for amplification of the open reading frame of Pem-CMG peptide. The

condition for PCR was the same as described earlier. The primers used were CDF and CDR, the sequences of which are given in figure 1.

Amplification of an open reading frame of Pem-CMG2 peptide

A cDNA synthesized with PRT primer was used as a template for amplification of the open reading frame of Pem-CMG2 peptide. The condition for PCR was the same as described earlier. The primers used were CDF-2 and PM1, the sequences of which are given in figure 1.

Amplification of a genomic fragment of *Pem-CMG* genes

Genomic DNA was prepared from abdominal muscle tissues of *P. monodon* by phenol/chloroform extraction method (15). About 150 ng of genomic DNA was used as a template for PCR amplification of the *Pem-CMG* sequences with the primers CMG-F and CMG-R for *Pem-CMG1* and primers CMG-F and CMG-R2 for *Pem-CMG2* (figure 2). The PCR reaction mixture (150 ng of genomic DNA template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 nM each primer, 200 mM each dATP, dCTP, dGTP, dTTP) was heated to 95°C for 5 minutes then, 2.5 units of AmpliTaq was added. Amplification was achieved by 35 successive cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes, followed by a 10 minute final extension at 72°C.

Cloning and Sequencing

PCR Amplification products were purified using GeneClean II kit (Bio101). The purified DNA fragments were digested with appropriate restriction before ligated to pUC18 vector predigested with corresponding enzymes. The ligation products were used to transformed *E. coli* JM109 and the recombinants were screened by restriction enzyme digestion. The nucleotide sequences of the recombinants were determined by the method of ABI PRISM™ Dye Terminator Cycle using ABI PRISM™ Model 377 automated DNA Sequencer (PE Applied Biosystems).

RESULTS

3'RACE cloning of cDNAs encoding CHH/MIH/GIH peptides

Five different 3' fragments that shared homology in nucleotide sequences to those of CHH/MIH/GIH from other crustaceans were obtained. Nucleotide sequence comparison showed that these 3'RACE clones shared 53 % identity in nucleotide sequences of their

coding regions whereas much lower level of identity was observed among their 3' untranslated regions (figure 3).

5' RACE cloning of cDNA encoding Pem-CMG peptide

Using three specific primers generated from the nucleotide sequence of the 3' RACE-5 fragment, a single band of amplification product of 370 bp (excluding G-rich tail) was obtained from 5' RACE and was subsequently cloned into pUC18. The nucleotide sequence analysis revealed that it contained cDNA insert homologous to the mRNA of CHH/MIH/GIH peptide family. However, there were diffenences in nucleotide at 4 positions in the region that the 5'RACE and the 3'RACE-5 fragment overlapped.

Amplification of an open *reading frame of Pem-CMG1* peptide

An eyestalk cDNA was synthesized with primer CMG-R that was designed from nucleotide sequences at the 3' end of the 3'RACE-5 fragment. This cDNA was used as a template for PCR with primer PM2 and CMG-R to amplify the 3' region of *Pem-CMG1* cDNA. The 5' region of *Pem-CMG1* cDNA was generated by PCR with PM3 and CMG-F, a primer that was designed from the sequence at the 5' end of the 5'RACE fragment (figure 1). Nucleotide sequences of the *3'Pem-CMG1* fragment was identical to that of the 3'RACE-5 fragment whereas nucleotide sequences of the *5'Pem-CMG1* was different from that of the 5'RACE fragment. An open reading frame for *Pem-CMG1* was cloned by amplification of the cDNA with primers CDF and CDR that contained the sequences from start codon and stop codon of *Pem-CMG1* (figure 1). The amino acid sequence deduced from *Pem-CMG1* is shown in figure 4.

Amplification of an open reading frame of Pem-CMG2 peptide

Oligonucleotide primer CDF-2 was designed from the start codon of 5'RACE fragment. The cDNA that was synthesized with PRT primer was amplified with CDF-2 and PM1 to obtain open reading frame as well as 3' untranslated region of *Pem-CMG2*. The deduced amino acid sequence of *Pem-CMG2* is shown in figure 4.

