EFFECT OF TOXIN GENE PROMOTER ON EXPRESSION OF CHLORAMPHENICOL ACETYLTRANSFERASE GENE (*cat*) IN *BACILLUS THURINGIENSIS*

SUPAT CHAREONPORNWATTANA

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ชื่อวิทยานิพนธ์ ผลของ Promoter จาก Toxin Gene ต่อการแสดงออกของยีน Chloramphenicol Acetyltransferase (*cat*) ใน *Bacillus thuringiensis*

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บทคัดย่อ

เมื่อนำ *Bam*H I-*Pst* I fragmentจากพลาสมิด pBTC1 ซึ่งมีส่วนของ Promoter จาก *cry IV* gene ที่สร้างสารพิษฆ่าลูกน้ำยุง และ มีน้ำหนักโมเลกุล 130 kDa ของเชื้อ *Bacillus thuringiensis* subsp. *israelensis* มาต่อกับพลาสมิดพาหะ pPL703 ซึ่งใช้สำหรับตรวจสอบส่วนที่เป็น promoter ของชิ้น DNA ทำให้ได้พลาสมิดสายผสม pPLC1 จากนั้นนำเข้า *Bacillus megaterium* สายพันธุ์ O-016 โดยวิธี protoplast transformation จากนั้นถ่ายทอดเข้าสู่ *B.thuringiensis* subsp. *israelensis* สายพันธุ์ c4Q2-72 และ 4Q2-72 โดยวิธี conjugation-like processโดยอัตราการถ่ายทอดพลาสมิดเท่ากับ 2.1×10^{-7} และ 1.7×10^{-7} ตามลำดับ (อัตราการถ่ายทอดเท่ากับจำนวน transconjugant ต่อจำนวน recipient ก่อน conjugate)เมื่อวิเคราะห์พลาสมิดจาก transconjugant ที่ได้โดยใช้ เอนไซม์ตัดจำเพาะ และ southern blot hybridization พบว่า พลาสมิด pPLC1 ที่อยู่ใน *B.thuringiensis* นั้นไม่มีการเปลี่ยนแปลงและมีชิ้นส่วน *Bam*H I-*Pst* I ที่มี promoter ของ *cry IV* gene ร่วมอยู่ด้วย DNA fragment ที่ต้องการอยู่ในพลาสมิด ระดับการแสดงออกของยีน chloramphenical

acetyltransferase (CAT) ใน crude extract จาก transformant และ transconjugant ที่เลี้ยงใน NBS medium (nutrient broth supplemented mineral) จนถึงระยะ mid exponential , T_0 , T_2 และ T_8 พบว่า ชิ้นส่วนของ promoter ซึ่งนำมาจาก *cry IV* gene นั้นแสดงคุณสมบัติเป็น post exponential promoter เมื่อเปรียบเทียบกับ transformant และ transconjugant ที่มี plasmid อนุพันธ์ เช่น pPL603 และ pPL703 และ จากการตรวจสอบจำนวน plasmid ต่อเซล (copy number) ของพลาสมิด pPLC1 และ อนุพันธ์ใน *B.thuringiensis* มีจำนวนใกล้เคียงกัน เมื่อศึกษาการคงอยู่ของ พลาสมิดสายผสม pPLC1 ใน transformant และ transconjugant โดย การเลี้ยงเชื้อในอาหารที่ไม่เติม kanamycin และมีการถ่ายเชื้อลงในอาหารเลี้ยงเชื้อใหม่ที่ไม่ใส่ยาทุกวัน แสดงให้เห็นว่า ไม่มีผลต่อการคงอยู่ของพลาสมิดในเซล tranconjugant แต่มีผลต่อการคงอยู่ของพลาสมิดใน transformant เมื่อเลี้ยงเชื้อใน สภาพดังกล่าว เป็นเวลาอย่างน้อย 4 สัปดาห์

Thesis Title	Effect of Toxin Gene Promoter on Expression of Chloramphenicol Acetyltransferase Gene (*cat*) in *Bacillus thuringiensis*
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ABSTRACT

A *Bam*H I-*Pst*I fragment harbouring the promoter region of *cry IV* gene encoding 130 kDa *Bacillus thuringiensis* subsp. *israelensis* mosquitocidal toxin protein from pBTC1 was cloned into the promoter probe plasmid vector, pPL703. The recombinant plasmid pPLC1 was transformed into *Bacillus megaterium* strain O-016 by protoplast transformation and subsequently was transferred from the transformant to *Bacillus thuringiensis* subsp. *israelensis* strain c4Q2-72 and 4Q2-72 by conjugation-like process with the frequency of 2.1×10^{-7} and 1.7×10^{-7} respectively (number of transconjugant per number of recipient before mating). The presence of cloned promoter region in the constructed plasmid was confirmed by restriction pattern and Southern blot hybridization. The expression level of chloramphenicol acetyltransferase gene (*cat*) was determined

by measuring the specific activity of the enzyme chloramphenicol acetyltransferase (CAT). The *cat* gene product was assayed in crude extracts obtained from lysozyme treated *B.megaterium* strain 0-016 transformants and *Bacillus thuringiensis* subsp. *israelensis* transconjugants. Both transformant and transconjugants were grown in NBS medium (nutrient broth supplemented with minerals) and harvested at the various growth phases, namely, mid exponential, T_0, T_2 and T_8. It was shown that the inserted promoter region from *cry IV* gene conferred the post exponential promoter activity according to the growth pattern when compared to those harbouring the relevant plasmid, pPL603, pPL703 and pTF6. There was no significant difference in term of plasmid copy number in all these plasmids in *Bacillus thuringiensis* host. Finally, the recombinant plasmid was stably maintained in *Bacillus megaterium* strain 0-016 transformant, but highly stable in *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 and 4Q2-72 transconjugant upon daily subculture for at least 4 weeks in LB broth medium without any selective pressure.

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LIST OF ABBREVIATIONS

bp	base pair
B.t.	*Bacillus thuringiensis*
B.t.i.	*Bacillus thuringiensis* subsp. *israelensis*
^{o}C	degree celcius
cat	chloramphenicol acetyltransferase gene
CAT	chloramphenicol acetyltransferase enzyme
Cm	chloramphenicol
DNA	deoxyribonucleic acid
DDT	dithiothreitol
et al	Et. alii (latin), and others
etc	Et. cetera (latin), other things
Fig.	figure
g	gram
hr	hour
kb	kilobase pair
kDa	kilodalton
Km	kanamycin
ug	microgram
ul	microlitre
M	molar
MW	molecular weight

LIST OF ABBREVIATIONS

(continued)

mg	milligram
ml	millilitre
mM	millimolar
min	minutes
N	normal
nm	nanometre
O.D.	optical density
ORI	origin of replication
Rf	rifampicin
rpm	revolution per minute
SDS	sodium dedocyl sulfate
sp.	species
subsp.	subspecies
Tris	Tris (hydroxymethyl) aminomethane
U	unit
UV	ultraviolet
v.	volt
wk	week

CHAPTER I

INTRODUCTION

The strategies which are usually employed for controlling the population of the insects could be classified into physical, chemical or biological methods. The use of chemical insecticides in controlling insects has a number of disadvantages, such as accumulation of the chemicals in the environment, insects that developed resistances to the certain chemicals and their toxicities toward non-target insects that could lead to an imbalance of the ecosystem. With these disadvantages, the biological control methods, which confer a specific toxicity to the particular group(s) of insects and demonstrate relatively low side effect, has been proven to be the better alternative for effective control of insect pests and vectors.

Microbes, including bacteria, fungi and viruses have been proposed as agents for use as biological insect control. Despite the relatively large number of insect pathogens, those products formulated with *Bacillus thuringiensis* are the ones that show the greatest potential as an effective biological controlling agents.

Bacillus thuringiensis is a gram positive aerobic spore - forming bacteria. It consists of a number of subspecies, most of which synthesizes proteinaceous parasporal crystalline inclusion that offers a selective toxicity against various groups of insects. Most of the described *Bacillus thuringiensis* strains produce insecticidal protein against lepidopteran insects (moths and butterflies), and a

few have been reported to confer the insecticidal activity against dipteran insects (mosquitoes and blackflies) (1). Recently, the strains producing coleopteran specific insecticidal protein have isolated (2,3). Because of the highly specific insecticidal action, *Bacillus thuringiensis* has received wide spread attention as a potential biological control agent (4,5,6).

Several strains and formulations of the biological control agents based on *Bacillus thuringiensis* subsp. *israelensis* (*B.t.i.*) have been reported to possess high level of biological activity against the larvae of mosquitoes both in the laboratory and in field conditions (7,8,9,10).

B.t.i. produces an irregular shape parasporal crystal which is very toxic to certain dipteran larvae such as *Aedes* and *Culex* (11). The major components of the parasporal body, encoded from different genes in 110 kb plasmid, are polypeptides with the molecular weight of approximately 28, 65, and 130 kDa (12,13,14). The 130 kDa and 65 kDa proteinhave shown larvicidal activity (15,16,17) except cytolytic activity has been observed in 28 kDa (38).

In spite of the presence of few copies of the toxin gene, *Bacillus thuringiensis* subsp. *israelensis* has been reported to produce such a large amount of proteinaceous toxin demonstrated as crystal formation. This high expression may be concerned with many criteria, e.g. the stability of 72 MDa plasmid and also that of relevant mRNA, the appropriate sigma factor that promote the transcription efficiency, also, the possibility of the strength of

the promoters of each toxin gene, which has been interesting.

Therefore, the objective of this study was to investigate the expression of CAT gene which was placed under control of promoter of 130 kDa toxin gene. This was achieved by constructing the recombinant plasmid harbouring the DNA fragment containing the promoter region of the 130 kDa toxin gene which was previously cloned in pBTC1 (18), and using promoter cloning plasmid pPL703 as a vector (19). The recombinant plasmid was introduced into *Bacillus megaterium* strain O-016 by protoplast transformation (20), and then the constructed plasmid was subsequently transferred from *Bacillus megaterium* strain O-016 to *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 and 4Q2-72 by conjugation-like process (21). It was hoped that this study might aid in the understanding of expression of toxin gene in *B.thuringiensis* host. Also, the investigation of chloramphenicol acetyltransferase enzyme (CAT) expression in various plasmids might lead to identification of appropriate plasmid expression vector for *B.thuringiensis.*

CHAPTER II

BACKGROUND

1. Biology of *Bacillus thuringiensis*

1.1 Identification

Bacillus thuringiensis (*B.t.*) is an aerobic gram-positive spore forming bacteria and classified in the family *Bacillaceae*. *Bacillus thuringiensis* has been utilized for the control of insect pests and demonstrated that it is highly toxic to larvae of lepidoptera, dipteran and coleopteran. Classification of *Bacillus thuringiensis* is summarized in the Table 1. The serotype of *Bacillus thuringiensis* with the exceptional larvicidal activity against mosquito larvae was first isolated from dead larvae of *Culex pipiens* Linn by Goldberg and Margalit in 1977 (11) from a pond in the Negev desert of Israel. The serotype was designated as *Bacillus thuringiensis* subsp. *israelensis* (*B.t.i.*). *Bacillus thuringiensis* was classified based on H-antigen as shown in Table 1 and *B.t.i.* was classified into serotype H-14. The nomenclature of *B.t.* is summarized in Table 2 (26) using *B.t.i.* as an example.

1.2 Toxin and Toxicity

B.t.i. is toxic toward mosquito and blackfly larvae. It has been demonstrated that such a bacterium affects about 72 species of mosquitoes (181). *B.t.i.* produces heat-labile delta-endotoxin

Table 1 Classification of *Bacillus thuringiensis* (*)

H-serotype	Subspecies	References
1	*thuringiensis*	Berliner (1911)
2	*finitimus*	Heimpel and Angus (1985)
3a	*alesti*	Toumanoff and Vago (1951)
3a·3b	*kurstaki*	Kurstak (1964)
4a·4b	*sotto*	Ishiwata (1901)
4a·4b	*dendrolimus*	Talalaev (1956)
4a·4c	*kenyae*	Noris and Burges (1963)
5a·5b	*galleriae*	Isakova (1958)
5a·5c	*canadensis*	de Barjac and Bonnefoi (1972)
6	*subtoxicus*	Heimpel and Angus (1958)
6	*entomocidus*	Steinhaus (1951)
7	*aizawai*	de Barjac and Bonnefoi (1963)
8a·8b	*morrisoni*	Norris (1964)
8a·8c	*ostrimiae*	Ren et al (1975)
9	*tolworthi*	Norris (1964)
10	*darmstadiensis*	Kreig , de Barjac and Bonnefoi (1968)
11a·11b	*toumanoffi*	Kreig (1969)
11a·11c	*kyushuensis*	Ohba and Aizawa (1979)
12	*thompsoni*	de Barjac and Thompson (1970)
13	*pakistani*	de Barjac et al. (1977)
14	*israelensis*	Goldberg and Margalit (1977)
15	*indiana*	Delucca , Simonson and Larson (1979)
16	*dakota*	Delucca, Simonson and Larson (1979)

Table 1 Classification of *Bacillus thuringiensis* (*) (continue)

H-serotype	Subspecies	References
17	*tohokuensis*	Iizuka et al. (1982)
18	*kumaotoensis*	Iizuka et al. (1982
19	*tochigiensis*	Iizuka etal. (1982)
20a20b	*yunnanensis*	Yu , Z. Huazhong Agri Coll. China (1984)
20a20c	*pondicheriensis*	de Barjac et al. 1990
21	*colmeri*	Dulucca, Palmgrenand, de Barjac (1984)
22	*shandongiensis*	de Barjac et al. 1990
23	*japonensis*	de Barjac et al. 1990
24	*neoleonensis*	de Barjac et al. 1990
25	*coreanensis*	de Barjac et al. 1990
26	*siloensis*	de Barjac et al. 1990
27	*mexicanensis*	Kronstad et al. 1983
ND	*wuhanensis*	Hubei Institute, China (1976)
ND	*fukuokaensis*	Yong et al. 1991

(*) : according to Dean DH 1984 (25)

Table 2 Nomenclature and name abbreviations using *Bacillus thuringiensis* subsp. *israelensis* as an example

Hierarchial status	Full name and best abbreviation	Definitive characters	Current numbers of each hierarchy
Genus	*Bacillus*	Morphology , Biochemistry	-
Species	*thuringiensis*(*B.t.*)	Morphology	-
Subspecies	*israelensis*(*B.t.i.*)	Flagella antigen HD-14	27 varieties 24 H-types
Crystal serotype	isr	Crystal antigen	ca. 28
Strain	None : arbitary numbers within varieties	Toxicity in groups of hosts	ca. 84
Isolate	e.g. HD-917* (de Barjac 1984**)	Isolation date, source, person who isolated it	900+
Mutant	e.g. HxD-197/1	Antibiotic resistance auxotrophy and pathogenicity etc.	0 to 50+ per strain

* Accesion number in the culture collection of Dulmage HT, USDA, Brownsville, Texas, USA

** Accession number of same isolate in collection of de Barjac H, Pasteur Institute, Paris, France

This data adapted from Burges HD 1984 (26).

located in the proteinaceous parasporal crystalline inclusion which is synthesized simultaneously with the formation of endospore during sporogenesis (27, 28). It has been shown that mosquito larvicidal activity is due to the toxin contained in the parasporal crystalline inclusion or crystal (12, 29).

The crystal that is ellipsoidal or dumb-bell shaped, is a bipyramidal aggregation of protein molecule which is composed mainly of 25-28, 65 and a doublet of 130 kDa polypeptide (12, 13, 14). It was reported by Ibarra and Federici (30) that the 130 kDa protein seen in SDS-PAGE in fact was composd of two polypeptides of approximately 126 and 135 kDa which could be observed separately in SDS-PAGE with good resolution and both were reported to contain larvicidal activity. The mosquitocidal activity as well as the cytolytic and hemolytic activity was attributed to 27 kDa protein (13, 15), whereas the 65 kDa protein (16) and 130 kDa protein (17) were reported to be attributed to the mosquitocidal activity.

1.3 Mode of action

The crystalline toxin is protoxin and only active after being digested by susceptible larvae, subsequently, activated to be the toxic moiety in alkaline environment (31). *In vivo* system, it was shown that the gut epithelium appeared to be the primary target of delta-endotoxin (32, 33). Upon exposure to the solubilzed toxin, cell death is associated with plasma membrane disruption, swelling, vacuolation, loss of cytoplasmic content and lysis (33, 34, 35). However, metabolic defect of epithelial cells is completed in 10-15

minutes after being treated and ion leaked from gut lumen to haemolymph and in 45 minutes after treatment, there is extensive separation of cell from basement membrane. Paralysis or death results from ionic imbalance in haemolymph. The fatty acid moieties of phospholipid on toxin lipid interaction by lipid dispersion and multilamellar liposome showed the ability of unsaturated phosphatidyl choline, sphingomyelin, and phosphatidylethanolamine to bind toxin. This indicated the specific requirement of unsaturated hydrocarbon chain for toxin-lipid association (36). The interaction between toxin and specific plasma membrane lipids causes detergent-like rearrangement of lipid leading to disruption of membrane integrity and eventual cytolytic (36). In addition, Gill et al. (37) reported that cytolytic activity of the solubilized toxin was probably dueto pore formation in cell membrane and this effect could be observed in mammalian cell as well (35).

2. Classification of the crystal protein gene

2.1 Diversity and classification of crystal protein gene

Up to present, DNA sequence have been reported for as many as 42 *Bacillus thuringiensis*crystal protein genes (38). Various sequences are identical or mostly identical and represent the same gene or a slight variant of the same gene. Those could be divided into 4 distinct crystal genes and 13 of these *cry* (crystal protein genes) specified a family of related insecticidal protein. These genes have been classified further into a minimum member of four

major classes and are characterized base on the structural similarity and the target insect spectra of the encoded protein.

These four major classes are: lepidoptera-specific (I), lepidotera and diptera-specific (II), coleoptera-specific (III), and diptera-specific (IV) genes. The rest of *Bacillus thuringiensis* crystal protein gene are encodedfor 27 kDa polypeptide which exhibits cytolytic activity against a variety of invertebrate and vertebrate cultured cells as well as erythrocytes (35) by interacting with the membrane phospholipid (36). The cytolytic effect may account for the mosquito killing activity of CytA. This gene has recently been designated as *cytA* (38), and CytA protein shows the unrelated properties with the other known *Bacillus thuringiensis* crystal protein toxins both in its amino acid sequence (38) and its mechanism of action. The genes assigned to four major *cry* classes and *cyt* class are listed in Table 3.

2.2 *cryI* genes : Toxins active against lepidopteran larvae.

The best studies aboutthe crystal protein mostly have been undertaken on lepidoptera specific crystal proteins. The 20 *cryI* sequences have been reported (Table 4) and six different genes (Table 3) have been categorized among the 20 sequences which all are encoded for 130 to 140 kDa proteins. These proteins are accumulated in cytoplasm as bipyramidal parasporal crystal protein during post-exponential phase of *Bacillus thuringiensis*. After being digested bythe susceptable insect larvae,the crystal protein is

Table 3 Insecticidal crystal protein genes of *Bacillus thuringiensis* (38)

Gene type	Host range*	# of amino acid	Predicted mol. mass. (kDa)	Other gene designations
cryIA(a)	L	1176	133.2	4.5 kb gene (39), *cryI-1* (40)
cryIA(b)	L	1155	131.0	5.3 kb gene (39), *kurhd1* (41), *bt2* (42), *cryI-2* (40)
cryIA(c)	L	1178	133.3	6.6 kb gene (39)
cryIB	L	1207	138.3	*cryA4* (53), type B (43)
cryIC	L	1189	134.8	type C (43), BTV1 (54), *Bta* (44)
cryID	L	1165	132.5	
cryIIA	L/D	633	70.9	P 2 gene (55), *cryB1* (56)
cryIIB	L	633	70.3	*cryB2* (56)
cryIIIA	C	644	73.1	*cryC* (49)
cryIVA	D	1180	134.4	130 kDa endotoxin gene (53), ISRH3 (46) 125 kDa endotoxin gene (45)
cryIVB	D	1136	127.8	130 kDa endotoxin gene (47), ISRH4 (48) 135 kDa endotoxin gene (45), *BtS* (59)
cryIVC	D	675	77.8	ORF1 (60)
cryIVD	D	643	72.4	*cryD* (61)
cytA	D/cytol**	248	27.4	27 kDa toxin gene (62)

* specified host range : L, Lepidoptera; D, Diptera; C, Coleoptera

** cytol : cytolytic and haemolytic

Table 4 Overview of reported crystal protein gene sequences

Crystal protein	*B. thuringiensis* subsp. and/or strains	References
cryIA(a)	*kurstaki* HD-1	63
	aizawai	40
	kurstaki HD-1	64
	sotto	65, 66
	entomocidus	67
cryIA(b)	*berliner* 171559	51, 68
	berliner 1715	42
	kurstaki HD-1	69
	kurstaki HD-1	41, 60
	aizawai IPL-7	64
	kurstaki HD-1	70
	kurstaki NRD-12	72
	aizawai IC-1	71
cryIA(c)	*kurstaki* HD-73	52
cryIB	*thuringiensis* HD-2	53
	entomocidus HD-110	38
cryIC	*entomocidus* 601	54
	aizawai HD-137	44
	entomocidus HD-110	38
cryID	*aizawai* HD-68	38
cryIIA	*kurstaki* HD-263	55
	kurstaki HD-1	56
cryIIB	*kurstaki* HD-1	56

Table 4 Overview of reported crystal protein gene sequences (continue)

Crystal protein	*B.thuringiensis* subsp. and/or strains	References
cryIIIA	*san diego*	57
	tenebrionis	46, 73, 74
	EG 2158	49
cryIVA	*israelensis*	58
	israelensis	48
cryIVB	*israelensis*	59
	israelensis	122
	israelensis	48
	israelensis	47
cryIVC	*israelensis*	60
cryIVD	*israelensis*	61
cytA	*israelensis*	62
	morrisoni PG-14	75
	israelensis	76
	morrisoni PG-14	77

From Hofte H and Whiteley HR (38)

solubilized in high pH condition (usually a range of 10-12), then protoxin is released and further cleaved to active toxin core fragment of 60-70 kDa by mid gut protease (78). It has been found that larvicidal activity is located in the amino-terminal half of the protein molecules in CryIA(b) (42) and CryIC (38) through N-terminal amino acid sequence of the trypsin activated toxin. The proteolytic site is highly conserved for the other CryIA and CryID protein and indicates that for these proteins the amino terminus of the toxin fragment is localized at the same position. The CryIB is very different from the the other CryI proteins in this region. Deletion analysis of several *cryI* gene [*cryIA*(a)(63), *cryIA*(b) (42,51), *cryIA*(c)(52), and *cryIC*(c)(44)] further confirmed that the carboxy-terminal halves of the protoxin is not absolutely required to manifest the toxic activity. Three of the *cryI* gene ; *cryIA(a)* , *cryIA(b)* , *cryIA(c)* were perviously designated as the 34.5 kb , 5.3 kb , and 6.6 kb genes respectively, on the basis of size by *Hind* III restriction fragment which contained the amino-terminal of the genes (39). The nucleotide differences between these three genes are localized mainly in a limited segment of the region encoding the toxic fragment (78). Recently, it was shown that *cryIB*, *cryIC*, and *cryID* genes are much different from each other and the *cryIA* genes.

A crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai* IC-1 is designated as a *cryIA(b)* gene although the protein has toxicity against two orders of insects depending on the source of proteolytic enzyme which regenerates toxin. These toxins are

lepidopteran specific and dipteran specific toxin (79, 80).

The IC-1 gene is included in the *cryIA(b)* subclass on the basis of structure of the gene product and amino acid sequence differs from the holotype by only four amino acids. The dominant feature ona comparative analysis of the deduced amino acid is that the carboxy-terminal half is highly conserved for all *cryI* genes. It is unclear whether the conserved sequence in the mentioned region reflects any functional significance. Interestingly, it has been found that the carboxy - half is rich in cysteine residues and these disulfide bonds have been proposed to be involved in the maintainance and the unusual solubility properties of the crystal. Therefore, the carboxy-terminal may be intimately involved in crystal formation (81).

Recently, the mechanism of insect resistance against CryIA(b) protein in *Plodia interpunctella* was proposed (182). It was shown that there was a correlation between toxicity and glycoprotein specific receptors on the brush border membrane ofthe insect midgut. The receptor binding with specificity determining region located within C-terminal half of toxic fragment is responsible for the spectrum of species susceptibility and one insect might be susceptible to several different toxic proteins, depending on the presence of the different receptors on the midgut wall. In this respect, it should be noted that the CryIA(b), CryI(c) and CryIC proteins have been demonstrate to consist of 2 structural domains (183, 184, 185).It seems probable that a two domain structure is a general feature of the most *Bacillus thuringiensis* toxin

proteins. These structural domains may represent functional domain, with C-terminal domain involved in specific binding whereas the N - terminus would be responsible for toxicity.

It was also demonstrated that the resistance of the mentioned insect is correlated with reduction in the affinity of toxin protein for its membrane receptor (186). Interestingly, the resistant strain was more sensitive to another crystal protein, as compared to the sensitive strain, apparently due to an increased binding capacity.

The distribution of crystal protein composition was conventionally studied by polyacrylamide gel electrophoresis (82, 83) and immunological reaction by polyclonal antibody against purified crystal protein (84). These methods exhibited that some crystal proteins contained more than one protein. The genetic approach became possible with the cloning of the crystal protein genes. The use of gene specific probes led to discovery that various subspecies of *Bacillus thuringiensis* contained one more closely related gene (39, 85).

Recently, an alternative approach was described in which monoclonal antibodies were used to identify single protein in the crystal preparation. Huber-Lukac et al. (86) described 10 monoclonal antibodies generated against purified crystal from *Bacillus thuringiensis* subsp. *kurstaki*. Investigation of the crystals of 14 *Bacillus thuringiensis* strains with these antibodies revealed an obvious difference in immunoreactivity. However, direct correlation was not made between the reactivity with certain

monoclonal antibodies and the presence of certain crystal protein types. Hofte et al. (43) used 35 monoclonal antibodies to distinguish the CryIA, CryIB and CryIC proteins in the crystal preparations in 29 strains 11 of serotypes. The CryIA is the most common crystal protein type and was present in all but one strain tested. The CryIB and CryIC are less common.

It should be noted that the monoclonal antibodies used in this study might not be able to detect all of the toxin in the crystal proteins due the subtle variation in amino acid sequence which might be responsible for the different host spectrum.

So, it is likely to use the monoclonal antibodies as a potent system for screening the newly isolated strain harbouring insecticidal proteins before using the time consuming bioassay process. The result obviously exhibited that many strains produced several crystal proteins simultaneously and the same or very similar crystal proteins occurred in *Bacillus thuringiensis* strains of the different varieties. Because most genes are located on a large conjugative plasmid (87), this mobility of crystal protein genes among the strains of *Bacillus thuringiensis* subspecies is not expected. However, the observed association of several *cryIA* genes (88, 89) and *cryIVB* (45) with insertion element (IS) (90) and/or transposon-like structures could contribute to their mobility.

The expressions of *cryI* genes have been studied in several reports.It has been found that crystal protein genes begin to express at about stage II of sporulation and continue until late sporulation (91, 92, 93, 94). For *cryIA(a)*, it is transcribed from the two

initiationsites, located about 16 base pairs apart. BtI which is activated early in sporulation ($t_0 - t_2$) by RNA polymerase containing sigma subunit of 35 kDa (95) as found in transcription of other toxin genes such as *cryIB, cryIIA* and *cytA*. The other, BTII, requires a second RNA polymerase containing another new sigma factor of 28 kDa. It has been demonstrated that several crystal protein genes harbour the strong terminator. Wong et al. (96) found that the presence of the terminator significantly enhanced the expression of gene by stabilizing crystal protein messenger RNA.

