CHARACTERIZATION OF CONJUGATION-LIKE GENE TRANSFER PROCESS IN *BACILLUS THURINGIENSIS* SUBSPECIES *ISRAELENSIS*

CHANPEN WIWAT

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ชื่อวิทยานิพนธ์ คุณสมบัติของขบวนการถ่ายทอดยีนโดยวิธี Conjugation-like ใน *Bacillus thuringiensis* subspecies *israelensis*

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บทคัดย่อ

จากการศึกษาการถ่ายทอดยีนส์โดยกระบวนการ Conjugation-like โดยวิธี broth mating พบว่าเชื้อ *Bacillus thuringiensis* subsp. *israelensis* สายพันธุ์ A084-16-194 สามารถส่งถ่ายพลาสมิด pBC16 และ pC194 ไปยัง *B. thuringiensis* สายพันธุ์ต่าง ๆ จำนวนทั้งหมด 25 subspecies อัตราการถ่ายทอดพลาสมิดอยู่ในช่วง 1.1×10^{-9} ถึง 9.8×10^{-5} นอกจากนี้ยังพบว่ามีการถ่ายทอดโครโมโซมใน *B. thuringiensis* จำนวน 10 subspecies จากทั้งหมด 25 subspecies ที่ได้ทำการทดลอง อย่างไรก็ดีการถ่ายทอดโครโมโซมมีอัตราการถ่ายทอดต่ำมาก (4.3×10^{-9} ถึง 3.7×10^{-7}) การถ่ายทอดพลาสมิดภายใน subspecies เดียวกัน 8 คู่ พบว่าอัตราการถ่ายทอดพลาสมิดสูงกว่าการถ่ายทอดพลาสมิดระหว่าง subspecies จากผลการทดลองบ่งชี้ว่าความสามารถในการถ่ายทอดทอดพลาสมิดระหว่าง subspecies เหล่านี้ไม่ได้ขึ้นต่อการดำรงอยู่ของพลาสมิดเฉพาะชนิดหนึ่งชนิดใด และยังไม่ได้ขึ้นต่อรูปแบบของพลาสมิดทั้งในเซลล์ตัวให้และตัวรับด้วย

ปรากฏการณ์การเกาะกลุ่มระหว่างเซลล์ 2 ชนิดของเชื้อ *B. thuringiensis* อย่างจำเพาะ ทำให้สามารถจำแนกเชื้อ *B.t.i* สายพันธุ์ต่าง ๆ ที่ทำการทดลองออกเป็น 2 กลุ่ม คือ กลุ่มที่ 1 (4Q2) และ กลุ่มที่ 2 (4Q272 และ c4Q272)

การถ่ายทอดพลาสมิด pBC16 ระหว่างสายพันธุ์ A084-16-194 และ c4Q272 สามารถถูกยับยั้งได้โดย 0.2 mM EDTA, 0.25 M $MgCl_2$, 0.5 M $MgSO_4$ และ 0.05 M $CaCl_2$ และสารเคมีเหล่านี้ในความเข้มข้นเดียวกันสามารถลดปริมาณ (EDTA และ $MgCl_2$) หรือยับยั้ง ($MgSO_4$) การเกาะกลุ่มของเซลล์ได้อีกด้วย

นอกเหนือจากการเกาะกลุ่มของเซลล์อย่างจำเพาะแล้ว ยังพบอีกว่าเชื้อสายพันธุ์ในกลุ่มที่ 1 (4Q2) ดื้อต่อยาเพนิซิลลินมาก นั่นคือสายพันธุ์ 4Q2 มีค่า MIC 12.5 มก. โดยที่สายพันธุ์ในกลุ่มที่ 2 (4Q272 และ c4Q272) มีความไวต่อยาเพนิซิลลินมากโดยมี MIC 0.06 และ 0.03 มคก. ตามลำดับ

ในการสกัดโปรตีนจากเชื้อ *B.t.i.* สายพันธุ์ต่าง ๆ ด้วย 6 M urea พบว่ามีโปรตีนที่มีน้ำหนักโมเลกุลสูงจากเชื้อสายพันธุ์ 4Q2 ซึ่งไม่พบในสายพันธุ์ 4Q272 และ c4Q272 จึงคาดหมายว่าเป็น S-layer protein จึงได้เตรียมแอนติบอดีย์ต่อ S-layer protein นั้น เพื่อใช้ทดสอบหาตำแหน่งของ S-layer protein บนเชื้อ *B.t.i.* โดยวิธี indirect immunofluorescent ความจำเพาะของแอนติบอดีย์ที่เตรียมได้นี้ทำการทดสอบยืนยันโดยวิธี Immunodiffusion และ Western blot

แอนติบอดีย์ต่อ S-layer protein ของ 4Q2 สามารถยับยั้งการถ่ายทอดพลาสมิด โดยกระบวนการ conjugation-like ระหว่าง *B.t.i.* สายพันธุ์ 4Q2-16 และ c4Q272 โดยอัตราการถ่ายทอดพลาสมิด pBC16 ลดลงจาก 9.7×10^{-6} เป็นน้อยกว่า 1×10^{-8} เมื่อไม่มีและมีแอนติบอดีย์ต่อ S-layer protein ของสายพันธุ์ 4Q2 และแอนติบอดีย์นี้ยังทำให้อัตราการถ่ายทอดพลาสมิด pBC16 โดยกระบวนการ conjugation-like ระหว่าง *B.t.i.* สายพันธุ์ A084-16-194 และ c4Q272 ลดลงด้วย โดยที่อัตราการถ่ายทอดพลาสมิดจะลดลงจาก 2.2×10^{-5} เป็น 1.2×10^{-6} เมื่อไม่มีและมีแอนติบอดีย์ตามลำดับ

ได้ทำการขยายยีนส์ที่ควบคุมการสร้าง S-layer protein จาก *B.t.i.* สายพันธุ์ 4Q2 โดยใช้ pBluescriptKS และ pUC12 เป็นดีเอ็นเอพาหะแล้ว transform เข้าสู่ *Escherichia coli* DH5α จากนั้นจึงใช้แอนติบอดีย์ต่อ S-layer protein เป็นสารทดสอบ ได้รับพลาสมิดลูกผสมที่มียีนส์ควบคุมการสร้าง S-layer protein 3 โคลน ได้แก่ pAC1, pAC2 และ pAC3 จากนั้นได้ทำการ subcloned พลาสมิดลูกผสม pAC1 ต่อไปจนได้ pAC11, pAC111 และ pAC112 ตามลำดับ และได้ทดสอบโปรตีนที่ควบคุมการสร้างโดยยีนส์ขนาดต่าง ๆ ด้วยวิธี Western blot

ทำการทดสอบหาลำดับนิวคลีโอไทด์ของยีนที่ควบคุมการสร้าง S-layer protein ขนาด 1.5 กิโลเบสที่อยู่ในพลาสมิดลูกผสม pAC111 โดยวิธี dideoxy chain-termination จากการวิเคราะห์ลำดับนิวคลีโอไทด์สามารถชี้บ่งตำแหน่งที่น่าจะเป็น initiation codon, -10, -35 และ Shine-Dalgarno sequence

ในการทำ Southern blot hybridization โดยใช้ดีเอ็นเอขนาด 5.2 กิโลเบสจาก pAC11 ติดฉลากด้วยไบโอตินเป็นดีเอ็นเอตรวจสอบได้บ่งชี้ว่ายีนที่ควบคุมการสร้าง S-layer protein อยู่บนโครโมโซมของ *B.t.i.* สายพันธุ์ 4Q2, 4Q272 และ c4Q272

Thesis Title: Characterization of Conjugation-like Gene Transfer Process in *Bacillus thuringiensis* subspecies *israelensis*

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ABSTRACT

The plasmids pBC16 and pC194 from *Bacillus thuringiensis* subsp. *israelensis* strains A084-16-194 were transferred to 25 subspecies of *B. thuringiensis* by a conjugation-like process using broth mating technique. The frequencies of transfer varied considerably between different mating pairs, ranging from 1.1×10^{-9} to 9.8×10^{-5}. Additionally, chromosomal transfer could also be demonstrated in ten *B. thuringiensis* subspecies with very low frequencies (4.3×10^{-9} to 3.7×10^{-7}). The matings within a group of eight subspecies gave higher frequencies of transfer than the matings between the subspecies indicating that the frequencies of transfer was higher in

the intrasubspecific than the intersubspecific transfers. The results indicated that the capability to transfer plasmids among these various subspecies did not depend on the presence of specific plasmids nor specific plasmid patterns in either donor or recipient cells.

The cell clumping phenomena appeared to be specific between certain pairs of *B. thuringiensis*. Among few strains being tested, wild type strain 4Q2 and its cured strains 4Q272 and c4Q272, clumping could be divided into two groups i.e. clumping group I (4Q2) and clumping group II (4Q272 and c4Q272).

