รายงานวิจัยฉบับสมบูรณ์

โครงการ การควบคุมการแสดงออกของ Mtx1 ใน *Bacillus sphaericus* และการเพิ่มผลผลิตของโปรตีน

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ศูนย์พันธุวิศวกรรมและเทคโลโลยีชีวภาพแห่งชาติ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

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Project Title : Regulation of Mtx1 expression in *Bacillus sphaericus* and its production improvement

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Mtx1 is a larvicidal toxin that is highly toxic to *Culex* species. It is expressed during vegetative phase of growth from *B. sphaericus*, but at very low level and rapidly degraded. In order to study regulation of *mtx1* expression, different promoter constructs were created in a way that stem loop would not form. GFP expression from Pmtx1 that could not form stem loop was about 2 times higher than WT Pmtx1 in *Bs*2297, while stem loop did not affect expression in *Bsu*168. These results suggest that stem loop structure plays a role in repression of *mtx1* in *Bs*2297. To obtain high production of Mtx1, 2 different hosts were used. The first host is *Bt*4Q7 which is a crystal deficient strain. The *mtx1* gene was cloned into pBCX (cyt2APro) containing a strong sporulation-specific *cyt2A* promoter that would redirect Mtx1 production into crystal protein inaccessible for proteases to degrade it. Mtx1 protein was produced from *Bt*4Q7 at low level, however *Bt*4Q7 (pBCX-cyt2APro-tMtx1) cell was toxic to *Culex* larvae. The second host is *B. subtilis* WB800 which lacks extracellular proteases. In this case, Mtx1was secreted directly into medium and protein degradation is minimizing in the absence of extracellular proteases. The secreted Mtx1 retained larvicidal activity.

Keywords: Mtx1, gene expression, *Bacillus sphaericus*, *Bacillus subtilis*

รหัสโครงการ: TRG5380013

ชื่อโครงการ: การควบคุมการแสดงออกของ Mtx1 ใน *Bacillus sphaericus* และการเพิ่มผลผลิตของโปรตีน

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โปรตีน Mtx1 จากแบคทีเรีย *B. sphaericus* มีความเป็นพิษจำเพาะอย่างสูงกับลูกน้ำยุงลาย โปรตีนนี้จะถูกผลิตในช่วงที่เซลล์ยังไม่สร้างสปอร์ในปริมาณที่น้อยมากและยังถูกทำลายอย่างรวดเร็ว ในการศึกษาการแสดงออกของโปรตีน Mtx1 โปรโมเตอร์ของยีนนี้ถูกสร้างขึ้นมาหลายแบบที่ไม่สามารถสร้างโครงสร้างแบบ stem loop ได้ โปรตีนเรืองแสงสีเขียวที่มาจากโปรโมเตอร์ที่ไม่สามารถสร้าง stem loop มีปริมาณมากกว่าที่มาจากโปรโมเตอร์ปกติประมาณ 2 เท่าในแบคทีเรีย *B. sphaericus* 2297 แต่โครงสร้าง stem loop ไม่มีผลต่อการแสดงของโปรตีนเรืองแสงสีเขียวในแบคทีเรีย *B. subtilis* 168 ผลการทดลองนี้แสดงให้เห็นว่าโครงสร้าง stem loop มีบทบาทสำคัญในการควบคุมการแสดงออกของโปรตีน Mtx1 ในแบคทีเรีย *B. sphaericus* 2297 เพื่อที่จะเพิ่มผลผลิตของโปรตีน Mtx1 แบคทีเรียสองชนิดถูกใช้เป็นเซลล์เจ้าบ้าน แบคทีเรียตัวแรกคือ *B. thuringiensis* 4Q7 ซึ่งเป็นสายพันธุ์ที่ไม่สร้างผลึกโปรตีน ยีน *mtx1* ถูกใส่เข้าไปในพลาสมิด pBCX (cyt2APro) ซึ่งมีโปรโมเตอร์ที่มีประสิทธิภาพสูงที่ทำงานในช่วงการสร้างสปอร์ ซึ่งโปรโมเตอร์นี้จะสร้างโปรตีน Mtx1 ให้เป็นผลึกโปรตีนที่ยากต่อการย่อยสลายเซลล์ *Bt*4Q7 สร้างโปรตีน Mtx1 ในปริมาณต่ำ แต่ว่าโปรตีนที่สร้างก็ยังมีความเป็นพิษกับลูกน้ำยุงลาย แบคทีเรียเจ้าบ้านตัวที่สองคือ *B. subtilis* WB800 ซึ่งไม่สามารถสร้างเอนไซม์ย่อยโปรตีนที่ถูกขับออกมานอกเซลล์ได้ ในกรณีนี้โปรตีน Mtx1 ถูกส่งออกมาสู่อาหารเลี้ยงเชื้อ และไม่ถูกย่อยสลาย และโปรตีน Mtx1 ยังมีความเป็นพิษกับลูกน้ำยุงลายอยู่

คำสำคัญ : Mtx1, **การแสดงออกของยีน**, *Bacillus sphaericus*, *Bacillus subtilis*

Introduction

Mosquitoes are vectors transmitting many serious diseases to human, such as malaria, dengue, and West Nile disease, which still are problems worldwide. *Bacillus thruingiensis* subsp. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) are known to produce the crystal proteins that are highly and specifically toxic to mosquito larvae. Four major proteins in *Bti* spores; Cry4A, Cry4B, Cry11A, and Cyt1A are very toxic to *Aedes* larvae (dengue vector) [1, 2]. On the other hand, *Bs* produces a binary toxin consisting of BinA (51 kDa) and BinB (42 kDa) that is highly toxic to *Culex* larvae (cause nuisance and vector of West Nile and filariasis) and *Anopheles* larvae (malaria vector) [3]. Both BinA and BinB are required for larvicidal activity [4].