In *E.coli, cryIA(a)* is transcribed from a site or at very near BtII (the second promoter of the crystal protein gene). The identity of the RNA polymerase responsible for this transcription has not been established. The expression of *cryIA(a)* gene in *E.coli* but not in *Bacillus subtilis*, is regulated negatively by a region of a DNA located at about a position -87 to -258 relative to BtI (the first promoter of the crystal protein gene). Deletion or interuption of this region enhances gene expression approximately 10 fold (96). The same experiment was also carried out in the *cryIA(b)* gene and yielded the increased expression as well (60). The biochemical basis for their regulatory mechanism has not been investigated.

Klier et al. (97) cloned the crystal protein gene from subspecies *thuringiensis* into *Bacillus subtilis*. It was shown that the expression occurred during sporulation but could not be detected in several *spo*$^-$ mutants. In agreement with this observation, fusion of *cryIA(a)* promoter to the promoterless *cat-86*

gene showed that expression in *Bacillus subtilis* occurred only in sporulation (98) and there was no CAT activity in *spoOA, spoOC, spoOF, spoOH, spoIIA,* and *spoIIE* mutants of *Bacillus subtilis* and conferred low activities in *spoOJ, spoIIC* and *spoIIIE* mutants when compared with the *spo*$^{+}$ strain. However, the dependence on sporulation may be related to the vector used in cloning because Shivarkumar et al. (99) reported that a crystal protein gene cloned in different plasmid was not regulated by sporulation.

2.3 *cryII* genes : Toxins active against lepidopteran and dipteran larvae

Many varieties of *Bacillus thuringiensis* which produce 130 kDa protoxins are designated as P_1 crystal protein and also some subspecies, for example *kurstaki, thuringiensis, tolwothi* and *kenyae,* also a cuboidal body composed of 65 kDa polypeptide previously designated as P_2 protein (100, 101, 102).

The first *cryIIA* gene was cloned from *Bacillus thuringiensis* subsp. *kurstaki* HD-263 and expressed in *Bacillus megaterium* (55). Cells producing the CryIIA protein were toxic for lepidopteran species, *Heliothis virescens* and *Lymantria dispar,* as well as for *Aedes aegypti*larvae of diptera. Widner et al. (56) cloned two related genes (*cryIIA* and *cryIIB*) from *Bacillus thuringiensis* subsp. *kurstaki* HD-1. Both genes encoded proteins of 633 amino acids with a predicted molecular mass of 71 kDa, slightly larger than the apparent molecular mass determined for P_2 proteins produced in *Bacillus thuringiensis.* Both genes were

expressed in *E.coli* and the recombinant proteins were purified. Although the two proteins are highly homologous (about 87% amino acid identity), they differ in their insecticidal spectra. CryIIA is active against both lepidopteran and dipteran species but CryIIB is toxic only to lepidopteran insects. The DNA sequence further indicates that *cryIIA* is the distal gene of an operon containing three reading frames (*orf1, orf2* and *cryIIA*). The gene products of *orf2* and *cryIIA* could be detected in the cuboidal crystals in several *Bacillus thuringiensis* subspecies, but there was no evidence to confirm the presence of *orf1* and *cryIIB* products in the crystal. The *orf2* gene product which is highly immunogenic has an unusual repeated structure. The function of *orf1* and *orf2* gene products are not known yet. Remarkably, comparison of the predicted amino acid sequence demonstrated that the *cryII* genes showed a rather limited homology to the other *cry* genes (38).

2.4 *cryIII* genes : Toxins active against coleopteran larvae

There are a number of other non-target insect orders which are not susceptable to the potent effects of lepidopteran-specific and dipteran-specific insecticidal proteins (103), for example, the agriculturally important groups of coleopteran (beetles). *Bacillus thuringiensis* subsp. *tenebrionis* was first described by Krieg et al. (104) and *Bacillus thuringiensis* subsp. *san diego* and *Bacillus thuringiensis* EG 2158 (49, 105) were also identified as coleopteran-specific toxin producing strains. Each of the strains produces rhomboidal crystals containing one major protein component.

Cloning and sequencing demonstrated the presence of the same crystal protein gene in all three strains. The *cryIIIA* is homologous to the toxin coding domain of the *cryI* and *cryIV* genes and lacks a region corresponding to the carboxy-terminal of these molecules. This insect control protein differs from other toxins in solubility, molecular weight of the crystal protein and immunological cross-reactivity.

Herrnstadt et al. (105) reported that crystal of *Bacillus thuringiensis* subsp. *san diego* was composed of a protein of molecular weight about 64 kDa and had no immunologically cross-reactivity with various *Bacillus thuringiensis* subsp. *kurstaki* crystal protein. Evidence showed a significant difference between the structure of the lepidopteran specific and coleopteran-specific insecticidal protein. Bernhard(106) found that *Bacillus thuringiensis* subsp. *tenebrionis* crystal protein contained composition of molecular weight of about 68 kDa and 50 kDa. The toxin gene located on a large plasmid was cloned into *E.coli* (107). The expression of the crystal gene product was recognized by Western blots and bioassays against larvae of Colorado Potato Beetle. The remarkable bands of crystal protein were expressed in *E.coli* with 74 and 68 kDa corresponding with those of *Bacillus thuringiensis* subsp. *tenebrionis* (108).

McPherson et al. (73) recently reported that the protoxin protein gene from the mentioned organism was cloned in *E.coli* and *P. fluorescens.* Two separated bands, demonstrated in *E.coli* resulted from distinct transcriptional initiation sites present in

the same open reading frame (*orf*) which could have also occurred in other prokaryotic systems as well. These proteins were expressed individually and showed the same lethality to the Colorado Potato Beetle larvae. A toxin gene from *Bacillus thuringiensis* subsp. *san diego* also has been cloned and reported (105) in *E.coli* which conferred a larger protein than the native protein.

2.5 *cryIV* and *cytA* : Toxins active against dipteran larvae

The *cryIV* class of the crystal protein genes is composed of a rather heterogenous group of dipteran-specific crystal protein genes. The *cryIVA, cryIVB, cryIVC, cryIVD* and *cytA* have been all isolated from the same 72 MDa plasmid present in *Bacillus thuringiensis* subsp. *israelensis*. These genes encoded for proteins with predicted molecular weight of 135, 128, 78 and 72 kDa, respectively. These proteins assemble together with the 27 kDa *cytA* gene product in ovoid crystal complexes. It was found that no single crystal component is as toxic as the crystal complex. One possible explanation for such a observation is that two or more proteins work synergistically, yeilding a higher toxic activity than would be expected on the basis of the specific toxicity of the individual proteins. The crystal complex with the same or a very similar protein composition has also been found in *Bacillus thuringiensis* subsp. *morrisoni* PG-14 (38). It was reported that solubilized crystals derived either from *Bacillus thuringiensis* subsp. *israelensis* (109) or from recombinant *E.coli* (49, 59, 110) or *Bacillus* strains (47, 49, 111) were toxic against larvae

of some mosquito species.

The feature of *cryIVA* and *cryIVB* is common to that of *cryI* genes. They also encode for 130 kDa protein which is later proteolytically converted into smaller toxic components. There is some controversy about the exact molecular weight of the toxic core fragment which ranges from 53 kDa (109) to 78 kDa (59) in different studies. The 3′ portions of these genes are almost identical to each other and highly similar to the 3′ portion of the *cryI* genes. This suggests that the toxic fragment of *cryIVA* and *cryIVB* is localized in the N-terminal halves. This was confirmed for the *cryIVB* gene product through deletion analysis (59, 110, 112).

The *cryIVC* gene, encoded for protein with a predicted molecular mass of 78 kDa, is composed of two adjacent open reading frames. The second open reading frame (*orf2*) is localized 45 base pair downstream from the stop codon of *orf1*. The *orf1* shows homology of the 5′ half of the other *cry* genes whereas *orf2* corresponds to the remaining carboxy-terminal part (60). This gene configuration has probably evolved through the insertion of a DNA fragment into a gene that otherwise would encode a approximately 130 kDa toxic peptide. The *cryIVC* could be introduced and expressed in either *E.coli*, *Bacillus subtilis* or cured *Bacillus thuringiensis* subsp. *israelensis* strain to produce the toxic protein of approximately 58 kDa which is presumably a proteolytic fragment of the *orf1* gene product. The region responsible for the toxic activity in *cryIVA*, *cryIVB* and *cryIVC* is highly divergent. Conserved amino acids are restricted mainly to five

sequence blocks which are also found in CryI and CryIIIA proteins.

The *cryIVD* gene encodes a 72 kDa protein (111) which is the major component of the *Bacillus thuringiensis* subsp. *israelensis* crystals (113). This crystal protein, different from all other known *cry*-coded proteins, is proteolytically converted into an active fragment of approximately 30 kDa (109, 114, 115). The exact localization of thisfragment in the intact protein has not been elucidated. Sequence comparisons show a rather limited homology to the other crystal proteins in a short region of the molecule (between codon 45 and 174).

The 27 kDa *cytA* gene product which shows no sequence homology with other crystal proteins, exhibits none or rather low toxic activity (76, 116). Moreover, the 27 kDa protein, purified from *Bacillus thuringiensis* subsp. *israelensis* crystals or from recombinant *Bacillus subtilis* clone, shows a cytolytic activity for a variety of invertebrate and vertebrate as well as mammalian cells (35, 76). The expression of the *cytA* gene in *E. coli* requires the presence of a segment of *Bacillus thuringiensis* subsp. *israelensis* DNA, encoded for 20 kDa polypeptide, which is located on the plasmid, but 4 kb upstream (116) whereas such a polypeptide is not required for *cytA* gene in *Bacillus subtilis* (76). The 20 kDa protein which is present in a small amount in crystal, and synthesized beginning about 2 hours after start of sporulation, provides the post-translational process to increase the amount of CytA produced. Previous studies of 20 kDa protein have shown that it does not significantly enhance the

transcription of *cytA*, stability of *cytA* mRNA or translational initiation. It is likely that the 20 kDa peptide plays a role in protecting CytA from protease degradation. A recent study was reported by Visick et al. (117) that CytA protein and the 20 kDa protein were coimmunoprecipitated from *E.coli* extracted by an antibody against each peptide which might be an indication of protein-protein interaction. Binding of these two proteins might be a mechanism of action in order to block protease susceptible site that led to a poor substrate for proteolysis. However, the result of further investigation showed thatthe amount of CytA produced was independent of the 20 kDa protein in cells with some mutation such as *rpoH*, *groEL* and *dnaK*, all of which reduced the ability of the cells to degrade abnormal protein (118, 119, 120, 121).

3. Cloning vector in *Bacillus subtilis*

There are a number of virtues of *Bacillus subtilis* being used as a common host for genetic manipulation. *Bacillus subtilis* is a gram-positive obligate aerobic bacilli which may offer a different internal environment for introducing genes due to the physiological and structural difference compared with those of *E.coli*. Moreover, *Bacillus subtilis* has beenused as a model to study cell differentiation because of its ability to sporulate and also used in fermentation for commercial purpose of exoenzyme production because of its ability to secrete the desired products from the cells. Therefore, *Bacillus subtilis*is obviously useful to be a host for expressing the cloned gene. Lastly,

this organism has never been found to be a causative agent in normal individuals either human or animal.

Many strains of *Bacillus subtilis* have demonstrated harbouring a number of cryptic endogenous plasmid (123). At the present time, most of plasmid vectors used in molecular cloning of *Bacillus subtilis* are derived from *Staphylococcus aureus*. Some of these plasmids (shown in Table 5) such as plasmid pC194 (124) pE194 (123, 125) and pUB110 (126, 127) have already been constructed.

A number of specialized vectors have been developed for a particular objective. William et al. (128) developed a promoter cloning vector pPL603, a pUB110 derivative which harboured the chloramphenicol acetyltransferse gene (*cat*) from *Bacillus pumilus* NCIB 8600. In this plasmid, *cat* gene was not expressed in vegetatively grown cell but expressed in stationary growth phase cell by selective transcripiton of 203 bp *Eco*RI-*Pst* I fragment (P_1) which conferred the post exponential promoter activity. Alternatively, P_1 fragment could be replaced with 21bp oligonucleotides from M13mp7 in order to obtain plasmid pPL703 which carried *cat* structural gene and completely lacked its own promoter (129, 130). These two plasmid have been used for detecting DNA fragment that gave promoter activity expressed in *Bacillus subtilis* (130, 131, 132, 133, 134). Some vectors were found to contain a strong promoter and used as an expression vector. For example, pTF6 (133), pPL708 (130,132, 135) and pPL608 (130, 136). With the plasmid vectors used in *Bacillus subtilis*, they also

Table 5 *Bacillus subtilis* cloning vectors

Plasmid	Source of plasmid	Antibiotic marker*	Reference
pBC16	*B.cereus*	Tc	141
pAB124	*B.stearothermophilus*	Tc	142
pT127	*S.aureus*	Tc	143, 144
pC194	*S.aureus*	Cm	143, 144
pC221	*S.aureus*	Cm	143, 144
pC223	*S.aureus*	Cm	143, 144
pUB110	*S.aureus*	Km	145
pUB112	*S.aureus*	Cm	143
pE194	*S.aureus*	Em	145
pPL603	*S.aureus*	Km	124
pPL608	*S.aureus*	Km, Cm	124
pPL703	*S.aureus*	Km	129, 130
pPL708	*S.aureus*	Km, Cm	129, 130, 132
pTF6	*S.aureus*	Km, Cm	133

* Antibiotic marker : Tc, Tetracycline ; Cm, Chloramphenicol
: Km, Kanamycin ; Em, Erythromycin

have been shown to replicate and express in other *Bacillus sp.*, for example, *Bacillus pumilus, Bacillus cereus, Bacillus megaterium* and *Bacillus thuringiensis* (137, 138, 139, 140), and common strategy in cloning genes into *Bacillus sp.* could be applied for manipulating genes into other desirable *Bacillus* hosts.

4. Transformation system in *Bacillus sp.*

Transformation system in *Bacillus sp.* have been studied by various groups (20, 147, 148, 149, 150, 151, 152, 153, 154). It seems that only the transformation system in *Bacillus subtilis* could demonstrate a successful performance with high frequency of tranformation (20, 153, 154) whereas a similar system in other microorganisms resulted in low frequency of tranformation.

In the past, genetic transformation was a process by which the bacterial cells uptake the DNA from the surrounding medium to obtain the alteration of genotype which then could be inherited. This strategy has been widely studied in different organisms since the transformation in *Pneumococcus* was discoverd in 1928. The mechanism of DNA uptake in a competent cell was proposed to involve specific receptors located on the cell membrane (158) which was comprised of DNA binding protein and the nuclease which degraded one strand of DNA duplex during the transformation across the membrane. The DNA processing in plasmid transformation involved two key steps, one was the DNA binding and the other was DNA uptake when DNA would be converted from duplex to a single stranded DNA. The DNA was cut

at the region of homology and simultaneously DNA molecule was introduced to the cell ; then it would tend to hybridize with complementary DNA(159).

For the studies in *Bacillus subtilis*, plasmid transformation system could be performed by using both competent and protoplast cells. In 1961, Anagnostopoulos and Spizizen (155) first described the competent cell transformation in *Bacillus subtilis* and followed by a number ofstudies from various investigators (153, 154, 156) in order to optimize the condition and improve transformation frequency. However, at the present time, the transformation of *Bacillus subtilis* competent cell still confers much lower efficiency (10^3 -10^4 transformants / ug DNA) than that conferred by *E. coli* (10^7 -10^8 transformants / ug DNA). Heirson et al. (157) improved the frequency of transformation in the vegetative cell from different *Bacillus thuringiensis* strains. The competency was induced by treating cells harvested from late-logarithmic cell in minimal medium (supplemented with 0.5 % cassamino acid) with 50 mM Tris-HCl pH 8.9 containing 30 % sucrose.

In 1979, Chang and Cohen (20) suggested the alternative method to introduce plasmid DNA to the cell of *Bacillus subtilis* by treating the protoplasts prepared from non-competent cells. The protoplast was generated by removing the cell wall from the lysozyme sensitive cell under hypertonic conditions; then after transformation, protoplasts were regenerated on enriched osmotic stabilizer medium in order to form the intact cell. With some modification, this method has been extensively used for other

Bacillus species (149, 150, 152,160, 161). However, some disadvantages also have been shown, for example, complicated and enriched regeneration medium (162), difficulties in generating protoplast from different *Bacillus* strains, handling of protoplast and its low stability.

For *Bacillus thuringiensis,* protoplast transformation has been modified from that previously described by Chang and Cohen. Many papers have reported about protoplast transformation in *Bacillus thuringiensis* using either plasmid pC194, pBC16 and pUB110 (150, 152, 160) but all demonstrated low frequency. This might be due to high resistance of cell wall to lysozyme. To overcome this problem, more lysozyme and longer incubation time were suggested to obtain more efficiency of cell wall hydrolysis. By this solution, the excess amount of lysozyme might lead to poor recovery of the protoplasts due to the binding of lysozyme to the protoplast membrane (163).

In 1987, Temeyer (164) suggested the use of endogenous autolytic system of *Bacillus thuringiensis* to obtain protoplasts, but the optimized condition for the enzyme activity was not obviously demonstrated. Crawford et al. (165) described the optimum condition for regenerating *Bacillus thuringiensis* subsp. *kurstaki* autoplast without lysozyme added. The optimum condition of autoplast formation was performed in 50 mM sodium acetate buffer pH 7.0 at 37°C with 10 % (v/v) polyethylene glycol as an osmotic stabilizer. The plasmid pC194 and pUB110 derivatives could be taken up and stably maintained in *Bacillus thuringiensis.* Rubinstein

et al. (166) reported a similar experiment for autoplast transformation. The autoplast transformation was formed after being cultured under rapid growth conditions (shifted to richer medium after being incubated in low glucose medium) followed by treatment in osmotic stabilizer buffer. The virture of the autoplast experiment involved the compromise between efficiency of regeneration and that of DNA uptake.

Recently, a new technique of transformation in *Bacillus sp.* which has been provided was eletroporation. Cells were transformed by a short pulse of strong electrical shock which led to the temporary and reversible breakdown of cell membrane and DNA uptake by transformed cell. In 1985, Schuter (167) described the optimum condition for electroporating intact *Bacillus thuringiensis* strains. The medium used was comprised of 400 mM sucrose, 1 mM $MgCl_2$ and 1mM phosphate buffer pH 6.0. The plasmid harbouring *kurhd1* protoxin gene was introduced and expressed in sporulated *Bacillus thuringiensis* subsp. *kurstaki* HD-1 (*cryB*$^-$) with the toxicity against insect larvae. By this procedure, the frequency was shown at 10^7 transformants / ug DNA. Mahillon et al. (168) proposed the use of electroporation for *Bacillus thuringiensis* in the presence of polyethylene glycol and the transformed cell suspension could be preserved at -70^oC in order to provide ready-made competent cells which conferred the efficiency of transformation between 10^2 - 10^5 transformants / ug DNA which depended on the strain of host and plasmid used. Bone et al. (169) introduced the plasmid containing gene encoded 27 kDa delta-endotoxin into acrystalliferous *Bacillus*

thuringiensis subsp. *israelensis* and subsp. *kurstaki*. Both transformants obtained by electroporation technique demonstrated the expression of the cloned toxin gene with the toxicity against *Aedes aegypti* larvae.

5. Conjugation-like process

Despite the fact that there was no obvious evidence about "classical conjugation" (involving conjugation tube, pilus) in the genus *Bacillus*, transfer of certain plasmid DNAs in *Bacillus thuringiensis* by cell mating was firstly reported by Gonzalez et al. (21). The plasmid harhouring crystal coding gene could be transferred between *Bacillus thuringiensis* subsp. *israelensis*, subsp. *kurstaki* and the two strains of the related species, *Bacillus cereus* during growth in the mixed culture and suggested in a preliminary study to be conjugation-like process. From this study, they could demonstrate transconjugants that were able to produce protoxin with identical antigenic properties to the donor strain. The heterospecific mating between *Bacillus subtilis* and *Bacillus thuringiensis* was also reported by Klier et al. (170). The plasmid harbouring the crystal gene (pBT42-1) cloned from *Bacillus thuringiensis* subsp. *berliner* 1715 in *Bacillus subtilis* was transferred to acrystalliferous *Bacillus thuringiensis* subsp. *kurstaki* or in a wild type strain of *Bacillus thuringiensis* subsp. *israelensis*. It was found that transconjugant cells were able to confer such a high expression of the crystal gene from *Bacillus thuringiensis* subsp.

berliner 1715. The crystal gene from chromosomal origin was integrated into chromosome and could not express. It was proposed that either transcription or translation might be suppressed. Lereclus et al. (171) reported the intergeneric mating experiments and showed that transfer of pAMB1 plasmid from *Streptococcus faecalis* to *Bacillus thuringiensis* could be performed by filter mating in agreement with conjugation-like system. This strain served as a plasmid donor in the mating system and demonstrated obvious differences between these two gram positive bacteria which did not obstruct plasmid transfer but the frequency of the transfer was considerably different depending on the *Bacillus thuringiensis* strain used as recipient. In 1987, Reddy et al. (172) reported the existence of self transmissible plasmid in four strains of *Bacillus thuringiensis.* These large transmissible (50-120 MDa) could mobilize pBC16 and other plasmids into *Bacillus cereus* and *Bacillus anthrasis* and were considered as conjugative plasmids. None of them encoded crystal toxin gene. DNA homology existed among Othese four plasmids but each displayed certain unique restriction fragments. Preliminary results indicated that part of homology in each plasmid could be attributed to the presence of the previously discovered *Bacillus thuringiensis* transposon, Tn4430 (173), the first transposon isolated from *Bacillus thuringiensis*.

6. Effect of Promoter sequence on gene expression

The promoter sequence is the DNA sequence which is specifically recognized and bound by the RNA polymerase enzyme and

subsequently initiated transcription. The transcriptional regulation can be achieved by modulating the efficiency which RNA polymerase productively interacts with the promoter sequence, i.e., strength of promoter. Therefore, the level of gene expression can be controlled by placing the gene under a weak or strong promoter. In *E.coli*, the promoter sequences reveal a remarkable homology in two regions located at position -10 and -35 bp from the transcriptional initiation site. The -10 region consists of 6 bp which form a consensus sequence TATAAT. The second consensus sequence, TTGACA, is centered around the -35 region. Gilbert (194) proposed that the -35 region is responsible for promoter recognition and that the -10 region is involved in the melting step which resulted in the formation of productive promoter complex. The nucleotide sequence of the intervening region appears to be unimportant. However, the precise distance between these two regions is crucial. The distance between -10 and -35 varies between 16 to 19 bp with 17 bp resulting in the maximum promoter strength. Mutations within -10 and -35 regions also result in a change of promoter strength. The transcription is normally initiated downstream from the -10 region, usually at a purine residue. In some cases, a more elaborate control mechanism may involve by other proteins for example CAP protein which facilitates the binding of RNA polymerase to the lac operon promoter (195). Despite this, most of the work has been performed in *E.coli*, and similar arrangements of these two highly conserved regions upstream from transcription initiation site have been identified in promoters of most prokaryotic systems. For gram

positive bacteria, *B.subtilis* which next to *E.coli* has been the second most studied genetic system. The transition of *B.subtilis* from vegetative growing cells to the sporulated cells is accompanied by changes in the predominant form of RNA polymerase (188). These modified RNA polymerases have novel sigma factors associated with the same core enzyme. Some of modified forms of RNA polymerase are reported to be present at a particular growth stage. The sporulation specific forms of RNA polymerase are presumably involved in the recognition of specific promoter sequence that control the expression of genes whose products are essential for sporulation. It was found in plasmid pPL603 that the endogenous promoter activity increases CAT activity during the late sporulation phase (132). The promoter activity was suggested to reside on 203 bp *Eco*R I-*Pst* I fragment. Therefore, these have evidences indicated that the promoter was highly regulated and under the control of sporulation (132, 188). *B.subtilis* RNA polymerase plays an important role in regulation of gene expression during growth and sporulation. The general features of *B.subtilis* RNA polymerase are similar to those reported for a number of prokaryotic cells. *B.subtilis* core RNA polymerase consists of 4 subunits, $\alpha_2\beta\beta'$, whereas holoenzyme contains an additional factor, i.e., sigma factor which plays an important role in promoter recognition. Although the *B.subtilis* enzyme is not extensively studied, it appears to function in a similar manner to that of *E.coli*'s enzyme. The holoenzyme complex involves in transcription initiation and promoter selection, then the

sigma factor is released from core enzyme which continues the elongation step. The remarkable finding in *B.subtilis* regarding the regulation of sporulation was the discovery of modified forms of RNA polymerase which appeared during the different stages of development. The first modified form of RNA polymerase discovered was the sigma-37 enzyme which is associated with sporulation. It appears that the sigma-37 enzyme is present in a small amount during vegetative growth and increases sharply to reach its maximum level during the early stationary phase then declines after the first two hours of sporulation whereas the sigma-55 enzyme is mainly found in the vegetative cell. The sigma-32 containing enzyme recognizes *spo* gene promoters and the time of its appearance is similar to sigma-37 enzyme, except that sigma-32 accounts for a smaller percentage of total RNA polymerase during early stages of sporulation (130). The minor form of modified RNA polymerase is sigma-28 containing enzyme which was detected in vegetative cell and enzyme was rapidly lost after the first hour of sporulation. The nucleotide sequences in -10 and -35 regions which are recognized by various form of RNA polymerase are listed below.

Holoenzyme	-35 region	-10 region
B.subtilis sigma-55	TTGACA	TATAAT
B.subtilis sigma-37	AGG-TT	GG-ATTG-T
B.subtilis sigma-32	AAATC	TA-TG-TT-TA
B.subtilis sigma-29	TT-AAA	CATATT
B.subtilis sigma-28	CTAAA	CCGATAT

CHAPTER III

MATERIALS AND METHODS

1.Chemicals and reagents

All chemicals used throughout this study were analytical grade or the purest grade available. All bacteriological media were obtained from Difco Laboratory.

Tris (hydroxymethyl) aminomethane, sodium chloride (NaCl), magnesium chloride ($MgCl_2 6H_2O$),dipotassium hydrogenphosphate(K_2HPO_4), potassium dihydrogenphosphate (KH_2PO_4), ammonium acetate, sodium acetate, acetic acid, iso-amyl alcohol,chloroform, phenol, absolute ethanol, hydrochloric acid, 8-hydroxyquinoline, sodium citrate, glucose, boric acid, calcium chloride ($CaCl_2$), and manganese chloride were purchaseed from Merck.

Ethidium bromide, dithriothreitol (DTT), sodium dedocyl sulfate (SDS), polyethylene glycol (PEG) MW 6000, disodium ethylene diamine tetraacetic acid (EDTA), sucrose, glycerol, bovine serum albumin (BSA), maleic acid, bromphenol blue, lysozyme, cesium chloride, agarode, ribonuclease A (RNAse A), acetyl coenzyme A, 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) and hexamine cobalt chloride were obtained from Sigma.

Coomassie brilliant blue G-250 was purchased from Bio Rad.

T_4 ligase and restriction enzymes were obtained from BRL. Antibiotics were bought from Sigma (rifampicin, tetracycline, ampicillin and chloramphenicol), Meiji (kanamycin) and Upjohn (spectinomycin). Nick translation system and Blu Gene Kit (non-radioactive nucleic acid detection system) were purchased from BRL.