The transfers of pBC16 plasmid from strain A084-16-194 into strain c4Q272 were found to be inhibited by 0.2 mM EDTA, 0.25 M $MgCl_2$, 0.5 M $MgSO_4$ and 0.05 M $CaCl_2$. These chemicals, at the same concentrations, could be demonstrated to reduce (EDTA and $MgCl_2$) or inhibit ($MgSO_4$) the extent of clumping between strain A084-16-194 and c4Q272.

Beside the clumping phenomena, it was also found that strain in clumping group I (4Q2) possessed high level of penicillin G resistance. Strain 4Q2 was resistant to penicillin G at MIC of 12.5 mg, but strains in clumping group II (4Q272 and c4Q272) were quite susceptible to penicillin G with MIC of 0.06 and 0.03 ug, respectively.

Extraction of S-layer protein by treatment with a 6 M urea indicated that there appeared to be extra-high molecular weight protein in the extracts obtained from

B.t.i. strain 4Q2. This protein band was found to be absent in strains 4Q272 and c4Q272. The antibody toward this S-layer protein was prepared and used for locating of S-layer protein on *B.t.i.* cell by using indirect immunofluorescent technique. Immunodiffusion reaction and Western blot analysis confirmed the specificity of the anti-S-layer protein antibody.

It was found that the antibody against 4Q2 S-layer protein inhibited the plasmid transfer via the conjugation-like process between *B.t.i.* strain 4Q2-16 and c4Q272. The frequencies of transfer of pBC16 plasmid was found to reduce from 9.7×10^{-6} to less than 1×10^{-8} in the absence and presence of anti 4Q2-S-layer protein antibody, respectively. This antibody could also found to reduce the frequencies of transfer of pBC16 plasmid via conjugation-like process between *B.t.i.* strain A084-16-194 and c4Q272. The frequencies of transfer between the two latter strains were found to reduce from 2.2×10^{-5} to 1.2×10^{-6} in the absence and presence of the antibody, respectively.

Using antibody detection technique, S-layer protein gene from *B.t.i.* strain 4Q2 was cloned in pBluescriptKS and pUC12 of *Escherichia coli* DH5α. Three positive clones namely pAC1, pAC2 and pAC3, containing the genes encoding for the S-layer protein were obtained. The pAC1 clone was subsequently subcloned to pAC11, pAC111 and pAC112. The presence of gene product was confirmed by using Western blot analysis.

The 1.5 kb fragment of the pAC111 plasmid was sequenced by using dideoxy chain-termination method. The nucleotide sequence showed the putative initiation codon (ATG), -10, -35 and Shine-Dalgarno sequence.

Southern blot hybridization using 5.2 kb fragment of the pAC11 as biotinylated probe indicated that the S-layer protein gene was located on chromosome of *B.t.i.* strains 4Q2, 4Q272 and c4Q272.

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LIST OF ABBREVIATIONS

^{o}C	degree celcius
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
et al	et alii (Latin), and other people
Fig	figure
g	gram
hr	hour
i.e.	id est (Latin), which is to say, in other words
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
l	liter
ug	microgram
ul	microliter
mA	milliampere
mg	milligram
ml	milliliter
mM	millimolar
M	molar
MIC	minimal inhibition concentration
min	minute
MW	molecular weight
Nal^{r}	nalidixic acid resistant
nm	nanometer

Pen^r	penicillin resistant
Pen^s	penicillin sensitive
Pen G	penicillin G
RNA	ribonucleic acid
Rif^r	rifampicin resistant
rpm	revolution per minute
SD	Shine-Dalgarno
SDS	sodium dodecyl sulphate
S-layer	surface layer
spp.	species
subsp.	subspecies
Tet^r	tetracycline resistant
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
V	volt
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

Bacillus thuringiensis is one of the most effective bacterial insecticides which is being used widely to control populations of insect pests and vectors (1-3). In contrast with chemical insecticides, the strains of this bacterium produce toxins with highly selective modes of action (1, 2) such that the toxic activity of particular isolate may be limited to very specific target insects and non-toxic to non-target insects or to other organisms including man (4). Also in contrast with many chemical insecticides, these biopesticides will not persist for an extended period in nature and, are thus, unlikely to destroy the environmental equilibrium. For these reasons, many strains of *B. thuringiensis* have become widely used as bioinsecticides for agriculture and for certain vectors of human diseases (4, 5).

More than 20 subspecies of *B. thuringiensis* produce different insecticidal toxins which can be categorized into three major groups; alpha-endotoxins, beta-exotoxins and delta-endotoxins (6). These toxins are encoded by various toxin genes located either on plasmids and/or on chromosomal DNA (2, 7). Further, each subspecies of *B. thuringiensis* has been found to harbor many cryptic plasmids (7, 8).

Presently, there are numerous attempts in trying to genetically improve strains of *B. thuringiensis*. There have been many studies on the subjects of cloning, characterizing and manipulating of toxin genes from *B. thuringiensis* via mutagenesis or gene transfer techniques (3, 9, 10-13). In order to widen the host range of *B. thuringiensis* strains by genetic manipulation techniques, one of the most effective means is the exploitation of conjugation-like gene transfer process which was first discovered by Gonzalez et al. in 1982 (14). Subsequently, there were many reports (15-19) on the use of this conjugation-like process for transferring plasmids in *B. thuringiensis*.

Despite these numerous examples of the conjugation-like transfer of plasmids and genes in *B. thuringiensis* and related bacteria, there has been no report on the attempt to elucidate the mechanism of this type of gene transfer.

The purpose of this study was, therefore, to elucidate possible factor(s) which might be involved in conjugation-like gene transfer process.

The strategy of this study was, firstly, to investigate the nature of the conjugation-like transfer of plasmids and chromosomal markers from *B. thuringiensis* subsp. *israelensis* to various subspecies of *B. thuringiensis* or among various strains of *B.thuringiensis*.

Secondly, studies were conducted to investigate the effect of various chemicals on conjugation-like gene transfer process.

Thirdly, attempts were made to study the S-layer protein and possible involvement on conjugation-like gene transfer process. The studies on S-layer protein also included molecular cloning and partially sequencing of S-layer protein gene.

CHAPTER II

BACKGROUND

1. Biology of *Bacillus thuringiensis*

Bacillus thuringiensis is a gram-positive, aerobic, endospore-forming bacterium which is closely related to *B. cereus* and *B. anthracis*. The principal property which is used to distinguish *B. thuringiensis* from *B. cereus* and *B. anthracis* is the presence of parasporal crystal during sporulation (20). The crystals or delta-endotoxins contain insecticidal activities which are effective against larvae of lepidopteran, dipteran or coleopteran (4, 6, 21). For example, *Bacillus thuringiensis* subsp. *kurstaki* possesses insecticidal activity against the larvae of butterflies and moths, the crystal toxin of *B. thuringiensis* subsp. *israelensis* is active against the larvae of mosquitoes and black flies (4), the endotoxin of *B. thuringiensis* subsp. *aizawai* displays a significant activity against the cotton leaf worms, *Spodoptera litteralis* (22) and the crystal of *B. thuringiensis* strain EG2158 is found to be toxic to larvae of Colorado potato beetle, *Leptinotarsa decemlineata* (23).

B. thuringiensis may be categorized into subspecies according to flagellar antigen as described by de Barjac (24) or delta-endotoxin shape, size and antigens as described by Krywienczyk and Angus (2, 25-27) or esterase pattern as described by de Barjac, Bonnefoi and

Norris (28). The classification based on H-antigen and plasmid profiles (29) are summarized in Table 1. Additional biochemical properties, such as the ability to utilize sucrose or cellobiose, presence or absence of arginine dehydrolase, has also been used in classification of *B. thuringiensis*, as in the division of serotype H-4a4b strains into two subspecies, *sotto* and *dendrolimus* (24).

In addition, there is a correlation between flagella serotype and crystal serotype. For example most *B. thuringiensis* subsp. *thuringiensis* (serotype H-1) isolates were of the *thu* crystal type (2) and most of *B. thuringiensis* subsp. *galleriae* isolates were of the *gal* crystal type (29). There is an exception to one crystal type of *B. thuringiensis* subsp. *kurstaki* isolates where there was a major division between k-1 and k-73 crystal types (25), and they also had a different activity for larvae. The identical crystals sometimes appeared in different H-serotypes; e.g. k-1 crystals were found in both *kurstaki* and *thuringiensis* subspecies. Furthermore, some isolates contained mixed crystal types.