Actually, *Bs* also produces mosquitocidal toxins (Mtx1, Mtx2, and Mtx3) during vegetative phase of growth [5-7]. Due to their low expression levels and susceptibility to protease, Mtx1 and Mtx2 cannot contribute to toxicity of spore formulation used in mosquito control [8]. However, Mtx1 has a high specific mosquitocidal activity and its mode of action is different from that of binary toxin, thus Mtx1 might prevent resistance occurred in *Culex* population treated by *Bs*. Structure and function of Mtx1 have been extensively studied, but its expression regulation is not thoroughly investigated. Information obtained here could be used to improve production of Mtx1 in *Bs*, thus help delaying or preventing resistance developed in mosquito larvae.

Toxic strains of *Bs* can be divided into high-toxicity strains (50% lethal concentration [LC_{50}] ~10^2 to 10^3 cells/ml) and low-toxicity strains (LC_{50} ~10^5 cells per/ml) [9]. Toxicity of highly toxic strains is due to the presence of binary toxin consisting of BinA and BinB [3]. BinB functions as binding component interacting with a receptor on

the target cell, while BinA exerts lethal activity with yet unidentified mechanism. Binary toxin is produced during sporulation phase as a parasporal crystal protein [3]. The low-toxicity stains, however, lack binary toxin, instead they produce soluble mosquitocidal toxins during vegetative growth. To date three kinds of mosquitocidal toxins have been identified (Mtx1, Mtx2, and Mtx3). Mtx2 and Mtx3 share some homology to each other and to other pore-forming toxins [5, 10], suggesting that they could kill the cells by forming pores.

Mtx1 is most well characterized toxin among those three mosquitocidal toxins. It is first identified in low-toxicity *B. sphaericus* strain SSII-1 as a 100-kilodalton protein [6]. Mtx1 has significant homology to ADP-robosylating toxins [11], and indeed it possesses ADP-ribosyltransferase activity [12]. It was shown that Mtx1 could ADP-ribosylate elongation factor TU (EF-Tu) in *E. coli* cell lysate [13], however, its relevant substrate in eukaryotic cells has not been elucidated. Mtx1 contains 870 amino acid residues with the 29 N-terminal residues functions as a signal peptide [6]. The mature Mtx1 without the putative signal peptide is proteolytically processed into a 27 kDa N-terminal fragment and a 70 kDa C-terminal fragment in the larvae gut [14], and ADP-ribosyltransferase activity lies in the smaller fragment.

While strain SSII-1 shows low and noticeably unstable toxicity, surprisingly, the purified recombinant 97 kDa form of Mtx1 (without its putative signal peptide) exhibits high activity against *C. quinquefasciatus* (LC_{50} ~15 ng/ml) and *A. aegypti* (LC_{50} ~50 ng/ml) mosquito larvae, comparable to that of binary toxin from highly toxic strains [14]. Therefore, low toxicity of strain SSII-1 could result from low production and/or instability

of the toxin. In fact, it was found that Mtx1 is degraded by subtilisin-like serine protease [15], also called sphaericase, during sporulation. When expressing Mtx1 in protease-deficient *Bs* strain, the sporulated recombinant bacteria showed toxicity as high as sporulated highly toxic strain [16]. In another study, the expression of Mtx1 was redirected to sporulation phase by utilizing binary toxin promoter. The recombinant bacteria showed toxicity during sporulation phase and then declined at later stages, indicating the Mtx1 degradation at the end of sporulation [8].

Low accumulation of Mtx1 in Bacilli was obviously observed in above studies, no matter the growth phase it being produced, since the presence of protein was detected only by Western blot analysis [8, 16]. This is in part due to the susceptibility of Mtx1 to degradation by protease. However, it seems that the low level of expression of Mtx1 itself also accounts for its low accumulation. Study of regulation of Mtx1 synthesis by translational *lacZ* fusion demonstrated that the expression was extremely low and limited to early exponential phase in *Bs*. Additionally, the expression in *Bs* was about eightfold lower than that in *B. subtilis*, suggesting a negative control in the original host [17]. The expression is drastically higher in *E. coli* cells [17]. This negative control phenomenon is consistent with the presence of A+T-rich inverted repeat sequence located between the

-10 region and the ribosome-binding site of the *mtx1* gene [6]. This inverted repeat is capable of forming hairpin loop structure, and prokaryotes are known to utilize posttranscriptional mechanism to control gene expression [18]. Therefore, it is most likely that there is an element exists in *Bs* to exert negative regulation over Mtx1 synthesis. The primary objective of this project is to study the regulation of Mtx1

expression. The gained knowledge could be used to improve production of Mtx1 in Bacilli.

There are several means to increase protein production; one is to utilize a strong promoter. Previous work showed that Mtx1 could be expressed during sporulation phase using the binary toxin promoter, but the expression level was still low [8]. Here, in this lab a strong sporulation specific promoter has been identified in *Bt* as a promoter of the gene *cyt2A*. Therefore, the Mtx1 will be expressed using this strong promoter in *Bt* to obtain high toxin production. In addition, since Mtx1 contains a putative signal peptide and probably be secreted by the cells, truncated *mtx1* gene will be fused with signal peptide from *Bacillus subtilis* and use this bacterium as a host for Mtx1 production.

Mtx1 purification for antibody production

GST-tMtx1 fusion protein was expressed from *E. coli* JM109 harboring pGEX-tMtx1 plasmid. The pGEX-tMtx1 contains truncated *mtx1* gene encoding amino acid 30 to amino acid 870. GST-tMtx1 was purified using column GSTrap and fusion protein was cleaved by enzyme thrombin to obtain tMtx1 protein. The protein was sent for antibody production at Kasetsart University.