Comparison of the amino acid sequences of Pem-CMG1 and Pem-CMG2

The deduced amino acid sequences of Pem-CMG1 and Pem-CMG2 were compared in figure 4. They were 44 % identical in the leading peptide region whereas higher level of identity (92%) was found in their mature peptides (figure 4). This result suggests that the two *Pem-CMG* cDNAs were transcribed from two different genes in CHH/MIH/GIH family. Comparison of the amino acid sequences of the putative mature Pem-CMG1 and Pem-CMG2 to the corresponding regions of CHH, MIH and GIH from other crustaceans revealed

that Pem-CMG1 was 43, 35, 32 and 31% identical to CHH from *P.japonicus,* MIH from *P.japonicus*, MIH from *Carcinus maenas* and GIH from *Homarus americanus*, respectively. Similar levels of identity could also be found between Pem-CMG2 and the other four peptides (figure 5).

Primary structure of *Pem-CMG* genes

Genomic fragment for *Pem-CMG1* and *Pem-CMG2*, designated as *g-CMG1* and *g-CMG2*, were amplified from genomic DNA of *P. monodon* with primers CMG-F and CMG-R and primers CMG-F and CMG-R2, respectively. These two genomic fragments contain nucleotide sequences from the first start codon (ATG) to the stop codon (TAG) of *Pem-CMG1* and *Pem-CMG2* genes.

Analysis of the nucleotide sequence of *g-CMG1* fragment cloned in pUC18 revealed that this fragment contained one intron that was 314 bp long. This intron separated the two exons in the coding region for the mature Pem-CMG peptide between the second and the third codons of ^{94}Arg (Figure 6). In contrast, *Pem-CMG2* fragment contained two introns. Intron 1, 405 bp long, interrupted the coding region for the leading peptide between ^{6}Leu and ^{7}Val, the second intron of 268 bp separated the mature peptide coding region between the second and the third codons of ^{94}Arg (figure 7). An exon-intron boundary was detected using Gene Finder program of Baylor College of Medicine (BCM) on the WWW. The splice junctions conformed to the splice donor and acceptor consensus sequence (16).

DISCUSSION

The 3' and 5' RACE strategy (17) was employed in order to clone a *Pem-CMG* cDNAs due to the lack of information on amino acid sequences of the peptides in CHH/MIH/GIH family in *P. monodon*. This technique requires only a single oligonucleotide primer which, in our case, was generated from the conserved amino acid sequence CEDCYN that is found conserved among the peptides in CHH/MIH/GIH family from a number of crustacean (1)

The open reading frame of two Pem-CMG peptides, Pem-CMG1 and Pem-CMG2, from *P. monodon* was successfully cloned. The deduced amino acid sequences from both *Pem-CMG1* and *Pem-CMG2* cDNAs reveal putative mature peptides comprising 74 amino acid residues. The Pem-CMG peptides of *P. monodon* shared the high degree of identity with CHH of *P. japonicus* whereas lower degrees of identity are found between Pem-CMG sequences and MIH reported for *P. japonicus* and *C. maenas*. The GIH of *H. americanus* also shows comparable degree of identity with both Pem-CMGs of *P. monodon*.

The gene for Pem-CMG1 of *P. monodon* (*g-CMG1*) contains only one intron that separates the coding sequence for the mature peptide whereas the gene encoding Pem-CMG2 (*g-CMG2*)contains two introns, one separates the leading pepetide coding sequences, the other interrupts coding seuqnece for the mature peptide. The structure of *Pem-CMG2* gene is similar to the primary structure of the genes encoding MIH in *Charybdis feriatus* and CHH-like peptide in *Metapenaeus ensis* that have recently been characterized (18,19). The *MIH* gene of *C. feriatus* contains two introns, intron 1 interrupts the sequence coding for signal peptide and the second intron separates the mature peptide sequence. Two introns were also found in *CHH*-like gene of *M. ensis.* They interrupt homologous regions to those of *C. feriatus MIH*, one in the signal peptide sequence and the other in the mature peptide sequence. The differences in the structure of genes in the same family among different species is not uncommon and may reflect their evolutionary pathway. It is, however, too early to draw any conclusion concerning gene structure of the member of CHH/MIH/GIH family because only little information has been obtained from a few species so far. The structure of the genes coding for other neuropeptides in CHH/MIH/GIH family from *P. monodon* as well as from other crustaceans needs to be explored. This would give a clue to answer the question of how these structurally different genes have evolved from related gene family.

The sequence information from several members of CHH/MIH/GIH family together with their biological activities suggest that the neuroendocrine regulation of related physiological activities in crustaceans is more complicated than previously thought. A complete understanding of these regulatory processes requires further information from molecular studies as well as physiologically studies to define more clearly the roles of individual peptide in this family.