2.Microorganisms and culturing procedures

Bacterial strain that were used in this study together with their properties and origins are listed below.

strain	plasmid	drug marker	source
E:coli DH5-∝	pBT8	Ap	our laboratory
B.sphaericus 2362(pBTC1)	pBTC1	TcStr	our laboratory
B.subtilis MI 111	none	none	our laboratory
B.subtilis MI 111(pTF6)	pTF6	Km(Neo)Cm	our laboratory
B.subtilis BR 151(pPL603)	pPL603	Km(Neo)Cm(spore)	PS Lovett*
B.subtilis BR 151(pPL703)	pPL703	Km(Neo)	PS Lovett*
B.megaterium O-016	none	none	our laboratory
B.megaterium O-016(pTF6)	pTF6	Km(Neo)Cm	our laboratory
B.megaterium O-016(pPL603)	pPL603	Km(Neo)	this study
B.megaterium O-016(pPL703)	pPL703	Km(Neo)	this study
B.megaterium O-016(pPLC1)	pPLC1	Km(Neo)	this study
B.t.i. c4Q2-72	none	Rf	DH Dean**
B.t.i. c4Q2-72(pTF6)	pTF6	RfKm(Neo)Cm	this study
B.t.i. c4Q2-72(pPL603)	pPL603	RfKm(Neo)	this study
B.t.i. c4Q2-72(pPL703)	pPL703	RfKm(Neo)	this study
B.t.i. c4Q2-72(pPLC1)	pPLC1	RfKm(Neo)	this study
B.t.i. 4Q2-72 (pPLC1)	pPLC1	RfKm(Neo)	this study

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All pure cultures of *E.coli* and *Bacillus sp.* were kept as stock cultures by streaking cultures on LB agar (1% tryptone, 0.5 % yeast extract, 0.5 % NaCl and 1.5 % Bacto Agar. For *Bacillus sphaericus*, antibiotic culturing media were used except that the agar was added at 2.0 % .

For drug resistant strains , the culture media were supplemented with either 50 ug/ml of ampicillin (Ap), 15 ug/ml of tetracycline (Tc), 20 ug/ml of chloramphenicol (Cm), 20 ug/ml of kanamycin (Km), or 50 ug/ml of rifampicin (Rf). The cultures were grown overnight at 37°C for *E.coli* or 30 °C for 1-2 days for *Bacillussp.* and subsequently kept at 4°C until used. For preparation of stock cultures, bacteria were grown in LB broth in rotary shaker until exponential phase. Subsequently, two volumes of cell culturecultures were mixed with 1 volume of 45 % glycerol and kept at -70°C in separated vials.

3. Culturing procedure for plasmid preparation

In general, basic growth medium in this study was LB broth medium which was composed of 1% tryptone, 0.5 % yeast extract and 0.5% NaCl.

The culture condition for plasmid preparation was initiated by inoculating culture from agar plate in LB broth containing an appropriate antibiotic(s) and incubated overnight in rotary shaker adjusted at 200rpm at 37 °C for *E.coli* or at 30 °C for *Bacillus sp.*. Subsequently, 1% of overnight culture was transferred to the medium with the same composition at the appropriate temperature as described previously to obtain mid-log phase culture for subsequent plasmid extraction.

4. Preparation of plasmid DNA

4.1 Small scale plasmid preparation

Plasmid from *E.coli* cells was extracted by rapid alkali-lysis method modified from that of Birnboim and Doly in 1979 (177). For small scale extraction, 1.5 ml of appropriately grown culture in LB broth containing the appropriate antibiotic was pipetted into Eppendorf tube and centrifuged in a microcentrifuge (Tomy Seiki Co, Japan) at 8000 rpm for 5 minutes. Cells were washed in 100 ul of solution I (50 mM glucose, 10 mM EDTA, and 25 mM Tris HCl pH8) and resuspended in 100 ul of the same solution containing 2 mg/ml of lysozyme and incubated at room temperature for 5-10 minutes; then, the tube was added with 200 ul of freshly prepared solution II (1% SDS, 0.2 M NaOH)and mixed immediately. The reaction mixture was maintained for 5 minutes at room temperature and then 150 ul of 3.0 M sodium acetate pH 4.8 was added. The tube was mixed by inverting

several times and further incubated on ice for 30 to 60 minutes.

The supernatant was collected by centrifugation at 10,000 rpm for 10 minutes and the clear supernatant was transferred to a new Eppendorf tube. Two volumes of cold absolute ethanol was added to precipitate the plasmid DNA and the tube was held at -20 °C for 1 hour. The DNA precipitate was collected by centrifugation at 10,000 rpm for 10 minutes and redissolved in 100 ul TE buffer (10mM Tris HCl and 1mM EDTA pH 7.5), then 50 ul of 7.5 M ammonium acetate was added to the DNA solution to make final concentration of 2.5 M. The plasmid DNA was reprecipitated with two volumes of cold absolute ethanol at -20°C for 1 hour and centrifuged as previously described. The pellet was washed once with 70% cold ethanol , dried in a dessicator and dissolved in 20 ul of TE buffer. RNA was removed by adding 1 ul of stock RNase (4 mg/ml) and incubated at 37°C for 30 minutes.

Plasmid extracted from *Bacillus sp.* was also prepared as described above for *E.coli* except that cells were incubated for a longer time (20-30 minutes) in solution I containing 4 mg/ml of lysozyme at 37°C.

4.2 Large scale plasmid preparation

For large scale preparation of plasmid DNA, the alkaline SDS method was also used but volume of the cell cultures and reagent solutions were increased and plasmids were purified by CsCl gradient ultracentrifugation (178).

The mixture of DNA solutions containing 4 ml of plasmid DNA,

4 grams of cesium chloride and 300 ul of 10 mg/ml ethhidium bromide was transferred to polyallomer tubes and were then centrifuged in a Hitachi HIMAC Centrifuge (model 70-P-72) at 54,000 rpm for 18 hours at 18°C (Rotor RPV 65T).

The covalently closed circular DNA was obtained by puncturing the tube at lower DNA band with needle connected to syringe under UV lamp. The ethidium bromide in the preparation was removed by extracting with water saturated iso-amyl alcohol and cesium chloride was removed by dialysing overnight in large volume of TE buffer pH 8.0 with two changes of TE buffer.

5.Quantitation of DNA

The method as described by Maniatis (178) was used to measure the amount of DNA or RNA in the preparation. For the quantitation of DNA, absorbance were taken at wavelength of 260 and 280 nm.

The OD_{260} allows the calculation of the concentration of nucleic acid in sample, an OD of 1 correspondes to approximately 50 ug/ml for double stranded DNA (178).

The ratio between the readings at 260 nm and 280 nm (OD_{260} / OD_{280}) provides an estimation for the purity of the nucleic acid. Pure preparation of DNA has OD_{260} / OD_{280} of 1.8. Sometimes , there is not enough DNA (less than 250 ng/ml) to assay spectrophotometrically, or DNA may be heavily contaminated with other UV absorbing substances that interfere with accurate analysis. A rapid method for estimating the amount of DNA in such sample is to utilize the UV induced fluorescence emitted by ethidium bromide

molecules intercalated into the DNA strands. Because the amount of fluorescence is directly proportional to the total amount of DNA, the quantity of DNA in the sample can be estimated by comparing the fluorescence yield of the sample with that of a series of standards. Approximately up to 10 ng of DNA can be detected by this method. This method was described by Maniatis (178).

6. Plasmid analysis

6.1 Restriction endonuclease digestion

Restriction of plasmid DNA was performed by using general buffer as previosly described by Maniatis et al, 1982 (178). Conditions for digestion and incubation periods were carried out according to the information given by manufacturers. The reactions were terminated by heating at 65°c for 15 minutes.

6.2 Agarose gel electrophoresis

For routine analysis of intact plasmid and DNA fragments, the horizontal type minigel electrophoresis chamber (Mupid, minigel electrophoresis set, Tokyo Co Ltd, Japan) was commonly used. A mixture of 5-10 ul of an appropriate amount of DNA solution and 3-5 ul of loading dye BJII solution (50% sucrose, 50 mM EDTA pH 8.0 and 0.05% bromphenol blue) was loaded into slot of the 0.7% agarose gel in Tris borate EDTA buffer (TBE: 89 mM Tris HCl,89mM boric acid and

2.5 mM EDTA pH 8.0).

Electrophoresis was routinely run at a constant voltage of 100 volts, at room temperature for 30 minutes or until the tracking dye ran off. The gel was removed and stained with an ethidium bromide solution (1-5ug/ml) for 10minutes and then destained in distilled water for 10-30 minutes. The DNA was visualized under a UV transilluminator(model TL-33 ultraviolet product Ltd, USA) and photographed.

To estimate molecular size of plasmid DNA fragment, seven fragments of lambda DNA digested with *Hind* III was used as a standard marker. Those fragments were 23.0, 9.4, 6.6, 4.5,2.3, 2.0 and 0.56 kb and used to determine unknown fragment size graphically, assuming a logarithmic relationship between molecular weight and electrophoretic mobility.

7. Protoplast transformation in *Bacillus megaterium* strain O-016

7.1 Protoplast preparation from *Bacillus megaterium* strain O-016

The procedure for protoplast preparation in *Bacillus megaterium* strain O-016 was similar to the method originally described by Chang and Cohen method (20) with some modifications. Bacteria were cultured overnight in 5 ml of LB broth at 30°C in shaker incubator at 200 rpm.

A 1% of overnight culture was inoculated into 50 ml of LB broth in 125 ml Erlenmeyer flask and was incubated at 30°C and

shaken at 200 rpm until reaching the mid exponential phase. Then, 20 ml of the culture was harvested and centrifuged (swing-rotor Kokusan H103N) at 3,000 rpm 10 minutes. The pellet was washed once in 10 ml of SMMP solution prepared by mixing equal volume of 2xSMM (1M sucrose /40 mM maleate pH6.5 /40 mM $MgCl_2$) with 4xPNB (penassay broth, Difco USA). Cells were once recentrifuged and resuspended in 1 ml SMMP solution containing 4 mg/ml lysozyme. The mixture was incubated for 30 minutes ina waterbathat 37°C. The appearance of protoplast was confirmed by phase contrast microscopy. Protoplast were washed in 10 ml of SMMP solution and centrifuged in a swinging rotor at room temperature at 1,800 rpm for 10 minutes. Protoplasts were immediately resuspended in 1 ml of SMMP solution.

The total viable cells were counted by mixing 50 ul of protoplast suspension with 450 ul SMMP solution and appropriate dilution wasspreaded on regeneration medium which enabled both protoplast and vegetative cell to grow. The number of viable vegetative cells(osmotic resistant cell or ORC)in theprotoplast suspension was determined by mixing 50 ul of suspension with 450 ul of 50 mM phosphate buffer and spreaded on LB agar in which only ORC could grow. The number of protoplast was the difference between total viable cells and ORC. The regeneration medium used in this experiment was DSL which consisted of 0.8 % Bacto Agar, 0.5 M sucrose, 0.5% glucose, 0.5% cassamino acid, 0.5% yeast extract, 0.5% NaCl, 1% tryptone, 20 mM $MgCl_2$, 0.35 % K_2HPO_4, 0.15 % K_2HPO_4 and 0.01% BSA.

7.2 Protoplast transformation of *Bacillus megaterium* strain O-016 with plasmid DNA

A 0.5 ml aliquots of washed protoplast was transferred to a sterilized centrifuge tube and plasmid DNA (1-3 ug) was added, and then 1 ml of 40 % polyethylene glycol 6000 (PEG) in 2 x SMM was added. The mixture was gently mixed and placed on ice for 2-3 minutes. The treated protoplasts were washed with 10 ml SMMP solution and centrifuged at the same condition (1,800 rpm for 10 minutes).

Protoplasts were resuspended in 1 ml SMMP solution and incubated at 30°C with gentle shaking for 2-3 hours and kanamycin (2ug/ml) was added to induced expression of kanamycin resistant gene. The cultures were further incubated for another 1 hour. The treated protoplasts were spread gently on regeneration medium containing kanamycin (20ug/ml) and incubated at 30°C for 1-3 days to allow kanamycin resistant transformant to develop. The number of protoplast and osmotic resistant cells were determined as described above.

8. Conjugation-like gene transfer procedure in *Bacillus thuringiensis* subsp. *israelensis*

Both donor and recipient cells from LB agar plate with appropriate antibiotics were separately inoculated in each 5 ml of LB broth tubes and aerated by shaking at 200 rpm at 30°C; then 50 ul of each overnight culture was transferred into separate tube of 5 ml LB broth medium.

The cultures were again incubated at the same condition for 3 to 4 hours or mid exponential phase. Then, they were ready for mating experiment which was performed by mixing 1 ml of each culture of donorandrecipient in sterilized tube and immediately filtered through millipore filter (0.45 um). The filter then was placed on LB agar and incubated at 30°C. After 6 hours of incubation, the filter was removed and resuspended in 1 ml of normal saline solution then 50 ul of cell suspension was spreaded on LB agar containingappropriateantibiotics. Thosewere then incubated 30°C for 48 hours and the number of transconjugants was scored.

The control experiment was carried out by the same procedure except only the donoror recipient cells were plated on LB agar containing antibiotics and the total number of parental strains were also determined by plating each culture of donor and recipient on LB agar containing appropriate antibiotic(s).

9. Cloning of *Bacillus thuringiensis* subsp. *israelensis* toxin gene promoter into *Bacillus megaterium* strain O-016

9.1 Restriction endonuclease digestion

A purified pBTC1 from *Bacillus sphaericus* 2362 was doubly digested completely with *Bam* HI and *Pst* I. Plasmid vector , pPL703 (4.9 kb), was also digested completely with the same enzymes.

9.2 Ligation of DNA fragment into the plasmid vector pPL703

After running electrophoresis in 1% NuSieve GTG agarose (FMC Bio product) for insert fragment, gel was stained with ethidium bromide, then the desired 400 bp *Bam* HI-*Pst* I fragment band was excised under UV transilluminator and put into Eppendorf tube. Remelt agarose slice by heating in waterbath at 68°C for 10 minutes (do not exceed 70°C), then the volume of melted agarose was determined and removed volume containing the required quantity of insert. Melted agarose was added with steriled deionized water (prewarm to 37°C) at least three times the volume of the melted agarose (the total volume of added water should not be over 30 ul). After being heated again in waterbath at 65°C for 10 minutes and chilled immediately on ice for 5 minutes, the insert mixture was added with the appropriate amount of prepared vector to obtain molar ratio of insert to vector of 5 : 1 and added the stock solution of 650 mM Tris HCl pH7.5, 10 mM ATP, 100 mM dithiothreitol (DTT), 100 mM $MgCl_2$, 10 mM hexamine cobalt chloride to make final concentration of 5 , 1 , 10 , 1 0 , 1 mM, respectively. Eventually, the ligation mixture was adjuted to appropriate volume with steriled deionized water to make final volume about 20-50 ul and 1-2 unit of T_4 DNA ligase was added. The reaction mixture was incubated at 15°C for overnight and ready for transformation experiment.

10. Recovery of 3.7 kb (2x1.85) *Xba* I fragment from pBT8 by using low melting temperature agarose

pBT8 was completely digested with *Xba* I and electrophoresed

in 0.7% agarose gel. After staining the gel in ethidium bromide, the 2 x 1.85 kb *Xba* I fragments were recovered by cutting slices of gel containing such DNA bands under UV light. The gel slice was laid on a new electrophoresis chamber and cover with 1% low melting temperature agarose. After the gel became hardening, electrophoresis was performed until the DNA band was completely moved from the hard gel into the low melting temperature gel. Usually it took about 10-15 minutes. After staining the low melting temperature gel containing the desired fragment was again cut under UV light, put into Eppendorf tube containing one or two volumes of TE buffer.

The gel was melted by heating in waterbath at 65°C for 10 minutes or until the low gel were completely melted. The melted gel was immediately removed by extracting twice with saturated phenol, and the agarose will be trapped between aqueous and phenol phase. The aqueous was collected and reextracted with diethyl ether twice to remove phenol residue (178). Diethyl ether was removed by evaporation in water bath at 60-65°C for 10-15 minutes. The DNA fragment were collected by ethanol precipitation as described previously.

11. Southern blot DNA-DNA hybridization

11.1 Southern transfer

DNAs from different strains of *Bacillus sp.* and DNA fragments were electrophoresed in 0.7% agarose gel and stained in ethidium bromide. After taking photographs, gels were denatured by

soaking in 1.5 M NaCl / 0.5 M NaOH for 20-30 minutes and was then neutralized by treating the denatured agarose gel in 3 M NaCl / 0.5 M Tris HCl pH 7.0 for 30-40 minutes.

The DNA from agarose gel was transferred to nitrocellulose or nylon membrane by Southern blot transfer technique as described previously (178,179).

After overnight transfer, the nitrocellulose membrane was washed with 2 x SSC (1 x SSC : 0.15 M NaCl, 0.015 M sodium citrate pH 7.0) the dried nitrocellulose membrane was placed between two pieces of filter paper and baked at 80°C for two hours.

11.2 Preparation of biotinylated DNA probes

1 ug of plasmid DNA was labelled with biotinylated-dATP (biotin-14-dATP) by nick translation (180) in the presence of unlabelled dTTP, dGTP and dCTP (no dATP) using BRL nick translation kit (BRL, USA) then the biotin-labelled DNA was isolated from free mononucleotide by ethanol precipitation and redissolved in 20 ul of TE buffer and probes were tested for intensity of labelling by comparing with the standard biotinylated DNA (200 pg/ul) available in Blue Gene Detection kit.

11.3 Prehybridization and hybridization with biotinylated DNA probe

After Southern blotting and baking the filter for 2 hours at 80°C, the filter was once soaked in 2 x SSC until uniformly hydrated and presoaked in 4 x SET (1 x SET : 0.15 M NaCl / 0.03 M Tris HCl pH 8.0 / 0.1 mM EDTA) for 10 minutes at room temperature.

For the prehydridization step, the filter was soaked in prehybridization solution (1ml/10cm^2 filter) containing 10xDenhardt's solution, 4 x SET, 0.5% SDS, 10 ug/ml heated denatured sheared calf thymus DNA (1 x Denhardt's solution : 0.02% Ficoll MW 400,000, 0.02% polyvinyl pyrollidone MW 360,000, 0.02% bovine serum albumin). The filter was incubated at 42°C in the sealed polypropylene bag with prehybridization solution for 2-4 hours.

For the hybridization step, after removing prehybridization solution, hybridization solution (1 ml/cm^2) containing 45% formamide, 2 x Denhardt's solution, 4 x SET, 0.5% SDS, 10 ug/ml heated denatured sheared calf thymus DNA and 100 ng/ml heated denatured biotinylated, DNA probe was added to the filter in the same bag then the filter was incubated at 42 °C overnight to achieve maximal hybridization.

For the post-hydridization step, the filter was washed twice with 250 ml of 2 x SSC / 0.1% SDS (v/v) for 3 minutes at room temperature, twice with 250 ml of 0.2 x SSC / 0.1% SDS (v/v) for 3 minutes at room temperature, twice with 0.16% SSC / 0.1% SDS (v/v) for 15 minutes at 50-55 °C with agitation and eventually rinsed the filter in 2 x SSC briefly at room temperature.

11.4 Filter blocking and colourimetric detection of biotin-labelled probe-target hybrid

The hybridized filter was rehydrated in blocking buffer 1 (0.1 M Tris HCl pH 7.5, 0.5 M NaCl) for 3 minutes with agitation and incubated for 1 hour at 65°C in buffer 2 (3% BSA in buffer 1) ,

dried at 80°C for 10-20 minutes, and rehydrated briefly in the same fresh solution. Filter was exposed to BRL streptavidin-alkaline phosphatase (SA-AP) conjugate in buffer 1 for 10 minutes (7 ul of SA-AP conjugate in 7 ml buffer 1 / 100 cm^2 filter) with agitation at room temperature. Filter was immediately washed twice with 20-40 fold greater volume of buffer 1 with agitation for 15 minutes and washed once with buffer 3 (0.1 M Tris-HCl pH9.5 / 0.1 M NaCl / 50 mM $MgCl_2$) at room temperature for 10 minutes.

For colour visualization, filter was incubated in the dye solution, 7.5 ml buffer 3 containing 33 ul nitroblue tetrazolium (NBT 75 mg/ml) and 25 ul of 5-bromo-4-chloro-3-indolylphosphate (BCIP 50 mg/ml), within a sealed hybridization bag. Colour development was processed in the dark or low light for 30 minutes to 3 hours. Longer incubation might result in increasing background. After colour development, filter was washed in 20 mM Tris HCl pH 7.5, 0.5 mM EDTA solution to terminate the reaction. Filter was kept dry and protected from strong light.

12. Assay for specific activity of enzyme chloramphenicol acetyltransferase (CAT) in *Bacillus sp.*

12.1 Growth of *Bacillus sp.*

Bacillus subtilis MI 111, *Bacillus subtilis* BR 151, *Bacillus megaterium* strain O-016 and *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 were purified by streaking on LB agar containing appropriate antibiotic and grown in NB broth overnight at

30°C with 200 rpm rotary shaker. 1% overnight cultures were inoculated in 250 ml Erlenmeyer flask containing 100 of NBS broth (nutrient broth supplemented with 1 mM $MgCl_2$, 0.7 mM $CaCl_2$, and 0.05 mM $MnCl_2$), then the cultures were incubated at 30°C with 200 rpm shaking. To observe cell growth, a 5 ml of each culture broth was taken at proper intervals for 15 hours and 24^{th} hour of incubation and determined the optical density (OD) at 600 nm by spectrophotometer (Shimadzu, UV-visible recording spectrophotometer) using uninoculated medium as the blank control and diluent for making dilution when absorbance of culture was over 0.8 .

12.2 Crude extracts from *Bacillus sp.*

Various strains of *Bacillus sp.* containing the relevant plasmid were purified and inoculated in NB broth overnight at 30°C with 200 rpm shaking. 1% of overnight cultures were transferred into 100 ml of NBS broth added with 2 ug/ml of chloramphenicol for induction of chloramphenicol acetyltransferrase gene (*cat*), then the cultures were incubated at 30°C with 200 rpm shaking.

20 ml of cultures were harvested at mid exponential, T_0, T_2 and T_8 according to the growth of each strain. The harvested cells were collected by refrigerated centrifugation at 8,000 rpm for 10 minutes and washed once with 0.01 M Tris HCl pH 7.8 . The pellets then were resuspended in 2 ml of the same buffer. Then, cell suspensions were added with 20 ug/ml lysozyme and incubated at 37°C for 20 minutes. The lysozyme treated cell suspensions were sonicated

for 10 seconds in ice bath. The supernatants were collected by centrifuging at 10,000 rpm for 10 minutes,then samples were ready for enzyme assay.

12.3 The quantitation of protein content in the samples

The amount of the protein in the samples was measured by using the method which involved the binding of coomasie brilliant blue G-250 as described previously by Bradford (23).

A 100 ul of the sample was added to 5 ml of protein reagent [Final concentration : 0.01% (w/v) coomasie brilliant blue G-250 , 4.7% (w/v)ethanol and 8.5% (w/v)phosphoric acid then mixed by either invertion or vortexing. The absorbace at 595 nm was measured after 2 minutes against reagent blank prepared from 100 ul of appropriate buffer mixed with 5 ml of protein reagent. The colour of the reaction is stable for 3 hours. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the amount of protein in unknown sample.

12.4 Assay of enzyme chloramphenicol acetyltransferrase (CAT) in sample

The method and calculation used for enzyme specific activity determination was described previously by Shaw (22). The reaction mixture was freshly prepared from the individual stock reagents to obtain the final concentration of each component as the following : 100 mM Tris HCl pH 7.8, 0.1 mM acetyl-CoA, 0.4 mg/ml 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and 0.1 mM chloramphenicol.

A 1.0 ml of working solution in 1 cm path length cuvett was added with 20 ul of sample at 37°C and mixed well. The absorbance of yellow colour increased in the reaction mixture was read at 15 secondintervals at 412 nm. The net change of absorbance per minute was calculated to give value for enzyme activity. The enzyme specific activities were expressed as micromole of chloramphenicol acetylated per minute per milligram protein (U/mg of protein).

13. Determination of relative plasmid copy number

Quantitation of plasmid copy number was done by inoculation bacterial strain harbouring appropriate plasmid in NB broth containing appropriate antibiotic and incubated at 30°C for overnight with shaking at 200 rpm; then 1 % overnight culture was transferred to NBS broth and grown at the same condition until reaching the exponential phase. Cells were collected and total cell count of each culture was performed by viable plate count technique. Known amount of cells were subjected to the alkali lysis method to obtain the crude recombinant plasmid which were further treated with phenol-chloroform extractionto remove contaminated protein. The plasmid DNA was electrophoresed at 100 volts in 0.7% agarose and stained in ethidium bromide. The intensity of bands in the sample were compared with those of known concentration plasmid which were used as standard.

14. Stability of recombinant plasmid pPLC1 in *Bacillus megaterium* strain O-016 and *Bacillus thuringiensis* subsp. *israelensis*

A loopful of cells from a single colony harbouring recombinant plasmid was grown in 5 ml LB broth containing kanamycin (20ug/ml) at 30°C overnight with 200 rpm shaking. The cultures were daily subcultured in 5 ml of fresh LB broth with or without kanamycin by 1% (v/v) inoculum of previous day cultures every day for 7 days and once a week for the second, the third and the fourth week. A suitable dilution was prepared and plated on LB agar with and without kanamycin to determine the number of viable cells and the number of drug resistant cells, respectively. Eventually,the colonies on kanamycin plates were randomly selected and extracted plasmid DNA to assure the presence of appropriate plasmids.

CHAPTER IV

RESULT

1) Protoplast transformation in *Bacillus megaterium* strain O-016 by plasmids pPL603 and pPL703

A strain of *Bacillus megaterium* strain O-016 was first isolated in 1984 by Loprasert, S. (174) and designated as *Bacillus* strain O-016. This strain was recently identified as *Bacillus megaterium* strain O-016 by de Barjac. The detailed biochemical reactions which led to the identification of this strain appeared in Annex A. *Bacillus megaterium* strain O-016 contained no plasmid, and was found to be very sensitive to lysozyme, and appeared to be suitable for use as host for protoplast transformation.

This study intended to use *Bacillus megaterium* strain O-016 asanintermediate host fortransferring genes into *Bacillus thuringiensis*. However, one must first develop efficient method for transforming *Bacillus megaterium* strain O-016. Since this bacterial strain has been found to be highly sensitive to lysozyme, thus, the most appropriate method for introducing gene into *Bacillus megaterium* strain O-016 might be via protoplast transformation technique. However, in order to exploit this technique effectively, one must also have appropriate conditions for regeneration of the transformed protoplasts. The medium used to regenerate protoplast of *Bacillus megaterium* strain O-016 was DSL medium which contained 0.5 M sucrose, 1 % tryptone and 0.5 %

NaCl. This formulation is similar to the composition of mDM-3 of Loprasert, S. (174) except that the sodium succinate was omitted. The detailed procedure for preparation of this regeneration medium appears in Materials and Methods section. As shown in Table 6, the regeneration frequency of *Bacillus megaterium* strain O-016 was found to be 7.0 % in DSL medium which was higher than the regeneration frequencies obtained by using media DM-3 or mDM-3 but very similar to the regeneration frequency obtained by using mDM-3T regeneration medium. By using protoplast transformation technique as described in Materials and Methods section, it was found that plasmids pPL603 and pPL703 could be introduced into *Bacillus megaterium* strain O-016. The number of transformants were determined by counting the number of kanamycin resistant colonies that appeared on DSL plates containing 20 ug/ml of kanamycin. Data as shown in Table 7 indicated that the frequency of transformation were 1.0×10^3 and 4.1×10^2 transformants per ug DNA of plasmids pPL603 and pPL703, respectively. In the control experiment where the plasmid was not added, the kanamycin resistant colony could not be detected. The transformation frequency obtained from plasmids pPL603 and pPL703 were found to be similar to the previous reported value that was obtained by using plasmid pTF6 (Line 1, Table 7).

In order to confirm the presence of relevant plasmids in the *B.megaterium* strain O-016 transformant, the kanamycin resistant colonies were randomly selected for the plasmid extraction, and the extracts were electrophoresed using 0.7% agarose gel. From eleven

Table 6 The regeneration frequency of *Bacillus megaterium* strain O-016 on various regeneration media.