Western blot analysis

Samples were loaded on 12% SDS-PAGE and then were transferred to nitrocellulose membrane with semi-dry blot set. The membrane was blocked with working buffer (PBS + 0.1% Tween) with 5% skim milk overnight. Then the membrane was incubated with buffer containing primary antibody against Mtx1 (1:3000) for 2 h. following by washing with buffer 3 times 10 min each. Then the membrane was incubated with secondary antibody conjugated with alkaline phosphatase following by washing with buffer 3 times. Finally color was developed by BCIP and NBT.

Cloning of *tMtx1* into pHT43

The *tMtx1* fragment was cut out from pGEM-tMtx1 with *Spe*I and *Aat*II and then inserted into pHT43 (MoBiTec) cut with *Xba*I and *Aat*II. The resulting plasmid was transformed into *B. subtilis* WB800 (extracellular proteases deficient strain).

Cloning of *tMtx1* into pBCX (cyt2APro)

The *tMtx1* fragment was amplified from pGEX-tMtx1 with primers pGEX-tMtx1F (5'-CCGGAGTCGACGGTAATGGCTTCAC-3'; containing *Sal*I site) and pGEX-tMtxR (5'-GCACTAGTGGTACCGAGCTCTC-3'; containing *Kpn*I site). The PCR product was inserted into pGEM-Teasy (Promega). Then *tMtx1* was subcloned into pBCX (cyt2APro) at *Sal*I and *Kpn*I sites. The resulting plasmid was transformed into *B. thuringiensis* 4Q7 (crystal minus strain).

Larvicidal toxicity assay against *Culex* larvae

Larvicidal toxicity assay was performed in 24-well plate. Five of second-instar *Culex quinquefasciatus* larvae (supplied from Institute of molecular biosciences, Mahidol University, Salaya campus) were placed in a well containing 1 ml of water. Then 1 ml of suspension of Mtx1 or media containing secreted Mtx1 was added into each well (20 larvae/concentration). The number of dead larvae was recorded after 48 hour incubation at room temperature.

Construction of inverted repeat deletions and no loop Pmtx1

Promoter region of *mtx1* gene was amplified using primers Pmtx1F (5'-CATACTTGTCGAATTCCTGACAGG-3'; containing *EcoR*I site) and Pmtx1R (5'-ACTCGACGGATCCATTAACCATG-3') and cloned into pAD123 plasmid containing promoterless *gfp* at *EcoR*I and *Sma*I sites to give plasmid pAD123-Pmtx1-gfp. Then the P*mtx1-gpf* fragment was amplified using primers pAD123-checkF (5'-CGTCTAAGAAACCATTATTATC-3') and gfpRev (5'- TCGAAGCTCGGCGGATTTGT-3').

The PCR product was digested with *Hind*III and then inserted into pBCX (cyt2APro) cut with *Sma*I and *Hind*III to replace *cyt2A* promoter region. The resulting plasmid pBCX-Pmtx1-gpf was used as template for Pmtx1 variants. Three constructs of deletions were generated (i) the whole inverted repeat was deleted (ΔIR1/2), (ii) the first half of inverted repeat was deleted (ΔIR1), and (iii) the second half of inverted repeat was deleted (ΔIR2). Primer A (5'-TAATAGTTATATATTTATTTTGAAGG-3') and B (5'-ACCAAAAAGAGGTGCAATTGATATG-3') were used to construct pBCX-ΔIR1/2-gpf, primer A and D (5'-CATAATTTAATAATAAAAAATAAAT-3') were used to construct pBCX-ΔIR1-gfp, primer C (5'-GTATTTAATAACATTAAATAAAAAT-3') and B were used to construct pBCX-ΔIR2-gfp. Primer D and E (5'-TAATATTATAGTTTATTATTGAATAATAGTTATATATTTATTTTGAAGG-3') were used to construct pBCX-no loop-gfp. Primer E was designed with the first half of inverted repeat was altered so that hairpin formation would not occur. Primers were designed to amplify the whole template plasmid, and then PCR products were self ligated (Fig.2). The plasmids containing different constructs were transformed into *B. sphaericus* 2297 (*Bs*2297) and *B. subtilis* 168 (*Bsu*168).

Fluorescent intensity measurement

Cells containing plasmid pBCX with Pmtx1 variants were grown in LB medium containing 10 μg/ml tetracycline at 37°C overnight. The overnight culture was diluted 1:100 into fresh LB with10 μg/ml tetracycline and the cells were grown at 37°C for 7 hour. Cells of OD_{600} = 1.0 was centrifuged and resuspended in 1 ml PBS and fluorescent intensity measurement was performed by JASCO FP-65000

spectrofluorometer. The fluorescent intensity was measured with excitation wavelength at 488 nm (band width 3 nm) and emission wavelength 500-600 nm (band width 3 nm).

Mtx1 purification and western blot analysis test

In this study the antibody against Mtx1 is needed for western blot analysis to evaluate Mtx1 production. Mtx1 protein was primarily purified as GST-tMtx1 fusion protein produced from plasmid pGEX-tMtx1 [19]. Then GST-tMtx1 was cleaved with enzyme thrombin to remove GST and the purified tMtx1 was used for antibody production raised in rabbit. The size of purified tMtx1 is ~97 kDa as expected (Fig. 1A). When the Mtx1 antibody was obtained, it was tested for activity and suitable dilution to be uses in western blot analysis. It was shown that the 1:3000 dilution of Mtx1 antibody clearly detected the protoxin form (97 kDa) and the activated form (70 and 27 kDa fragments) of Mtx1 (Fig. 1B). Mtx1 is sensitive to protease degradation and usually degraded to active fragments at 70 and 27 kDa upon long storage. The other observed bands could be the further degraded Mtx1 products. Therefore, the 1:3000 dilution of Mtx1 antibody was used for western blot analysis throughout this project.