Furthermore, nucleotide sequences have been reported for various *B. thuringiensis* crystal protein genes. Several sequences are identical or nearly identical (30) and represented the same gene or slight variant of the same gene. They could be categorized into 14 distinct crystal protein genes and 13 of these crystal

protein genes (*cry*) specificity a family of related insecticidal protein. These 13 genes have been further divided into four major classes and several subclasses characterized on the basis of the insecticidal spectra of the crystal proteins and structural similarity (30). The four major classes are lepidoptera-specific (*cryI*), lepidoptera- and diptera-specific (*cryII*), coleoptera-specific (*cryIII*) and diptera specific (*cryIV*) genes. One crystal protein gene of *B. thuringiensis* codes for a 27-kDa protein that exhibits cytolytic activity against a variety of invertebrate and vertebrate cells, in addition, this gene is designated as a *cytA* for cytolytic crystal protein (30). In addition, each class of *cry* genes could be divided into various subclasses based on the similarity of nucleotide sequences. The *cryI* genes were divided into *cryIA*, *cryIB*, *cryIC*, *cryID*, *CryIE* and *cryIF*, the *cryII* genes were divided into *cryIIA*, and *cryIIB*, the *cryIII* genes were divided into *cryIIIA*, *cryIIIB*, and *cryIIIC* and the *cryIV* genes were grouped into *cryIVA*, *cryIVB*, *cryIVC* and *cryIVD*. The *cryIA* genes were further divided into *cryIA* (a), *cryIA* (b) and *cryIA* (c) based on the location of *Hin*dIII site within the coding region and a second 5' flanking *Hin*dIII site (30).

1.1 The plasmid profile of *B. thuringiensis*

Most subspecies of *B. thuringiensis* possess a large number of plasmids (Table 1) differing in the number and sizes of the plasmids (2). Some subspecies, such as

Table 1 Characteristics including number of plasmids, location of delta-endotoxin genes and crystal protein gene types of various subspecies of *Bacillus thuringiensis*.

Species	H-serotype	No. of plasmids*	Location of -endo-toxin genes	Crystal protein genes	Reference(s)
thuringiensis	1	7, 10, 17	plasmid/chromosome	*cryIB*	31, 32, 33, 223
finitimus	2	2	plasmid/chromosome		34
alesti	3a	6, 10, 12	plasmid		31, 32, 33, 35
kurstaki	3a3b	6, 8, 11, 12	plasmid	*cryIA* (a) *cryIA* (b) *cryIA* (c) *cryIIA* *cryIIB*	48 49 50 51 52
sotto	4a4b	3	plasmid	*cryIA* (a)	33, 53
dendrolimus	4a4b	4	chromosome		37
kenyae	4a4c				46
galleriae	5a5b	3, 4, 5	plasmid		31, 32, 33, 36, 46
canadensis	5a5c				
entomocidus	6	4	chromosome/plasmid	*cryIA* (a) *cryIB* *cryIC*	7, 38, 54, 30 30 30
subtoxicus	6	2	plasmid/chromosome		36, 37
aizawai	7	8, 12	plasmid	*cryIA* (a) *cryIA* (b) *cryIC* *cryID*	23, 39, 40, 36, 55 46, 56 30
pacificus	7				
morrisoni	8a8b	5	plasmid	*cytA***	33, 57
tenebrionis				*cryIIIA*	58
ostrinae	8a8c	4	plasmid		31
nigeriensis	8b8d				59
tolworthi	9	6, 9	plasmid		33, 36
caucasicus	10a				45

Table 1. (Continued)

Species	H-serotype	No. of plasmids*	Location of -endo toxin genes	Crystal protein genes	Reference(s)
darmstadiensis	10	4	plasmid		41
toumanoffi	11a11b	5	plasmid		33
kyushuensis	11a11c		chromosome		41
thompsoni	12	4	plasmid		31, 32
pakistani	13				46
israelensis	14	6, 9	plasmid	*cryIVA* *cryIVB* *cryIVC* *cryIVD* *cytA***	43, 44, 60 61 49 238 62
dakota	15				46
indiana	16				46
tohokuensis	17				46
kumamotoensis	18				46
tochigiensis	19				46
yunnanensis	20a20b				46
pondicheriensis	20a20c				59
colmeri	21	6	plasmid		31
shandongiensis	22				59
japonensis	23				59
neoleonensis	24				59
coreanensis	25				59
siloensis	26				59
mexicanensis	27				33
wuhanensis	-	4			33
fukuokaensis	N.D.	3			45

* Data collected from different articles which reports different number of plasmids of the same subspecies.
** This gene encoded 27-KDa protein that showed a cytolytic activity for a variety of in vertebrate cells.

thuringiensis, *kurstaki*, *alesti* and *aizawai* possess as many as 12 plasmids in various sizes (31-33, 35, 36, 39, 40), but some subspecies such as *subtoxicus* or *finitimus* possess only two different types of plasmids (34, 36, 37).

There is a variation in plasmid profiles of some subspecies reported from different laboratories. These variations may depend on the methods of preparation of plasmids, particularly in the recovery of large plasmids (>100 MDa). The differences may also be because of variations in plasmids profiles in different isolates of the same subspecies (7). Such intraserotype difference may originate by means of interstrain plasmid transfer in both plasmid-plasmid and plasmid-chromosome recombination (6). There is evidence for the presence of insertion sequence (IS) in large plasmids which are related to protoxin genes (63, 64); therefore, the transfer of plasmids via cell mating may contribute to variations in plasmid profiles for different isolates with the same serotype (2).

1.2 Genetics of *B. thuringiensis* and its gene transfer

1.2.1 Transformation in *B. thuringiensis*

Transformation is a process by which a cell takes up naked DNA from the surrounding medium and incorporates it to the recipient host. The technique used for plasmid transformation in *B. thuringiensis* has been carried out by protoplast transformation (3, 10, 65-67),

autoplast transformation (68, 69), and more recently, by electroporation (11, 12).

Protoplast transformation in *B. thuringiensis* has been modified from the method of protoplast transformation in *B. subtilis* originally described by Chang and Cohen (65). Because of the high resistance of *B. thuringiensis* cell wall to lysozyme, a high concentration of lysozyme at 15 mg/ml (66) or a combination of lysozyme and achromopeptidase (70) have been used to obtain an efficient cell wall hydrolysis. However, the frequency of transformation was still very low, ranging from 10^{-7} to 10^{-4} transformants per no. of protoplasts (66). The low efficiency transformation may result from the excess lysozyme treatment which leads to undesirable effect such as interference with cell wall regeneration due to strong binding of lysozyme to protoplast membrane (67). The regeneration frequency of *B. thuringiensis* protoplast has been found to be very low (69). Low frequency of transformant had been reported for plasmid pC194, pBC16, or pUB110 which are all small plasmids of 2.9, 4.5 and 4.5 kb, respectively (3, 9, 10, 66).

It has been suggested that the existence of certain factor(s) associated with cells during rapid growth may confer an apparent sensitivity to lysozyme (2); also, they could be related to the autolytic removal of the cell wall in this bacteria with generation of *B.*

thuringiensis autoplast by induction its endogenous enzyme. The experiments were successfully demonstrated by Crawford et al. (68), and Rubinstein and Sanchez-Rivas (69). However, the transformation frequency by this method was still quite low, ranging from 10^{-6} to 10^{-5} transformants per regenerated autoplasts when transformed with pC194, and must use very high concentration of DNA, approximately 10 ug at a time (68).

Recently, a new method for transforming *B. thuringiensis* using electroporation technique has been reported. Electroporation technique has been employed to introduce DNA into many types of eukaryotic cells, and into some bacterial strains such as *Escherichia coli* with high transformation frequencies, i.e. 10^8 cells/ug of DNA (71). In electroporation, a high voltage electric discharge through a cell suspension results in a transient permeability of the cell membrane, allowing entry of DNA into the cells. There are a few reports on transformation of *B. thuringiensis* by electroporation (11, 12, 72), but only small plasmids, such as pC194, pBC16, pUB110, pGKV2, and pBD64 ranging in size 2.9, 4.5, 4.5, 4.6 and 4.8 kb, respectively, could be introduced into *B. thuringiensis* using this technique at frequencies approximately 10^1 to 10^6 transformants/ug DNA (11, 12, 72). The electroporation frequencies of transforming plasmid pHV33, 7.3 kb, into *B. thuringiensis* HD1 was less than 50 transformants/ug DNA (72), electroporation of plasmid

pGKV2, 4.6 kb into *B. thuringiensis* subsp. *israelensis* strain 4Q272 was found to be only 10^2 transformants/ug DNA (11). Also plasmid pTV1, ~12.4 kb could not be shown to transfer into *B. thuringiensis* subsp. *israelensis* by electrophoration technique (12). Furthermore, even small plasmids, pUB110 (4.5 kb) and pMK4 (5.6 kb), could not be electroporated into every subspecies of *B. thuringiensis* such as *morrisoni*, *aizawai*, and *kurstaki* (12). These evidences demonstrated that there were limitations for using electroporation as a tool for introduction of DNA into *B. thuringiensis* subsp. *israelensis* particularly involving large plasmids.