Regeneration medium	Total viable count(CFU/ml)	ORC[a] (CFU/ml)	Total protoplast (CFU/ml)[b]	Regenerated[c] cell(CFU/ml)	Regeneration frequency[d]
DSL	$1.2x10^9$	$9.6x10^6$	$1.2x10^9$	$8.4x10^7$	7.0
DM	$4.5x10^7$	$6.0x10^2$	$4.5x10^7$	$1.9x10^6$	4.2*
mDM-3	$4.5x10^7$	$6.0x10^2$	$4.5x10^7$	$2.4x10^6$	5.3*
mDM-3T	$4.5x10^7$	$6.0x10^2$	$4.5x10^7$	$3.1x10^6$	6.9*

(a) Osmotic resistant cell (ORC) which was determined by counting the number of colonies of lysozyme treated cell which grew on LB agar.

(b) The number of total protoplast was determined by substracting the number of ORC from the number of total viable count.

(c) The regenerated cell was determined by determining the number of colonies which grew on DSL medium.

(d) Regeneration frequency was determined by using the following calculation

$$\frac{100 \times [\text{ regenerated cell} - \text{ORC }]}{[\text{ total viable count} - \text{ORC }]}$$

* Data taken from Chawala, U 1990 (161)

Table 7 Frequency of transformation of various plasmids in *Bacillus megaterium* strain O-016.(a)

Plasmid	Number of colonies on DSL media containing Km	Transformation frequency (transformants / ug DNA)
pTF6(b)	6.4×10^3	3.2×10^3
pPL603	2.0×10^3	1.0×10^3
pPL703	8.1×10^2	4.1×10^2
without plasmid	ND(c)	ND

(a) Protoplasts were generated by suspending cells in SMMP solution containing 4 mg/ml of lysozyme at 37°C for 30 minutes. Two microgram of plasmid DNA was used for each transformation experiment. The transformants were selected on DSL medium supplemented with 20 ug/ml of Km.

(b) Data taken from Chawala, U 1990 (161)

(c) ND = Not detectable

randomly picked kanamycin resistant *B.megaterium* strain O-016 clones which were transformed with plasmid pPL603, they were all found to contain plasmids which comigrated with the purified plasmids pPL603. The plasmid pPL603 used as marker was extracted and purified from *Bacillus subtilis* BR 151. The plasmid patterns of *B.megaterium* strain O-016 receiving plasmid pPL603 was shown in Figure 1. One transformant was designated as *B.megaterium* strain O-016 (pPL603) was selected for further study. For *Bacillus megaterium* strain O-016 which was transformed with plasmid pPL703, eleven clones of kanamycin resistant transformants were also randomly picked for plasmid extraction. All of them were also found to harbour plasmids which migrated to the same position with the purified plasmids pPL703. The plasmid pPL703 used as marker was extracted and purified from *Bacillus subtilis* BR 151. The plasmid patterns of *B.megaterium* strain O-016 receiving plasmid pPL703 was shown in Figure 2. One transformant was designated as *B. megaterium* strain O-016 (pPL703) was also selected for further study. Furthermore, as will be shown in a latter section, both *B. megaterium* strain O-016 (pPL603) and *B.megaterium* strain O-016 (pPL703) could be shown to express chloramphenicol acetyltransferase activities.

2) Transfer of plasmid pPL603, pPL703 and pTF6 from *Bacillus megaterium* strain O-016 into *Bacillus thuringiensis* subsp. *israelensis* c402-72 by conjugation-like process

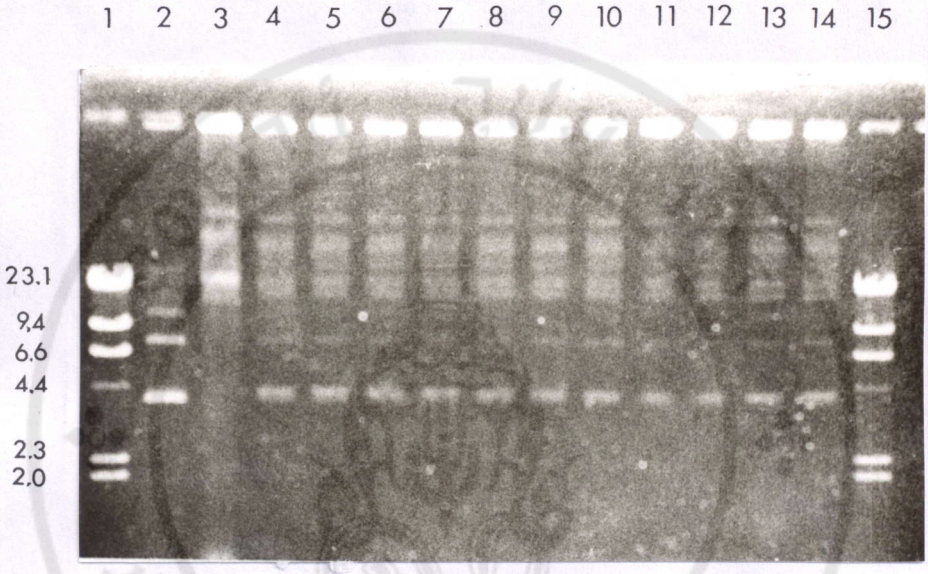

Figure 1 Agarose gel electrophoresis of plasmid pPL603 extracted from *Bacillus megaterium* strain O-016 transformants. Plasmids were extracted from *Bacillus megaterium* strain O-016 (lane 3), or from various transformants of *Bacillus megaterium* strain O-016 (lane 4 through 14). Lane 2 contained purified plasmid pPL603 which was extracted from *Bacillus subtilis* BR 151. Lanes 1 and 15 contained *Hind* III digested λ DNA.

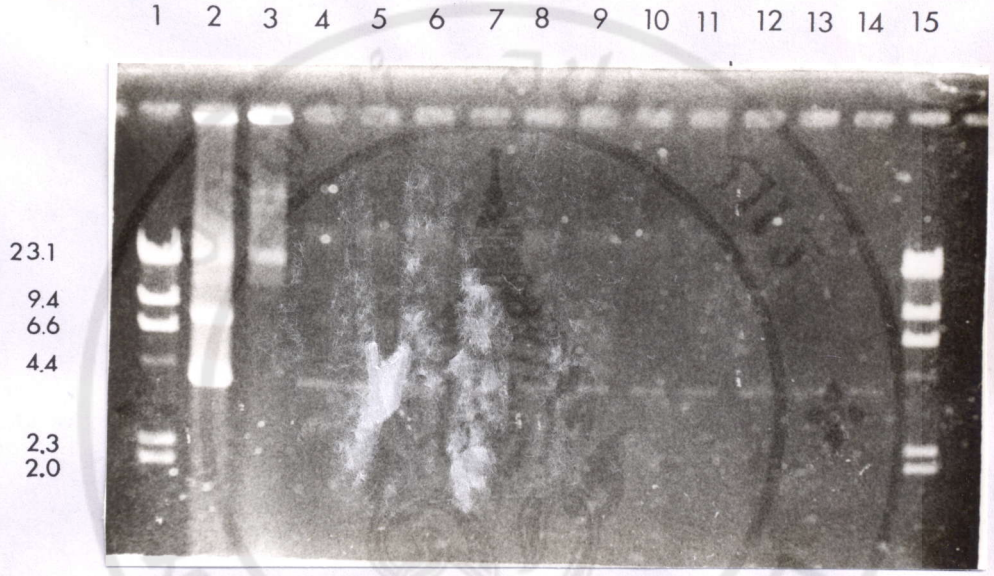

Figure 2 Agarose gel electrophoresis of plasmid pPL703 extracted from *Bacillus megaterium* strain O-016 transformants. Plasmids were extracted from *Bacillus megaterium* strain O-016 (lane 3), or from various transformants of *Bacillus megaterium* strain O-016 (lane 4 through 14). Lane 2 contained purified plasmid pPL703 which was extracted from *Bacillus subtilis* BR 151. Lanes 1 and 15 contained *Hin*d III digested λ DNA.

One each of stable transformants of *Bacillus megaterium* strain O-016 which harboured plasmid pPL603, pPL703 and pTF6 was selected for using as donors for transferring the appropriate plasmid into *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 via conjugation-like process. Three transformants were designated as *B.megaterium* strain O-016 (pPL603), *B.megaterium* strain O-016 (pPL703) and *B.megaterium* strain O-016 (pTF6) for those which carried plasmid pPL603, pPL703 and pTF6, respectively. In the following experiments, a rifampicin resistant strain of *B.t.i.* c4Q2-72 was used as the recipient. The donor and recipient cultures were mixed in equal volume and filtered through sterilized millipore filter. The millipore membrane containing the mating mixture was placed on an LB agar plate and the mating was allowed to take place for 6 hours as previously described in the Materials and Methods section. After the mating, transconjugants were selected on LB agar containing both kanamycin and rifampicin at the concentration of 20 and 50 ug/ml, respectively. The results as shown in Table 8 indicated that the frequencies of transfer of plasmid pPL603, pPL703 and pTF6 from relevant transformants of *Bacillus megaterium* strain O-016 to *Bacillus thuringiensis* c4Q2-72 were 2.8×10^{-8}, 5.0×10^{-8} and 3.2×10^{-8}, respectively. The rate of transfers was expressed in term of the ratio between number of transconjugant per one recipient. As shown in the Table 8, the rate of plasmid transfers was found to be relatively low regardless of the types of plasmid used. The numbers of transconjugant obtained were between 25-50 colonies in all three mating pairs.

Table 8 Frequency of plasmid transfers from *Bacillus megaterium* strain O-016 harbouring indicated plasmids to *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 via conjugation-like process.

Mating pair	Number of cell (CFU / ml)		Number of transconjugant	Frequency of transfer[a]
	B.megaterium strain O-016	*B.t.i.* c4Q2-72		
*B.megaterium*O-016(pPL603)x*B.t.i.*c4Q2-72	9.4×10^8	8.7×10^8	25	2.8×10^{-8}
*B.megaterium*O-016(pPL703)x*B.t.i.*c4Q2-72	1.2×10^9	9.9×10^8	50	5.0×10^{-8}
*B.megaterium*O-016(pTF6) x*B.t.i.*c4Q2-72	1.7×10^9	1.0×10^9	32	3.2×10^{-8}

[a] Frequency of transfer = $\frac{\text{Number of transconjugant}}{\text{Number of recipient (Before mating)}}$

In order to demonstrate the existence of relevant plasmids in transconjugants, anumberof kanamycin and rifampicin resistant colonies from each mating pair were randomly selected for plasmid extraction. Extracts of all five *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 transconjugants obtained from the mating pair involving plasmid pPL603 could be shown to harbour additional plasmids which comigrated with plasmid pPL603 (Figure 3). Extracts of all five *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 transconjugants obtained from the mating pair involving plasmid pPL703 could be shown to harbour additional plasmids which comigrated with plasmid pPL703 (Figure 4) and, extracts of all eleven *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 transconjugants obtained from the mating pair involving plasmid pTF6 could be shown to harbour additional plasmids which comigrated with plasmid pTF6 (Figure 5). As shown in Figure 3, 4, 5, all *B. thuringiensis* subsp. *israelensis* c4Q2-72 transconjugants contained appropriate plasmids which migrated to the same position in agarose gel electrophoresis with pPL603, pPL703 and pTF6 extracted from the respective donors i.e. *Bacillus megaterium* strain O-016 (pPL603) *Bacillus megaterium* strain O-016 (pPL703) and *Bacillus megaterium* strain O-016 (pTF6) (Figure 3, 4 and 5).

3) Restriction analysis of plasmid pPL603, pPL703 and pTF6 in *Bacillus megaterium* strain O-016 transformants *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 transconjugants

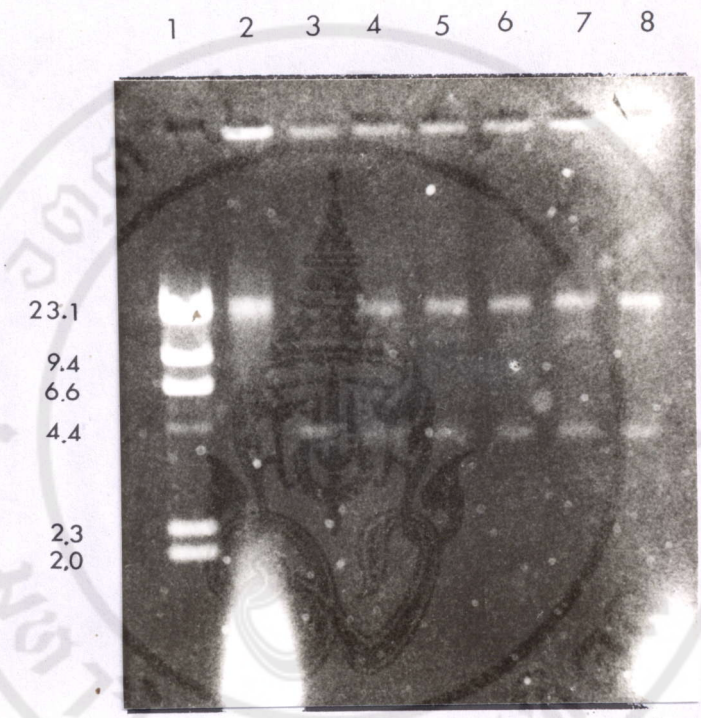

Figure 3 Agarose gel electrophoresis of plasmid pPL603 extracted from *B.t.i.* c4Q2-72 transconjugants. Plasmids were extracted from *B.t.i.* c4Q2-72 (lane 2), *B.megaterium* strain O-016 harbouring pPL603 (lane 3), or *B.t.i.* c4Q2-72 transconjugants (lane 4 through 8). Lane 1 contained *Hin*d III digested λ DNA.

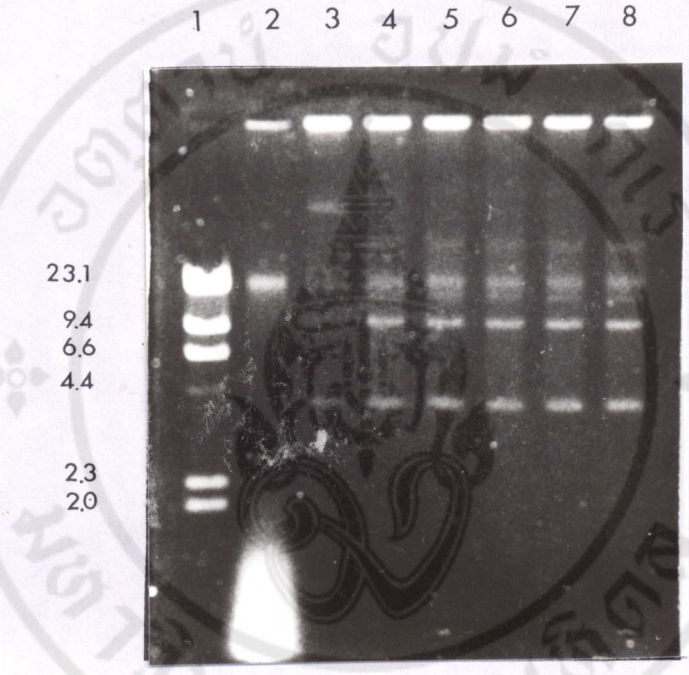

Figure 4 Agarose gel electrophoresis of plasmid pPL703 extracted from *B.t.i.* c4Q2-72 transconjugants. Plasmids were extracted from *B.t.i.* c4Q2-72 (lane 2), *B.megaterium* strain O-016 harbouring pPL703 (lane 3), or *B.t.i.* c4Q2-72 transconjugants (lane 4 through 8). Lane 1 contained *Hin*d III digested λ DNA.

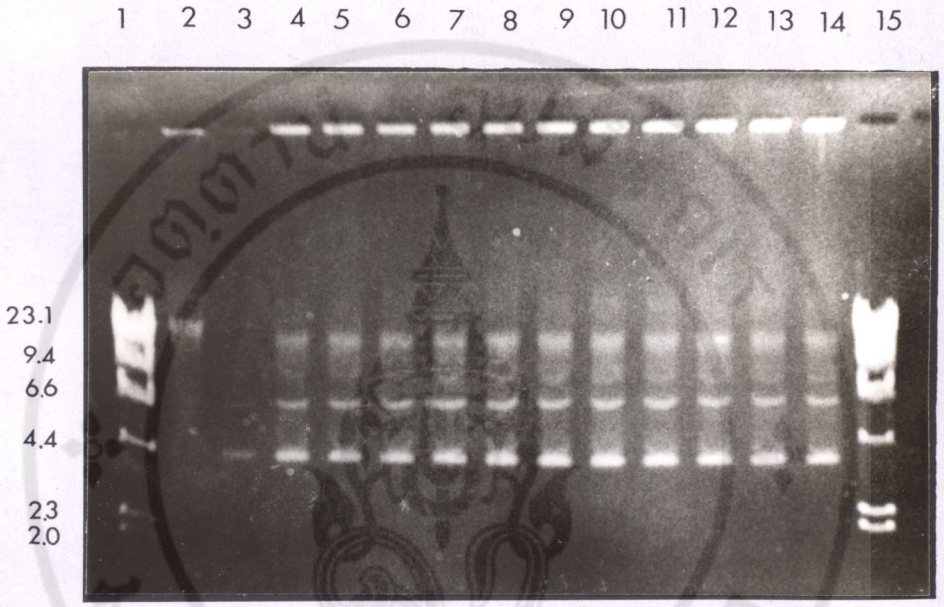

Figure 5 Agarose gel electrophoresis of plasmid pTF6 extracted from *B.t.i.* c4Q2-72 transconjugants. Plasmids were extracted from *B.t.i.* c4Q2-72 (lane 2), *B. megaterium* strain O-016 harbouring pTF6 (lane 3),or *B.t.i.* c4Q2-72 transconjugants (lane 4 through 14). Lanes 1 and 15 contained *Hin*d III digested λ DNA.

To ensure that the kanamycin traits in *Bacillus megaterium* strain O-016 transformants and *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 transconjugants were resulted from the presence of the plasmids pPL603, pPL703 or pTF6, The plasmids from all relevant strains were analysed. After extraction, the restriction pattern of the digested plasmids were compared by using gel elctrophoresis with those obtained from the original hosts. The schematic diagram together with the method for construction of pPL603, pPL703 and pTF6 appears in Figure 6. As evidenced from Figure 6, all three plasmids possessed a single site for *Eco*R I and *Bgl* II restriction. Thus, if the plasmids were completely digested with *Eco*R I and *Bgl* II, they would result in two fragments i.e. 2.9 and 2.2 kb for pPL603, 2.7 and 2.2 kb for pPL703 and 2.8 and 2.2 kb for pTF6. The pattern of unrestricted or *Eco*RI and *Bgl*II restricted plasmids in *Bacillus subtilis* host was shown in Figure 7. Whereas the pattern of those plasmid *Bacillus megaterium* strain O-016 and *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 were shown in Figure 8 and 9, respectively. Regardless of the hosts, the unrestricted plasmid pPL603, pPL703 and pTF6 appeared to migrate to the same position in the agarose gel. The restriction patterns of the plasmid from the transformants and transconjugants were compared and proved to be pPL603, pPL703 and pTF6 by identical restriction patterns with those plasmids isolated from *Bacillus subtilis*. Each double digested plasmid by these two restriction enzymes (*Eco*R I and *Bgl* II) produced two DNA fragments of 2.9 and 2.2 kb for pPL603, 2.7 and 2.2 kb for pPL703

Plasmid Construction & Restriction Map

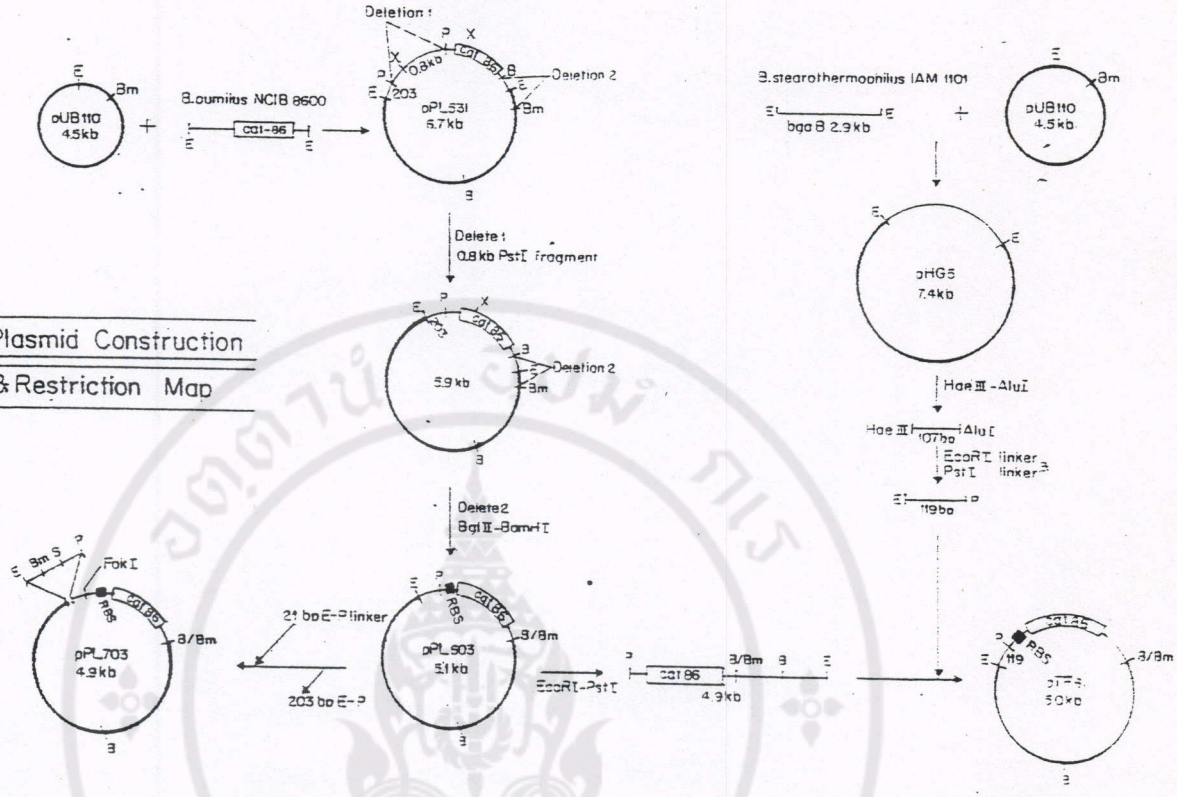

Figure 6 Schematic diagrem of plasmid pPL603 and its derivatives pPL703 and pTF6. Plasmid pPL603 was derived from pPL531 which contained *cat-86* gene from *Bacillus pumilus* NCIB 8600. The plasmid pPL531 was internal derived from ligation of plasmid pUB110 and *cat 86* gene. Two fragments were deleted from pPL531 which to resulted in plasmid pPL603. Plasmid pPL703 was derived from pPL603 by replacement of the 203 bp *Eco*R I-*Pst* I fragment which conferred post exponential promoter activity with a 21 bp oligonucleotide from phage M13mp7 harbouring unique *Bam*H I and *Sal* I cloning sites. Plasmid pTF6 was constructed from 4.9 *Eco*R I - *Pst* I fragment of pPL603 and the 107 bp *Hae* III - *Alu* I fragment (converted to *Eco*R I and *Pst* I site by linkers) from pHG5 which carried promoter activity of *bgaB* gene from *Bacillus stearothermophilus* IAM 11001 (132. 133, 135). The abbreviation in the figure are as following. E, *Eco*R I; P, *Pst* I; B, *Bgl* II; Bm, *Bam*H I; X, *Xba* I; S, *Sal* I

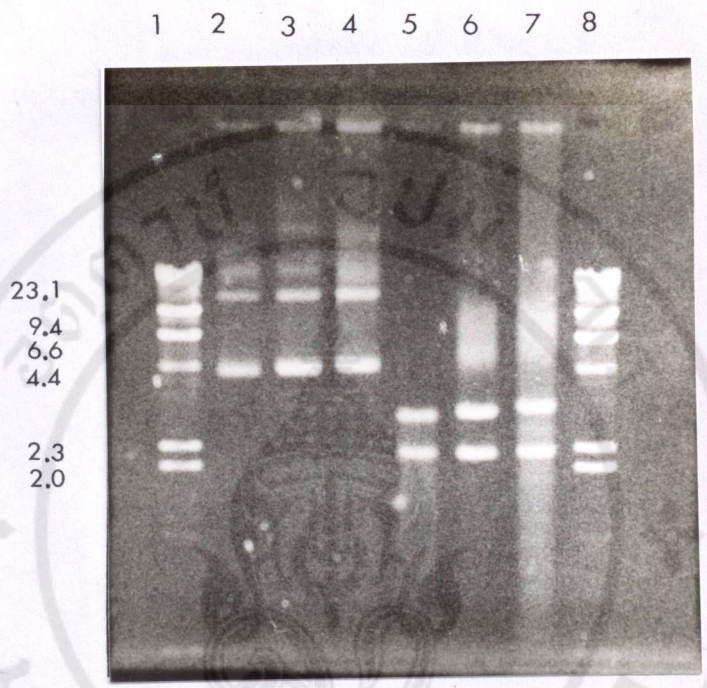

Figure 7 Agarose gel electrophoresis of unrestricted and *Eco*R I -*Bgl* II restricted preparations of pPL703, pTF6 and pPL603 from *B.subtilis*. The plasmids were extracted from *B.subtilis* containing pPL703 (lane 2), pTF6 (lane 3) or pPL603 (lane 4) and subjected to agarose gel electrophoresis. The corresponding plasmid preparations, pPL703 (lane 5), pTF6 (lane 6) or pPL603 (lane 7) were restricted with *Eco*R I and *Bgl* II prior to subjecting to agarose gel eletrophoresis. Lanes 1 and 8 represented *Hin*d III digested λ DNA.

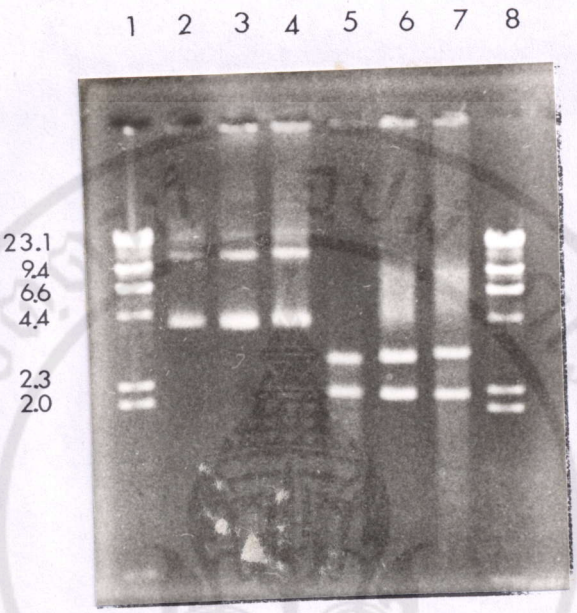

Figure 8 Agarose gel electrophoresis of unrestricted and *Eco*R I-*Bgl* II restricted preparations of pPL703, pTF6 and pPL603 from *B.megaterium* strain O-016 transformants. The plasmids were extracted from *B.megaterium* strain O-016 containing pPL703 (lane 2), pTF6 (lane 3) or pPL603 (lane 4) and subjected to agarose gel electrophoresis.The corresponding plasmid preparations, pPL703 (lane 5), pTF6 (lane 6) or pPL603 (lane 7) were restricted with *Eco*R I and *Bgl* II prior to subjected to agarose gel electrophoresis. Lanes 1 and 8 represented *Hin*d III digested λ DNA.