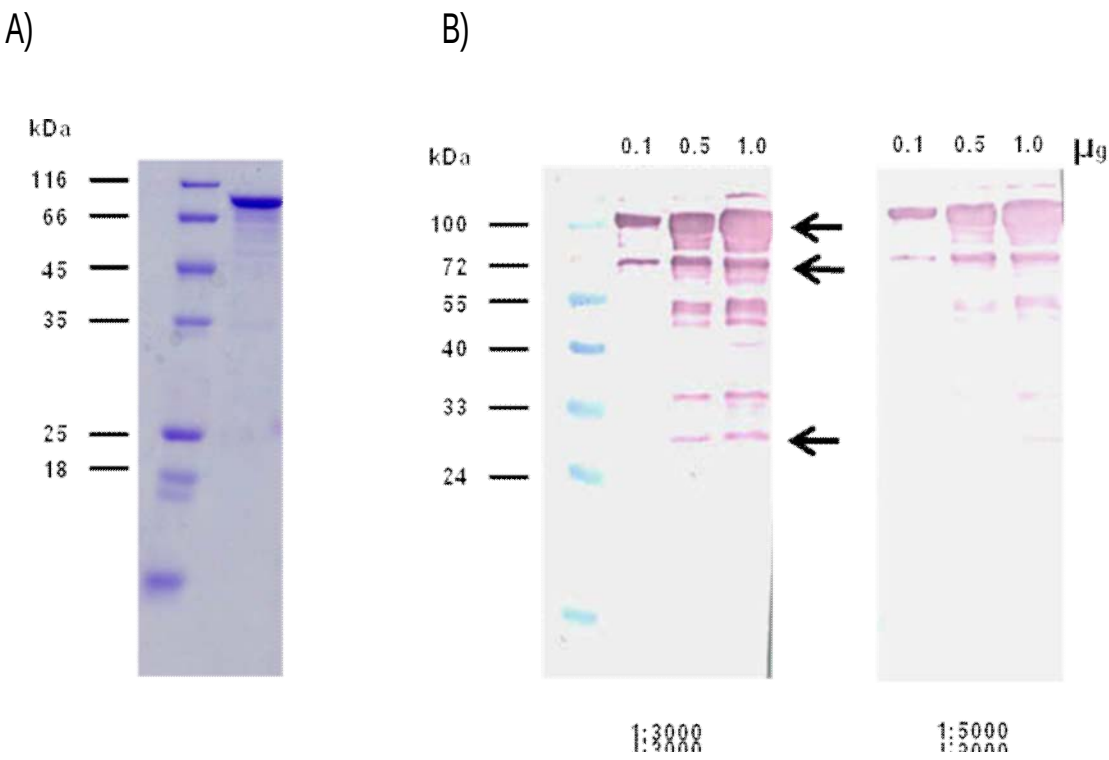


Figure 1. Mtx1 purification and western blot analysis test. A) Purified tMtx1 after removal of GST analysed on 12% SDS PAGE. B) Western blot analysis test on antibody against Mtx1 raised in rabbit. Two dilutions (1:3000 and 1:5000) of serum were tested against purified tMtx1 at concentration 0.1, 0.5, and 1.0 µg. The arrows from top to bottom indicate the protoxin form of Mtx1 at 97 kDa, and activated fragment at 70 kDa and 27 kDa, respectively.

Role of inverted repeat in regulation of *mtx1* expression

Previous study showed that expression level of *lacZ* fused with *mtx1* promoter (Pmtx1) in *B. sphaericus* cells is about 8-fold lower than that in *B. subtilis* cells. With the presence of inverted repeat upstream of ribosome binding site of *mtx1* gene, these evidences suggest that there may be a repression mechanism in *B. sphaericus* [17]. To determine whether inverted repeat upstream of *mtx1* gene is involved in expression of Mtx1, variants of Pmtx1 region were constructed including three different deletions of inverted repeat and Pmtx1 that cannot form hairpin loop (Fig. 2B). The resulting plasmids (pBCX-ΔIR1/2-gfp, pBCX-ΔIR1-gfp, pBCX-ΔIR2-gfp, and pBCX-no loop-gfp) were transformed into *Bs*2297 and *Bsu*168.

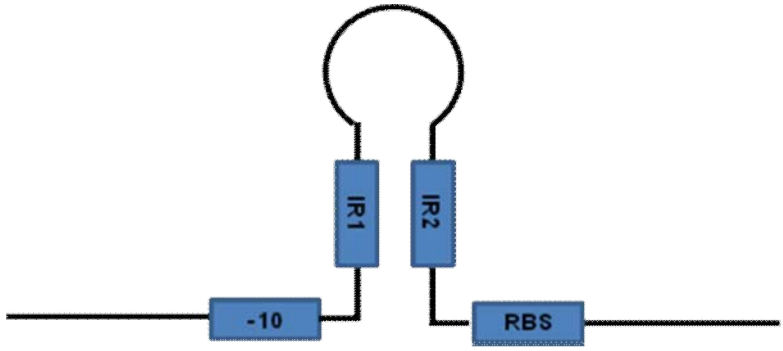
Fluorescent intensity measurement illustrated that in *Bs*2297 cells expression of *gfp* of three inverted repeat deletions was about 2 times higher than that of WT *mtx1* promoter, while GFP intensity of no stem loop Pmtx1 was slightly higher than deletion constructs (Fig. 3A). On the other hand, *Bsu*168 cells containing inverted repeat deletions and no stem loop Pmtx1 showed GFP intensity similar to that of WT *mtx1* promoter (Fig. 3B). These results suggest that inverted repeat of *mtx1* promoter only affect *gfp* expression in *Bs*2297 but not in *Bsu*168.