1.2.2 Transduction in *B. thuringiensis*

Some generalized transducing phages specific for *B. thuringiensis* have been isolated (73-84) as shown in Table 2. However, the studies emphasized their morphology and specificity or their application for serving as a taxonomic tool by bacteriophage typing (83, 84). Some of these transducing phages could transfer chromosomal genes in certain subspecies of *B. thuringiensis* (76). The J7W-1 (77) and TP-21 (80) were found as prophages in plasmid DNA of *B. thuringiensis* subsp. *sotto* and *kurstaki* respectively. The information related to these generalized transducing phages is still limited; therefore, the potential use of them as a genetic manipulation tool will need further extensive studies.

Table 2. Transducing phages in various *B. thuringiensis* subspecies.

Transducing phage	Subspecies of *B. thuringiensis*	Reference
phage 63	*gelechiae* (serotype 1)	73
phage 64	*gelechiae*	74
CP 54 Ber	*berliner* 1715 (serotype 1)	75
TP13, TP18	*aizawai* (serotype 7)	76
J7W-1	*sotto* (AF101) (serotype 4a4b)	77
TG-2	*galleriae*	78
TP-21	*kurstaki*-HD1	80
GT-8	*subtoxicus*	81
phage I, II	*finitimus*	84
phage II, III	*morrisoni*	84

1.2.3 Conjugation-like process

Despite the existence of a tremendous amount of information on the mode of gene transfer via conjugation transfer in gram negative bacteria, there have been few reports on the similar process in gram positive bacteria. In gram positive bacteria, there has been no evidence of sex pili as in gram negative bacteria. However, the gene transfer via cell to cell contact has been reported. In 1974, Clewell et al. (85) discovered that the streptococcal plasmid pAMB1 could transfer erythromycin and lincomycin resistance markers from *Streptococcus faecalis* to *B. subtilis* by filter membranes mating. Later, in 1981, Landman et al. (86) found that *B. subtilis* could transfer pAMB1 and chromosomal markers to an auxotrophic *B. subtilis* recipient. However, in 1982, Landman et al. (87) also reported that mating could occur in the absence of pAMB1. Therefore, they reported that mating mechanism seem to be a form of intact cell fusion and quite different from the F-mediated conjugation system of *E. coli*.

Recently, there were several reports on the evidence of gene transfer in gram positive bacteria via conjugation-like process for example, in *B. thuringiensis, B. cereus, B. anthracis, B. megaterium* and *Lactobacillus sp.* (14, 16, 17, 19, 88).

2. Factors involved in conjugation and conjugation-like gene transfer process.

It was well documented that F plasmid, a conjugative plasmid, possesses the ability to transfer itself, other plasmids and some part of chromosomes between strains of *E. coli* by conjugation. However, in gram negative cocci, such as neisseria, or in gram positive bacteria especially in *B. thuringiensis*, the factors involved in this mode of gene transfer process are not clear.

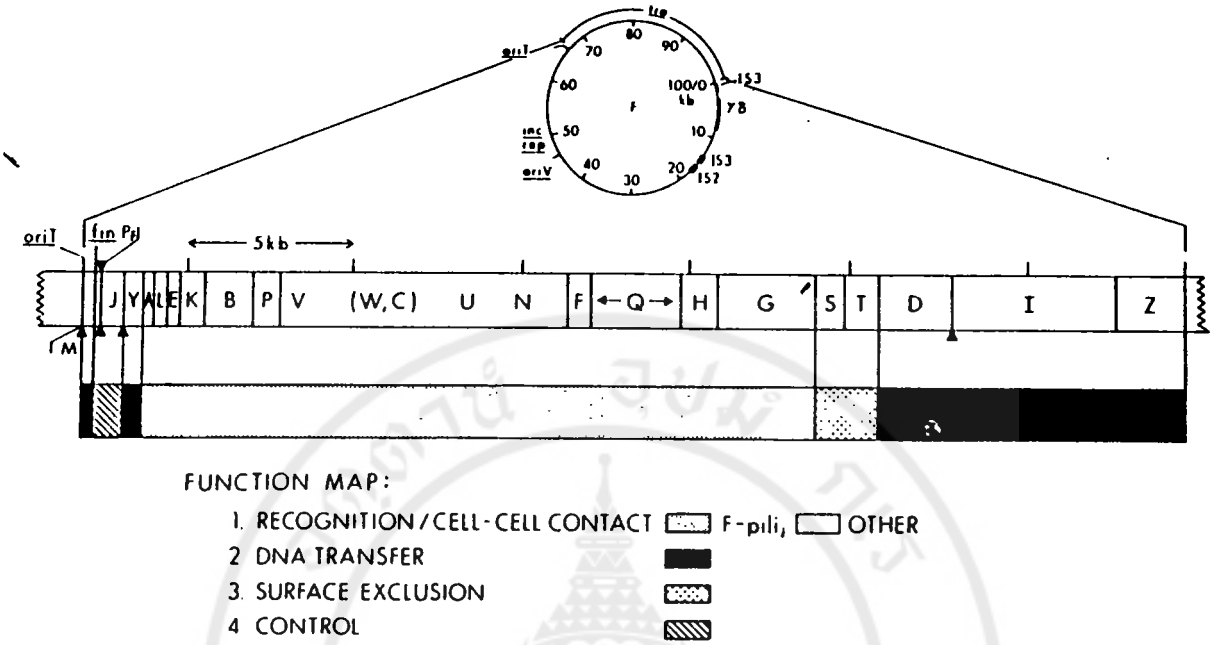
2.1 Conjugation gene transfer in *E. coli*

Conjugation is a natural means of gene transfer process. It seems to be clear that conjugation in *E. coli* requires the following:

a) Two mating types, donors (males) harboring F plasmid (F^+) and recipients (females) which are lacking the F factor (F^-).

b) Cell to cell contact via F-pilus, long (up to 20 um), thin (8 nm in diameter) and tubular structure which extends from the bacterial cell surface.

F plasmids (Fig. 1) possess *tra* region comprising 21 genes code for proteins which are essential for transferring copies of F by conjugation, and also four insertion sequences are known to be involved in the conjugal transfer of genes from the bacterial chromosome. Sex pili are coded by F plasmid, and play an important role in conjugation. Cells containing conjugative



FUNCTION MAP:

1. RECOGNITION / CELL-CELL CONTACT F-pili, OTHER
2. DNA TRANSFER
3. SURFACE EXCLUSION
4. CONTROL

Fig. 1 Genetic map of the F plasmid DNA transfer (*tra*) region. The upper part of the figure shows the F plasmid, approximately 100 kb. The position and extent of the *tra* region are indicated in relation to the different insertion sequence (IS2, IS3 and gamma-delta) and to the region containing the origins of vegetative replication (*ori V*), and other functions required for replication (*rep*) and incompatibility (*inc*). The role of each of the tra proteins in the overall process of conjugal DNA transfer is indicated in the map.

plasmids are unable to transfer DNA if they fail to produce sex pili either because of a mutation in a *tra* gene or because of repression of the *tra* operon. Attachment of an antibody to sex pili also inhibits conjugation. F plasmid, occasionally, integrated into the chromosome (Hfr), and mobilized the chromosome so that it could be transferred during cell-to-cell contact. The F plasmids may be excised from the chromosome while incoporating chromosomal genes. Such F plasmids containing chromosomal genes (F′) can be transferred at high frequency to recipients.

Conjugation process in *E. coli* can be divided into four major steps (89):

2.1.1 Initiation of DNA transfer.

It was found that DNA transfer and conjugative DNA synthesis are initiated at a specific site on the plasmid called *Ori T* (origin of transfer) in response to an unidentified signal generated by mating pair formation.

2.1.2 Strand separation and single strand transfer

After nicking at *Ori T*, the two strands of a plasmid must be unwound to allow transmission of a single strand to the recipient cell. The strand transferred with the 5′ terminus leading into recipient cell (90).

2.1.3 Conjugative DNA synthesis.

Transfer of a single strand of plasmid DNA is normally associated with synthesis of a replacement

strand in the donor cell and of a complementary strand in the recipient via a process of rolling circle replication.

2.1.4 Circularization of transferred DNA

The rolling-circle model for DNA transfer (91) hypothesized transfer linear single stranded DNA of longer than a unit length. The model raised the possibility that circularization occurs in the recipient cell by recombination between homologous DNA regions.

2.2 Conjugation gene transfer between *Neisseria spp*.

Little is known concerning the mechanism involved in cell contact between the donor and recipient during conjugation in *Neisseria gonorrhoeae*. In *N. gonorrhoeae*, a plasmid-encoded pilus has not been identified, and gene transfer only occurs when cells are on a solid surface (92). Genco and Clark (93) have found that the formation of stable mating pairs during conjugation in *N. gonorrhoeae* and *N. cinerea* appears to require the presence of a specific conjugal receptor that consists of both lipopolysaccharide (LPS) and outer-membrane protein because the purified LPS from *N. cinerea* Con^+ and Con^- strains inhibited conjugation when added to the mixture (93).