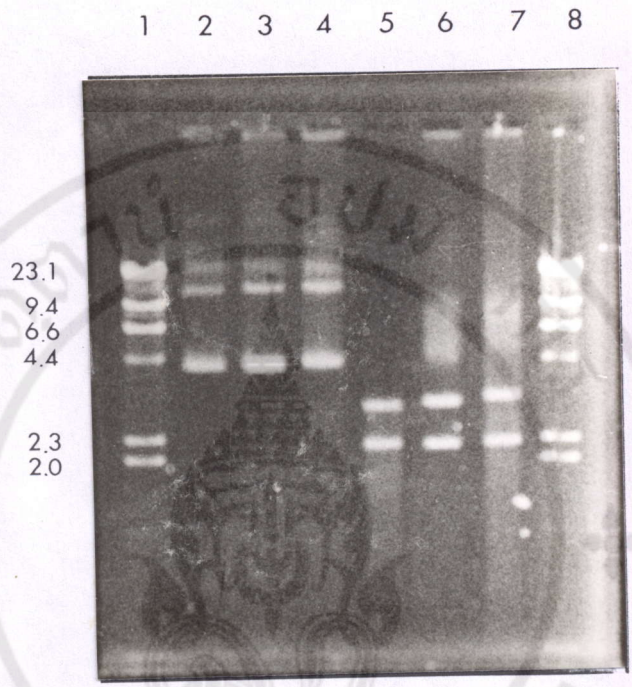

Figure 9 Agarose gel electrophoresis of unrestricted and *Eco*R I-*Bgl* II restricted preparations of pPL703, pTF6 and pPL603 from *B.t.i.* c4Q2-72 transconjugants. The plasmids were extracted from *B.t.i.* containing pPL703 (lane 2), pTF6 (lane3) or pPL603 (lane 4) and subjected to agarose gel elctrophoresis. The corresponding plasmid preparation , pPL703 (lane 5), pTF6 (lane 6) or pPL603 (lane 7) were restricted with *Eco*R I and *Bgl* II prior to subjecting to agarose gel electrophoresis. Lanes 1 and 8 represented *Hin*d III digested λ DNA.

and 2.8 and 2.2 kb for pTF6 in all three hosts, *B. subtilis, B. megaterium* strain O-016 and *B.t.i.* c4Q2-72, being examined as shown in Figure 7, 8 and 9. Basing on the fact that plasmids pPL603, pPL703 and pTF6 contained *Eco*R I and *Bgl* II as unique site, it could be calculated that the three plasmids had the sizes of 5.1, 4.9 and 5.0 kb, respectively in all three hosts i. e. *Bacillus subtilis, Bacillus megaterium* strain O-016 and *B.t.i.* c4Q2-72 (Figure 7, 8 and 9). Thus, it might be concluded that the three plasmids (pPL603, pPL703 and pTF6) could be presented in all hosts, *B.subtilis, B.megaterium* strain O-016 or *B.t.i.* c4Q2-72 without any alterations.

4) Expression of chloramphenicol acetyltransferase gene (*cat*) in *Bacillus subtilis, Bacillus megaterium* strain O-016 transformants and *B.t.i.* c4Q2-72 transconjugants

It has been previously known that the expression of chloramphenicol acetyltransferase (*cat*) gene depends largely on the growth phase of the organism harbouring the gene. Thus, it is important to determine to the growth curve of the three hosts organism, i.e. *B. subtilis, B. megaterium* strain O-016, *B. thuringiensis* subsp. *israelensis*. The growth curves for *B. subtilis* MI 111 and BR151 *B.megaterium* strain O-016 and *B. thuringiensis* subsp. *israelensis* c4Q2-72 were determined and illustrated in Figure 10. These growth curves were used as references for determination of growth phase of various organism

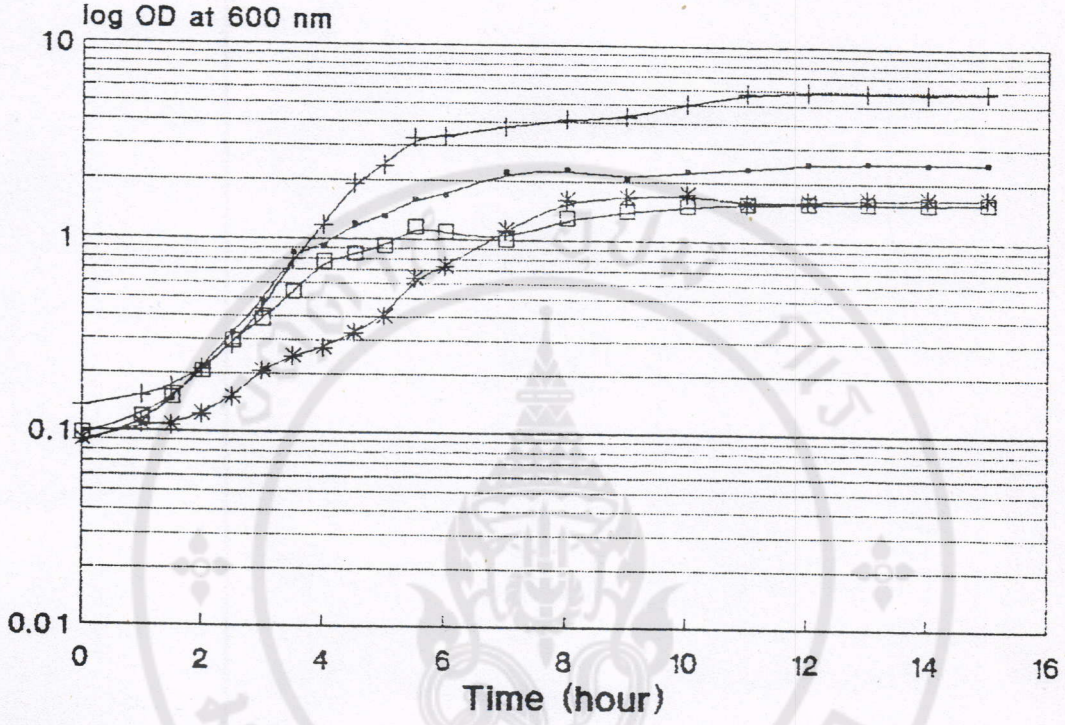

Figure 10 Growth curves of various bacterial cultures. *Bacillus subtilis* strain MI 111 (□) and BR 151 (*), *Bacillus megaterium* strain O-016 (+) and *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 (·). Each culture was inoculated with 1% inoculum of overnight cultures and grown in NBS broth at 30 °C. At designated time interval, samples were taken. The optical density values were measured at wavelength 600 nm and logalithmic values of OD were plotted against time.

employed in this study. The appropriate strains of *B.subtilis*, *B. megaterium* strain O-016 transformants and *B.t.i.* transconjugants were grown in 100 ml NBS broth containing 2 ug/ml of chloramphenicol for induction at 30°C with 200 rpm shaking to the designated growth phases i.e. mid log, T_0 and T_8 as judged by the growth curve shown in Figure 10. Samples were taken and cells were harvested during mid log, T_0 and T_8. Twenty millilitres of cell cultures were washed and resuspended in 2 ml of 0.01 M Tris HCl pH7.8 added with 20 ul of 10 mg/ml lysozyme. The lysozyme treated cell suspensions were sonicated and centrifuged to partition the unbroken cell. The supernatants obtained from centrifugation were used for determination of chloramphenicol acetyltransferase (CAT) activities. The level of expression was determined by measuring the specific activities of the enzyme chloramphenicol acetyltransferase (CAT) obtained from crude extracts. CAT specific activities were expressed as unit per milligram of protein in sample (U/mg protein).

The results as tabulated in Table 9 showed the relationships between the growth phases and the specific activities of enzyme chloramphenicol acetyltransferase in various *Bacillus subtilis* strains harbouring either pPL603, pPL703 or pTF6.

For plasmid pTF6, which harboured a strong promoter from thermostable β-galactosidase I gene of *Bacillus stearothermophilus* IAM11001, the CAT specific activities of *Bacillus subtilis* obtained from mid log, T_0 and T_8 cultures were 1.81, 8.52 and 5.31 U/mg protein, respectively. *Bacillus subtilis* BR 151 harbouring pPL603 conferred very low CAT specific activities of 0.10

and 0.04 U/mg protein from mid log and T_0 cultures, respectively. The CAT activities of 0.50 U/mg protein was found in culture from T_8 extract. The increase of CAT activity in the T_8 culture might be due to the post exponential promoter in the plasmid. In *B.subtilis* strains harbouring the promoterless plasmid pPL703, it could be demonstrated that very low background CAT specific activities of 0.04, 0.04 and 0.06 U/ mg protein from the extracts obtained from mid log, T_0 and T_8, respectively. *B.subtilis* host conferred low CAT specific activities of 0.06, 0.08 and 0.04 U/mg protein from the same three type of culturing condition (Table 9).

In *Bacillus megaterium* strain O-016, transformants harbouring plasmid pTF6 obtained from mid log,T_0 and T_8 cultures were found to have CAT activities of 7.30, 8.02 and 2.44 U/mg protein, respectively. Whereas *B.megaterium* strain O-016 transformants harbouring pPL603 were shown to confer relatively lower specific activities of 0.10, 0.17 and 0.17 U/mg protein from mid log, T_0 and T_8 cell cultures, respectively. The CAT specific activities of the host *B.megaterium* strain O-016 itself and *B.megaterium* strain O-016 (pPL703) conferred very low CAT activities (0.08, 0.02 and 0.02 U / mg protein) from mid log, T_0 and T_8 cultures, respectively (Table 10).

In transconjugants obtained from *B.t.i.* c4Q2-72 containing plasmid pTF6, the CAT specific activities were found to be 3.38, 5.11 and 4.85 U/mg protein from cultures harvested at mid log, T_0 and T_8 (Figure11) whereas the *B.t.i.* c4Q2-72 which harboured pPL603 conferred CAT specific activities of 0.04, 0.09

and 0.15 U/mg protein. The *B.t.i.* c4Q2-72 harbouring pPL703 was shown to have very low level of CAT activity from all three phase of growth whereas 0.08, 0.07 and 0.06 U/mg protein from pPL703 harbouring *B.t.i.* cells in the serial cultures. *B.t.i.* host which harboured no plasmid conferred very low CAT background activities of 0.04, 0.09 and 0.05 U/mg protein from the same type of cultures.

In the exponential phase of all three hosts being examined, it was found that plasmid pTF6 conferred the highest CAT specific activity and was obtained from mid log culture of *Bacillus megaterium* strain O-016 whereas *Bacillus subtilis* showed the highest CAT specific activitiy when culture was harvested at onset of stationary phase. TheCAT specific activties seemed to be decreased at T_8 growth phase in all of those three *Bacillus* hosts.

For plasmid pPL603, chloramphenicol acetyltransferase gene was poorly expressed in all three *Bacillus* hosts obtained from mid log and T_0 cultures. However some activity could be observed in *Bacillus megaterium* strain O-016 and *B.t.i.* c4Q2-72 in T_8 cultures. In *Bacillus subtilis,* CAT specific activities were found to increase to 0.50 U/mg protein whereas slightly increased in *Bacillus megaterium* strain O-016 and *B.t.i.* c4Q2-72.

All of *Bacillus* hosts, namely *Bacillus subtilis, Bacillus megaterium* strain O-016 and *B.t.i.* c4Q2-72, which carried plasmid pPL703, chloramphenicol acetyltransferase genes were poorly expressed because it lacked a promoter. Therefore, very low background enzyme activities could be observed throughout the growth phase of

Table 9 Activities of chloramphenicol acetyltransferase in *Bacillus subtilis* MI 111 and BR151 harbouring either pTF6 , pPL603 or pPL703

Growth phase	CAT specific activity			
	B.subtilis MI 111 host	*B.subtilis* MI 111 (pTF6)	*B.subtilis* BR 151 (pPL603)	*B.subtilis* BR 151 (pPL703)
mid log	0.06	1.81	0.10	0.04
T_0	0.08	8.52	0.04	0.04
T_8	0.04	5.31	0.50	0.06

Table 10 Activities of chloramphenicol acetyltransferase in *Bacillus megaterium* strain O-016 harbouring pTF6, pPL603 or pPL703

Growth phase	CAT specific activity			
	B.megaterium O-016 host	*B.megaterium* O-016 (pTF6)	*B.megaterium* O-016 (pPL603)	*B.megaterium* O-016 (pPL703)
mid log	0.08	7.30	0.10	0.06
T_0	0.03	8.02	0.17	0.06
T_8	0.02	2.44	0.17	0.08

Table 11 Activities of chloramphenicol acetyltransferase in *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 harbouring pTF6, pPL603 or pPL703

Growth phase	CAT specific activity			
	*B.t.i.*c4Q2-72 host	*B.t.i.* (pTF6)	*B.t.i.* (pPL603)	*B.t.i.* (pPL703)
mid log	0.04	3.38	0.04	0.08
T_0	0.09	5.11	0.09	0.07
T_8	0.05	4.85	0.15	0.06

each culture regardless of type of the hosts.

There appeared to be some variation in the expression of *cat* gene activities with regards to the difference in the types of plasmids as well as the types of the hosts.

5) Construction of recombinant plasmid pPLC1 and protoplast transformation into *Bacillus megaterium* strain O-016

It has been well documented that *B.t.i.* could produce delta endotoxin in substantially large quantity, despite the fact that *Bacillus thuringiensis* subsp. *israelensis* harboured only few copies of 110 kb plasmid on which the delta endotoxin gene is located. The amount of proteinaceous toxin has been previously calculated to be as high as about 10 % of cellular protein. There were many reasons being proposed to account for such high expression of delta endotoxin gene in *B.t.i.*. One possible factor which might lead to such high expression was the promoter strength of the delta endotoxin gene. In order to test this assumption,a hybrid vector was constructed to composed of the promoter region of 130 kDa delta endotoxin protein gene and the *cat* gene of the plasmid pPL703.

The method for construction of a recombinant plasmid containing promoter of the delta endotoxin gene was as follows. A 400 bp of *Bam*H I-*Pst* I fragment of pBTC1 (Figure 11) which contained the promoter region of 130 kDa mosquitocidal toxin gene was prepared by purifying plasmid pBTC1 from *Bacillus sphaericus* strain 2362 which was then doubly digested with restriction enzyme *Bam*H I

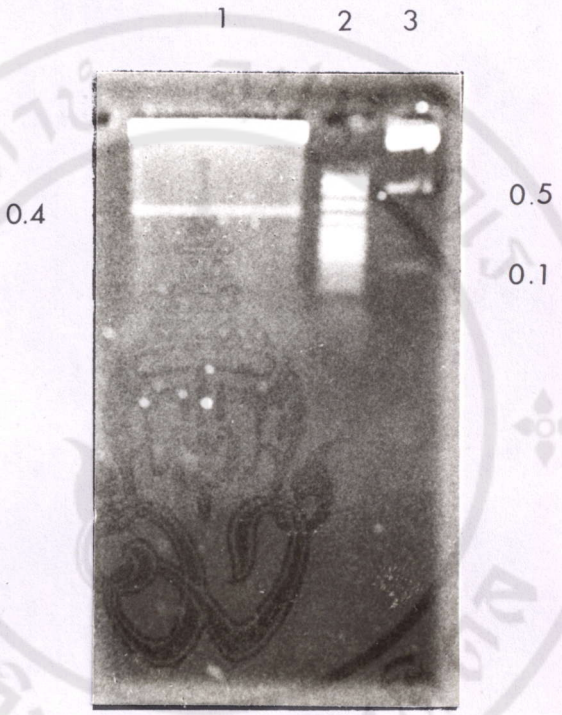

Figure 11 Nusieve GTG agarose gel electrophoresis of pBTC1 extracted from *Bacillus sphaericus* strain 2362. The plasmids were doubly digested with *Bam*H I and *Pst* I (lane 1). Lanes 2 and 3 represented *Hpa* II digested pBR322 and *Hin*d III digested λ DNA, respectively.

and *Pst* I. The restricted products was electrophoresed in agarose gel as shown in Figure 11. The 400 bp promoter fragment from excised DNA band (Lane 1,Figure 11) was ligated into promoter cloning vector, pPL703 at *Bam*H I-*Pst* I site as described in Materials and Methods section. The schematic diagram of the recombinant plasmid pPL703 and the method for construction of a hybrid plasmid pPLC1 is outlined in Figures 12 and 13. Subsequently, *B.t.i.* toxin gene promoter which controlled expression of *cat* gene pPLC1 could be determined by measuring CAT specific activities.

This new recombinant plasmid was designated as pPLC1. After overnight ligation at 15°C, the plasmid pPLC1 (Figure 13) was introduced into *Bacillus megaterium* strain O-016 by using protoplast transformation technique. After 1-3 days of incubation on DSL regeneration medium, there were a number of kanamycin resistant clones appeared. Twenty kanamycin resistant clones from regeneration medium were randomly picked up, purified and subjected for plasmid extraction. Out of twenty clones, five clones were found to harbour the plasmid which had larger molecular size than that of plasmid pPL703. The larger size of plasmids found were presumably contained the ligated fragment. As indicated in Figure 14, the five clones were found to contain plasmid which migrated to a position slightly higher than pPL703. One of these clones was selected, purified and designated as *Bacillus megaterium* strain O-016 (pPLC1) was used for analysis of plasmid by using restriction enzymes and hybridization.

6) Analysis of plasmid pPLC1 in *Bacillus megaterium* strain O-016

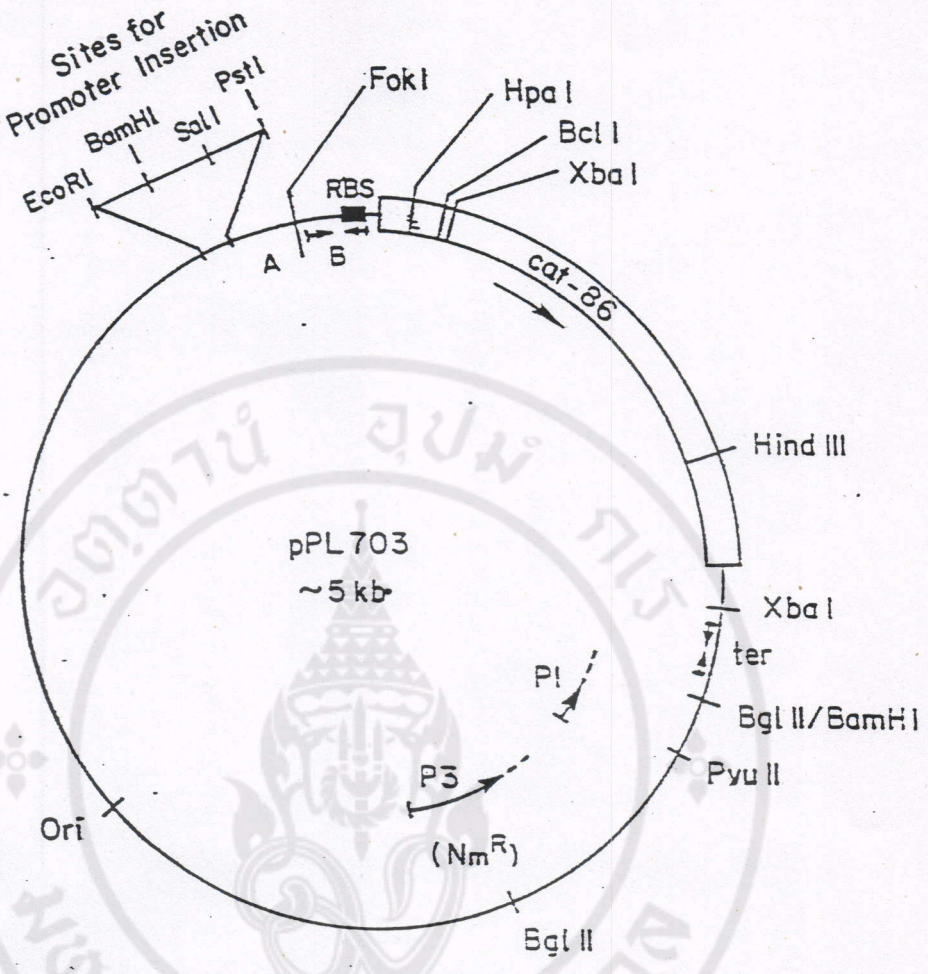

Figure 12 Schematic diagram of promoter cloning plasmid pPL703. The *cat-86* which was previously cloned from *Bacillus pumilus* NCIB 8600 is located on 1250 bp *Pst* I-*Bgl* II fragment, inserted between the *Eco*R I and *Bam*H I sites of pUB110 by using a 21 bp *Eco*R I-*Pst* I synthetic linker. The 144 bp of DNA between the *Pst* I site and the translation initiation codon for *cat-86*, contains the signals for chloramphenicol and amicetin inducibility, is devided by *Fok* I cleavage site. Nm^r is the selectable marker which confers neomycin and kanamycin resistance. RBS ; ribosome binding site

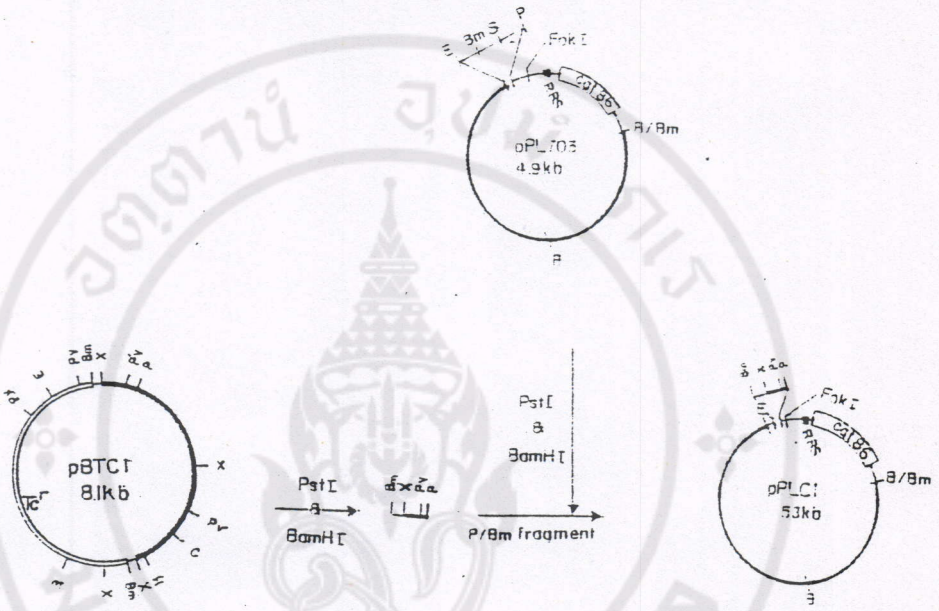

Figure 13 Schematic diagram of pPLC1 plasmid construction. Plasmid pBTC1 from *Bacillus sphaericus* strain 2362 , contained *Bacillus thuringiensis* subsp. *israelensis* 130 kDa crystal toxin gene, was double digested with *Bam*H I and *Pst* I. The *Bam*H I-*Pst* I harbouring the promoter region of crystal toxin gene was inserted between *Bam*H I and *Pst* I sites of promoter cloning vector pPL703. The abbreviation in the figure are as following.

RBS; ribosome bindind site; E, *Eco*R I; P, *Pst* I; B, *Bgl* II Bm, *Bam*H I; X, *Xba* I; S, *Sal* I

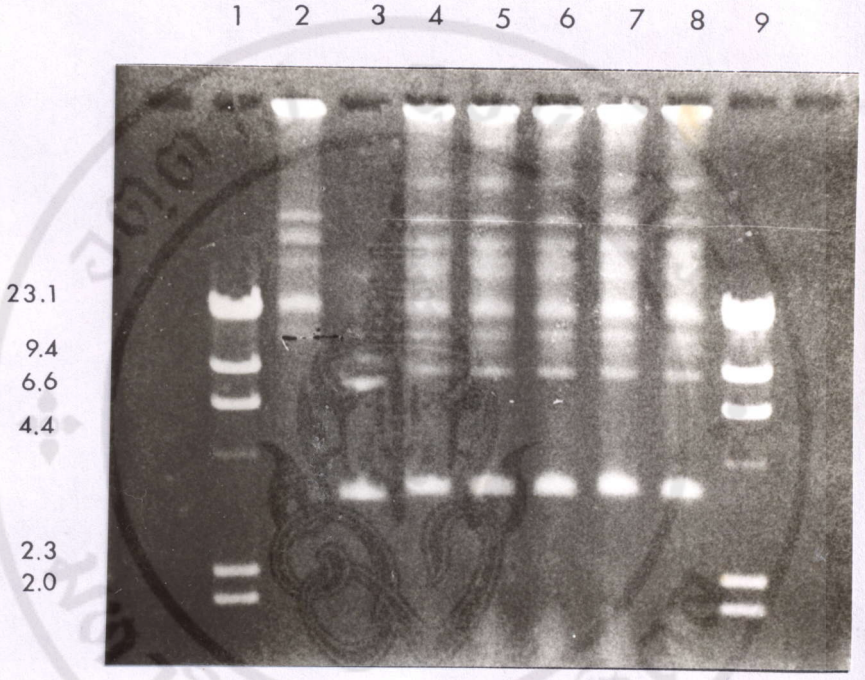

Figure 14 Agarose gel electrophoresis of recombinant plasmids extracted from *B.megaterium* strain O-016 transformants. Plasmids were extracted from *B.megaterium* strain O-016 (lane 2), and from 5 transformants of *B.megaterium* strain O-016 (lane 4 through 8). Lane 3 contained purified plasmid pPL703 which was extracted from *B.subtilis* BR 151. Lane 1 and 9 represented *Hin*d III digested λ DNA.

(pPLC1) transformants

Analysis of plasmid pPLC1 in *Bacillus megaterium* strain O-016 which was transformed with plasmid pPLC1 and designated as *Bacillus megaterium* strain O-016 (pPLC1), was undertaken. The recombinant plasmid was extracted, purified, digested with appropriate restriction enzymes and subjected to agarose gel electrophoresis. Data as shown in Figure 15 indicated that the unrestricted and *Pst* I restricted pPLC1 showed an increase in the size of the fragments when compared with those of pPL703. The plasmid pPL703 was found to contain only one *Pvu* II site whereas *Pvu* II digested pPLC1 showed two DNA bands corresponding to 1.3 kb and 4.0 kb fragments. The generation of these two fragments for *Pvu* II digestion was most likely due to the presence of additional *Pvu* II site in the cloned fragment. One additional *Xba* I site was alsofound in the recombinant plasmid and led to the decreasing molecular size of *Xba* I digested pPLC1 when comparing to the original plasmid pPL703. Also, doubly digested pPLC1 with *Bam*H I and *Bgl* II lead to the presence of two DNA fragments of sizes 3.1 kb and2.2 kb instead of the two fragments with 2.7 kb and 2.2 kb from *Bam*H I-*Bgl* II digested pPL703 (Figure 15). Thus, it appeared that the pPLC1 plasmid which was transformed into *B.megaterium* strain O-016 (pPLC1) possessed additional piece of DNA fragment which presumably to be the 400 bp promoter region from *Bacillus thuringiensis* subsp. *israelensis* delta endotoxin gene from plasmid pBTC1. The existence of the promoter region in the cloned

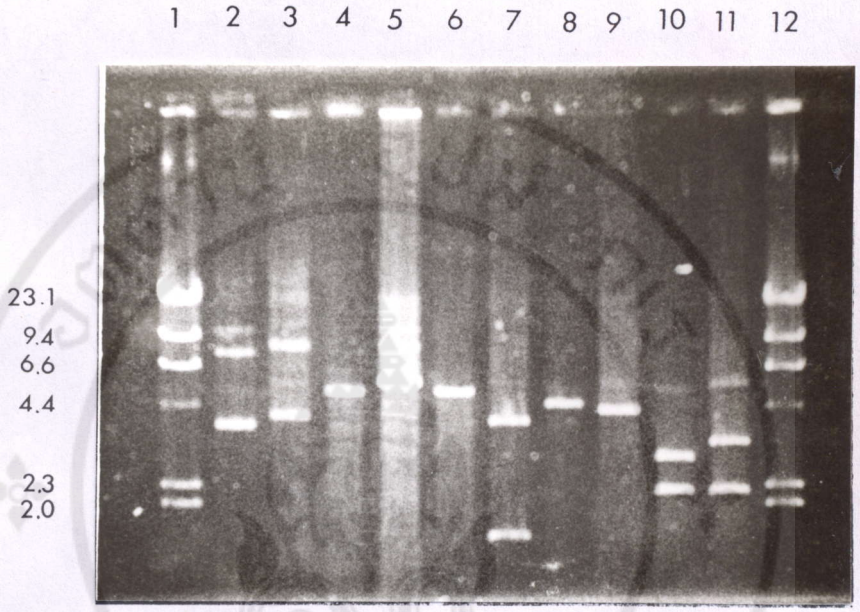

Figure 15 Agarose gel electrophoresis of unrestricted and restricted preparations of pPL703 and recombinant plasmid pPLC1 extracted from transformants of *Bacillus megaterium* strain O-016. The plasmids were extracted from *B.megaterium* strain O-016 containing pPL703 (lane 2) and pPLC1(lane 3).The corresponding plasmid preparations, pPL703 and pPLC1 were restricted with enzyme *Pst* I (lanes 4 and 5), *Pvu* II (lanes 6 and 7), *Xba* I (lanes 8 and 9) and *Bam*H I-*Bgl* II (lanes 10 and 11). Lanes 1 and 12 represented *Hin*d III digested λ DNA.

fragment could be confirmed by hybridization of plasmid pPLC1 isolated from *B.megaterium* strain O-016 with 3.7kb *XbaI* fragment from *B.t.i.* toxin gene as shown in latter section.