When the *mtx1* gene is transcribed, a stem loop was predicted to form by IR1 and IR2 region (Fig. 2A) and this structure may repress the gene expression. The stem loop forming was predicted using RNAfold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The observed derepression of the three deletion constructs (ΔIR1, ΔIR2, and ΔIR1/2) illustrates that both IR1 and IR2 are important for repression of *mtx1*

gene. To confirm that the stem loop structure plays a role in this repression, IR1 sequence was altered so that a stem loop would not form. The gene expression of this construct was abolished suggesting that the stem loop formation is essential for this repression.

In this study the expression level of *mtx1* in *B. subtilis* was about 4-fold higher than that is *B. sphaericus*, while the previous work [17] showed 8-fold higher of expression level in *B. subtilis* cells. This could due to the difference in vector, medium and *B. sphaericus* strain used in the experiments (pBT, NYSM and *Bs*2362 in previous work). Intriguingly, stem loop structure did not affect expression of *mtx1* gene in *B. subtilis* cells. However, in *E. coli* DH5α cells GFP signal could not be detected from Pmtx1 and all variants (data not shown) suggesting that this system did not work in *E. coli* DH5α strain. Another plasmid or *E. coli* strain could be tested to see the expression pattern and compare to *Bs*2297 and *Bs*168. The information gained from *E. coli* would help us to interpret whether there is another element beyond stem loop structure (i.e. protein binds to stem loop) involve in gene repression of *mtx1*. However, only 2-fold increase of GFP intensity observed when stem loop is eliminated could just arise from stem loop structure alone interfering with gene expression.

A)



B)

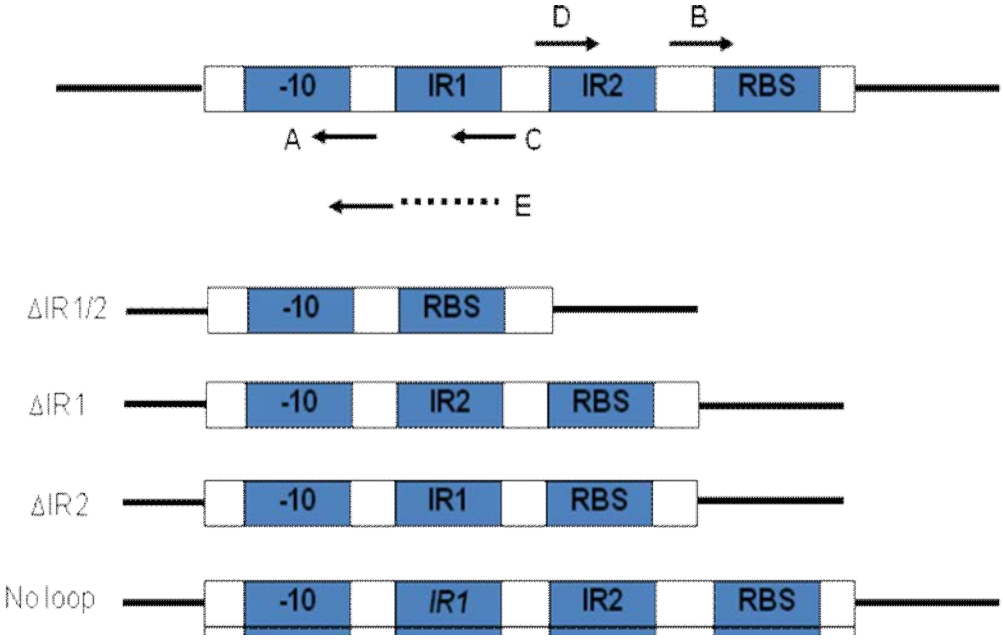
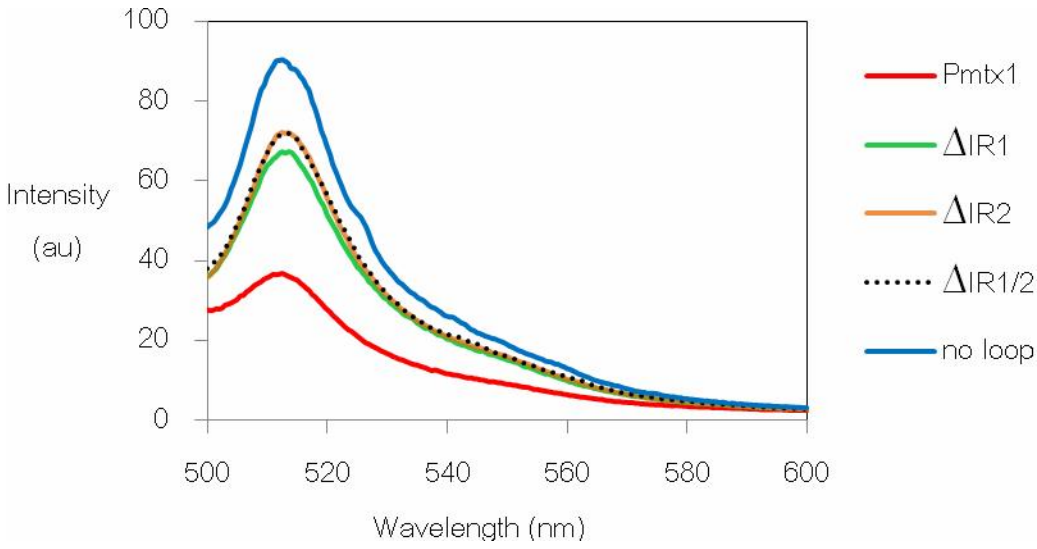


Figure 2. A) Predicted stem loop formation at Pmtx1. B) Diagram of different constructs of Pmtx1. Promoter region of *mtx1* gene including -10 sequence (-10), the first half of inverted repeat (IR1), the second half of inverted repeat (IR2), and ribosome binding site (RBS) are shown. *IR1* represents altered sequence at IR1 repeat. Arrows with letter A, B, C, D, and E are primers used to construct Pmtx1 variants.