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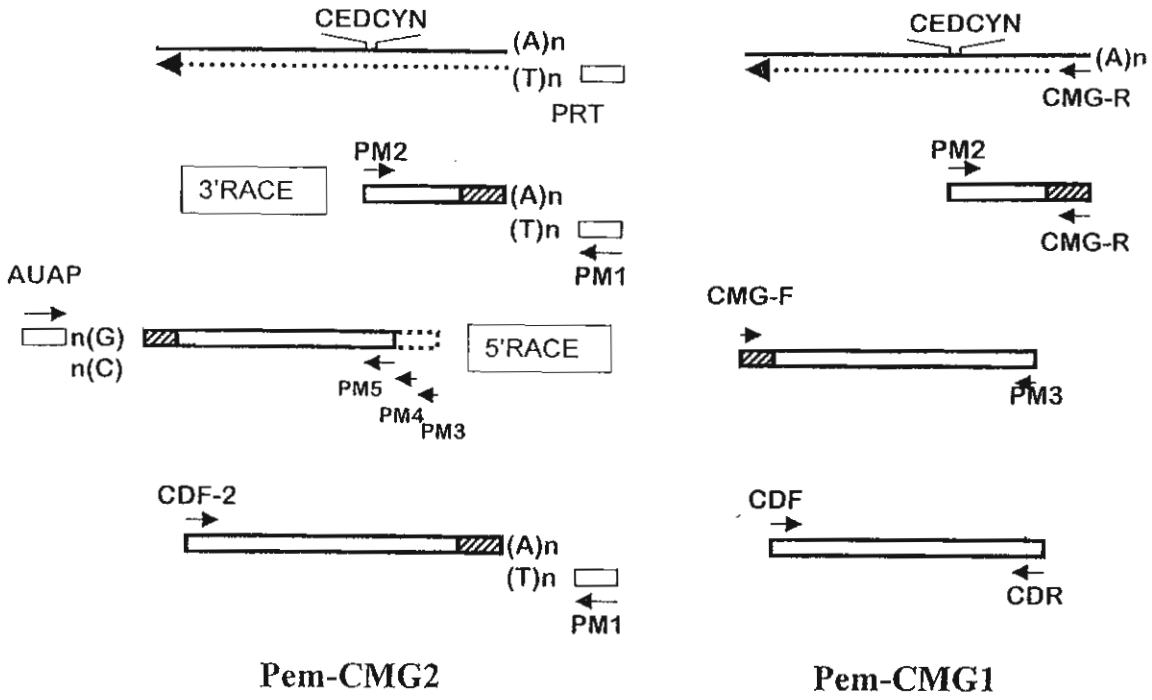

Figure 1. Schematic diagram showing the strategies used for cloning *Pem-CMG1* and *Pem-CMG2* cDNA and the primers used in cDNA synthesis and PCR. Hatched boxes represent 5' and 3' untranslated regions. Detail of the primers used is given below.

3'RACE primers

PRT : 5' – CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTT – 3'
PM1 : 5' – CCGGAATTCAAGCTTCTAGAGGATCC – 3'
PM2 : 5' – CCGGAATTCTGYGAAGAYTGYTACAAC – 3'

5'RACE primers

AAP : 5' – GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG – 3'
AUAP : 5' – GGCCACGCGTCGACTAGTAC – 3'
PM3 : 5' – CCGGAATTCCCTTTGACGAGGCCGGAAC – 3'
PM4 : 5' – CCGAAGCTTGTCCACGCAGTAGAG – 3'
PM5 : 5' – CCGGAATTCACCTCGTTGTGGAAACAG – 3'

open reading frame

CDF : 5' – CCGAAGCTTCATATGGTTGCCGTTGGAC – 3'
CDF-2 : 5' – CGGGATCCATGGTTGCGGTCCGATTGG – 3'
CDR : 5' – CGGGATCCCTACTTGCCGAGCCTCTA – 3'

Figure 2. Schematic diagram showing the strategy used for amplification of *Pem-CMG* genomic fragments and the primers used. Dotted area represent 5' and 3' untranslated regions. Primers CMG-F and CMG-R were used to amplify g-CMG1 fragment. Primers CMG-F and CMG-R2 were used for amplification of g-CMG2 fragment. Detail of the primers used is given below.