7) Transfer of plasmid pPLC1 from *B.megaterium* strain O-016 (pPLC1) to *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 and 4Q2-72 via conjugation-like process

One of the transformants of *B.megaterium* strain O-016 (pPLC1) which harboured plasmid pPLC1 was employed as a donor for transferring the pPLC1 plasmid into *B.t.i.* c4Q2-72 and 4Q2-72 via conjugation-like process. The details describing the conjugation-like gene transfer process appeared in Materials and Methods section. As shown in Table 12, the frequencies of transfer of pPLC1 from *B. megaterium* strain O-016 (pPLC1) to *B.t.i.* c4Q2-72 and 4Q2-72 were found to be 2.1×10^{-7} and 1.7×10^{-7} respectively. as shown in Table 12. The *B.t.i.* transconjugants were selected on LB agar plates containing 20 ug/ml of kanamycin and 50 ug/ml of rifampicin. After overnight incubation on these plates, nine transconjugants were randomly picked from from matingpair *B. megaterium* strain O-016 (pPLC1) and *B.t.i.* c4Q2-72 and five transconjugants were randomly picked from mating pair *B.megaterium* strain O-016 (pPLC1) and *B.t.i.* 4Q2-72. The plasmids were extracted from these transconsjugants and were analysed by agarose gel electrophoresis as shown in Figure 16 and 17.

Both strains of *B.t.i.* c4Q2-72 and 4Q2-72 transconjugants

Table 12 Frequency of plasmid pPLC1 transfer from *Bacillus megaterium* strain O-106 (pPLC1) to *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 and 4Q2-72 via conjugation-like process.

Mating pair	*B.megaterium* O-016(pPLC1)(CFU/ml)	*B.t.i.* (CFU/ml)	Number of transconjugant	Frequency[a] of transfer
B.megaterium x *Bti*c4Q2-72	9.1×10^{8}	4.7×10^{8}	99	2.1×10^{-7}
B.megaterium x *Bti*4Q2-72	7.8×10^{8}	2.5×10^{8}	43	1.7×10^{-7}

(a) Frequency of transfer = $\frac{\text{number of transconjugant}}{\text{number of recipient (before mating)}}$

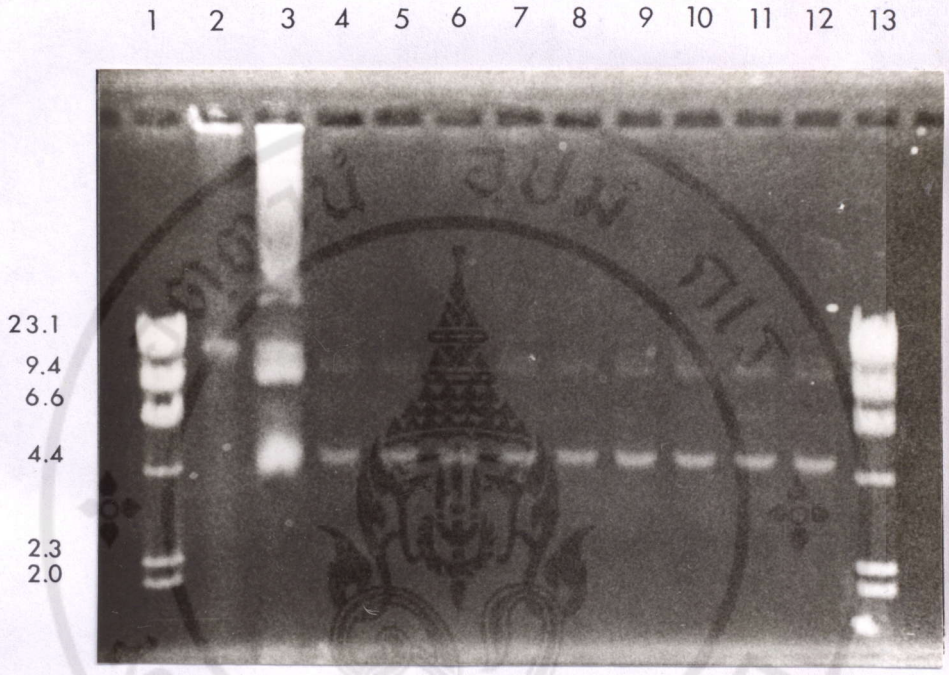

Figure 16 Electrophoresis patterns of plasmids from various transconjugants. Plasmid pPLC1 was transferred from *B.megaterium* strain O-016 (pPLC1) to *B.t.i.* c4Q2-72 via conjugation-like process. Plasmids were extracted from *B.t.i.*c4Q2-72 (lane 2), *B.megateriun* strain O-016 (pPLC1) harbouring pPLC1 (lane 3) and *B.t.i.* transconjugants (lane 4 through 12). Lanes 1 and 13 contained *Hind* III digested λ DNA.

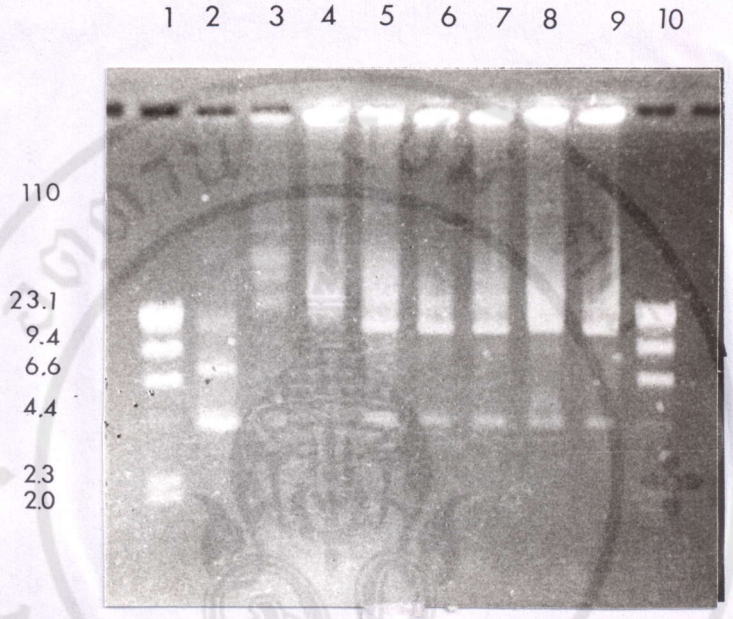

Figure 17 Electrophoresis pattern of plasmids from various transconjugants. Plasmid pPLC1 was transferred from *B.megaterium* strain O-016 (pPLC1) to *B.t.i.* 4Q2-72 via conjugation-like process. Plasmids were extracted from *B.megaterium* strain O-016 harbouring pPLC1 (lane 3), *B.t.i.* 4Q2-72 (lane 4) or *B.t.i.* 4Q2-72 transconjugants (lane 5 through 9). Lane 2 contained purified pPLC1 from *B.megaterium* strain O-016.Lanes 1 and 10 represented *Hind* III digested λ DNA.

which were transferred with pPLC1 showed the plasmids which comigrated with the plasmid pPLC1 extracted from *B.megaterium* strain O-016 (pPLC1). The plasmids from both strains of *B.t.i.* transconjugants were also extracted and proved to be pPLC1 by restriction analysis. The restriction patterns of the plasmid extracted from transformants and transconjugants restricted with *Pst* I, *Pvu* II and *BamH* I-*Bgl* II gave exactly the same restriction patterns as shown in Figure 18. Thus, it appeared the plasmid pPLC1 could be transferred from *B.megaterium* strain O-016 to *B.thuringiensis* subsp. *israelensis* without any alteration.

8) Hybridization of the plasmid pPLC1 in *Bacillus megaterium* strain O-016 (pPLC1) and *B.thuringiensis* transconjugants with 3.7 *Xba* I fragment from *B.t.i.* toxin gene

In order to ensure the presence of *B.t.i.* 130 kDa toxin gene promoter fragment in plasmid pPLC1 in *B.megaterium* strain O-016 transformant and *B.t.i.* transconjugants, the 3.7 kb *Xba* I fragment from plasmid pBT8 was used as a probe to detect the relevant fragment in the plasmid pPLC1.

The purified and linearized plasmid pPLC1 from *B.megaterium* strain O-016 (pPLC1), *B.t.i.* c4Q2-72 (pPLC1) and 4Q2-72 (pPLC1) were prepared and subjected to agarose gel electrophoresis. After electrophoresis, the DNA bands in agarose gel were transferred to nitrocellulose paper and subjected to hybridization with biotin-labelled 3.7 kb *Xba* I fragment. The detailed procedure used in

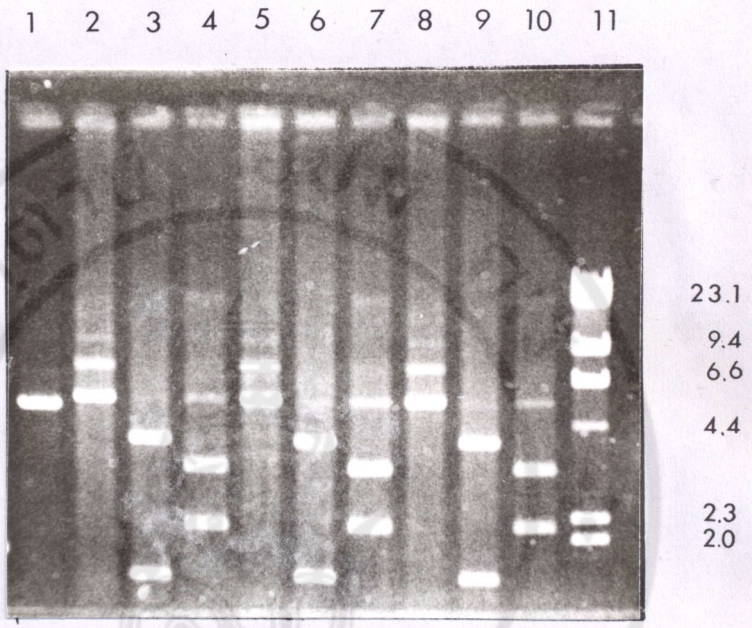

Figure 18 Agarose gel electrophoresis of restricted products of pPLC1 extracted from various hosts. Plasmids pPLC1 which were extracted from *B.megaterium* strain O-016 (pPLC1) (lanes 2, 3 and 4), from *B.t.i.* c4Q2-72 (pPLC1) (lanes 5, 6 and 7) or from *B.t.i.* 4Q2-72(pPLC1) (lanes 8, 9, 10) were restricted with enzymes *Pst* I (lanes 2, 5, 8), *Pvu* II (lanes 3, 6, 9), and *Bam*H I-*Bgl* I (lanes 4, 7, 10). Lane 1 contained *Pst* I digested pPL703 and lane 11 represented *Hin*d III digested λ DNA.

hybridization is described in Materials and Methods section. It was shown that the plasmid pPLC1 from either *B.megaterium* strain O-016 transformant, *B.t.i.* c4Q2-72 and 4Q2-72 transconjugants conferred positive bands that represented the existence of the cloned fragment from *B.t.i.* delta endotoxin gene. The plasmid pPL703 did not react with the probe as shown in Lane 2 of Figure 19. The plasmid pBT8 which contained the toxin gene reacted positively with the probe (Lane 3, Figure 19). Thus, it was cleared that the recombinant plasmid contained the promoter region of *B.t.i.* toxin gene in pPL703 and could be transferred into *B.megaterium* strain O-016, *B.t.i.* c4Q2-72 and *B.t.i.* 4Q2-72.

9) Expression of chloramphenicol acetyltransferase gene (*cat*) in *Bacillus megaterium* strain O-016 (pPLC1) and *B.t.i.* (pPLC1)

The *B.megaterium* strain O-016 (pPLC1) transformant and *B.t.i.* (pPLC1) transconjugants were grown to mid log, T_0, T_2 and T_8 as determined by the growth curve of each culture as shown in the Figure 10. After appropriate cultivation period, the cells were harvested and the crude extracts were prepared. The crude extracts were used for determination of chloramphenicol acetyltransferase activities. The relationships between the growth phases and specific activities of chloramphenicol acetyltransferase in *B.megaterium* strain O-016 (pPLC1) and *B.t.i.* (pPLC1) strains harbouring recombinant plasmids pPLC1 were observed.

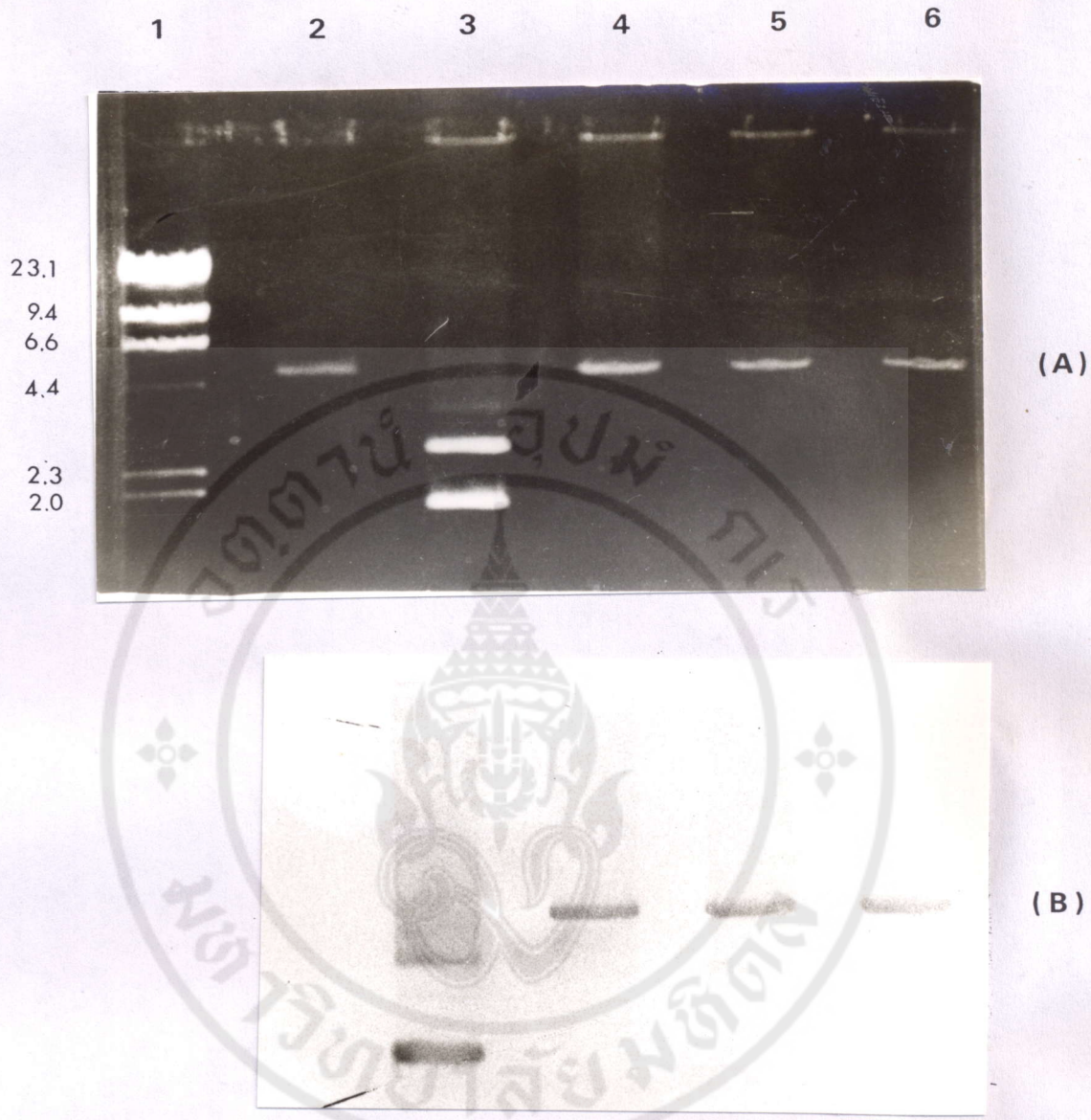

Figure 19 Southern hybridization of plasmid pPLC1, from *B.megaterium* strain O-016 (pPLC1), *B.t.i.* c4Q2-72 (pPLC1) and 4Q2-72 (pPLC1) with biotinylated 3.7 kb *Xba* I fragment of delta endotoxin gene probe. Photograph labelled A showed an agarose gel electrophoresis pattern of the *Pst* I restricted pPLC1 from *B.megaterium* strain O-016 (pPLC1)(lane 4), from *B.t.i.* c4Q2-72 (pPLC1)(lane 5) or *B.t.i.* 4Q2-72 (pPLC1)(lane 6). Lanes 1, 2 and 3 represented *Hind* III digested λ DNA, *Pst* I digested pPL703 and *Xba* I digested pBT8, respectively. Photograph labelled B represented Southern hybridization resulting from the transfer of the DNA in the gel from (A)to nitrocellulose and hybridized with 3.7 kb *Xba* I fragment of delta endotoxin gene probe.

The cell cultures were harvested during mid log , end of exponential phase (T_0), 2 hours (T_2) and 8 hours (T_8) after the onset of sporulation. The CAT activities in the crude extracts, expressed as unit per milligram cellular protein, were determined as described previously. *Bacillus megaterium* strain O-016 (pPLC1) showed very low CAT specific activities of 0.02, 0.01, 0.05 and 0.18 U/mg protein from crude extract from cultures harvested at mid log, T_0, T_2 and T_8, respectively. Whereas, CAT specific activities from the various stages of growth, i.e., mid log, T_0, T_2 and T_8 obtained from *B.t.i.* c4Q2-72 (pPLC1) were 0.08, 0.12, 0.19 and 0.48 U/mg protein, respectively. *B.t.i.* 4Q2-72 (pPLC1) possessed CAT specific activities of 0.16, 0.10, 0.15 and 0.46 U/mg protein from cells harvested at mid log , T_0, T_2 and T_8 phase of growth, respectively (Table 13). The CAT specific activities in *B.t.i.* c4Q2-72 and 4Q2-72 host were shown to be very low.

10) Copy number of plasmids pPL603, pPL703, pTF6 and pPLC1 in *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 and 4Q2-72

Since it has been documented that the numbers of plasmid varied considerbly in different hosts, the number of the plasmid in each cell might lead to the varying level of expression of certain gene located in the plasmid. The copy number of plasmid in each cell has been demonstrated to affect the level of transcription and translation efficiency (187). Therefore, the copy number of plasmids

Table 13 Activities of chloramphenicol acetyltransferase in various *Bacillus* hosts harbouring plasmid pPLC1

Growth phase	CAT specific activity			
	B.megaterium strain O-016 (pPLC1)	*B.t.i.* c4Q2-72 (pPLC1)	*B.t.i.* 4Q2-72 (pPLC1)	*B.t.i.* 4Q2-72 host
mid log	0.02	0.08	0.16	0.04
T_0	0.01	0.12	0.10	0.03
T_2	0.05	0.19	0.15	0.03
T_8	0.18	0.48	0.46	0.01

Table 13 The relationships between the growth phases and the specific activities of chloramphenicol acetyltransferase enzyme in various *Bacillus* hosts harbouring pPLC1. Cells were cultured in 100 ml NBS broth containing 2 ug/ml of chloramphenicol for induction at 30°C with shaking. Samples were taken and cells were harvested during mid log, T_0, T_2 and T_8 according to the pre-determined growth curve. The 20 ml of cell cultures were washed and resuspended in 2 ml of 0.01 M Tris - HCl pH 7.8 . The lysozyme treated cell suspensions were sonicated and supernatants obtained by centrifugation were used for determination of enzyme activities. CAT activities were expressed as unit per milligram of cellular protein (U/mg protein).

pPL603, pPL703, pTF6 and pPLC1 in the two *B.t.i.* hosts were determined to find out whether there were any differences in the copy number of plasmids among the various host strains employed in this study.

The method used for determination of plasmid copy number was as previously described in Materials and Methods section. The concentration of the plasmids were estimated by comparing with known plasmid standard in gel electrophoresis. As shown in Figure 20, the plasmid pSP72, pTF6, pPL603, pPL703, pPLC1 (*B.t.i.* c4Q2-72) and pPLC1 (*B.t.i.* 4Q2-72) were estimated to be 400, 600, 200, 300, 200 and 100 ng, respectively per 10 ul of loading. Calculation of plasmid copy number were tabulated in Table 14. The results as shown in Table 14 showed that plasmid pPL603, pPL703, pTF6 and pPLC1 were presented in *B.t.i.* c4Q2-72 host at about 30-45 copies per cell and *B.t.i.* 4Q2-72 transconjugants contained approximately 44 copies per cell. Thus, there appeared to be only slight variation in the copy number of various plasmids in *B.t.i.* hosts. It should noted that the copy number of various plasmids reported in this study reflected only the relative number and should be used only to compare the number of plasmids in various strains in this study.

11) Stability of recombinant plasmid pLC1 in *B.megaterium* strain O-016 and *B.thuringiensis* subsp. *israelensis* c4Q2-72 and 4Q2-72

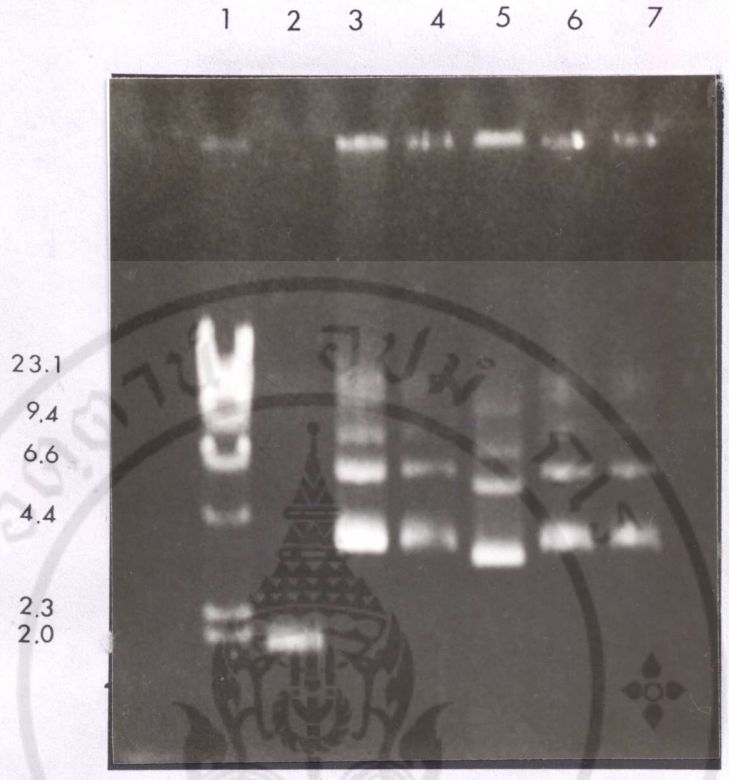

Figure 20 Agarose gel electrophoresis of various plasmids obtained from *B.t.i.* transconjugants for estimating DNA concentration by the intensity of EtBr stained plasmids. The appropriate amount of plasmids extracted from *B.t.i.* c4Q2-72 (pTF6)(lane 3), *B.t.i.* c4Q2-72 (pPL603)(lane 4), *B.t.i.* c4Q2-72 (pPL703)(lane 5), *B.t.i.* c4Q2-72 (pPLC1) (lane 6) and *B.t.i.* 4Q2-72 (pPLC1) (lane 7) was electrophoresed and compared with plasmid pSP72 extracted from *E.coli* (lane 2) as known plasmid standard. The estimated DNA concentrations were used as basis for calculation as shown in Table 14. Lane 1 represented *Hin*d III digested λ DNA.

Table 14 The relative copy number of plasmids pPL703, pPL603, pTF6 and plasmid pPLC1 in *Bacillus thuringiensis* subsp. *israelensis* strains[a].

Determination	*B.t.i.* strains				
	c4Q2-72 (pTF6)	c4Q2-72 (pPL603)	c4Q2-72 (pPL703)	c4Q2-72 (pPLC1)	4Q2-72 (pPLC1)
viable cell / ml	1.10×10^{9}	2.84×10^{8}	4.18×10^{8}	2.80×10^{8}	1.30×10^{8}
tot.cell extracted	3.30×10^{9}	0.85×10^{9}	1.25×10^{9}	0.84×10^{9}	3.90×10^{8}
vol DNA loaded (ul)	10	10	10	10	10
DNA estimated (ug)	0.65	0.20	0.30	0.20	0.10
total vol of DNA	10	10	10	10	10
total DNA (ug)	0.65	0.20	0.30	0.20	0.10
ug of DNA per cell	1.82×10^{-10}	2.35×10^{-10}	2.40×10^{-10}	2.38×10^{-10}	2.56×10^{-10}
size of plasmid(bp)	5069	5153	4971	5371	5371
MW of plasmid (Da) (bp x 660)	3.35×10^{6}	3.40×10^{6}	3.28×10^{6}	3.54×10^{6}	3.54×10^{6}
relative plasmid copy number	33	42	44	41	44

(a) The 3 ml of bacterial cell cultures were used for plasmid extraction and the estimations were performed in 0.7% agarose gel electrophoresis and compared with the known plasmid standard.

