J.Sci.Soc.Thailand, 22(1996) 121-130

THE EFFECT OF *cryIVB* TERMINATOR FRAGMENTS ON EXPRESSION OF THE CHLORAMPHENICOL ACETYLTRANSFERASE GENE IN *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS*

NANTAREE SIRICHOTPAKORN,[a] WATANALAI PANBANGRED,[a] SOMSAK PANTUWATANA[b] AND AMARET BHUMIRATANA[a]

[a] *Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.*

[b] *Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.*

(Received May 21, 1996)

ABSTRACT

A ClaI-BamHI fragment harbouring the terminator region of cryIVB gene encoding 130 kDa δ-endotoxin protein from Bacillus thuringiensis *subsp.* israelensis *was cloned into* E. coli *cloning vector pGEM7-Zf(+) to allow introduction of useful deletion sites and T7 primer region for sequence analysis. The new recombinant plasmid was designated as pGBT8 which was subjected to further manipulation leading to the construction of three additional clones. The first clone harboured plasmid pGBT8.1 which contained 221 bp of distal portion of cryIVB gene and its terminator. The second clone harboured plasmid pGBT8.2 which contained 127 bp of completed cryIVB terminator. The third clone harboured plasmid pGBT8.3 which contained 86 bp of the cryIVB gene or only half of cryIVB terminator. All three clones containing various fragments of cryIVB terminator were further subcloned into the multicloning sites of pTFM6 vector which located at the position 5' to the cat-86 terminator. Thus, three double terminators constructs were obtained and designated as pTT1, pTT2, pTT3 respectively. In order to study the effects of single cryIVB terminator, a derivative of pTT2 where cat-86 terminator was deleted was constructed and designated as pTT2Δ. All the recombinant plasmids were first transformed into* Bacillus subtilis *MI113 and subsequently transformed into* Bacillus thuringiensis *c4Q272 using electroporation technique. The presence of cloned terminator regions in the newly constructed plasmids was confirmed by restriction patterns and Southern blot hybridizations. The expression levels of chloramphenicol acetyltransferase gene (cat) was determined by measuring the specific activity of the enzyme chloramphenicol acetyltransferase (CAT). The cat gene's products were assayed in crude extracts of both* B. subtilis *MI113 and* B. thuringiensis *subsp.* israelensis *c4Q272 grown in nutrient broth supplemented with minerals and harvested at the various growth phases, namely, mid log, at the on-set of sporulation (T_0), and 8 h after on-set of sporulation (T_8). By comparing the CAT activities in* B. subtilis *and* B. thuringiensis *hosts harbouring various plasmid constructs, it could be concluded that cryIVB terminator had a stimulatory effect on chloramphenicol acetyltransferase gene expression in* B. thuringiensis *subsp.* israelensis *c4Q272.*

INTRODUCTION

Among various entomopathogenic micro-organisms, *Bacillus thuringiensis* has been one of the most successful and widely used microbial insecticides. Advantages of microbial based biopesticides over synthetic organic insecticides include the high degree of specificity toward target insects, no harmful effects toward human, animals and beneficial insects and relatively less "polluting" to the environment. The availability of genetic engineering techniques for use in strain improvement together with the increased awareness of environmental conditions by the public sector toward pesticide usage, have led to dramatic increase in biological insecticide research by both academic and industrial sectors.[1-3]

Bacillus thuringiensis is a gram-positive soil bacterium which produces parasporal crystalline inclusions during sporulation. These inclusions consist of proteins exhibiting highly specific insecticidal activities.[4,5] The amount of crystal protein production was about 20 to 30% of the total dry weight in sporulated culture. A number of factors have been proposed to account for the high level of δ-endotoxin production in *B. thuringiensis*, for example, stable inheritance of the δ-endotoxin genes,[6] gene dosage,[7] strength of the transcription promoter,[6] the efficiency with which the transcribed message is translated and the stability of the mRNA transcript.[8] It has been proposed that the stability of mRNA may result from the putative transcriptional termination of a toxin gene (a stem-loop structure) which acts as a positive retroregulator and subsequently increases the half life of the mRNA. Basing on the fact that the crystal proteins are usually produced in large quantity, it is quite interesting to note that the potential terminator sequences found

downstream of the different *cry* genes are frequently highly conserved.[1,9] The fusion of such putative terminator with the 3′ end of the heterologous genes might enhance the half life of their transcripts and consequently their expression levels. The objective of this study was to investigate the effect of terminator of *cryIVB* gene which encoded for 130 kDa δ-endotoxin in *Bacillus thuringiensis* subsp. *israelensis* (*B.t.i.*) on the expression of chloramphenicol acetyltransferase gene (*cat-86*), a reporter gene. Various plasmid constructs were achieved by constructing the recombinant plasmids harbouring various sizes of terminator region of the *cryIVB* gene. The levels of chloramphenicol acetyltransferase enzyme (CAT) expression of these various constructs were examined in both *B. subtilis* and *B.t.i.* hosts.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture media