A)



B)

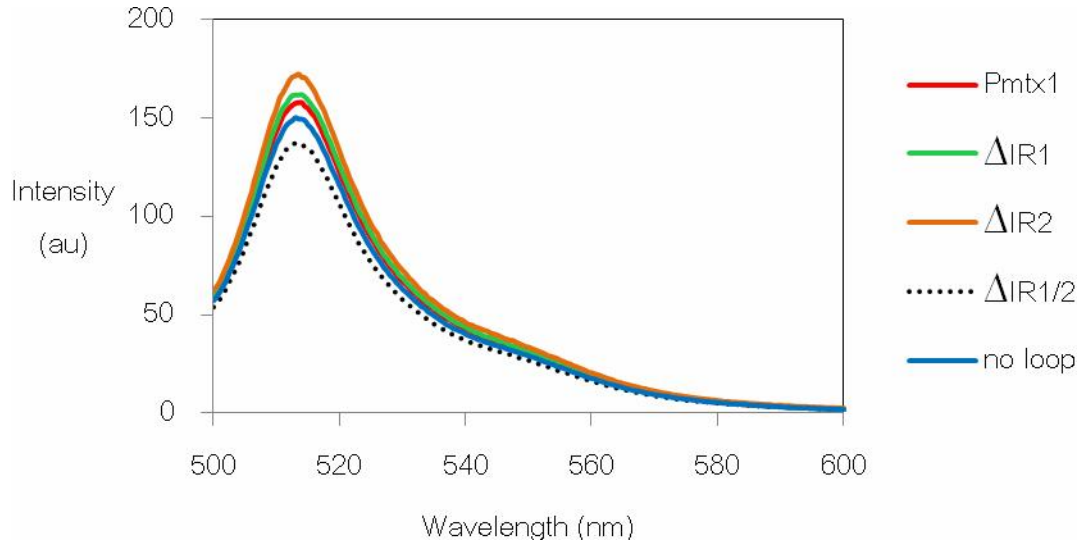


Figure 3. Fluorescent intensity of *Bs*2297 (A) and *Bsu*168 (B) containing different Pmtx1 constructs. Graphs were generated from average values from 2 independent experiments. Red line is WT P*mtx1*, green line is ΔIR1, orange line is ΔIR2, black dot line is ΔIR1/2, and blue line is Pmtx1 with no stem loop.

Mtx1 production from *Bt*4Q7 (pBCX-cyt2APro-tMtx1) and its larvicidal activity

The previous study showed that protein Mtx1 could be expressed during sporulation phase in *Bt*4Q7 using *bin* promoter and the recombinant cells were toxic to *Culex* larvae [8]. We aim to increase Mtx1 production during sporulation phase by using promoter of *cyt2A* gene. It is sporulation specific that was identified in the lab showing to improve protein expression up to 6 times. *Bt*4Q7 cells harboring pBCX-cyt2APro-tMtx1 were grown in 25 ml of CCY medium at 30°C until complete sporulation [20]. Culture was centrifuged and pellet was resuspended in 1 ml of water. These concentrated spore/protein complex suspension was tested for larvicidal activity against *Culex* larvae (Table 1). It was shown that *Bt*4Q7 (pBCX-cyt2APro-tMtx1) was toxic to *Culex* larvae, while *Bt*4Q7 containing vector without *mtx1* gene did not kill the larva. These results demonstrate that Mtx1 was produced by *Bt*4Q7 and the protein is active.

Table 1. Toxicity of *Bt*4Q7 containing pBCX-cyt2APro-tMtx1 against *Culex* larvae reported as percentage of dead larvae.

Strains	Protein suspension volume (μl)				
	100	50	25	12.5	6.25
*Bt*4Q7 (pBCX-cyt2APro)	0	5	5	5	5
*Bt*4Q7 (pBCX-cyt2APro-tMtx1)	100	100	100	95	75

Western blot analysis showed that *Bt*4Q7 (pBCX-cyt2APro-tMtx1) produced protein at ~97 kDa which is consistent with the size of truncated Mtx1 (Fig.4). However, Mtx1 protein could not be observed on SDS-PAGE, but only detected by western blot analysis. This implies that Mtx1 is produced at very low level. Although *cyt2A* promoter could induce high expression level of other proteins, but it appears that this is not the case for Mtx1 protein. It is possible that the produced Mtx1 is gradually degraded by proteases.