2.3 Conjugation gene transfer in *Streptococcus faecalis*

Streptococcus faecalis does not possess any sex pili, therefore, conjugation does not depend on sex

pili as in *E. coli*. The donor cells harbor conjugative plasmid(s) that produce a protein adhesion on the surface that cause aggregation with recipients cells. In addition, the recipients cells excrete small peptide sex pheromone that each elicit a mating response in donor cells carrying the corresponding conjugative plasmid.

2.3.1 Sex pheromones and pheromone inhibitors

A plasmidless strain of *S. faecalis* excretes multiple peptide pheromones (94) which are specific for different conjugative plasmids of *S. faecalis*. When a recipient acquires such a specific plasmid such as pAD1 (Fig. 2), it shuts down the production of the related pheromone, cAD1. However, the recipient continues to excrete pheromones specific for other plasmids; therefore, different pheromones are known to be produced by single types of cells.

Plasmid containing cells excrete a unique peptide that behaves as a competitive inhibitor of a certain pheromone (95), such as iAD1 is inhibitor of cAD1, and there is evidence that iAD1 is encoded by the plasmid pAD1 (96).

Various pheromones and some related inhibitor peptides have been purified and characterized by Suzuki and co-workers (97), (Table 3). The pheromones are hydrophobic octa- or hepta- peptides, containing at least one hydroxyamino acid residue which could serve as a modification site for the shutdown of endogenous pheromone

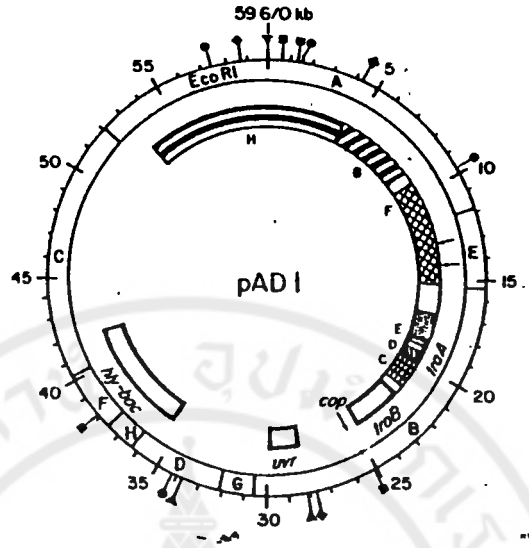


Fig. 2 Physical and functional map of pAD1. Regions important for transfer are *traA*, *traB* and *C* through *H*.

Table 3. Amino acid sequences of some sex pheromones and related inhibitors

Pheromone or inhibitor	MW (Da)	peptide	Reference
cPD1	912	H-Phe-Leu-Val-Met-Phe-Leu-Ser-Gly-OH	97
cADI	818	H-Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly-OH	100
cAM373	733	H-Ala-Ile-Phe-Ile-Leu-Ala-Ser-OH	101
eCF10	789	H-Leu-Val-Thr-Leu-Val-Phe-Val-OH	102
iPD1	828	H-Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser-OH	103
iAD1	846	H-Leu-Phe-Val-Val-Thr-Leu-Val-Gly-OH	104

activity when the corresponding plasmid is present (98). Deletion of one amino acid from the N-terminal or C-terminal residue of pheromone, i.e. cPD1, resulted in reduced activity or diminished its activity completely (99). Such evidences imply means that the full length of peptide of pheromone is important to its activity, and the specificity was determined by N-terminal sequence (99).

2.3.2 Aggregation between donor and recipient in *S. faecalis*

The pheromone responsed donor cells synthesize a proteinaceous substance(s) that appears on the cell surface (105) and facilitates the formation of mating aggregates on random collision with recipient bacteria. This induced substance or aggregation substance (AS), appears as a layer of hairlike structures (106, 107) by using scanning electron microscopy. The aggregation substances are proteins ranging from 74,000 to 157,000 Da (108-111).

Aggregation of donors and recipients resulted from interaction between "binding substance" (BS) which is present on the recipient surface and "aggregation substance" (AS) on donor (94). The binding substance is composed of lipoteichoic acid (LTA) because low concentrations of added LTA are able to inhibit the aggregation (94). In addition, the antiserum raised against the inducible pPD1 was able to inhibit aggregation of induced pPD1-containing cells (98).

2.3.3 Plasmid transfer

When donors are exposed to pheromone for 60 min and then mated with recipients for 10 to 15 min, the transfer occurred only in the direction from induced to uninduced cells (112). Interestingly, when both two types of cells were induced, transfer occurred in both directions but at frequencies significantly reduced as in the case where transfer was from an induced to uninduced strain (113). Therefore, entry (surface) exclusion also appears to be induced by pheromone and its activity was specific in entrance of a certain plasmid. Thus, when the cells were exposed to only cPD1 prior to mating, only the pPD1 derivative transferred; on the other hand, when the cells were induced with cAD1 only, the pAD1 derivative was transferred (111).

The transposon mutagenesis was used to analyse the pheromone-responding plasmid pAD1 and pCF10 (114, 115). It was indicated that insertions in regions D, E, F, G, and H, of pAD1 greatly reduced or eliminated the ability of pAD1 to transfer in broth; insertions in region H allowed cells to undergo inducible aggregation, but plasmid transfer was not detectable in broth. Insertions in the G and H region, inducible surface proteins could be detected. Region F insertion cells did not undergo induced aggregation even though AD130 and AD153 were produced, but AD74 and AD157 were missing but DNA transfer still occurred. Ike and Clewell (115),

Ehrenfeld and Clewell (116), Weaver and Clewell (117) have proposed that products encoded by *tra A* and *tra B* act to negatively control the pheromone response.

In addition, E-region inserts block all aspects of the pheromone response (116), and the E region may contain the beginning of an operon.

2.4 Conjugation-like gene transfer in *B. thuringiensis*

In 1982, Gonzalez et al. (14) discovered effective plasmid transfer system among strains of *B. thuringiensis* and *B. cereus* via cell mating. This mode of gene transfer has been named a conjugation-like process. The plasmid transfer was deoxyribonuclease resistant and required cell to cell contact (118). In 1983, Klier et al. (15) reported the heterospecific mating between *B. subtilis* and *B. thuringiensis* strain and the transfer of the cloned crystal genes of plasmid and chromosomal origins from *B. thuringiensis* subsp. *berliner*. The toxin gene of subsp. *berliner* was transferred via conjugation-like process to an acrystalliferous *B. thuringiensis* mutant of subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis*. The transcipient cells were found to express the crystal gene from *B. thuringiensis* subsp. *berliner* at a high level. Klier et al. (15) also found that when the crystal gene of the chromosomal origin was transferred into the recipient, an acrystalliferous *B. thuringiensis* subsp. *kurstaki*, the toxin gene might be integrated into

the chromosome and did not express which suggested that transcription or translation of the chromosomal copy might be repressed.

Later, Lereclus et al. (38) reported the intergeneric mating experiments and showed that transfer of the pAMB1 plasmid from *S. faecalis* to *B. thuringiensis* occurred under conditions in agreement with a conjugation-like system. They demonstrated that obvious differences between these two gram positive bacteria were the different frequencies of transfer that depended on the *B. thuringiensis* strain used as recipient.

In 1984, Gonzalez and Carlton (43) reported that the 75-MDa plasmid implicated in *B. thuringiensis* subsp. *israelensis* (*B.t.i.*) toxin production by curing was found to be transferred efficiently into a plasmid-free, Cry^- *B.t.i.* recipient strain, converting it to Cry^+. A Spo^+ Cry^+ *B.t.i.* strain was generated that carried the 75-MDa plasmid alone, indicating that this plasmid was both necessary and sufficient for toxin production. Battisti et al. (17), and Reddy et al. (19) demonstrated the transfer of plasmids by mating from *B. thuringiensis* subspecies to *B. anthracis* and *B. cereus*. Transfer of the selectable tetracycline resistant plasmid pBC16 and other plasmids occurred during mixed incubation in broth. Reddy, Battisti and Thorne (19) believed that plasmids, pXO11 and pXO12, found in *B. thuringiensis* were responsible for plasmid mobilization, because *B. anthracis*

and *B. cereus* transcipients inheriting either pXO11 or pXO12 became effective donors.

In 1986, Loprasert et al. (16) demonstrated that the plasmid pC194 from *B. megaterium* 0016, (formerly reported as *B. thuringiensis* 0016) and pBC16 from *B. cereus* could be transferred to *B.t.i.* by using the conjugation-like process.

In 1987, Koehler and Thorne (18) demonstrated that the plasmid pLS20 of *B. subtilis* (natto) promoted transfer of the pBC16 from *B. subtilis* (natto) to the *Bacillus* spp., *B. anthracis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis* and *B. thuringiensis* by using conjugation-like process.