B. thuringiensis subsp. *israelensis* strain c4Q272 was obtained from the Bacillus Genetic Stock Center (Ohio State University). *B. subtilis* MI111 (*arg*-15 *leu*A8 $r_M m_M$), and the plasmid pTF6 (a 1.7kb, *Eco*RI-*Hpa*I fragment of pHG5 containing the promoter of *bgaB* gene inserted in *Eco*RI-*Pst*I site of pPL603) have been described previously.[10] *B. subtilis* MI113 (*arg*-15, *trp*C2 *hsm*M, *hsr*M) was obtained from Prof. T. Imanaka (Biotechnology research laboratory, Osaka University). *E. coli* DH5α (F- f80d lacZΔm15Δ(lacZYA-argF) U19 recA1 endA1hsdR17(r^-_k, m^+_k) supE44 1-thi-1gyrA96 relA1 (BRL)) and the plasmid pGEM7 (*E. coli* cloning vector harbouring multicloning sites in the *lacZ* gene) were used in plasmid manipulations involving deletion and nucleotide sequencing. Bacteria were grown in Luria Bertani (LB) medium or Penassay broth (Difco Laboratories). Where indicated, ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), rifampicin (50 μg/ml) or kanamycin (10 μg/ml) were supplemented to the media.

Isolation of DNA, transformation, and Southern blot DNA-DNA hybridization

Preparation and transformation of plasmids DNA from *E. coli* were performed followed the standard protocol.[11] Preparations of plasmid from *B. subtilis* MI111, MI113 and *B. thurigiensis* subsp. *israelensis* strain c4Q272 were performed by alkaline lysis method as described by Birnboim and Doly.[12] Transformations of *B. subtilis* MI113 and *B. thuringiensis* subsp. *israelensis* strain c4Q272 were performed by electroporation method as described by Shoda *et al.*[13] Southern analysis was carried out followed the standard protocol.[11] Dig labelling and detection kit obtained from Boehringer Mannheim was used in hybridization experiment. Restriction endonucleases, and T4 DNA ligase were obtained from either Bethesda Research Laboratories (BRL), Biolab (New England), or Boehringer Mannheim.

Plasmid deletion and DNA sequencing

Nested sets of deletion were generated with exonuclease III enzyme (BRL) as described in standard method.[11] DNA sequencing was carried out by the dideoxy chain termination method of Sanger *et al.*[14] For the chain termination method, the sequencing kit was obtained from Pharmacia and [35s]αdATP (Amersham Corp).

CAT enzyme assays

The CAT enzyme was assayed by the colorimetric procedure described by Shaw[15] and protein was measured by the Bradford procedure[16] using protein assay kit from BioRAD. CAT specific activities were expressed as unit per milligram protein, and 1 unit is equivalent to the amount of enzyme which releases 1 μmole of acetylated chloramphenicol per minute under the standard assay conditions.

RESULTS

Construction of derivatives of pGBT8 plasmids containing various length of terminator fragments from *cryIVB* gene

To facilitate the construction of various length of terminator fragments from *cryIVB* gene of *B. thuringiensis* subsp. *israelensis* (*B.t.i.*), plasmid pGBT8 was constructed by cloning a 600 bp *Cla*I and *Bam*HI fragment from plasmid pBT8[17] containing *cryIVB* terminator into pGEM7 cloning vector (Fig. 1(a)). Deletion and sequencing analysis of pGBT8 plasmid was performed in order to select the clones with appropriate deletion fragments. Three representative clones were selected. The first clone harboured plasmid pGBT8.1 which composed of pGEM7, 96 bp at the 3′ end of the structural *cryIVB* gene and a completed terminator fragment of the *cryIVB* gene containing 123 bp. The second clone harboured plasmid pGBT8.2 which composed

only of the 123 bp of *cryIVB* terminator fragment which represented the completed *cryIVB* terminator region. The third clone harboured plasmid, pGBT8.3 which contained 86 bp of terminator fragment which represented only a portion of the *cryIVB* terminator region. The physical maps of plasmids pGBT8.1, pGBT8.2, and pGBT8.3 are shown in Fig. 1(b).