It is important that if one wants to employ the plasmid pPLC1 as a possible vector for introducing genes into *B.thuringiensis* for the purpose of genetically improving the organism, this plasmid must be stably maintained in *B.thuringiensis* host. Thus the stability of plasmid pPLC1 in *B.thuringiensis* was investigated. Cultures of *Bacillus megaterium* strain O-016 (pPLC1) and *Bacillus thuringiensis* subsp. *israelensis* c4Q2-72 (pPLC1) and 4Q2-72 (pPLC1) containing recombinant plasmids pPLC1 were daily subcultured in LB broth with and without selective pressure i.e. kanamycin for 4 weeks. The appropriate dilution of each culture was spreaded on LB agar plates to determine the total viable cell and on LB agar supplemented with kanamycin (20 ug/ml) to determine the total number of kanamycin resistant clones which presumably harbouring the plasmid pPLC1. Results as indicated in Figure 21 demonstrated that the recombinant plasmid, pPLC1, could be stably maintained in *B.t.i.* c4Q2-72 and 4Q2-72 during the first week. Subsequently, the number of kanamycin resistant transconjugants were gradually decreased in the last three weeks until reaching about 30 % for *B.t.i.* c4Q2-72 and 29 % for *B.t.i.* 4Q2-72. However, the plasmid pPLC1 plasmid appeared to be rapidly lost from *B.megaterium* strain O-016 (pPLC1) within 3 days of daily subculturing therefore: the kanamycin used in LB agar was decreased to 10 ug/ml. Nonetheless, the kanamycin marker in the pPLC1 plasmid also appeared to be rapidly lost within 5 days of daily subculturing even if the concentration of the kanamycin was lower to 10 ug/ml. The similar experiments were performed by cultivating relevant strains in the presence of kanamycin (20 ug/ml)

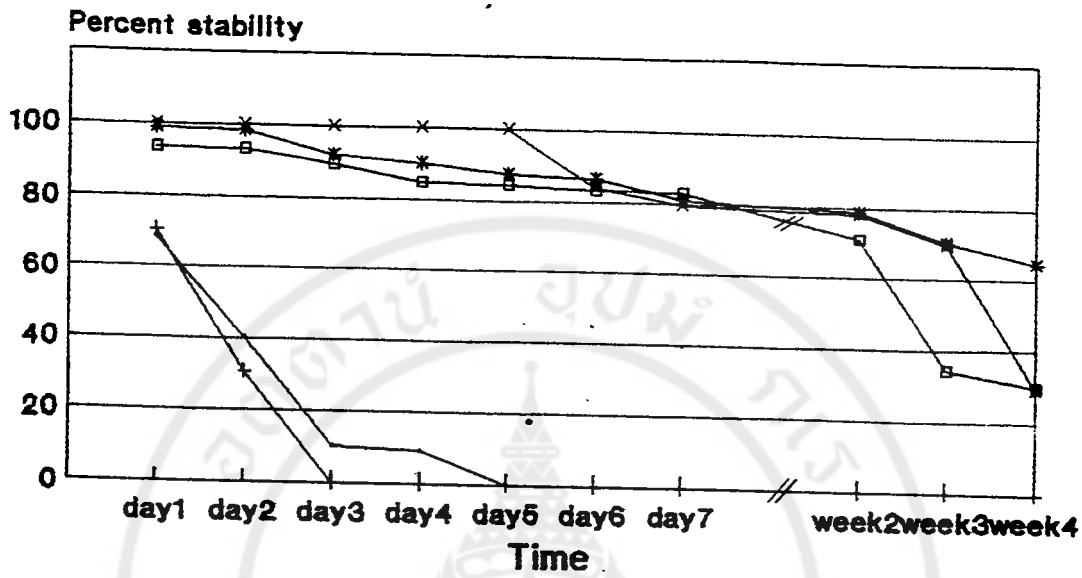

Figure 21 Stability of recombinant plasmid pPLC1 in various hosts. Cultures were daily subcultured LB broth with and without kanamycin and plated on LB agar with and without kanamycin. The number of bacteria was determined everyday for a week and once a week for the second, third and fourth week. The symbols in the figure were as follows. *B.megaterium* strain O-016 (pPLC1) plated on LB agar with 10 ug/ml Km (·), *B.megaterium* strain O-016 (pPLC1) plated on LB agar with 20 ug/ml Km (+), *B. megaterium* strain O-016 added with 20 mg/ml of Km for selective pressure during daily subculturing (*), *B.t.i.* c4Q2-72 (pPLC1)(□) and *B.t.i.* 4Q2-72 (pPLC1)(x).

for selective pressure in LB broth when the transformant was subcultured throughout the experiment. The result as shown in Figure 20 indicated that the plasmid was found to be stably maintained for the entire 4 weeks period of the experiment. In order to assure the presenceof plasmids in kanamycin resistant clones, the kanamycin resistant colonies from transformants and transconjugants were randomly selected for plasmid extraction to confirm the existence of the recombinant plasmid. All were found to harbour the pPLC1 plasmids.

CHAPTER V

DISCUSSION

The recent advances in the molecular biology of the crystal protein genes of *Bacillus thuringiensis* subsp. *israelensis* have been reported by many investigators (18, 38, 39, 45, 47, 48, 61, 62, 63, 122). The accumulation of delta endotoxin in the form of crystal or inclusion parasporal body in the sporulated cell demonstrated very high level of production of the protein. It was shown by DeanD.H.(25) that delta endotoxin deposited within the *B.thuringiensis* cell as crystal and released from parental cell only after its lysis following sporulation. As high as 20-30 % of the cellular protein synthesizing activity during sporulation was devoted to the production of the toxin. Therefore the control mechanism(s) which lead to such a high level of expression of these toxin genes have been the subject of numerous studies and reports (29, 191, 192). There are many criteria that have been reported to affect the expression of a certain gene, for example copy, number of the gene,the strength of promoter or/and terminator that may control the transcriptional efficiency. The increased amount of mRNA has also been reported to lead to high level of gene expression by increasing the rate of binding between mRNA and ribosome (187). However, the delta endotoxin gene located on 110 kb plasmid of *B.t.i.* has been reported to be presence is low number, thus, the high level of gene expression of the *B.t.i.* toxin might be regulated by the influence of factor(s) effecting gene expression and / or

translation rather than the effect of gene copy number. Reported by Trisrisook M (18) indicated that the 130 kDa toxin gene from pBTC1 containing its own promoter and terminator was poorly expressed in *E.coli*. The gene product could only be detected *E.coli* by Western blot technique but not by protein staining technique indicating that only low level of gene product was expressed. However, when the 130 kDa toxin gene from *B.t.i.* was transferred into *B. sphaericus* host, the protein could be detected by protein staining (18). Both of these studies implied that in order to obtain good expression of the 130 kDa toxin gene, the gene must be in appropriate host, perhaps, in *B.t.i.* itself. The study on expression of certain gene(s) in *B.t.i.* host has been limited by the lack of efficient gene transfer system in *B.t.i.*. Transfer of foreign genes into *B.t.i.* via protoplast transformation was found to be rather difficult. This might be due to the high level of lysozyme resistance in *B.t.i.* that resulted in the difficulties for protoplast formation and subsequently lead to low frequency of regeneration of protoplast. The interference in cell wall regeneration has been suggested to be due to the strong binding of the lysozyme to the protoplast membrane (189). Using electroporation as a technique for introducing gene into *B.t.i.* , there were a few reports available on transformation by this technique (167, 168, 169) but only small plasmids were documented to be introduced into *B.thuringiensis* at approximate frequency of 10^1 - 10^6 transformant / ug DNA. Crawford et al (165) and Rubinstein et al (166) reported that

transformation of DNA might be achieved bythe autolytic removal of the cell wall in *B.thuringiensis* autoplast. However, transformation by this technique was quite low ranging from 10^{-6} - 10^{-5} transformants per regenerated autoplast. Using another alternative approach, Heierson et al(157) reported that transformation could be achieved by using the induction of competence in *B.thuringienisis* strains by treatment of 50 mM Tris-HCl buffer pH 8.9 containing 30 % sucrose. Again, it was found that the very low transformation frequency of 6.7 x 10^{-7} was obtained in transformation of pBC16 into *B.thuringiensis* subsp. *israelensis*. Based on these observations, it was concluded that it appeared to be still very difficult to introduce the plasmid DNA directly into *B.t.i.*. One of the most efficient gene transfer system in *B.t.i.* has been by the use of conjugation-like gene transfer technique. However, this method has been limited to the use of transferring genes among *B.t.* strains themselves, and does not allow for use in introducing heterologous genes(s) into *B.t.* strains. Loprasert et al. (175) reported that *B.megaterium* strain O-016 could be employed as an intermediate bacterium for introducing plasmids into *Bacillus thuringiensis* subsp. *israelensis*. *B.megaterium* strain O-016, a lysozyme sensitive strain, could be easily generated into protoplasts which was easily to be transformed and eventually achieved high frequency of transformation. Subsequently, the gene from *B.megaterium* strain O-016 can be transferred into *B.t.i.* by conjugation-like gene transfer technique. Using this strategy in this study, *B.megaterium* strain O-016 was employed as

an intermediate strain for transferring pTF6, pPL603, pPL703 and pPLC1 into *B.thuringiensis* subsp. *israelensis* via conjugation - like process. It was found that high rate of protoplast formation of various plasmids (pTF6, pPL603, pPL703, pPLC1) in *Bacillus megaterium* strain O-016 could be achieved by generating protoplasts in 4 mg/ml of lysozyme at 37°C for 30 minutes (161). The transformed protoplasts of *B.megaterium* strain O-016 were regenerated in DSL media. It has been reported that development of suitable formulations of regeneration media was very important to assure the high rate of transformation. This study improved the conditions for regeneration of protoplasts. By using modified DM-3 (mDM-3) media. The type and molarity of osmotic stabilizers such as sucrose and sodium succinate had been found to affect the regeneration frequency of protoplasts. In order to improve the regeneration medium to be used for obtaining regenerated protoplast of *B.megaterium* strain O-016, 0.5 M sucrose and 1% tryptone were used in mDM-3T to provide higher regeneration frequency. In this study, the regeneration media used was that of DSL which contained 0.5 M sucrose, 1 % tryptone, 0.5 % yeast extract and 0.5 % NaCl. The regeneration frequency of *B.megaterium* strain O-016 obtained from DSL regeneration medium was 7.0 % which sligthly higher than those obtained from DM-3, mDM-3 and mDM-3T media as shown in Table 6. It was found that the plasmids pTF6, pPL603 and pPL703 could be introduced into *B. megaterium* strain O-016 by protoplast transformation technique with frequency of 3.2×10^3 (161), 1.0×10^3

and 4.0×10^2 transformants per ug plasmid DNA, respectively. These transformation frequencies found in this study were similar to those previously reported by Loprasert et al (175) and Chawala, U. (161). It was also confirmed in this study that the transformed plasmids in various transformants still retained the same restriction patterns (Figure7-9) as those plasmid isolated from original host, i.e. *B. subtilis*. The plasmids in *B.megaterium* strain O-016 could be transfer into *B.t.i.* by using conjugation-like gene transfer process. The optimum condition for transferring plasmid DNA between *B.megaterium* strain O-016 and *B.t.i.* c4Q2-72 by filter technique was previously described by Mongkolsamai(unpublished data). The highest number of transconjugant was achieved when the donor and recipient cultures were mixed in the equal ratio for 6 hours. When similar procedure were conducted in this study, the rate of transfer of plasmids pPL603, pPL703 and pTF6 from *B.megaterium* strain O-016 into *B.t.i.* c4Q2-72 were found to be 2.8×10^{-8}, 5.0×10^{-8} and 3.2×10^{-8}, respectively. These frequencies were found to be slightly lower than that obtained from the conjugation-like gene transfer from *B. megaterium* strain O-016 harbouring plasmid pTF6 and pHV33 to *B.t.i.* strain A-084 as reported by Chawala U(161). For conjugation-like gene transfer *B.cereus* harbouring plasmid pBC16 and *B.t.i.* A-084, it was found to confer much higher frequency of transfer (174). Perhaps, this might due to the close relation between these two bacteria.

In this study the activity of chloramphenicol acetyltransferase was used as a marker for comparing the level of

cat gene expression. The *cat-86* gene was used as an indicator because this gene was well characterized and its gene product was easy to be assayed. Moreover, it contained the preceding sequence of *cat-86* structural gene which was suggested to be a strong ribosome binding site. *B.megaterium* or *B.thuringiensis* hosts which contained appropriate plasmids were grown in NBS medium added with sub-inhibitory level of chloramphenicol (2ug/ml) in order to induce the expression of *cat-86* gene. The nature of inducibility of the *cat-86* gene has been the subject of many studies (130, 132, 135). Ambulos et al. (135) reported the sequence essential to the chloramphenicol inducible expression of *cat-86* gene resided on 144 bp regulatory region that intervened between *cat-86* structural gene and cloned promoter fragment. The important element within 144 bp segment consisted of a pair of inverted repeat that immediately preceded *cat* coding region and span the ribosome binding site for the gene. Because of the location of inverted repeat *cat-86* transcripts were predicted to sequester the ribosome binding site in a stable stem-loop structure which could block translation of mRNA and acted as a weak transcriptional termination signal. Hence, chloramphenicol which was added for induction of gene expression, was believed to play a major role in destabilization of mRNA stem-loop resulted in increasing of translational efficiency. The destabilization of mRNA was proposed to be involved with essential upstream element in 144 bp (135) and binding of chloramphenicol to ribosome, enabling the ribosome to function related to disruption of the RNA stem-loop. Byeon and Weisblum (190) suggested that

chloramphenicol bound to 50S ribosomal subunit might uncovered sequence in 23S rRNA. Such sequences in the later subunit were nearly identical to the ribosome binding site and theoretically compete with the RNA stem-loop formation thereby freeing *cat-86* ribosome binding site.

For the expression of the plasmid pTF6 harbouring strong promoter of *B.stearothermophilus* IAM 11001 β-galactosidase (*bga B*) in the various *Bacillus* hosts, it was found that *B. subtilis*, *B.megaterium* strain O-016 and *B.t.i.* c4Q2-72 containing pTF6 constitutively expressed high level of chloramphenicol acetyltransferase enzyme (CAT). The extracts obtained from T_0 phase cultures were found to demonstrate the highest level of *cat-86* gene product when compared with those of mid-exponential extracts, The level of CAT enzyme were found to decrease at 8 hours after the onset of stationary phase i.e. T_8 in all of three *Bacillus* hosts. When we studied the published nucleotide sequence (133), it was found that the position -35 and -10 of *bgaB* promoter were TTGACA and TAATT, respectively. These two regions were recognized by sigma-55 of RNA polymerase which was mainly found in vegetative cell and decreased in sporulated cell (130, 133). Therefore, the decrease of *cat* gene expression of pTF6 in all *Bacillus* hosts should be due to lacking of appropriate sigma subunit of RNA polymerase. The highest level of *cat-86* gene expression, 8.52 U/mg protein, was obtained from T_0 phase extract of *B.subtilis* carrying pTF6 whereas the non - plasmid harbouring *B.subtilis* possessed very low background CAT activity throughout its

growth cycle.

All of the three *Bacillus* hosts harbouring plasmid pPL603 which contained *cat-86* isolated from *Bacillus pumilus* NCIB 8600 and its endogenous post exponential promoter located on *Eco*R I-*Pst* I fragment. The plasmid pPL603 was found to be under sporulation control. The CAT activities obtained from exponential and T_0 cultures were found to apparently low and appeared to increase at 8 hours after cessation of exponential phase with different level of expression among the hosts. At T_8 phase, CAT activity was greatly increased to 0.50 U/mg protein in *B. subtilis* whereas slightly increased in *B.megaterium* strain O-016 and *B.t.i.* c4Q2-72. These might be due to the need of appropriate sigma subunit directly involved in RNA polymerase using for promoter selection and transcriptional initiation as previously reported in *B.subtilis* (130). Perhaps, such a sigma factor is lacking in *B. megaterium* strain O-016 and *B.t.i.*. In addition, the promoter cloning replicon, pPL703, which was lacked of promoter conferred very low endogenous CAT activities in all of three *Bacillus* hosts throughout their growths. Inthe plasmid pPLC1 containing *B.t.i.* toxin gene promoter, it was also found that *B.thuringiensis* subsp. *israelensis* both c4Q2-72 and 4Q2-72 strains conferred higher CAT activities of 0.08, 0.12, 0.19, 0.48 and 0.16, 0.10, 0.15, 0.46 u/mg protein, respectively from exponential, T_0, T_2 and T_8 cultures compared with those obtained from *B.megaterium* strain O-016 harbouring such a plasmid which conferred the CAT activities of 0.02, 0.01, 0.05 and 0.18 U/mg protein from the same

serial cultures. The highest expression of *cat* gene in pPLC1 plasmid was found in *B.thuringiensis* hosts might result from the fact that *B.t.i.* could recognize their own promoter better than *B.megaterium* strain O-016 used as an intermediate strain for conjugation-like gene transferby more proper transcriptional system. In both *B.t.i.*strains, i.e. c4Q2-72 and 4Q2-72, it was found that *cat-86* gene was poorly expressed at exponential and the onset of stationary phase. However, the CAT activities were found to increase significantly at T_2 phase ofits growth stage which was reported to be the beginning of mosquitocidal protein synthesis in *B.t.i.* strains (193). The increased CAT specific activities at T_2 was suggested to be due to the increased transcription of the *cat* gene by sporulation related transcription (130). At T_8 phase more than two fold of CAT activity was observed over vegetative growing cells. From the published nucleotide sequence of *B.t.i.* toxin gene (47),it was found that the position -35 and -10 of the promoter region were AGTTT and AGATTGT, respectively which were found to be consensus with AGGTT and GTATTGT recognized by sigma-37 factor of RNA polymerase (130). This sigma-37 subunit was mainly available in post exponential growing cell. This led to the lower expression in exponential stage cell and higher expression in post exponential growing cell. Therefore, it should be noted that *B.t.i.* 130 kDal toxin gene promoter worked as post exponential promoter similar to the previously reported 203 bp *Eco*R I-*Pst* I fragment in pPL603 (132) expressed in *B.subtilis* BR 151 (Table 9). Moreover, the recognition of the toxin gene promoter might also involved with a

series of gene responsible in spore formation (*spo*) as proposed in *B. subtilis* harbouring pPL603 (188). It was demonstrated that some of *spo* mutant strains of *B.subtilis* conferred poor *cat-86* gene expression. Some of early *spo* gene products (6 of *spo0* genes and *spoIIE* gene) were directly or indirectly essential to gene transcription and blocks in *spoIIE* gene also permitted anintermediate level of gene expression whereas the later functioning spore genes (*spoIIB, spoIIF, spo IIA* and *spoIIC*) presumably enhance either transcription and translation of *B. subtilis*. Moreover, the sporulation specific form of RNA polymerase which involved directly in the promoter selection was not detected in a mutant blocked in *spo0A* (188). It was reported by Klier et al. (97) that the expression of crystal protein gene from *B.thuringiensis.* subsp. *thuringiensis* appeared only during sporulation in *B.subtilis* and absence in some *spo*$^-$ mutants. With this observation, Klier et al (97) concluded that the fusion gene between *cry IA(a)* promoter and the promoterless *cat-86* gene showed that *B.subtilis* conferred CAT activity only in post exponential phase. No CAT activity could be detected in *spo0A, spo0C, spo0F, spo0H, spoIIA* or *spoIIE* mutants (188). The *spo0J, spoIIC* and *spoIIIE* of *B.subtilis* exhibited low CAT activities than that of *spo*$^+$ strain. However, Shivakumar et al.(99) reported that the dependence on sporulation gene might be related to the vector used in cloning because it was found that a crystal protein gene cloned in different plasmid was not regulated by sporulation. This study reported

that the CAT specific activities obtained from *B.t.i.* harbouring pPLC1 (containing 130 kDa promoter) were much less than those obtained from *B.t.i.* harbouring plasmid pTF6 (containing *bgaB* gene promoter). It was well established that, the factor determined the level of gene expression in prokaryotic system was considered at two levels. Firstly, the transcriptional level was concerned by the strength of the promoter, DNA sequence specifically recognized and bound by RNA polymerase and by termination process. Secondly, the translational level, ribosome binding site that played the important role in binding with ribosome. The degree of homology between Shine Dalgano sequence and 16S rRNA was though to be crucial in translational initiation efficiency (135). To analyze *cat* gene expression between pPLC1 and pTF6, it should be noted that the terminator sequence which suggested to be rho protein independent and "AGGAGG" sequence of RBS which perfectly homology with the sequence "UCUUUCCUCC" in 16S rRNA of gram positive bacteria were common in both plasmids. Thus the two plasmid, pPLC1 and pTF6, appeared to differ only in the promoter region.

Interestingly, the unique feature of *bgaB* gene promoter in pTF6 suggested to be a factor determined its strength was the upstream sequences of promoter region. The mentioned "upstream activator" sequences consisted of 39 bp rich in adenine and thymine which absented in *B.t.i.* toxin promoter might play an important role in the begining of transcription by destabilizing the DNA helix (133). Furthermore, even the sequence between position -35 which was responsible for promoter recognition of RNA

polymerase and position -10 which involved in melting step led to the formation of productive promoter complex were appeared to be unimportant but decreasing or increasing of the intervening distance between these two position was crucial (187). The plasmid pTF6 had the intervening nucleotide in the preferable range about 16-19 bp but not in plasmid pPLC1. The longer intervening nucleotide in pPLC1 also could lead to lower expression pPLC1, it should be noted that the cloned promoter had one more RBS of the toxin gene and translational initiation sequence but 144 bp resided between multiple cloning site and *cat-86* coding sequence contained about 12 translation termination codons in all three reading frame. This virtue resulted in the absence of the fusion protein therefore the translation of *cat* gene in pPLC1 harbouring cell were initiated from the RBS of the vector as found in pTF6. It was also of interest to note that the high expression of CAT activity in pTF6 occured during the vegetative phase whereas that of pPLC1 occured during sporulation phase. Recently, there has been additional factor which was found to involve inregulation of gene expressionin *B.t.i.*. Visick et al. (117) reported that gene encoded for 20 kDal just 4 bp upstream from gene encoded for 28 kDa cytolytic protein of 110 kb plasmid could increase the persistence of CytA protein. The evidence elucidated the exist of protein-protein interaction between those two protein by coimmunoprecipitation obtained from the whole cell extracts with antibody against CytA and / or with antibody against the 20 kDa protein. This protein-protein interaction was found to play a role

in protecting protein degradation because it was shown in further investigation (177) that the amount of CytA produced became independent of the 20 kDa protein in the cell. Mclean et al. (116) reported in the previous study of 20 kDa protein that such a protein did not significantly increase the transcription of *cytA* or the stability of mRNA but exerted its effect after the initiation of translation. By this explanation, the lower expression of pPLC1 in *B.t.i.* c4Q2-72 might be due to lacking of the essential fragment for gene expression either for promoter recognition or for stabilizing gene product. Therefore, the plasmid pPLC1 was transferred to another *B.t.i.* strain ie. 4Q2-72 which contained intact 110 kb plasmid via conjugation-like gene transfer. The data shown in the Table 13 demonstrated that there was no significant difference in both the pattern of gene expression and the level of CAT activities in *B.t.i.* strain c4Q2-72 and 4Q2-72. Thus, this implied that it appeared to be no suggested effect by the presence of 110 kb plasmid for the expression of *cat* gene in pPLC1.

It has been known that not only the promoter strength that determined the level of transcription but also the number of copy of the plasmid. The increased mRNA could increase, the rate limiting step of gene expression, the binding of mRNA to ribosome (187). Therefore, the experiment for relative plasmid copy number determination was performed. In this study, the result showed that there was no significant different among the relative number of copy of plasmids harboured in *B.t.i.* strains i.e. pPL603, pPL703, pTF6 and pPLC1. Thus, the differencein *cat* expressions obtained

from various plasmid in *B.t.i.* strains should not be resulted from the difference in the relative copy number of plasmids.

It was also previously reported that the level of gene expression also depended on the stability of plasmid which contained the cloned gene. The reduction of gene product could be due to the instability of the plasmid particularly when cells were grown in non selective condition. It was found in this study that plasmid pPLC1 could not be stably maintained in *B.megaterium* strain O-016 in non selective culturing condition. *B.megaterium* strain O-016 conferred very low CAT activities throughout of its growth compared with *B.t.i.* strains that could stably maintain plasmid pPLC1.

It was also shown in this study that the cloned *B.t.i.* toxin gene promoter did increase the level of *cat* gene expression in *B.t.i.* however, this expression appeared to be lower than the level obtained from plasmid pTF6. This lower level of expression might be due to low promoter strength itself or due to the lack of essential regulatory region which was not cloned in this experiment. Therefore, the further works that might lead to the improvement of gene expression in *B.t.i.*might include, for example, site directed mutagenesis technique that might enhance the promoter strength, and insertion of synthetic A-T rich oligonucleotide that might be able to facilitate transcription by destabilizing DNA helix (133). Furthermore, there has been report concerning the importance of terminator sequence of insecticidal protein gene in stabilizing mRNA (38) and subsequently lead to increasing of the toxin gene. Thus the construction of a recombinant plasmid to include the

terminator sequence might also be included in the future strategy to increase the gene expression in *B.thuringiensis* subsp. *israelensis*.

CHAPTER VI

SUMMARY

The progress in genetic engineering has provided the potential to improve the expression of bacterial strains. In this study, attempts were made to demonstrate the strength of the *B.t.i.* toxin gene promoter which might be used for enhancing the expression of the desirable gene in *B.thuringiensis* host. Such a promoter was expected to be a strong promoter as evidenced by the naturally accumulation of insecticidal protein as an inclusion body in a number of *B.t.* strains. Thus, the plasmid containing the *B.t.i.* toxin gene promoter, i.e. plasmid pPLC1, was constructed by using the virtue of promoter cloning vector, pPL703. From the result, it was shown that very low background activities of chloramphenicol acetyltransferase (CAT), the enzyme used as a marker were obtained from all three *Bacillus* hosts harbouring pPL703 throughout their growth. The plasmid pPL603 which contained the post exponential promoter demonstrated low CAT activities in *B.megaterium* strain O-016 and *B.t.i.* whereas higher CAT activities was obtained from lysate extracted from T_8 culture of *B.subtilis*. The cloned *B.t.i.* toxin protein promoter in pPLC1 was inserted preceding *cat-86* and CAT specific activities were used to determine the effect of promoter. It was shown that *B.t.i.* which contained pPLC1 harbouring toxin gene promoter conferred higher CAT activities than that harboured promoterless version replicon pPL703. Moreover, the result also showed the post exponential promoter activity of the

cloned region by correlation between the sporulation of the growth cycle and levels of enzyme chloramphenicol acetyltransferase. However, this toxin promoter was not as strong as expected when compared with the strong promoter previously isolated from *bgaB* gene of *Bacillus stearothermophilus* IAM 11001 in plasmid pTF6. All of three *Bacillus* hosts harbouring pTF6 possessed high CAT activities. Therefore, it appaered that the promoter from *bgaB* gene in pTF6 was stronger than toxin gene promoter isolated from *B.t.i.* toxin gene. However, these two promoters appeared to function at different growth phase, the *bgaB* gene promoter in plasmid pTF6 enhanced CAT activity in vegetative phase whereas the toxin gene promoter in plasmid pPLC1 enhance CAT activity in sporulation phase. In order to enhance the expression of desirable gene, e.g. gene encoded for enzyme or insecticidal protein, the promoter of *bgaB* gene in plasmid pTF6 was recommended to be applied to yield a large amount of gene product.

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APPENDIX

Identification of *Bacillus megaterium* strain O-016

Bacillus megaterium strain O-016 was first isolated and designated as *Bacillus sp.* strain O-016 in 1984 by Loprasert, S (174). This strain was found to harbour no plasmid, was very sensitive to lysozyme treatment at very low concentration for example 2 mg/ml of lysozyme could be introduced to generate as high as 100% protoplast with in 30 minutes at 37°C and the regeneration frequency of such a strain was much better than that of *Bacillus thuringiensis.* The transformation frequency ranges between 10^2 - 10^3 transformants / ug DNA and most of plasmid DNAs with drug resistant phenotype could be stably maintained and retained the restriction pattern as native DNA (161,175). Therefore, *Bacillus megaterium* strain O-016 showed the promise to be suitable host, used for introducing plasmid DNA by protoplast transformation.

Further studies were carried out on gene transfer system from *Bacillus megaterium* strain O-016 to *Bacillus thuringiensis* strains by conjugation-like process. The mating experiment was performed on the filter membrane for 6 hours to optimize the condition. The rate of transfer among these two species ranges between $10^{7-}10^8$.