Oligonucleotide primers

CMG-F : 5' – CGGAATTCTCAGTGCAGAGGGAGAGCC – 3'
CMG-R : 5' – CGGAATTCATTTCTCTTATTTTTATTAGACAGG – 3'
CMG-R2 : 5' – GCGGATCCCTGCTTTATGAAGACACTG – 3'

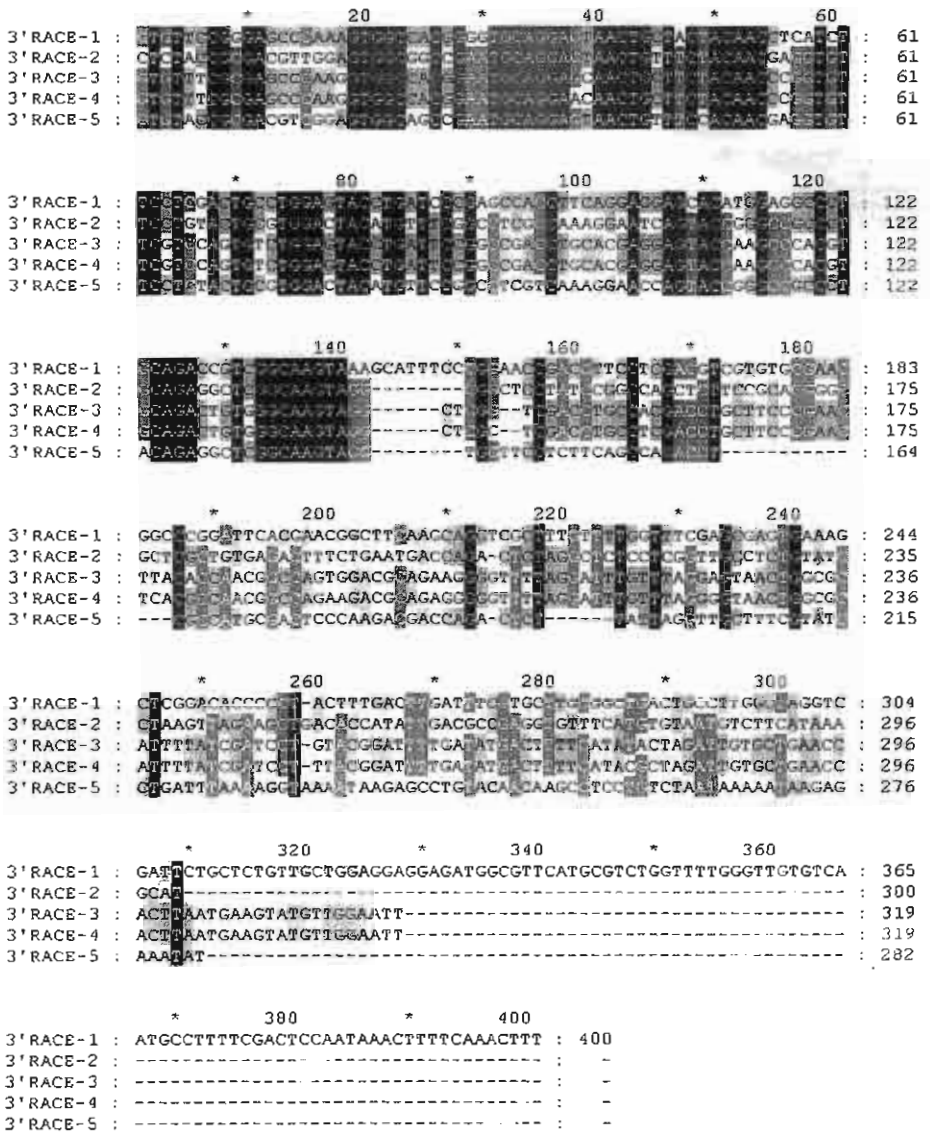

Figure 3. Comparison of nucleotide sequences among 3'RACE fragments. The sequence of PM2 primer is excluded from the 5' end of all fragments. The positions where the nucleotides in all five sequences are identical are highlighted (black). Dark grey and light grey area represent the positions where nucleotides are found identical in four and three sequences, respectively.

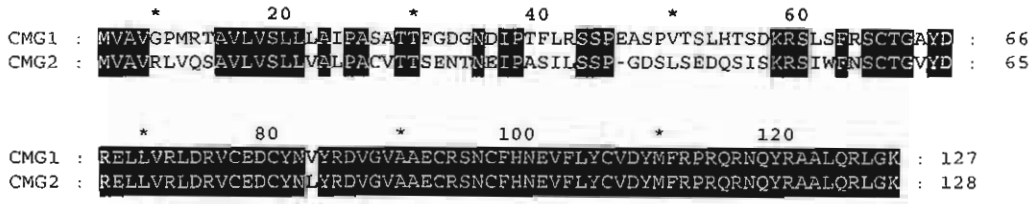

Figure 4. Comparison of the amino acid sequences deduced from *Pem-CMG1* and *Pem-CMG2*. The identical amino acid residues between the two sequences are highlighted