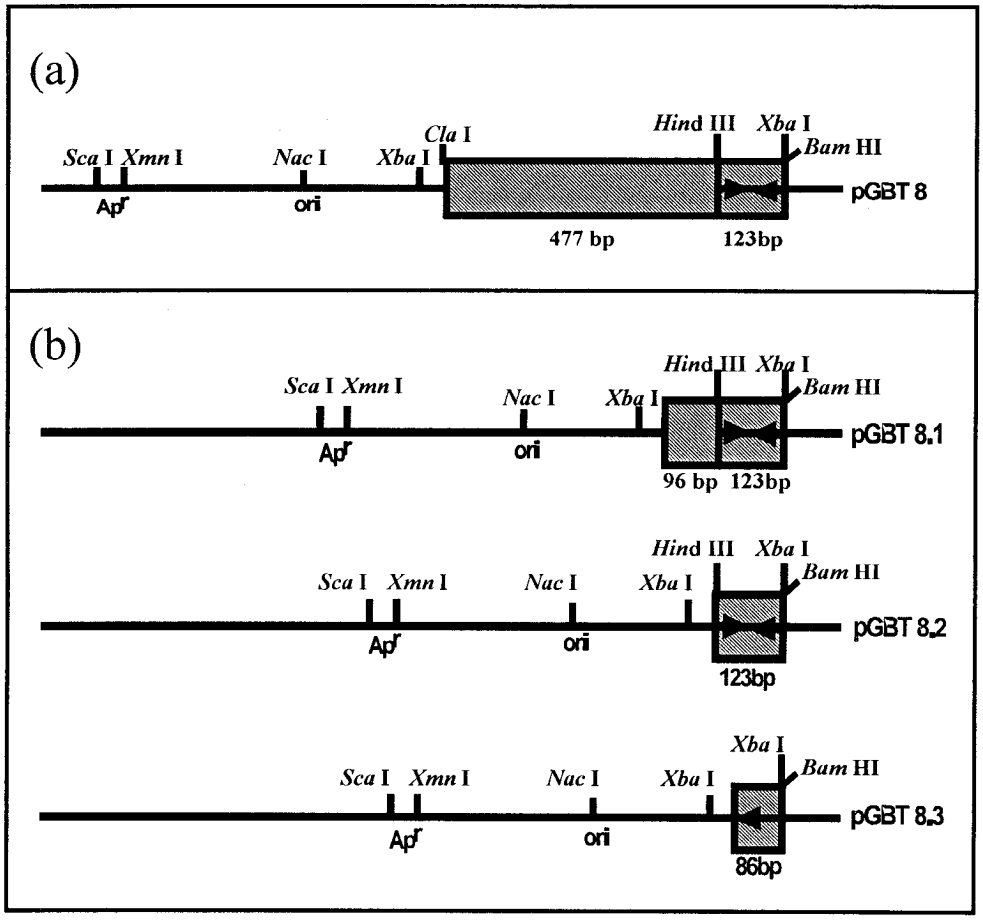

Fig. 1 Schematic representation of plasmid pGBT8 and its derivatives. The plasmid pGBT8 (a) was constructed from plasmid pGEM7 with the addition of a 600 bp *Cla*I- *Bam*HI fragment representing 3′ end of *cryIVB* gene. Physical maps of pGBT8.1, pGBT8.2, and pGBT8.3 which were nested sets of deletion of plasmids derived from pGBT8 were shown in (b). The plasmids pGBT8.1, pGBT8.2 and pGBT8.3 contained various fragments of 3′ end of *cryIVB* gene. The shaded box (■) indicated the *cryIVB* gene. The dark facing arrows (➡⬅) showed the inverted repeat sequences which represented *cryIVB* terminator and single dark arrow (⬅) showed half of *cryIVB* terminator. The abbreviations are as follows, Ap^r, ampicillin resistance gene; ori, origin of replication.

Construction of terminator cloning vector

To facilitate the assessment on the effect of *cryIVB* terminator sequence on heterologous gene expression in *B. subtilis* and *B.t.i.* hosts, a system involving the attachment of various fractions of terminator on to a reporter gene, the chloramphenicol acetyltransferase gene (*cat-86*) was constructed. For convenience in achieving desire plasmid constructs, the vector modification were carried out in *E. coli* host prior to transforming into *B. subtilis* and *B.t.i.* The strategies for construction of plasmid pTFM6 were as follows. Firstly, a 1.6 kb *Eco*RI-*Pvu*II fragment of pTF6 containing *cat-86* gene was ligated into pBR322 vector. Then, a 95 bp *Apa*I-*Nsi*I fragment of pGEM7 was inserted in the 3′ end of *cat-86* gene in order to introduce the essential terminator cloning sites. Finally, the 1.5 kb *Pst*I-*Pvu*II fragment of pTF6 was replaced by the previous similar fragment

where multicloning site was inserted. Therefore a new plasmid vector namely pTFM6 harbouring the reporter gene (*cat-86* gene), the multicloning site at distal of *cat-86* gene and a *Bacillus* origin of replication, was used in further experiments for insertions of various fragments of *cryIVB* gene terminator. The detailed map of plasmid pTFM6 is illustrated in Fig. 2.

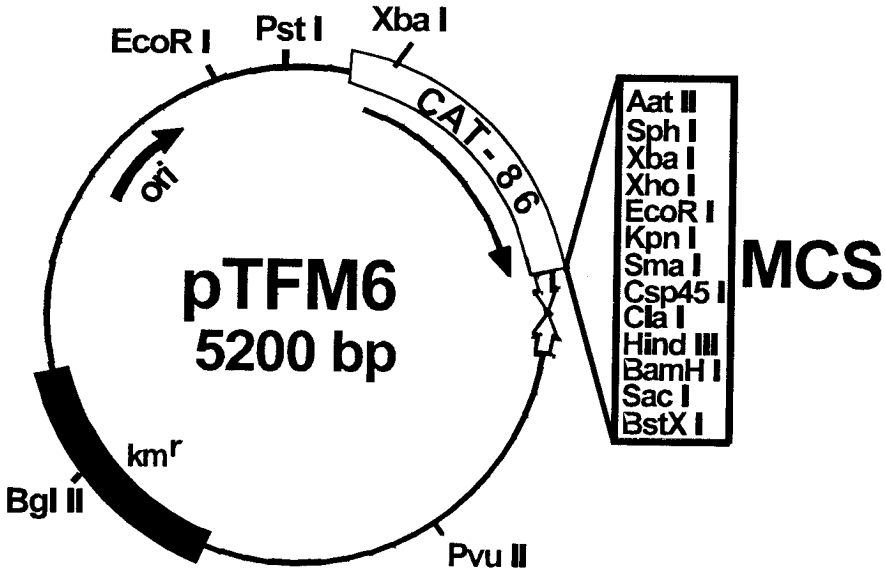

Fig. 2 Schematic representation of terminator cloning plasmid pTFM6. The plasmid contained 600 bp of *cat-86* gene (□) and 95 bp of multicloning sites inserting between *cat-86* stop codon and *cat-86* terminator (⇨⇦). The light facing arrows following *cat-86* designate inverted repeat sequences which represented the *cat-86* terminator. The long arrow designates the direction of *cat-86* transcription. The short arrow labelled *ori* designated the direction of replication of the plasmid. The abbreviations were as follows: km^r, kanamycin resistance gene; MCS, multicloning site; ori, origin of replication.