Chapman and Carlton (118) also demonstrated that a Cry^+ streptomycin sensitive donor strain of *B. thuringiensis* could be transferred to a cry^- streptomycin-resistant recipient by the mating technique in nutrient broth. The transfer 75 MDa plasmid was inhibited by streptomycin (100 ug/ml), nalidixic acid (50 ug/ml) and rifampicin (12 ug/ml) but resistant to DNase. The authors concluded that the plasmid transfer in *B. thuringiensis* fitted the classical definition of conjugation because their experiments indicated that protein, RNA and DNA synthesis in donor were required and also DNA synthesis in recipient was also required.

3. S-layer protein

Many strains of eubacteria and archaebacteria possess regular arrays of subunits as the outermost component of their cell envelopes named "surface layer protein" (S-layer protein) as shown in Table 4. S-layers composed of protein or glycoprotein subunits have the ability to assemble into two dimensional arrays both in the presence and absence of surfaces suitable for adhesion (119). Being located at the cell surface, S-layers may be directly involved in the interactions between the cell and its environment; therefore, various functions of S-layer proteins have been proposed and as detailed below. However, more information will be needed to support and clarify these proposals.

3.1 Location of S-layer protein

S-layer protein is located on the outermost part of the bacteria, but its attachment sites on bacterial cell wall are different depending on the type of bacterial cells. The S-layer protein can be categorized into 3 types depending on cell wall structure.

In archaebacteria which have no peptidoglycan layer possess only a two-layered envelope structure. The S-layer protein is closely associated with the cytoplasmic membrane (119).

Gram-positive bacteria have a rigid cell wall composed of very thick peptidoglycan, whereas S-layers are located on the external part of peptidoglycan (119) and is

Table 4. Characteristics of S-layer protein of various gram-positive bacteria.

Organism	Lattice*	MW	Ion requirement for assembly	% CHO	Reference(s)
Acetobacterium woodii, strain WB1	O	-			119
Bacillus alvei, strain 183	H	-			119
B. anthracis	H	-			119
B. brevis 47	H	150, 130	Mg^{2+}	ND	166
B. cereus ATCC 4342	S	-			184
B. fastidiosus	S	-			119
B. macroides	S	-			130
B. megaterium	S	-			119
B. polymyxa NCIB4747	S	-			185
B. psychrophilus, strain W16A	S	-			130
B. schlegelii DSM 2000	S	-			131
B. sphaericus, NTCC 9602	S	142		0.4	163
" " , 9602 (variant)	S	120			163
" " , P-1	S	140			164
Bacillus sp., CIP 76-111	H	255			132
B. stearothermophilus (29 strains)	H,S,O	80-170		+	119, 140, 141, 147
B. subtilis	S	-			119
B. thuringiensis 4045	-	92			119
Clostridium aceticum, DSM 1496	S	-			119
C. botulinum type A 190L	P	195			119
C. formioaceticum DSM 912	S	-			119
C. lentoputrescens ATCC 17791	O	-			119
C. novyi	P	-			119
C. polysaccharolyticum	P	-			133
C. sporogenes	S	-			119

Table 4 (Cont'd)

Organism	Lattice*	MW	Ion requirement for assembly	% CHO	Reference(s)
C. tetani	P	-			119
C. thermoautotrophicum	S	-			134
C. thermohydrosulfuricum, L111-69	H	140,		9	199
C. thermosaccharolyticum, D120-70	S	140		3	199
Corynebacterium diphtheriae, C4	S	-			119
Deinococcus radiodurans strain Sark	-	115		1	209
Lactobacillus acidiophilus, ATCC 4357	P	43			119
L. brevis, ATCC 8287	O	51			157
L. buchneri ATCC 4005	H	55			135
L. casei	H	-			136
L. fermenti NCTC 7230	O	52			119
L. helveticus ATCC 10797	P	51			119
Sporosarcina ureae, ATCC 13881	S	150	Mg^{2+}		161
Thermoanaerobacter ethanolicus	H	-			119

* O = oblique

H = Hexagonal

P = Periodic structures but not further characterized

S = Square

\+ = containing carbohydrate but the percentage did not reported

attached to outer membrane in gram-negative (119), as shown in Fig. 3. Several organisms have been observed to possess the presence of double S-layers composed of identical or different subunit species such as *B. brevis*, *Bacillus* sp. strain KL-1 and *Pyrobaculum organotrophum* which are composed of double layers of periodic S-layer structure of the outermost layer and the inner most layer as well (120, 121).

3.2 Ultrastructure of S-layer protein

S-layers have been identified on the surface of cells as a regular arrangement of subunits by electron-microscopic technique. By application of the freeze-fracture technique, it has been confirmed that S-layers completely cover the cell surface (122). S-layers with oblique (p2), square (p4), and hexagonal (p6) symmetry have been identified (Figs. 4-6). The morphological units consist of two, four or six monomers, respectively, and exhibit center-to-center spacings in the range of 5 to 32 nm (123). However, in bacilli the lattices are uniformly aligned over the cylindrical part of the cell but exhibit numerous faults and random orientation at the cell poles and septation sites (124, 125), because S-layers cannot cover spherical surfaces by simple bending (126-128). Taylor et al. (128) proposed that, in S-layers with hexagonal symmetry, closed containers may be generated by introducing pentagons in the place of hexagon.

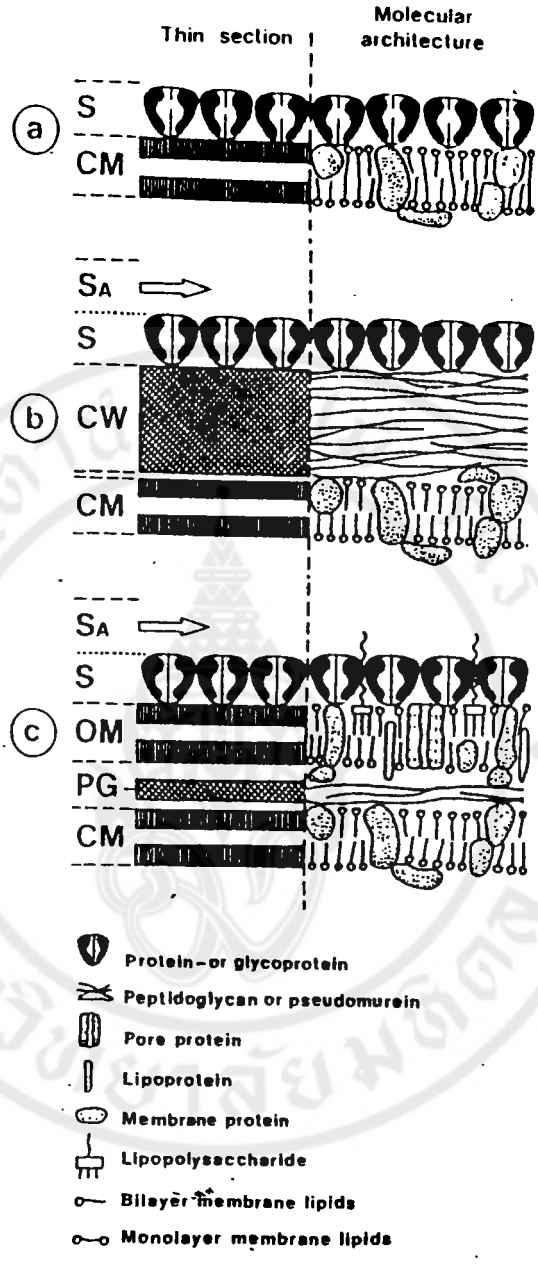


Fig. 3 Schematic drawing of the three categories of bacterial cell envelope containing S-layers.

(a) cell envelope of archaebacteria,

(b) cell envelope of gram-positive bacteria,

(c) cell envelope of gram-negative bacteria.

CM: cytoplasmic membrane; CW: cell wall;

OM: outer membrane; PG: peptidoglycan;

S: S-layer; SA: indicates the possibility of additional S-layer.