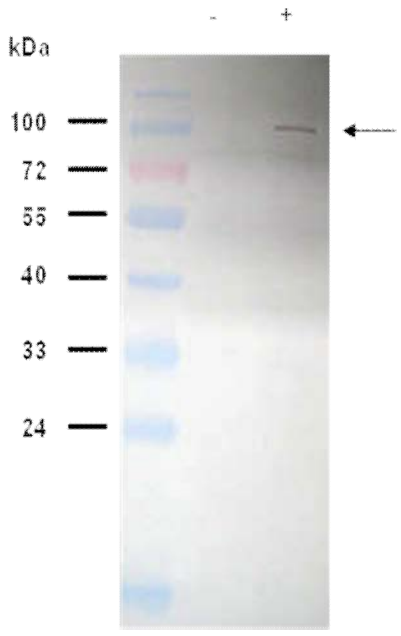


Figure 4. Western blot analysis of *Bt*4Q7 (pBCX-cyt2APro-tMtx1). 20 μl of protein-spore suspension was loaded. (-) represents sample from *Bt*4Q7 (pBCX-cyt2APro) and (+) represents sample from *Bt*4Q7 (pBCX-cyt2APro-tMtx1). Arrow indicates tMtx1 protein.

Mtx1 production from *B. subtilis* WB800 (pHT43-tMtx1) and its larvicidal activity

Mtx1 contains a putative signal peptide and is probably secreted outside the cells. Therefore it is interesting to utilize *B. subtilis* as a host because *B. subtilis* is suitable for expression of secreted protein. One of the reasons that make *B. subtilis* a good host for secreted protein because an extracellular proteases deficient strain, WB800, was constructed to minimize protein degradation. Therefore it was expected that the secreted Mtx1 would be stable. WB800 (pHT43-tMtx1) cells were grown in LB at 37 °C and samples were collected at different times and were subjected to western blot analysis (Fig. 5). It was shown that at early growth phase (5 h after induction), tMtx1 protein was present both in the cells and in the media. At stationary phase, majority of tMtx1 was secreted into media. Almost 2 days after induction (44 h), tMtx1 could be detected in the media and majority of the protein was present as protoxin form indicating no degradation of the tMtx1.

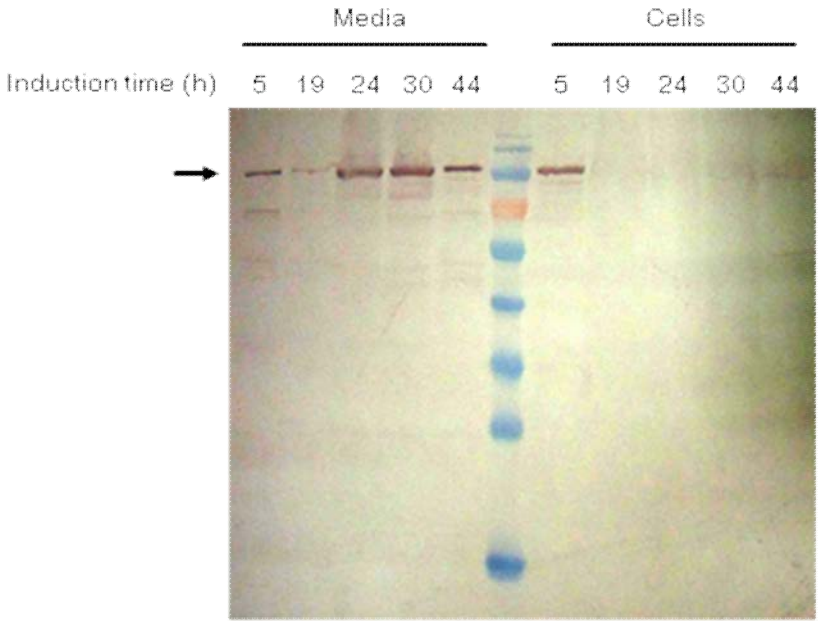


Figure 5. Western blot analysis of WB800 (pHT43-tMtx1). A) Samples were collected at 5, 19, 24, 30, and 44 hours after induction with 1 mM IPTG. 1 ml of cell culture was centrifuged and supernatant fraction was precipitated with TCA (10% final) before loading on SDS-PAGE. Arrow indicates tMtx1 protein.

Next, larvicidal activity of WB800 (pHT43-tMtx1) was tested against *Culex* larvae to determine whether secreted tMtx1 protein is biologically active. Cells were collected at 24 hours after IPTG induction and supernatant fraction was tested for larvicidal activity. Since we could not determine the amount of secreted tMtx1, 2-fold serial dilution of culture supernatant was performed in the assay. Culture media of WB800 (pHT43-tMtx1) was more toxic to *Culex* larvae compared to culture media of WB800 (pHT43) (Table 2). It is interesting that culture media of WB800 (pHT43) was also toxic to *Culex* larvae. There is a study reporting that culture supernatant of *B. subtilis* is toxic to larval and pupal stages of mosquitoes and this due to surfactin secreted by the cells [21, 22]. Therefore, it is likely that WB800 also secrets surfactin that is toxic to larvae.

Table 2. Toxicity of WB800 (pHT43-tMtx1) against *Culex* larvae reported as percentage of dead larvae. Values presented here are the average from seven independent experiments.

Strains	Dilution of culture media					
	1/16	1/32	1/64	1/128	1/256	1/512
WB800 (pHT43)	68	58	9	5	1	2
WB800 (pHT43-tMtx1)	94	78	61	36	11	10

To evaluate stability of tMtx1 protein, culture medium was stored at 4°C for 5 and 12 months and western blot was performed again (Fig. 6). It appeared that majority of tMtx1 was not degraded after 1 year storage. Larvicidal activity of these long storage samples was also tested, unfortunately, activity of secreted tMtx1 drastically decreased (Table 3). These results imply that although tMtx1 was not degraded, it lose its active conformation upon long period of storage.