Subcloning of three *cryIVB* terminator fragments into plasmid pTFM6

Various segments of the terminator region of *cryIVB* δ-endotoxin gene were linked to the terminal part of the *cat-86* structural gene in pTFM6 plasmid. The *Xho*I-*Bam*HI fragment of all three plasmids, pGBT8.1, pGBT8.2 and pGBT8.3 were ligated into pTFM6 plasmid between the 3′ end of *cat-86* structural gene and *cat-86* terminator. Three new plasmids were obtained. The first plasmid which was designated as pTT1 contained a portion of 3′ end of *cryIVB* toxin gene, *cryIVB* terminator and *cat-86* terminator. The second plasmid which was designated as pTT2 contained *cryIVB* terminator and *cat-86* terminator. The third plasmid which was designated as pTT3 contained only half of *cryIVB* terminator and *cat-86* terminator. Furthermore, in the attempts to compare the effect between *cat-86* terminator and *cryIVB* toxin terminator on the expression of *cat-86* gene, the plasmid composing only of *cryIVB* toxin terminator was constructed by deletion of *cat-86* terminator in pTT2 plasmid resulting in a new derivative plasmid lacking *cat-86* terminator which was designated as pTT2Δ. The schematic representation of these plasmids appears in Fig. 3. These plasmids were transformed into *B. subtilis* MI113 and subsequently to *B.t.i.* hosts.

In order to confirm the presence of relevant plasmids in *B. subtilis* and *B.t.i.* hosts, Southern hybridizations were carried out in all the strains obtained using undeleted *cryIVB* terminator fragment from pBT8 as probe. Also, the presences of the *cat-86* genes in all the constructs were confirmed by the used of labelled *cat-86* terminator fragment from pTF6 plasmid as probe. The Southern hybridization data as illustrated in Fig. 4 confirmed the presence of the *cryIVB* terminator in all the transformants of *B. subtilis* and *B.t.i.* hosts. Similar experiments also confirmed the presence of the *cat-86* genes in all the transformants of *B. subtilis* and *B.t.i.* hosts.

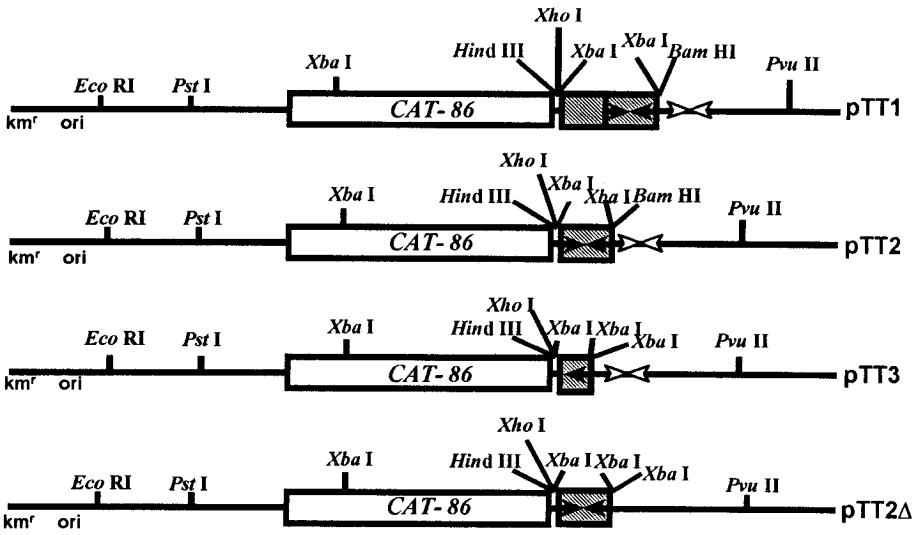

Fig.3 The restriction maps of plasmids pTT1, pTT2, pTT3 and pTT2Δ. These plasmids were constructed from pTFM6 terminator cloning vector by insertion of various sizes of *cryIVB* terminator fragment between *cat-86* stop codon and *cat-86* terminator. The dark facing arrows (➡⬅) indicated the *cryIVB* terminator and the light facing arrows (⇨⇦) indicated the *cat-86* terminator. The single arrow (⬅) represented half of *cryIVB* terminator. The shaded areas (▩) represented *cryIVB* gene fragments, and the light area (□) represented *cat-86* gene.