From the above reaction with two properties of *Bacillus megaterium* strain O-016, It is possible to manipulate the plasmid DNA via *Bacillus megatrium* strain O-016 as an intermediate strain, into

Bacillus thuringiensis which is very difficult to be transferred. This strain has been employed by many studies (174, 161) as a mean to transfer DNA plasmid into *Bacillus thuringiensis.*

In 1990, *Bacillus sp.* strain O-016 was identified by de Barjac et al. based on API system (176) as a strain close to *Bacillus megaterium* strain O-016 and designated as MEG'005 under the I.E.B.C. Number (International Entomopathogenic *Bacillus* centre) according to the following microscopic and biochemical tests :

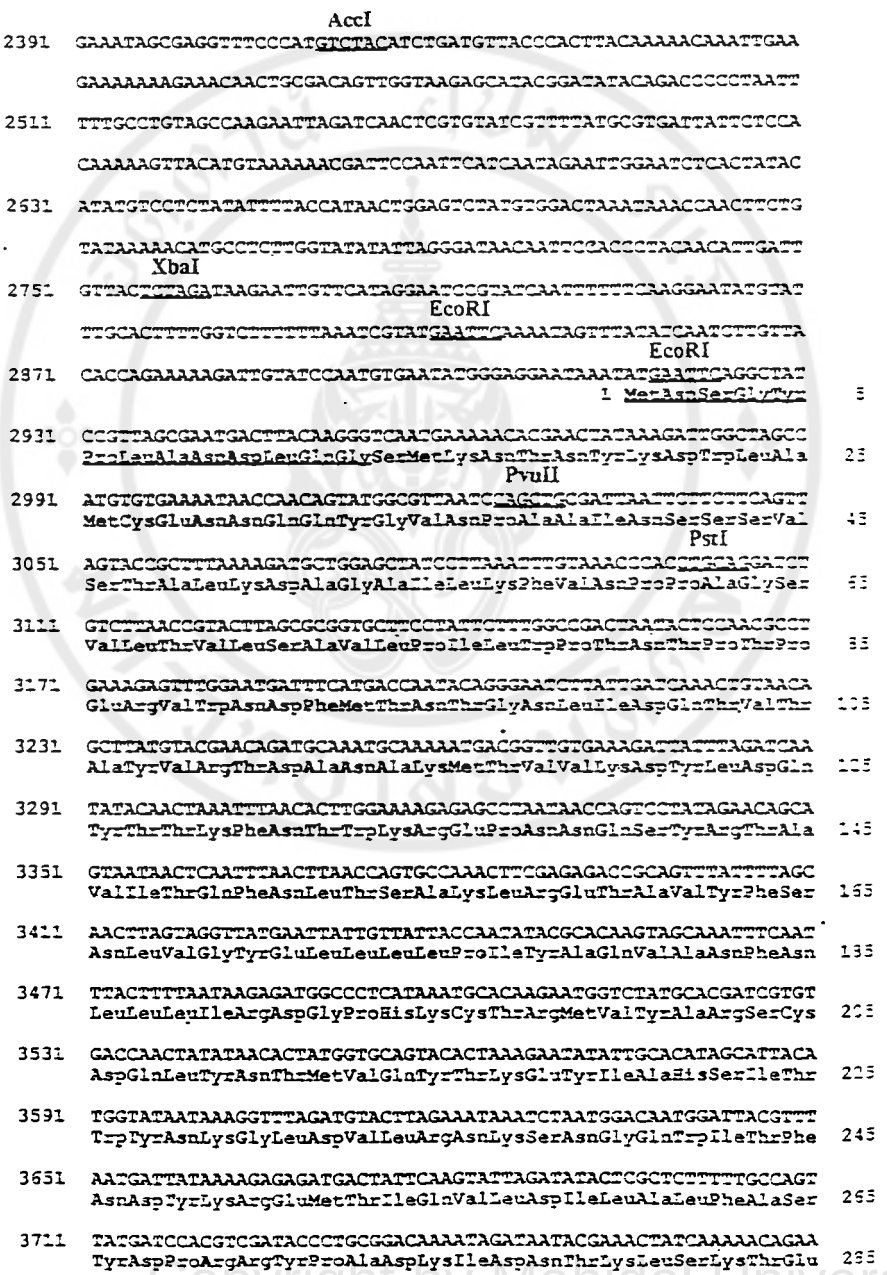
width of rod	> 0.9 um
sporangium	not swollen
oval shape spore , no crystal	

ONPG -	LCD -	AMC -
Urease -	ODC -	Gelatin -
ADH -	Citrate -	NO_2 -
TDA -	H_2S -	N_2 -
Glycerol +	Inositol +	Inuline W
Erythritol -	Mannitol +	Melezitose VW
D-Arabinose -	Sorbitol +	D-Raffinose W
L-Arabinose +	A-Methyl-D-Mannoside -	Amidon W
Ribose +	A-Methyl-D-Glucoside -	Glycogen +
D-Xylose +	N-Acetyl-Glucosamine +	Xylitol -
L-Xylose -	Amygdaline +	ß-Gentiobiose +
Adonitol -	Arbuline +	D-Turanose -
ß-Methyl-Xylosidase -	Esculine +	D-Xylose -
Galactose -	Salicine +	D-Tagatose _

D-Glucose +	Cellobiose +	D-Fucose -
D-Fructose +	Maltose +	L-Fucose -
D-Mannose +	Lactose -	D-Arabitol -
Rhamnose +	Saccharose +	Gluconate -
Dulcitol -	Trehalose +	2-Ceto Gluconate -
5-Ceto Gluconate -		

+	:	Positive Reaction
-	:	Negative Reaction
W	:	Weak Positive Reaction
VW	:	Very Weak Position Reaction

The nucleotide sequence of the gene coding for a 130-kDa mosquitocidal protein of *Bacillus thuringiensis* subsp. *israelensis*(47)



The nucleotide sequence of the gene coding for a 130-kDa mosquitocidal protein of *Bacillus thuringiensis* subsp. *israelensis*.

(continue)

```
3771  TTTACAAGAGAGATTTATACAGCTTTAGTAGAATCTCCTTCTAGTAAATCTATAGCAGCA
      PheThrArgGluIleTyrThrAlaLeuValGluSerProSerSerLysSerIleAlaAla   305

3831  CTGGAGGCAGCACTTACACGAGATGTTCATTTATTCACTTGGCTAAAGAGAGTAGATTTC
      LeuGluAlaAlaLeuThrArgAspValHisLeuPheThrTrpLeuLysArgValAspPhe   325

3891  TGGACCAATACTATATATCAAGATTTAAGATTTTTATCTGCCAATAAAATTGGGTTTTCA
      TrpThrAsnThrIleTyrGlnAspLeuArgPheLeuSerAlaAsnLysIleGlyPheSer   345

3951  TATACAAATTCTTCTGCAATGCAAGAAAGTGGAATTTATGGAAGTTCTGGTTTGGTTCAA
      TyrThrAsnSerSerAlaMetGlnGluSerGlyIleTyrGlySerSerGlyLeuValGln   365

4011  ATCTATCTCATCAAATTCAACTTAATTCTAATTGTTATAAAACTTCTATCACAGATACTA
      IleTyrLeuIleLysPheAsnLeuIleLeuIleValIleLysLeuLeuSerGlnIleLeu   385

4071  GCTCCCCCTCTAATCGAGTTACAAAAATGGATTTCTACAAAATTTGATGGTACTCTTGCC
      AlaProProLeuIleGluLeuGlnLysTrpIleSerThrLysPheAspGlyThrLeuAla   405

4131  TCTTATAATTCAAATATAACACCAACTCCTGAAGGTTTAAGGACCACATTTTTTGGATTT
      SerTyrAsnSerAsnIleThrProThrProGluGlyLeuArgThrThrPhePheGlyPhe   425

4191  TCAACAAATGAGAACACACCTAATCAACCAACTGTAAATGATTATACGCATATTTTAAGC
      SerThrAsnGluAsnThrProAsnGlnProThrValAsnAspTyrThrHisIleLeuSer   445

4251  TATATAAAAACTGATGTTATAGATTATAACAGTAACAGGGTTTCATTTGCTTGGACACAT
      TyrIleLysThrAspValIleAspTyrAsnSerAsnArgValSerPheAlaTrpThrHis   465

4311  AACATTGTTGACCCTAATAATCAAATATACACAGATGCTATCACACAAGTTCCGGCCGTA
      AsnIleValAspProAsnAsnGlnIleTyrThrAspAlaIleThrGlnValProAlaVal   485

4371  AAATCTAACTTCTTGAATGCAACAGCTAGAGTAATCAAGGGACCTGGTCATACAGGGGGG
      LysSerAsnPheLeuAsnAlaThrAlaArgValIleLysGlyProGlyHisThrGlyGly   505

4431  GATCTAGTTGCTCTTACAAGCAATGGTACTCTATCGGGAGGCAGAATGGAGATTCAATGT
      AspLeuValAlaLeuThrSerAsnGlyThrLeuSerGlyGlyArgMetGluIleGlnCys   525

4491  AAAACAAGTATTTTTAATGATCCTACAAGAAGTTACGGATTACGCATACGTTATGCTGCA
      LysThrSerIlePheAsnAspProThrArgSerTyrGlyLeuArgIleArgTyrAlaAla   545
                                        XbaI
4551  AATAGTCCAATTGTGATTGAATGTGATCATATGTATTACAAGGAGTTTCTAGAGGAACAA
      AsnSerProIleValIleGluCysAspHisMetTyrTyrLysGluPheLeuGluGluGln   565

4611  CGATTAGTACAGAACTACGTTTCAAGACCTAATAATATAATACCTACAGATTTAAAATAT
      ArgLeuValGlnAsnTyrValSerArgProAsnAsnIleIleProThrAspLeuLysTyr   585

4671  GAAGAGTTTAGATACAAAGATCCTAATGATGCAATTGTACCGATGAGATTATCTTCTAAT
      GluGluPheArgTyrLysAspProAsnAspAlaIleValProMetArgLeuSerSerAsn   605

4731  CAACTGATAACTATAGCTATTCAACCATTAAACATGACTTCAAATAATCAAGTGATTATT
      GlnLeuIleThrIleAlaIleGlnProLeuAsnMetThrSerAsnAsnGlnValIleIle   625

4791  GACAGAATCGAAATTATTCCAATCACTCAATCTGTATTAGATGAGACAGAGAACCAAAAT
      AspArgIleGluIleIleProIleThrGlnSerValLeuAspGluThrGluAsnGlnAsn   645

4851  TTAGAATCAGAACGAGAAGTTGTGAATGCACTGTTTACAAATGACGCGAAAGATGCATTA
      LeuGluSerGluArgGluValValAsnAlaLeuPheThrAsnAspAlaLysAspAlaLeu   665

4911  AACATTGGAACGACAGATTATGACATAGATCAAGCCGCAAATCTTGTGGAATGTATTTCT
      AsnIleGlyThrThrAspTyrAspIleAspGlnAlaAlaAsnLeuValGluCysIleSer   685

4971  GAAGGAATTATATCCAAAGAAAAAATGCTCTTATTAGATGAAGTTAAAAATGCGAAACAA
      GluGlyIleIleSerLysGluLysMetLeuLeuLeuAspGluValLysAsnAlaLysGln   705
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The nucleotide sequence of the gene coding for a 130-kDa mosquitocidal protein of *Bacillus thuringiensis* subsp. *israelensis*.

(continue)

```
5031  CTTAGTCAATCTCGAAATGTACTTCAAAACGGGGATTTTGAATCGCGTACGCTTGGTTGG
      LeuSerGlnSerArgAsnValLeuGlnAsnGlyAspPheGluSerArgThrLeuGlyTrp  725

5091  ACAACAAGTGATAATATCACAATTCAAGAAGATGATCCTATTTTTAAAGGGCATTACCTT
      ThrThrSerAspAsnIleThrIleGlnGluAspAspProIlePheLysGlyHisTyrLeu  745

5151  CATATGTCTGGGGCGAGAGACATTGATGGTACGATATTTCCGACCTATATATTCCAAAAA
      HisMetSerGlyAlaArgAspIleAspGlyThrIlePheProThrTyrIlePheGlnLys  765
                                  AccI
5211  ATTGATGAATCAAAATTAAAACCGTATACACGTTACCTAGTAAGGGGATTTGTAGGAAGT
      IleAspGluSerLysLeuLysProTyrThrArgTyrLeuValArgGlyPheValGlySer  785

5271  AGTAAAGATGTAGAACTAGTGGTTTCACGCTATGGGGAAGAAATTGATGCCATCATGAAT
      SerLysAspValGluLeuValValSerArgTyrGlyGluGluIleAspAlaIleMetAsn  805
           PvuII
5331  GTTCCAGCTGATTTAAACTATCTGTATCCTTCTACCTTTGATTGTGAAGGGCTAATCGTT
      ValProAlaAspLeuAsnTyrLeuTyrProSerThrPheAspCysGluGlyLeuIleVal  825

5391  GTGAGCGTCCGCTGTGCCGCTAACATTTGGGACACTTCTGATATGTTGTATTCATGCCAA
      ValSerValArgCysAlaAlaAsnIleTrpAspThrSerAspMetLeuTyrSerCysGln  845

5451  TATGATACAGGGAAAAAGCATGTCGTATGTCAGGATTCCCATCAATTTAGTTTCACTATT
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5511  GATACAGGGGCATTAGATACAAATGAAAATATAGGGGTTTGGGTCATGTTTAAAATATCT
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5571  TCTCCAGATGGATACGCATCATTAGATAATTTAGAAGTAATTGAAGAGGGCCCAATAGAT
      SerProAspGlyTyrAlaSerLeuAspAsnLeuGluValIleGluArgGlyProIleAsp  905

5631  GGGGAAGCACTGTCACGCGTGAAACACATGGAGAAGAAATGGAACGATCAAATGGAAGCA
      GlyGluAlaLeuSerArgValLysHisMetGluLysLysTrpAsnAspGlnMetGluAla  925

5691  AAACGTTCGGAAACACAACAAGCATATGATGTAGCGAAACAAGCCATTGATGCTTTATTC
      LysArgSerGluThrGlnGlnAlaTyrAspValAlaLysGlnAlaIleAspAlaLeuPhe  945

5751  ACAAATGTACAAGATGAGGCTTTACAGTTTGATACGACACTCGCTCAAATTCAGTACGCT
      ThrAsnValGlnAspGluAlaLeuGlnPheAspThrThrLeuAlaGlnIleGlnTyrAla  965
                ClaI
5811  GAGTATTTGGTACAATCGATTCCATATGTGTACAATGATTGGTTGTCAGATGTTCCAGGT
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5871  ATGAATTATGATATCTATGTAGAGTTGGATGCACGAGTGGCACAAGCGCGTTATTTGTAT
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5931  GATACAAGAAATATTATTAAAAATGTTGATTTTACACAAGGGGTAATGGGGTGGCATGTA
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5991  ACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTGGTTCTATCTAATTGG
      ThrGlyAsnAlaAspValGlnGlnIleAspGlyValSerValLeuValLeuSerAsnTrp 1045

6051  AGTGCTGGCGTATCTCAAAATGTCCATCTCCAACATAATCATGGGTATGTCTTACGTGTT
      SerAlaGlyValSerGlnAsnValHisLeuGlnHisAsnHisGlyTyrValLeuArgVal 1065

6111  ATTGCCAAAAAAGAAGGACCTGGAAATGGGTATGTCACGCTTATGGATTGTGAGGAGAAT
      IleAlaLysLysGluGlyProGlyAsnGlyTyrValThrLeuMetAspCysGluGluAsn 1085

6171  CAAGAAAAATTGACGTTTACGTCTTGTGAAGAAGGATATATTACGAAGACAGTAGATGTA
      GlnGluLysLeuThrPheThrSerCysGluGluGlyTyrIleThrLysThrValAspVal 1105

6231  TTCCCAGATACAGATCGTGTACGAATTGAGATAGGCGAAACCGAAGGTTCGTTTTATATC
      PheProAspThrAspArgValArgIleGluIleGlyGluThrGluGlySerPheTyrIle 1125
                                                        HindIII
6291  GAAAGCATTGAATTAATTTGCATGAACGAGTGATTAATAAAAAATACCTAAAGCTTTAAA
      GluSerIleGluLeuIleCysMetAsnGluStop  1135

6351  AACCCTGGAGAAAGTTTTCTCCATGGTTTTTAATTTCTGCATTTATTAATTCTGGTACAA

                          XbaI
6411  AAAATATATAGAAAACATAAAAAATAGATATCTAGA  6446
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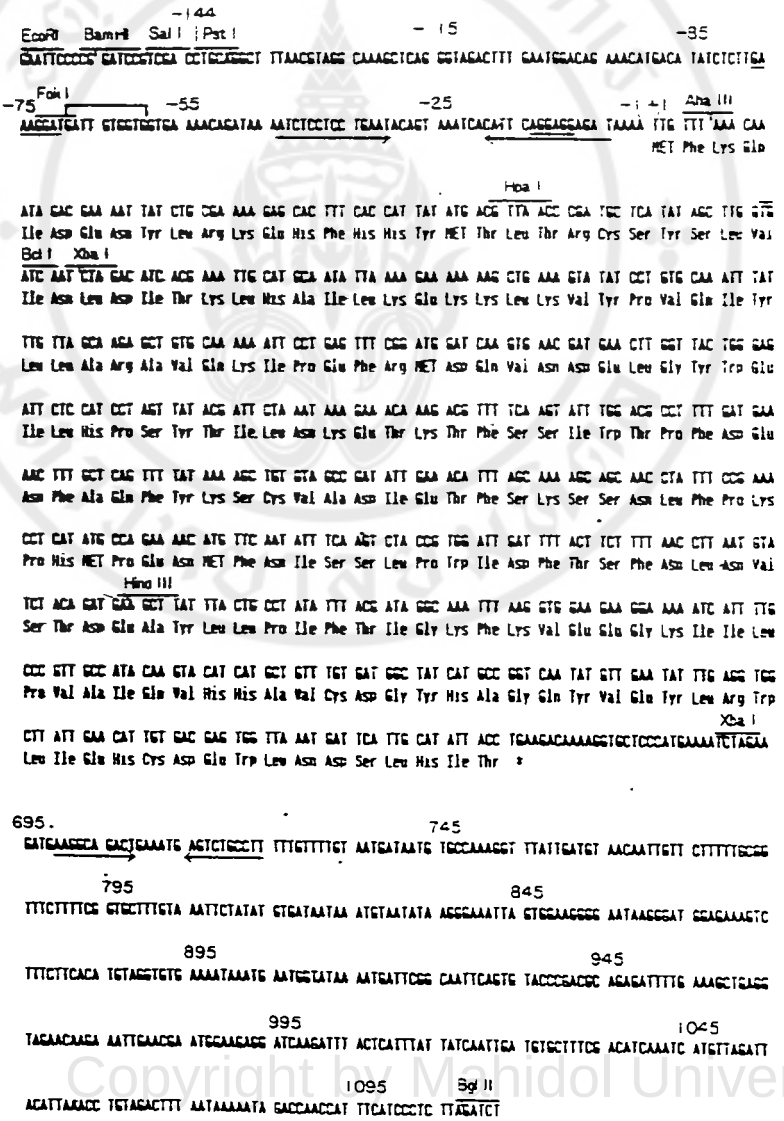
The nucleotide sequence of *Bacillus pumilus* NCIB 8600 chloramphenicol acetyltransferase gene (*cat-86*) (135).



The nucleotide sequence of *Bacillus stearothermophilus* IAM 11001 *bgaB* gene (133).

```
(AluI)
HindIII  10        20        30        40        50        60        70        80        90        100       110       120
AAGCTTTTCCCAAAACCATAGGTCTTTTTGGGAGTGTTCCCAGTGGTGATGGTTTGGAGTTCTTGTTTTAGTGATTTTACTGTATCCTTCATAAATTTGTCCTCCGAACATTGTTGAATC

                                                                                                                    240
ACTTGTTCTTAGGTTGAGGATTAGGTGCATTTTTTATACATTTATTAAAGAAGAAGGGCTTATTAATAAGGGTTATACTAAAAACTGAATCTTGTTTTTATCTACTCTGAAAATACCTAA

                                                                                                  HaeIII                360
ACTCCTAAATGCACCAATTTCACGATGTTCGACAGAAAAATATTTTTATCCTGCCAATGACGACGAAATTTTTCCGTTGCGAGGGCCTATATATTTGGTTGTTTTTAATTAAAAAATATA
                    HincII                                                                                  AluI
TATTTATTTAGTAAAATATTGTTGTTGACAAATACTAAATTTTAACTTAATTTATAATTAAACGAAAATTAGCTAGGGGGAATAATTATGAATGTGTTATCCTCAATTTGTTACGGAGGA
                    -35                 -10           mRNA                                    MetAsnValLeuSerSerIleCysTyrGlyGly

                                                                                                                    600
GATTATAACCCAGAGCAATGGCCAGAGGAAATTTGGTATGAAGATGCTAAGTTGATGCAAAAAGCGGGGGTGAATTTAGTATCTTTAGGGATTTTCAGTTGGAGCAAGATCGAACCGTCT
AspTyrAsnProGluGlnTrpProGluGluIleTrpTyrGluAspAlaLysLeuMetGlnLysAlaGlyValAsnLeuValSerLeuGlyIlePheSerTrpSerLysIleGluProSer

                                                                                                                    720
GATGGAGTGTTCGACTTTGAATGGCTAGACAAGGTTATAGATATACTATATGACCACGGTGTTTATATTAACTTGGGGACGGCGACTGCAACTACTCCAGCTTGGTTTGTAAAAAAGTAT
AspGlyValPheAspPheGluTrpLeuAspLysValIleAspIleLeuTyrAspHisGlyValTyrIleAsnLeuGlyThrAlaThrAlaThrThrProAlaTrpPheValLysLysTyr

                                                                                                                    840
CCAGATTCTTTGCCGATCGATGAAAGCGGTGTCATTCTCTCGTTTGGCAGTAGACAACATTATTGTCCTAATCATCCTCAATTAATTACGCACATAAAGAGACTTGTGAGGGCTATAGCA
ProAspSerLeuProIleAspGluSerGlyValIleLeuSerPheGlySerArgGlnHisTyrCysProAsnHisProGlnLeuIleThrHisIleLysArgLeuValArgAlaIleAla

                                                                                                                    960
GAACGGTATAAAAATCATCCGGCACTCAAAATGTGGCATGTTAATAATGAGTATGCATGTCACGTTTCCAAGTGTTTTTGTGAGAATTGTGCTGTCGCGTTTAGAAAGTGGCTAAAGGAA
GluArgTyrLysAsnHisProAlaLeuLysMetTrpHisValAsnAsnGluTyrAlaCysHisValSerLysCysPheCysGluAsnCysAlaValAlaPheArgLysTrpLeuLysGlu

                                                                                                                    1080
AGATATAAAACAATCGATGAATTAAATGAACGTTGGGGTACAAACTTTTGGGGACAGCGATACAATCATTGGGATGAAATTAATCCCCCTAGAAAGGCACCAACTTTTATTAATCCATCC
ArgTyrLysThrIleAspGluLeuAsnGluArgTrpGlyThrAsnPheTrpGlyGlnArgTyrAsnHisTrpAspGluIleAsnProProArgLysAlaProThrPheIleAsnProSer

                                                                                                                    1200
CAAGAACTTGATTACTACCGTTTTATGAATGACTCAATTCTCAAGTTGTTTTTAACAGAAAAGGAAATTTTACGTGAGGTAACACCAGATATTCCAGTATCAACTAATTTCATGGGTTCA
GlnGluLeuAspTyrTyrArgPheMetAsnAspSerIleLeuLysLeuPheLeuThrGluLysGluIleLeuArgGluValThrProAspIleProValSerThrAsnPheMetGlySer

                                                                                                                    1320
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PheLysProLeuAsnTyrPheGlnTrpAlaGlnHisValAspIleValThrTrpAspSerTyrProAspProArgGluGlyLeuProIleGlnHisAlaMetMetAsnAspLeuMetArg

                                                                                                  HpaI
                                                                                                                    1440
AGTTTAAGAAAAGGTCAACCGTTTATTTTGATGGAGCAGGTAACCTCACATGTTAACTGGCGCGATATTAATGTTCCAAAACCGCCAGGTGTAATGCGTCTATGGAGTTATGCAACTATT
SerLeuArgLysGlyGlnProPheIleLeuMetGluGlnValThrSerHisValAsnTrpArgAspIleAsnValProLysProProGlyValMetArgLeuTrpSerTyrAlaThrIle

                                                                                                                    1560
GCCCGTGGTGCAGATGGTATTATGTTTTTCCAGTGGCGTCAAAGTAGAGCAGGAGCTGAAAAATTCCACGGTGCAATGGTGCCCCACTTTTTGAACGAGAATAATAGAATTTATAGGGAA
AlaArgGlyAlaAspGlyIleMetPhePheGlnTrpArgGlnSerArgAlaGlyAlaGluLysPheHisGlyAlaMetValProHisPheLeuAsnGluAsnAsnArgIleTyrArgGlu

                                                                                                                    1680
GTTACACAGTTAGGGCAAGAGCTGAAAAAGTTAGATTGTTTGGTCGGATCTAGAATCAAGGCAGAGGTCGCGATCATTTTTGATTGGGAAAACTGGTGGGCTGTCGAACTAAGTTCCAAA
ValThrGlnLeuGlyGlnGluLeuLysLysLeuAspCysLeuValGlySerArgIleLysAlaGluValAlaIleIlePheAspTrpGluAsnTrpTrpAlaValGluLeuSerSerLys

                                                                                                                    1800
CCACATAATAAACTAAGATATATTCCTATAGTTGAAGCTTATTATAGGGAATTATATAAACGTAATATTGCTGTCGATTTTGTAAGGCCATCTGATGATCTAACAAAATACAAAGTAGTT
ProHisAsnLysLeuArgTyrIleProIleValGluAlaTyrTyrArgGluLeuTyrLysArgAsnIleAlaValAspPheValArgProSerAspAspLeuThrLysTyrLysValVal

                                                                                                                    1920
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IleAlaProMetLeuTyrMetValLysGluGlyGluAspGluAsnLeuArgGlnPheValAlaAsnGlyGlyThrLeuIleValSerPhePheSerGlyIleValAspGluAsnAspArg

                                                                                                                    2040
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ValHisLeuGlyGlyTyrProGlyProLeuArgAspIleLeuGlyIlePheValGluGluPheValProTyrProGluThrLysValAsnLysIleTyrSerAsnAspGlyGluTyrAsp

                                                                                                                    2160
TGTACGACGTGGGCGGACATAATCCGATTAGAAGGGGCAGAACCTCTAGCGACATTTAAGGGGGATTGGTATGCAGGACTTCCGGCGGTTACACGTAACTGCTACGGTAAAGGAGAGGGG
CysThrThrTrpAlaAspIleIleArgLeuGluGlyAlaGluProLeuAlaThrPheLysGlyAspTrpTyrAlaGlyLeuProAlaValThrArgAsnCysTyrGlyLysGlyGluGly

                                                                                                                    2280
ATTTACGTCGGTACTTATCCAGATAGTAATTATTTAGGCAGGCTTTTAGAACAGGTTTTCGCTAAACATCATATTAATCCCATTCTTGAAGTAGCTGAAAATGTAGAGGTGCAACAAAGA
IleTyrValGlyThrTyrProAspSerAsnTyrLeuGlyArgLeuLeuGluGlnValPheAlaLysHisHisIleAsnProIleLeuGluValAlaGluAsnValGluValGlnGlnArg

                                                                                                                    2400
GAGACTGATGAATGGAAGTATTTGATTATCATCAATCATAATGATTACGAAGTGACGCTGTCACTGCCAGAAGATAAGATATACCAGAATATGATTGATGGGAAATGTTTTCGAGGAGGT
GluThrAspGluTrpLysTyrLeuIleIleIleAsnHisAsnAspTyrGluValThrLeuSerLeuProGluAspLysIleTyrGlnAsnMetIleAspGlyLysCysPheArgGlyGly

                                                                                                                    2520
GAATTGAGGATTCAAGGGGTTGATGTAGCAGTGTTAAGAGAGCATGATGAAGCCGGGAAGGTTTAGAGAAGTCTCGTTCCGACAGTTGGCAACATAATATGCATAAGATGACAATGTCTA
GluLeuArgIleGlnGlyValAspValAlaValLeuArgGluHisAspGluAlaGlyLysValStop

2533
TAAACATTGGATC
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