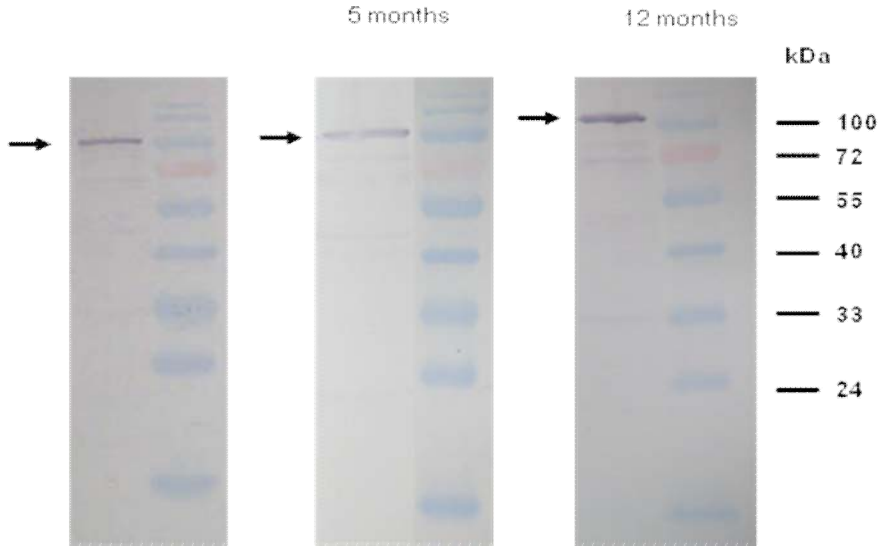


Figure 6. Stability of secreted tMtx1 from WB800. Western blot analysis of samples stored at 4°C for 5 and 12 months. Arrow indicates tMtx1 protein.

Table 3. Toxicity of WB800 (pHT43-tMtx1) against *Culex* larvae after long storage. Values are reported as percentage of dead larvae. ND is not determined.

WB800 (pHT43-tMtx1)	Dilution of culture media						
	1/8	1/16	1/32	1/64	1/128	1/256	1/512
0 month	ND	90	75	75	60	5	0
5 months	ND	35	35	5	5	0	0
12 months	45	35	10	0	5	0	ND

There are different strategies to improve heterologous proteins expression from *B. subtilis*. PrsA is an extracellular chaperone that helps refold secreted mature protein into an active conformation, and signal peptidases (Sip) that remove mature protein from signal peptide can be a rate limiting of protein secretion process. It was shown that PrsA protein is essential for efficient secretion of some exoproteins and overproduction of PrsA enhances the secretion of certain protein [23, 24]. With engineered increasing protein synthesis, SipS and SipT function to increase the secretion capacity [25, 26]. Therefore these 3 proteins; PrsA, SipS, and SipT were co-expressed with Mtx1 to determine whether they can improve Mtx1 production. The plasmid pAD123-PrsA, pBCX (cyt2A)-SipS, and pBCX (cyt2A)-SipT was individually transformed into WB800 (pHT43-tMtx1). Culture supernatant of these cells was collected and subjected to western blot analysis to evaluate Mtx1 production. The results showed that Mtx1 expression level from all three co-expression cells was comparable to WB800 (pHT43-tMtx1) (Fig. 7) suggesting that PrsA, SipS, and SipT did not improve Mtx1 production from *B.subtilis*. Larvicidal activity of these co-expression

cells was similar to WB800 (pHT43-tMtx1) (data not shown). It is consistent with western blot result.

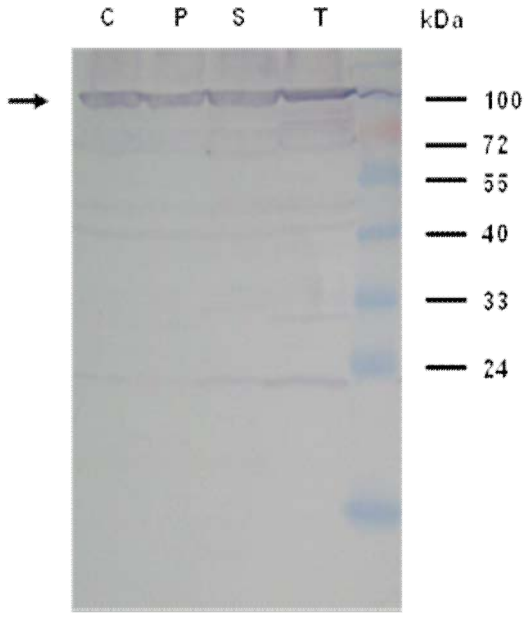


Figure 7. Mtx1 production from WB800 (pHT43-tMtx1) with co-expression of PrsA, SipS, and SipT. C represents sample of WB800 (pHT43-tMtx1). P, S, and T represent sample of WB800 (pHT43-tMtx1) with co-expression of PrsA, SipS, and SipT, respectively. Arrow indicates tMtx1 protein.

Conclusions

Mtx1 protein from *B. sphaericus* has high activity against mosquito larvae, but it is not in use because of its low expression and instability. There is inverted repeat present upstream of ribosome binding site of *mtx1* gene and stem loop structure formed by this inverted repeat was proved to plays a role in repression of Mtx1 in *B. sphaericus*. In order to improve Mtx1 production, a strong sporulation-specific promoter was use to express Mtx1 in *Bt*4Q7. The active Mtx1 was successfully produced, but the expression level was still low. In addition, *B. subtilis* WB800 was used as a host to secrete tMtx1 into medium. The secreted tMtx1 was not degraded but lost larvicidal activity upon long storage.

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- จัดเตรียม manuscript “Repression mechanism of Mtx1 and its production from alternative Bacilli hosts”

2. การนำผลงานวิจัยไปใช้ประโยชน์

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3. อื่นๆ

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