Expression of chloramphenicol acetyltransferase gene (*cat*) in *B. subtilis* and *B.t.i.* hosts

The chloramphenicol acetyltransferase gene was employed as marker to examine the effect of *cryIVB* terminator on gene expression. The levels of CAT activities were determined during various phases of growth namely, at mid log (m), at the on-set of sporulate (T_0), and 8 h after sporulate (T_8) in both *B. subtilis* MI113 and *B.t.i.* hosts containing each plasmid which was designated as pTF6, pTFM6, pTT1, pTT2, pTT3, and pTT2Δ (Table 1 and Table 2). The presence of CAT activities in all the hosts under investigation indicated that all the plasmids which contained the same vegetative promoter from thermostable β-galactosidase I gene of *B. stearothermophilus* IAM 11001 conferred high CAT specific activities in the exponential phase of growth in *B. subtilis* MI113 and *B.t.i.* hosts. It was observed that in *B. subtilis* host (Table 1) the highest enzyme activities could be observed at the on-set of sporulation phase (T_0) and CAT activities decreased after 8 h after the on-set of sporulation (T_8). These observations were true for all cultures regardless of the type of the plasmids being presence in each host.

As expected, the insertion of multicloning site into the pTF6 plasmid did not interfere with the expression of CAT activities as evident in the similarity in the pattern of CAT activities in the *B. subtilis* hosts containing either pTF6 or pTFM6 (Table 1). The presences of various constructs of complete *cryIVB* terminators (pTT1, pTT2, pTT3) did not have much effect on the expression of *cat-86* in *B. subtilis*. However, the activities of CAT appeared to decrease somewhat, to the level of 11.90 units/mg protein if only half of the *cryIVB* terminator was inserted (pTT3). It was, also, of interest to note that the complete *cryIVB* terminator could substitute for the *cat-86* terminator as illustrated by the similar levels of CAT activities in hosts with plasmids pTT2 and pTT2Δ.

When *B.t.i.* was used as host for various plasmid constructs, it was found that the chloramphenicol acetyltransferase genes were well expressed in all four recombinant plasmids containing various fragments of *cryIVB* terminator as well as the plasmid pTF6. Data as illustrated in Table 2 indicated that the level of expressions of CAT activities appeared to be generally higher when *B.t.i.* was used as host than those of *B. subtilis*. These observations might be due to their strong vegetative promoter of thermostable β-galactosidase I gene as well as the presence of fragments of *cryIVB* terminator which could be better functioned in *B.t.i.*