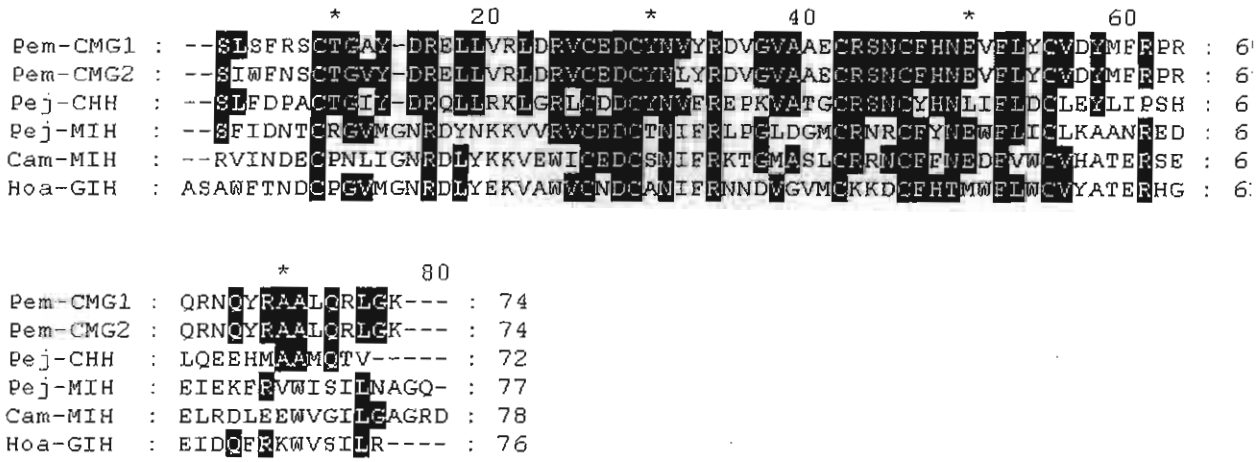

Figure 5. Amino acid sequence alignment among the peptides in CHH/MIH/GIH family. The positions where the amino acids in Pem-CMG1 or Pem-CMG2 are identical to those of any other sequences are highlighted.

```
1    :TCAGTGCAGAGGGAGAGCCTGGAAGTTGCTGACCGTCGCTCCCGATCTGC:  50
51   :CTCTACTCTAAAGATATGGTTGCCGTTGGACCGATGCGGGCAGCTGTCCT: 100
101  :GGTGTCCCTGCTGTTGGCAATCCCGGCCTCTGCCACCACCTTCGGAGACG: 150
151  :GAAATGACATTCCAACGTTCCTCCGTTCTTCCCCAGAAGCCTCTCCTGTG: 200
201  :ACTTCCCTTCACACCTCAGACAAACGCAGCCTATCCTTCAGGTCTTGCAC: 250
251  :GGGCGCCTACGACCGCGAACTCCTTGTAAGGCTCGACCGCGTGTGCGAAG: 300
301  :ACTGCTACAACGTGTACCGCGACGTCGGAGTGGCAGCCGAATGCAGGTAA: 350
                                                    ----
351  :CTTATTACTTTGCAGTAACCCACCCAGTTGTGTTGTGATTAAAGACTATT: 400
      ---------------------- Intron ----------------------
401  :GTAGAAGCGTATTAGTATACCATCTATTACTTTAGTATATCATCTATTAC: 450
      ---------------------- Intron ----------------------
451  :TATCATCTATCGGACAATGATTTCATACTGAGTTTGTTATCCATGGAGCT: 500
      ---------------------- Intron ----------------------
501  :TTCGTTAACATCGCCTGTTGCCTAAAAAGCCTCAATAATTATAAAATGTA: 550
      ---------------------- Intron ----------------------
551  :TAAACAGAATATACTTTATTCCTTATATCTCTTTATTCACATAGTTATTT: 600
      ---------------------- Intron ----------------------
601  :TTTCACATTTCTCCTGTACGACCTACCAGTGGAACTACATAACATTATTC: 650
      ---------------------- Intron ----------------------
651  :CTTCCTTCAGGAGTAACTGTTTCCACAACGAGGTGTTCCTCTACTGTGTG: 700
      ----------
701  :GACTACATGTTCCGGCCTCGTCAAAGGAACCAGTACCGGGCCGCCCTACA: 750
751  :GAGGCTCGGCAAGTAGGTGGTTCCTCTTCAGCCAGACCTCGCCATGCGAC: 800
801  :TCCCAAGACGACCAGACTCTTATTAGGTTGCTTTCGTATCCTGATTTAAA: 850
851  :AGGTAAAATAAGAGCCTGTACAACAAGCCTCCTGTCTAATAAAAATAAGA: 900
901  :GAAATAT                                           : 907
```