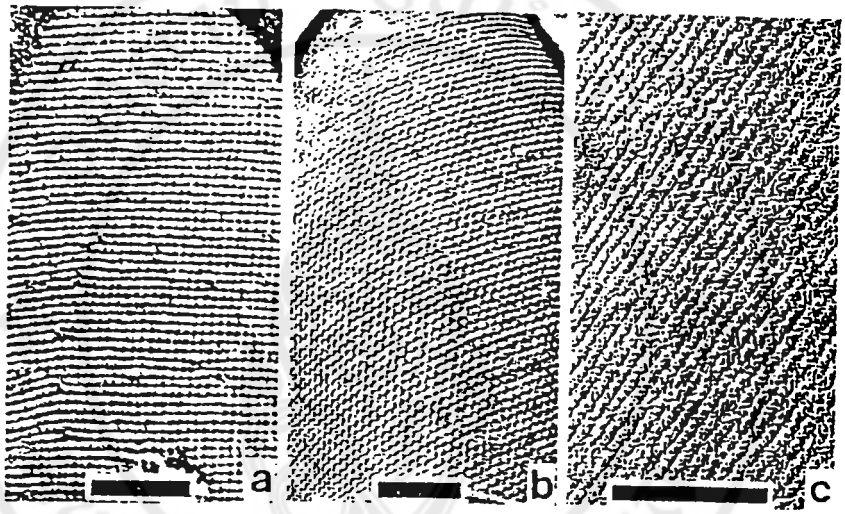


Fig. 4 Electron micrographys of replicas of freeze-etched preparation of S-layer proteins from various microorganisms: (a) *Desulfotomaculum nigrificans* (square), (b) *Clostridium thermohydrosulfuricam* (hexagonal), and (c) *Bacillus stearothermophilus* (oblique).

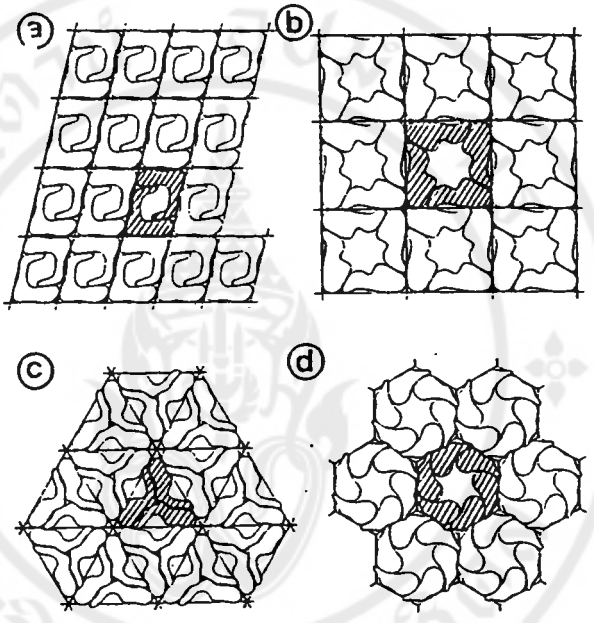


Fig. 5 Schematic drawing of the three types of S-layer proteins observed on bacteria: (a) oblique (P2), (b) square (P4), (c) hexagonal (P3), and (d) hexagonal (P6).

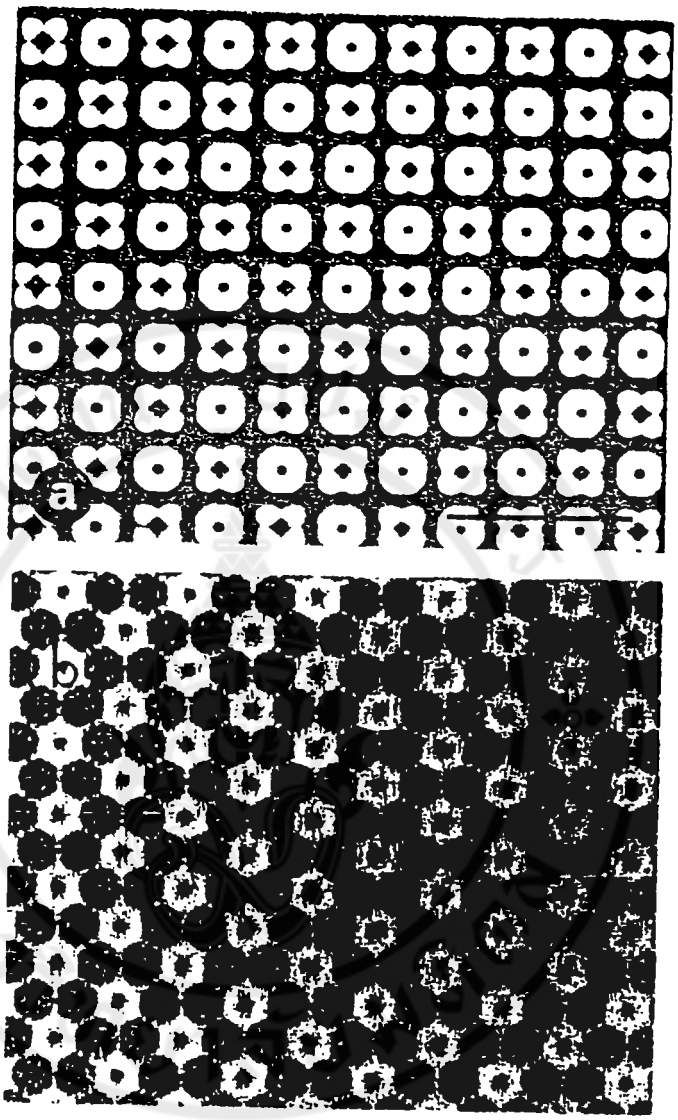


Fig. 6 Computer image of S-layer protein (a) square, and (b) hexagonal.

The data available showed that most S-layers of archaebacteria have hexagonal symmetry; in eubacteria, square or oblique lattices are frequently observed (123). Due to their crystalline nature, S-layers exhibit uniform pore morphologies, and individual lattices can display more than one pore size, which is estimated to be between 2 to 6 nm (123, 129).

3.3 Chemical composition of S-layer

The amino acid compositions reported for S-layers of various thermophilic and mesophilic bacteria are similar interm of the percentage of acidic or basic, polar or non-polar, and hydrophobicity factor (Table 5). Most S-layers contain a high proportion of acidic and hydrophobic amino acids and sulfur-containing amino acids are low (119). However, the S-layer protein of the thermophilic and acidophilic, *Sulfolobus acidocaldarius*, possesses low amounts of both acidic and basic amino acids but high levels of hydroxyl-containing amino acids (137). Therefore, the protein is resistant to detergents and high temperature (138).

The S-layer protein of methanogen *Methanospirillum hungatei* shows unusual resistance to a wide range of detergents, chaotropic agents, oxidizers, proteases, and glycosidases (139). However, even the molecular weight of the monomer protein is unknown.

Some S-layer proteins are glycosylated, and chemical analysis on S-layers of *B. stearothermophilus*,

Table 5. Characteristics including molecular weight and amino acid composition of S-layer protein from various thermophilic and mesophilic bacteria.

Organism	Growth temp (°C)	MW (kDa)	Amino acid (% residue) Acidic	Basic	[OH][1]	non-polar	[S][2]	Ho[3]	Reference
A. salmonicida A450	25	49	21.7	9.8	13.0	55.3	0.1	1007	213
A. salmonicida V75/93	27	54	20.5	13.1	16.2	50.2	0	981	188
A. radiodurans, Sark	30	115	25.3	9.6	18.5	45.5	1.1	663	209
D. radiodurans, R1	30	104	24.9	6.3	23.8	43.7	1.3	848	172
Acinetobacter sp. MJT/F5/199A	30	65	26.5	7.5	12.2	53.8	0	668	171
A. serpens VHA	37	140	20.1	4.8	20.3	54.7	0.2	927	168
C. fetus 23D	37	98	19.6	7.4	18.3	53.5	1.2	943	159
S. ureae ATCC 13881	30	150	23.3	11.8	14.1	50.0	0.8	925	161
B. sphaericus P1	37	140	21.1	8.0	22.1	48.6	0.1	973	164
H. halobium	30	-	27.9	7.4	21.0	42.8	0.9	790	214
H. salinarium ATCC 19700	37	200	33.2	4.2	22.6	39.1	0.8	763	174
B. stearothermophilus 3a/NRS 2004	60	94-165	19.4	13.1	18.9	48.5	0.1	1070	149
B. stearothermophilus 3c/NRS 1536	60	112	22.1	12.7	15.5	49.7	0	1022	119
B. stearothermophilus PV72	60	122	21.4	13.2	16.0	49.4	0	1010	119
B. stearothermophilus E4-65	60	100	20.8	13.4	17.6	48.3	0	1077	119
D. nigrificans NCIB 8395	60	85-130	21.1	14.1	12.6	52.3	0	1061	141
D. nigrificans NCIB 8706	60	94	20.1	12.1	17.1	50.2	0.6	1060	141
D. nigrificans B200-71	60	85	22.4	10.1	19.3	47.6	0.6	1091	141
D. nigrificans T206-71	60	130	21.7	10.4	16.9	50.2	0.8	1065	141
C. thermosaccharolyticum D120-70	62	140	24.8	8.4	18.0	48.8	0	956	199
C. thermohydrosulfuricum L111-69	70	140	20.3	9.9	16.9	51.9	1.0	1092	199
B. thuringiensis	30	91.4	18.6	10.8	ND	21.2	ND	ND	157

[1][OH] = Hydroxyl group-containing amino acids

[2][S] = Sulfur-containing amino acid

[3]Ho = Hydrophobicity factor

Desulfotomaculum nigrificans and *C. thermohydrosulfuricum* has shown that the proteins are glycosylated (140, 141). The glycoprotein of *Halobacterium halobium* contains both O-glycosidic and N-glycosidic linkage glycans (142-144). One of the glycans, containing galactose, galacturonic acid, N-acetylglucosamine and 3-O-methylgalacturonic acid, is linked to the protein via asparaginyl-N-acetylgalactosamine (145). Another glycan, composed of glucuronic acid-containing oligosaccharide units is attached to the protein via asparaginylglucose (144). The S-layer protein of *H. salinarum* possesses non-sulphated glycan (146).