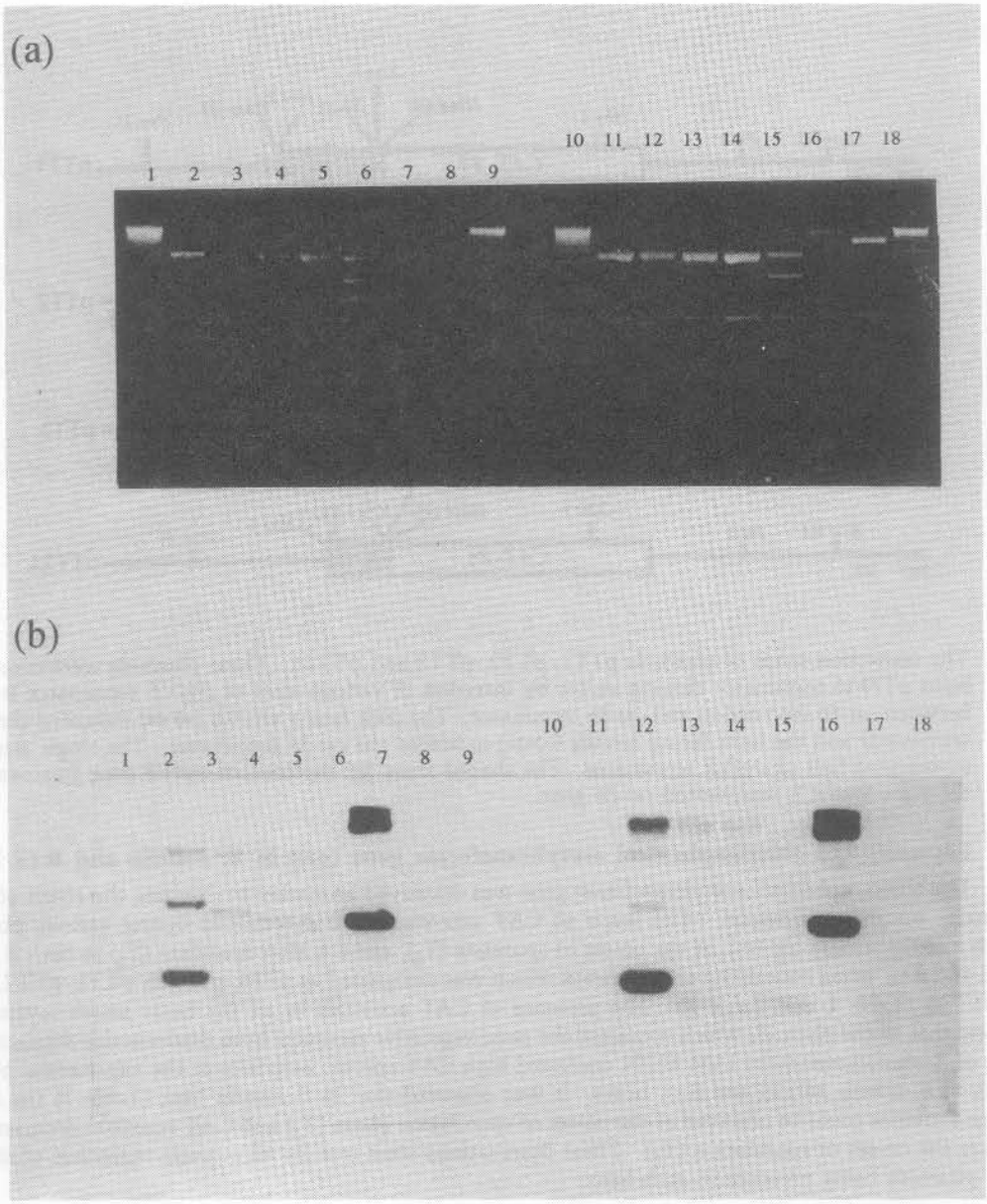

Fig. 4 Southern hybridization of plasmids pTT1, pTT2, pTT3 and pTT2Δ obtained from *B. subtilis* MI113 and *B. thuringiensis* subsp. *israelensis* c4Q272 hosts with 11 d-UTP labelled *Cla*I-*Bam*HI fragment of 3′ end of *cryIVB* toxin gene probe. Photograph (a) on the right side, (lane 10 through 18) showed agarose gel electrophoresis patterns of *Eco*RI-*Bgl*II-*Xba*I restricted pTT1 (lane 12), pTT2 (lane 13), pTT3 (lane 14), pTT2Δ (lane 15) obtained from *B. subtilis* MI113 hosts. Photograph (a) on the left side, (lane 1 through 9) showed agarose gel electrophoresis patterns of *Eco*RI-*Bgl*II-*Xba*I restricted pTT1 (lane 3), pTT2 (lane 4), pTT3 (lane 5) pTT2Δ (lane 6) obtained from *B. thuringiensis* subsp. *israelensis* c4Q272 hosts. Plasmid pTFM6 was digested with *Eco*RI-*Bgl*II-*Bam*HI which provided as negative control (lanes 2 and 11). Plasmid pBT8 was digested with *Cla*I-*Bam*HI which provided as positive control (lanes 7 and 16). Plasmid pTF6 was digested with *Xba*I-*Pvu*II (lanes 8 and 17). Lanes 1 and 10 represented *Pst*I digested λ DNA. Lanes 9 and 18 represented *Hind*III digested λ DNA. Photograph (b) showed Southern hybridization resulting from the transfer of the DNA from the gel shown in photograph (a) to the nylon membrane and hybridized with 11 d-UTP labelled *Cla*I-*Bam*HI fragment of 3′ end of *cryIVB* toxin gene probe.

Table 1 Activities of chloramphenicol acetyltranferase in *Bacillus subtilis* strain MI113 harbouring pTF6, pTFM6, PTT1, pTT2, pTT2Δ or pTT3 at various phases of growth.

Growth Phase	CAT specific activity (U/mg protein)					
	pTF6	pTFM6	pTT1	pTT2	pTT2Δ	pTT3
Mid log (M)	8.46	8.57	7.17	10.00	8.52	7.98
At the on-set of sporulation (T_0)	17.28	21.92	18.49	16.04	18.60	11.90
Eight hours after the on-set of sporulation (T_8)	12.90	13.30	11.03	12.90	12.88	8.16

Table 2 Activities of chloramphenicol acetyltranferase in *Bacillus thuringiensis* subsp. *israelensis* c4Q272 harbouring pTF6, pTT1, pTT2, pTT2Δ and pTT3 at various phases of growth.

Growth Phase	CAT specific activity (U/mg protein)				
	pTF6	pTT1	pTT2	pTT2Δ	pTT3
Mid log (M)	12.53	14.35	14.20	11.00	9.74
At the on-set of sporulation (T_0)	22.67	22.63	25.90	25.15	22.70
Eight hours after the on-set of sporulation (T_8)	14.75	20.07	23.56	24.67	15.99

It was further found that for all plasmid constructs the level of CAT activities were higher at the on-set of sporulation (T_0) than the mid log cultures (Table 2). The highest specific activities were obtained from *B.t.i.* cultures harvested at the on-set of sporulation (T_0) of all the plasmids being examined. The CAT specific activities at various phases of growth of pTT1, pTT2, pTT2Δ seemed to be slightly increased when compared to the CAT specific activities of pTF6. The specific activities of the cultures harvested after 8 h after the on-set of sporulation were found to decline rapidly in *B.t.i.* crude extracts of cultures harbouring pTF6 and pTT3, whereas in *B.t.i.* harbouring pTT1, pTT2 and pTT2Δ the activities were only slightly decreased. However, when the CAT activities were measured at 8 h after the on-set of sporulation *i.e.* T_8, the hosts with complete *cryIVB* terminators (pTT1, pTT2 and pTT2Δ) possessed higher CAT activities than those with out *cryIVB* terminator (pTF6) or those with only half of the *cryIVB* terminator (pTT3).

When *cat-86* terminator was deleted from plasmid construct (pTT2Δ), it was found that there appeared to be no difference in the expression of CAT activities. This observation was similar to the one made earlier when *B. subtilis* was used as host for plasmid pTT2Δ. Again, the presence of only *cryIVB* terminator seemed to be sufficient for high expression of CAT activities in *B.t.i.* hosts. The presence of complete *cryIVB* terminator appeared to lead to the maintain of high CAT activities at T_8 only in *B.t.i.* hosts but not when *B. subtilis* were used as hosts. The CAT activities could not be maintained at high level at T_8 when the incompleted *cryIVB* terminator were presence or when the entire *cryIVB* terminator was absence in both the *B. subtilis* and *B.t.i.* hosts.