Figure 6. Nucleotide sequence of *g-CMG1* fragment. The 5' and 3' untranslated regions are underlined. The intron sequence is indicated by dashed line.

```
1     :TCAGTGCAGAGGGAGAGCCTCGAAGTCGGTGGTCCTTGTTCCTTCTGCTC:   50
51    :TCTGTCGAGTCCAGCGAAATGGTTGCGGTCCGATTGGTAAAGGAGGTTTA:  100
                                                 ----------
101   :AGTTCAGGCGGATTTAACTCTTGGTTAAAGTGCAGAGCTTATCCTGTGCA:  150
       ---------- Intron 1 ----------
151   :AATATTGTTTTTTTTCGTATAGGGGATCCGATTTTTTTTTTTTTCTTTAT:  200
       ---------- Intron 1 ----------
201   :TAATTTTCCAATGCTTCTGACGCTGTATGCATTATTCATTTGTCTTGTGT:  250
       ---------- Intron 1 ----------
251   :GTCCCCTCCGAACAAAACTGATTGATTCGAGAGTAGTAGTAAAGCGAACA:  300
       ---------- Intron 1 ----------
301   :TAGGAAGTTTTTCTAAACATCGAAGTCATAATCATTATATCAATACGGCC:  350
       ---------- Intron 1 ----------
351   :ACAAAATCCTGTAAGGGTCTCAGGACTGACCTCGGACATGAATAATTTCA:  400
       ---------- Intron 1 ----------
401   :GCAAGGTCCAATTTTTTTCTTAGTTACAGATGTTGATACTTCTTGGAAAC:  450
       ---------- Intron 1 ----------
451   :AAACCGTCCTCTGAAGTTAACTCCTCTCCCTCTGCCAACAGGTGCAGTCA:  500
       ---------- Intron 1 ----------
501   :GCTGTTCTTGTGTCCTTGCTGGTGGCACTTCCGGCCTGTGTCACAACTTC:  550
551   :TGAAAACACGAATGAAATACCGGCGTCCATTCTTTCTTCCCCTGGGATTC:  600
601   :CCTAACAGAGACCAAAGCATAAGCAAACGTTTATATTGTTCAATTCTGGC:  650
651   :ACGGGGTCTACGACCGCGAACTTCTTGTAAGGTTCGACCGCGTGTGCGAA:  700
701   :GACTGCTACAACTTCTACCGGACGTCGGAGTGGCGGCCGAATGCAGGTAT:  750
                                                     ----
751   :GTTATTCTAATTCTTACATAAAAATGTCAACAACCACTTTATACAGCAGC:  800
       ---------- Intron 2 ----------
801   :GAGTATCTGTAATCGCCTGTCAAGATCAGATGTGATAGTATGCTGGGTAA:  850
       ---------- Intron 2 ----------
851   :ATACTGTTATCATTTAATAGGTCGATTTTTTAATCACTATAAAACCTTTA:  900
       ---------- Intron 2 ----------
901   :TTTAGATTCCAAGGATCTGAGTAAAATCAACTTTATTTCCCTTTTCTCTT:  950
       ---------- Intron 2 ----------
951   :GCATTCTATTTTCATTCCTTCTTTACCTACTTACATGGTTACATAGAACC: 1000
       ---------- Intron 2 ----------
1001  :TTTACATATTGCAGGAGTAACTGTTTCCACAACGAGGTGTTCCTGTACTG: 1050
       ----------
1051  :CGTGGACTACATGTTCCGGCCTCGCCAGAGGAACCAGTACCGGGCCGCCC: 1100
1101  :TCCAGAGGCTCGGCAAGTAGGCGGTTCGTCTTCGGTCAACCCTTCCTGCG: 1150
1151  :GGGACGCTCGCCGTGAGATTTTTTCTAGGCTCTCCTTCGGTTGCTTCTGT: 1200
1201  :ATCCTAAGTTAGAAACGGATAACATATTGACGTCGAGGTGTTTCATGTAC: 1250
1251  :AGTGTCTTCATAAAGCAG                                : 1268
```

Figure 7. Nucleotide sequence of *g-CMG2* fragment. The 5' and 3' untranslated regions are underlined. The sequencesof the two introns are indicated by dashed line.