DISCUSSION

In *B. thuringiensis* subsp. *israelensis*, the mosquitocidal toxin synthesis has been reported to begin at T_2 (*i.e.* 2 h after on-set of sporulation) stage of growth and sporulation.[18,19] It was found that the regulation of this δ-endotoxin synthesis was sporulation specific event. The high level of crystal protein synthesis in *B. thuringiensis* has been found to be regulated by distinct mechanisms at transcriptional and post transcriptional level.[1] The termination sequence also has been shown to involve in the high level of δ-endotoxin production. Stable stem-loop structures associated with termination sequences have been shown to prolong the message half life through decreasing ribonuclease activities.[20] The efficient and faithful termination is an important factor in optimising gene expression.[21,22] The rate of particular protein synthesis would depends on the steady state level of mRNA in the cell which is a reflection of endoribonuclease and 3′ exoribonuclease attack, which in turn results from difference in mRNA sequence and structure as well as the association of macromolecules such as ribosomes with the messenger RNA.[23,24] According to the important role of the transcription terminator, the 130 kDa *cryIVB* gene terminator of *B.t.i.* was the subject of this study. Series of recombinant plasmids harbouring various sizes of *cryIVB* terminator fragments were constructed and the effects of the terminator were determined by comparing the specific activity of chloramphenicol acetyltransferase enzyme in both *B. subtilis* MI113 and *B.t.i.* hosts. The expression of *cat-86* gene could be detected since the early stage of growth in all cultures of *B. subtilis* MI113 and *B.t.i.* containing various derivatives of the plasmids. This might be due to the fact that the *bgaB* promoter of pTF6 which was included in all plasmid constructs being investigated in this study, were recognized by σ^{43} which directed the RNA polymerase to start transcription in the vegetative stage of cell growth. Results obtained in this study indicated that the CAT activities in *B.t.i.* hosts were generally found to be higher than the CAT activities obtained from *B. subtilis* harbouring those plasmids. Such results might be due to the efficiency of *bgaB* promoter, the stability of *cat* mRNA transcripts or the stability of CAT protein were better in *B.t.i.* than *B. subtilis* MI113. The highest level of CAT activities were obtained at the on-set of sporulation phase (T_0) of both host cultures regardless of the types of the plasmid presence in the hosts. This might resulted from the maximum accumulation of the translated protein at this stage. The presence of half *cryIVB* terminator lead to the decrease in CAT activities obtained from both *B. subtilis* and *B.t.i.* hosts (those harbouring plasmids pTT3). The effects of terminator in sporulation phase were determined after 8 h of the on-set of sporulation phase (T_8). The results showed that the levels of CAT activities were markedly decreased in T_8 stage of *B. subtilis* harbouring various plasmids. Thus, *cryIVB* terminator in plasmid pTT1, pTT2, pTT2Δ and pTT3 did not help in maintaining the level of CAT activities in *B. subtilis* MI113 hosts. It might be possible that, in *B. subtilis,* the mRNA was degraded by ribonuclease at the same rate with or without the presence of *cryIVB* terminator in addition to the *cat-86* terminator. In contrast, the CAT activities at T_8 stage in *B.t.i.* cultures harbouring complete *cryIVB* terminator (pTT1, pTT2, pTT2Δ) were found to be similar to the T_0 stage. Thus, the fragments containing the *cryIVB* terminator in pTT1, pTT2 and pTT2Δ might help to maintain the level of CAT activities in the sporulation phase of *B.t.i.* regardless of the variation in the *cryIVB* terminator constructs. Although *cryIVB* terminator could confer to the stability of the mRNA in T_8 stage, the degradation of CAT protein might still occur. Thus, the CAT activities of *B.t.i.* cultures containing plasmid with *cryIVB* terminator were not found to increase after the T_0 stage. The level of CAT activity obtained from T_8 stage of *B.t.i.* harbouring pTF6 and pTT3 was found to decrease from the level obtained at the T_0 stage. It was shown that either *cat* mRNA or CAT protein was rapidly degraded in *B.t.i.* hosts containing plasmids pTF6 or pTT3. Although plasmid pTT3 contained half of *cryIVB* terminator, it was found not to be able to stabilize the mRNA transcripts. Thus, the presence of both *cat-86* terminator and half of *cryIVB* terminator might not be able to protect mRNA from ribonuclease digestion. Furthermore, the additional *cryIVB* gene sequence in plasmid pTT1 did not seem to interfere with the efficiency of transcription termination. The deletion of *cat-86*

terminator (pTT2Δ) from the double terminator construct (pTT2) did not lead to the difference in the expressions in CAT activities in either *B. subtilis* or *B.t.i.* hosts. Thus, the presence of *cryIVB* terminator alone might be sufficient for the expression of *cat-86* gene in both *B. subtilis* and *B.t.i.* hosts.

From the results, it could demonstrate that the *bgaB* promoter activity functioned well in both *B. subtilis* MI113 and *B.t.i.* hosts since the high level of CAT activities were reported in the early stage of cell growth until reaching the maximum at the on-set of stationary phase. Judging from the levels of CAT activities in *B. subtilis* and *B.t.i.* hosts, complete *cryIVB* terminator seemed to be able to stabilize *cat* mRNA in sporulation phase only in *B.t.i.* hosts but not in *B. subtilis* hosts. And, it was shown that half of *cryIVB* terminator could not stabilize *cat* mRNA in the sporulation phase of both hosts as determined by lower CAT activity in pTT3. This results indicated the stage as well as the host specificity toward terminator function. On the contrary, *cat-86* terminator appeared to function more efficiently in the vegetative stage until the on-set of stationary phase of both *B. subtilis* MI113 and *B.t.i.* hosts as evident by lower CAT activity in pTF6 at T_8. However, the CAT activities were maintained at high levels in *B.t.i.* hosts containing plasmids pTT1, pTT2 and pTT2Δ which possessed complete *cryIVB* terminator at the end of *cat-86* gene. The importance of complete *cryIVB* terminator in leading to high expression of CAT activities was also supported by low level of CAT in the construct with only half *cryIVB* terminator at the end of *cat-86* gene (pTT3). From these results, by using *cat* gene as reporter gene, it implied the important role of *cryIVB* terminator in stabilizing mRNA at sporulating stage which reflected higher CAT activity at such stage of growth in *B. thuringiensis* hosts. Whereas, constructs which lack of *cryIVB* terminator (pTF6) or incomplete *cryIVB* terminator (pTT3) produced CAT activities at much lower levels.

ACKNOWLEDGEMENTS

This research was supported by UNDP/World bank/WHO Special Program for Research and Training in Tropical Diseases (TDR), a program-based grant No. TDR/ISG/PRCB/88, and by National Center for Genetic Engineering and Biotechnology, grant No. BT-38-06-SDM-14-32. The authors thank Prof. T.W. Flegel for critically reading of this manuscript.

REFERENCES

1. Lereclus, D., Delecluse, A. and Lecadet, M. M., in ***Bacillus thuringiensis, an Environmental Biopesticide: Theory and Practice***, Entwistle, P. F, Bailey, M. J. Higgs, S., eds. (1993), pp. 37, John Wiley & Sons.
2. Wiwat, C., Panbangred, W. and Bhumiratana, A. (1990) ***J. Industrial Microbiol.* 6**, 19.
3. Bhumiratana, A., in "Local Production of *Bacillus sphaericus*", ***Bacterial Control of Mosquitos and Black Flies***, Sutherland, D. and de Barjac, H., eds. (1991), Rutgers University press. U.S.A.
4. Whiteley, H. R. and Hofte, H. (1989) ***Microbiol. Rev.* 53**, 242.
5. Wiwat, C., Panbangred, W., Mongkolsuk, S., Pantuwatana, S. and Bhumiratana, A. (1995) ***Current Microbiol.* 30**, 69.
6. Ge, A. Z., Pfister, R. M. and Dean, D. H. (1990) ***Gene* 93**, 49.
7. Shivakumar, A. G., Vanags, R. I., Wilcox, D. R., Katz, L., Vary, P. S. and Lawrena, J. (1989) ***Gene* 79**, 21.
8. Priest, F. G. (1992) ***J. Appl. Bacteriol.* 72**, 357.
9. Wong, H. C. and Chang, S. (1986) ***Proc. Natl. Acad. Sci.* 83**, 3233.
10. Hirata, H., Fukazawa, T., Negoro, S. and Okada, H. (1986) ***J. Bacteriol.* 165**, 722.
11. Maniatis, T., Fritsch, E. F. and Sambrook, J. ***Molecular Cloning: a Laboratory Manual*** (1982), Second Edition, Cold Spring Harbor Laboratory Press, New York.
12. Birnboim, H. C. and Doly, J. (1979) ***Nucl. Acid. Res.* 7**, 151.
13. Matsuno, Y., Ano, T. and Shoda, M. (1992) ***J. Ferm. Bioeng.* 73**, 261.
14. Botterman, J., Peferoen, M., Hofte, H. and Joos, H. (1989) ***European. Patent.*** Appl. 0 358 557 A2.
15. Shaw, W. V. (1975) ***Method. Enzymol.* 43**, 737.
16. Bradford, M. M. (1976) ***Anal. Biochem.* 72**, 2488.
17. Trisrisook, M., Pantuwatana, S., Bhumiratana, A. and Panbangred, W. (1990) ***Appl. Env.Microbiol.* 56**, 1710.

18. Lecadet, M. and Dedonder, R. (1971) ***Eur. J. Biochem.* 23**, 282.
19. Somerville, H. J. (1971) ***Eur. J. Biochem.* 18**, 226.
20. Mc Laren, R. S., Newbury, S. F., Dance, G. S. C., Canston, H. C. and Higgins, C. F. (1991) ***J. Mol. Biol.* 211**, 81.
21. Venetia, A., Saunders, V. A. and Saunders, J. R. ***Microbial Genetics Applied to Biotechnology*** (1987), First Edition, pp. 219, Croom Helm Ltd., London.
22. Wilson, K. S. and von-Hippol, P. H. (1995) ***Proc. Natl. Acad. Sci.* 92**, 8793.
23. Belesco, J. G. and Higgins, C. F. (1988) ***Gene* 72**, 15.
24. Di Mari, J. F. and Bechofer, D. H. (1993) ***Mol. Microbiol.* 7**, 705.