Research Output จากโครงการ

6. Research Output จากโครงการ

6.1 ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

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6.3 การเสนอผลงานในที่ประชุมวิชาการในประเทศ

1. Senapin, S., Panyim, S. and Tungpradabkul, S. A new type of flagellin gene in Pseudomonas putida. The 24[th] Congress of Science and Technology of Thailand, 19-21 October 1998, Bangkok, Thailand.

2. Boonchuey, C., Boonyawan, B., Panyim, S. and Sonthayanon, S. A complete cDNA sequence of phosphopyruvate hydratase from black tiger prawn (Penaeus monodon) The 24[th] Congress on Science and Technology of Thailand, 19-21 October 1998, Bangkok, Thailand.

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10. Phucharoen, J. Butraporn, R. and Panyim, S. Production and Characterization of cyclinE transgenic mice and cyclinE transfected fibroblast : functional studies of cyclinE in vivo and in vitro. 25th Congress on Science and Technology of Thailand, 20-22 October 1999, Pitsanulok, Thailand.

11. Chaicherdsakul, T., Boonchouy, C., Udomkit, A., Wilairat, P., Panyim, S. and Sonthyanon, B. Partial characterization of mitochondrial DNA of black tiger prawn (Penaeus monodon) 25th Congress on Science and Technology of Thailand, 20-22 October 1999, Pitsanulok, Thailand.

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6.4 การประชุมวิชาการอณูชีววิทยาประจำปี

1. "งานวิจัยด้านอณูชีววิทยา ครั้งที่ 1" 25 กันยายน 2540 ณ สถาบันอณูชีววิทยาและพันธุศาสตร์ มหาวิทยาลัยมหิดล ศาลายา มีผู้เข้าร่วมประชุม 42 คน และผู้เสนอผลงาน 10 คน

2. "งานวิจัยด้านอณูชีววิทยา ครั้งที่ 2" 25 กันยายน 2541 ณ สถาบันอณูชีววิทยาและพันธุศาสตร์ มหาวิทยาลัยมหิดล ศาลายา มีผู้เข้าร่วมประชุม 59 คน และผู้เสนอผลงาน 10 คน

3. “งานวิจัยด้านอณูชีววิทยา ครั้งที่ 3” 1 ตุลาคม 2542 ณ สถาบันอณูชีววิทยาและพันธุศาสตร์ มหาวิทยาลัยมหิดล ศาลายา มีผู้เข้าร่วมประชุม 79 คน และผู้เสนอผลงาน 9 คน

รายชื่อที่ปรึกษาโครงการ
และคณะผู้ร่วมวิจัย

รายชื่อที่ปรึกษาโครงการและคณะผู้ร่วมวิจัย

ชื่อ - นามสกุล	ตำแหน่งวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
1. ดร. สกล พันธุ์ยิ้ม	ศาสตราจารย์	ศาสตราจารย์	ชีวเคมี	คณะวิทยาศาสตร์	มหิดล	หัวหน้าโครงการ	หัวหน้าโครงการ
2. ดร. ชนันท์ อังศุธนสมบัติ	อาจารย์	ผู้ช่วยศาสตราจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักวิจัย	นักวิจัย
3. ดร. วิภา จึงจตุพรชัย	อาจารย์	รองศาสตราจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักวิจัย	นักวิจัย
4. ดร. สุณี เกิดบัณฑิต	อาจารย์	ผู้ช่วยศาสตราจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักวิจัย	นักวิจัย
5. ดร. บุรชัย สนธยานนท์	อาจารย์	ผู้ช่วยศาสตราจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักวิจัย	นักวิจัย
6. ดร. อภินันท์ อุดมกิจ	อาจารย์	อาจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักวิจัย	นักวิจัย
7. ดร. จรรยาภรณ์ ฟู่เจริญ	อาจารย์	อาจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักวิจัย	นักวิจัย
8. ดร. วิเชษฐ ลีลามานิตย์	อาจารย์	ผู้ช่วยศาสตราจารย์	ชีวเคมี	คณะเภสัชศาสตร์	มหิดล	นักวิจัย	นักวิจัย
9. ดร. มธุรส พงษ์ลิขิตมงคล	ผู้ช่วยศาสตราจารย์	รองศาสตราจารย์	ชีวเคมี	คณะวิทยาศาสตร์	มหิดล	นักวิจัย	นักวิจัย
10. ดร. วิไล หนุนภักดี	อาจารย์	ผู้ช่วยศาสตราจารย์	ชีวเคมี	คณะวิทยาศาสตร์	มหิดล	นักวิจัย	นักวิจัย
11. ดร. สุมาลี ตั้งประดับกุล	อาจารย์	ผู้ช่วยศาสตราจารย์	ชีวเคมี	คณะวิทยาศาสตร์	มหิดล	นักวิจัย	นักวิจัย
12. ดร. ศราวุธ จิตรภักดี	อาจารย์	อาจารย์	ชีวเคมี	คณะวิทยาศาสตร์	มหิดล	นักวิจัย	นักวิจัย
13. น.ส. บุญญรักษ์ บุญญวรรณ	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	นักวิจัย : มหาวิทยาลัยเชียงใหม่
14. นายคำรภ รัตนสุดา	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	นักวิจัย : มหาวิทยาลัยมหิดล
15. น.ส. เพ็ญนภา มานิตย์โชติพิสิฐ	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	อาจารย์ : มหาวิทยาลัยรังสิต

รายชื่อที่ปรึกษาโครงการและคณะผู้ร่วมวิจัย

ชื่อ - นามสกุล	ตำแหน่งวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
16. น.ส. ประวีณา กิตติกุล	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	อาจารย์ : จุฬาลงกรณ์มหาวิทยาลัย
17. น.ส. กรรณาภรณ์ วีรชาโต	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	อาจารย์ : จุฬาลงกรณ์มหาวิทยาลัย
18. นายจงรักษ์ กิตติวรการ	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	อาจารย์ : มหาวิทยาลัยมหิดล
19. น.ส. ฐนียา ดวงจินดา	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	นักวิจัย : ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
20. น.ส. กุลศิริ เจริญศิลป์	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
21. น.ส. จันทนา วงศ์สันติชน	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
22. นายนพคุณ มูลสิน	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
23. นายประพฤติดี ปิยะวิริยะกุล	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	อาจารย์ : จุฬาลงกรณ์มหาวิทยาลัย
24. นายเริงวิทย์ บุญโยม	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	อาจารย์ : มหาวิทยาลัยนเรศวร
25. นายสุนทรต์ ชูลักษณ์	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	อาจารย์ : มหาวิทยาลัยบูรพา
26. น.ส. ศรินทิพย์ เรืองเวทดี	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาโท	ศึกษาต่อปริญญาเอก
27. ม.ล. เสาวรส สวัสดิวัฒน์	ไม่มี	ไม่มี	ชีวเคมี	คณะวิทยาศาสตร์	มหิดล	นักศึกษาปริญญาเอก	นักวิจัย : มหาวิทยาลัยมหิดล
28. นายปานันท์ กาญจนภูมิ	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาเอก	นักศึกษาปริญญาเอก
29. นายวันชัย อัศวลาภสกุล	ไม่มี	ไม่มี	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาเอก	นักศึกษาปริญญาเอก

รายชื่อที่ปรึกษาโครงการและคณะผู้ร่วมวิจัย

ชื่อ - นามสกุล	ตำแหน่งวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
30 น.ส. พงโสภี อัตศาสตร์	นักวิจัย	นักวิจัย	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	นักศึกษาปริญญาเอก	นักศึกษาปริญญาเอก
31. นางเพียงจันทร์ สนธยานนท์	นักวิจัย	นักวิจัย	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	ผู้ช่วยวิจัย	ผู้ช่วยวิจัย
32. นางจุฑามาศ ศิริปาณี	นักวิทยาศาสตร์	นักวิทยาศาสตร์	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	ผู้ช่วยวิจัย	ผู้ช่วยวิจัย
33. นายสมภพ ลีตะชีวะ	นักวิจัย	นักวิจัย	สถาบันอณูชีววิทยาและพันธุศาสตร์		มหิดล	ผู้ช่วยวิจัย	ผู้ช่วยวิจัย
34. น.ส. ชนิกานต์ บุญช่วย	นักวิทยาศาสตร์	นักวิทยาศาสตร์	สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี		มหิดล	ผู้ช่วยวิจัย	ผู้ช่วยวิจัย
35. นายอำนาจ ชะนะมา	นักวิทยาศาสตร์	นักวิทยาศาสตร์	สถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี		มหิดล	ผู้ช่วยวิจัย	ผู้ช่วยวิจัย