The S-layer of *B. stearothermophilus* NRS 2004/3a possesses two types of glycan chains: one chain contains trisaccharide repeating units of rhamnose linked to an asparagine residue of protein (147), and other chain is composed of tetrasaccharide repeating units of glucose, N-acetylglucosamine, and di-acetyl mannuronic acid (148). *C. thermohydrosulfuricam* S-layer is composed of disaccharide repeating units of rhamnose and mannose (149).

3.4 Dynamics of S-layer protein

A diversity of S-layers within the same species and/or the absence or presence of S-layers has been reported for some bacteria, i.e. *P. acidovorans* (150), *A. serpens* (Koval), *Acenitobacter* spp. (151). *B. aneurinolyticus* (152), *Lactobacillus* spp. (153), *B. sphaericus* (154, 155) and *Sulfolobus* spp. (156).

The amount of S-layer protein from *B. thuringiensis* varied depending on the age of the culture (157). Using electron microscopy of early exponential phase cells revealed only patches of S-layer. As the culture matured to mid-exponential, the cells became completely coated, and at the stationary phase, so much S-layer was produced that it was beginning to slough-off. The S-layer proteins of *B. brevis* 47, both middle wall protein and outer wall protein, are shed from the bacterial surface at the stationary phase (158). In addition, Luckevich and Beveridge (157) reported that on repeated subculturing of *B. thuringiensis* 4045 in liquid medium, the yield of protein was significantly reduced, but subculture back to solid medium renewed production of S-layer.

Comparing the first 10 to 30 amino acids from N-terminal of various S-layer proteins from *B. thuringiensis* 4045, *A. salmonicida, A. hydrophila* TF7, *B. brevis* 47, *D. radiodurans* Sark, *H. Halobium, C. fetus* VC119, there appeared to be no homology in the sequence of these amino acids (157).

3.5 Isolation of S-layer protein

A variety of bacterial S-layers has shown that most are composed of a single protein species, sometimes covalently linked to carbohydrate chain such as S-layer of *B. stearothermophilus, Desulfotomaculum nigrificans* and *C. thermohydrosulfuricum* (140, 141, 146, 159). Sodium

dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) of various bacterial S-layer proteins demonstrated that the molecular masses of the monomers vary from 40,000 to 220,000 Da with a predominant number greater than 100,000 Da (119). Thus, for many bacteria, the S-layer protein ranks with the largest proteins of the cells (138). The subunits of most S-layers interact with each other and with the underlying cell envelop components through noncovalent forces, including hydrogen bonding, ionic bonding and hydrophobic interactions (63). In addition, some S-layers require monovalent or bivalent cations for structural integrity (119).

Common procedures for solubilization of S-layer protein involve treatment with chaotropic agents, detergents or metal-chelating agents (123).

Some organisms shed excessive S-layer material that can be harvested from the growth medium (139). Most techniques for isolation and purification of S-layers involve the mechanical disintegration of cells, and differential centrifugation to separate the cell wall fragments (160). In gram-positive bacteria, the crude cell wall preparations are frequently treated with detergent (e.g. Triton X-100) to dissolve membrane contaminants (160). S-layer fragments are obtained either by digestion with lysozyme (160, 161), or by treatment with low concentration of chaotropic agents (e.g. 0.5 M urea, 1 to 2 M guanidine hydrochloride; both can loosen

the bonds to the supporting layer without dissociating the lattice (157, 162). High concentration of urea, guanidine hydrochloride, formamide and sodium dodecyl sulfate (SDS) (157, 163-166) and chelating agents can directly disintegrate S-layer subunits from cell walls. Low pH treatment of intact cell walls may cause a reversible denaturation of S-layer protein subunits without detaching them from the supporting layer (163, 167).

In gram-negative bacteria, S-layers can be removed from the outer membrane of isolated cell enveloped fragments by treatment with low concentrations of urea or guanidine hydrochloride (163, 167, 168), metal chetating agents such as EDTA, EGTA (123, 168-171), SDS (172).

In archaebacteria, S-layers attached to the membrane, can be removed by treatment with Triton-X-100 or SDS, change in pH and the ionic strength, or extraction with organic solvent (146, 173-175).

Cell wall of gram-positive bacteria contains less protein than gram-negative bacteria's, it is feasible to extract the S-layer protein from the cell wall of gram-positive bacteria without much contamination with other proteins. However, a few S-layers, particularly thermophilic archaebacteria, exhibit high stability to common denaturants, suggesting that most of the protein subunits are covalently linked (123) such as in *Thermoproteus* spp. (176), *Methanotrix concilii* (177) or *Methanospirillum hungatei* (178) and *Deinococcus radiodurans* (172).

Some investigators demonstrated that some pure S-layer preparations reveal more than one band on SDS-PAGE especially in thermophilic Bacillaceae (123). This can be attributed to (a) the presence of two, closely associated S-layers composed of different subunit species (166, 179); (b) one S-layer protein latice with stoichiometrical amounts of one or more associated protein species (180); (c) proteolytic cleavage of S-layer subunits *in vivo* or in the course of the S-layer isolation procedure (172, 181); and (d) the microheterogeneity of the carbohydrate residue(s) in S-layer glycoprotein subunits (182, 183).

3.6 Functions of S-layer protein

S-layers might have a broad spectrum of functions depending on the habitat of the bacteria. Several studies of S-layers still emphasized the structure, chemistry and assembly, but very few studies elucidated their biological roles. S-layers have proposed to have the following functions:

3.6.1 Molecular-sieve

There are some reports indicating that S-layer proteins provide the organisms with a selective advantage in their natural competitive habitat (123). However, some S-layers are frequently lost on prolonged cultivation under laboratory conditions. Because laboratory conditions made optimal for rapid growth, the S-layer deficient mutants outgrow the wild-type strain (123).

Due to the pore size between subunit of S-layer protein of eubacteria which have a pore diameter in the range of 2-3 nm,the pores allow the passage of small molecules such as nutrients and metabolic degradation products, while protecting from larger particles such as lysozyme and bacteriophages (184-186). However, some strains of *B. stearothermophilus* were sensitive to lysozyme, probably, because its pores diameter was approximately 4.5 nm. Interestingly, most S-layers *in situ* are highly resistant to proteases but become degradable upon removal of the supporting layer (123, 160).

3.6.2 Physical barriers

There were evidences indicating that *Aquaspirillum serpens, A. sinuosum*, and *Aeromonas salmonicida*, possess S-layer array external to the outer membrane and were resistant to the attachment and invasion by bacterial predator *Bdellovibrio bacteriovorus* (187). The role of S-layer of *A. salmonicida*, which causes a fish disease, is to provide the organisms with an external protective barrier against fish defence mechanisms such as the bactericidal activity of serum complement (188, 189) as well as S-layer on *Campylobacter fetus* (190, 191), a causative agent of infectious abortion in farm animals, which protected the cells from the host immune response.

In addition, S-layer ultrafiltration membrane (SUM) of *B. stearothermophilus* or *C.*

thermohydrosulfuricum which is prepared by deposition of the native fragments of S-layer suspended in distilled water, on the surface of smooth nucleation track membranes or on the surface of open-celled foam-like microfiltration membrane, were resistant to 0.1 N HCl, or 0.1 N NaOH for 170 hr at 20°C, resistant to organic solvents (i.e. methanol, ethanol, propanolol-1, propanol-2, butanol-1, n-butylacetate, methylenechloride, carbontetrachloride, acetone, dimethyl sulfoxide, dimethyl formamide, toluene and cetonitrile), and also resistant to high concentrations of chaotropic agents (0.5. M guanidine hydrochloride) for 72 hr at 20°C (122).

3.6.3 Morphogenetic functions

A morphogenetic function of S-layers seems to have been demonstrated only in archaebacteria (175), in which the S-layers are the only component of the cell wall. For instance, *S. acidocaldarius* (175, 192) and *Methanococcus* species (193), these porous, closed glycoprotein containers have sufficient strength to withstand changes in the osmotic pressure while allowing enough flexibility to form narrowly curved surfaces. *Halobacterium*, the S-layers provide no protection against conditions of low ionic strength (194, 195), but they determine the shape of the cell at high salt concentrations (196); therefore, a morphogenetic function of S-layers is particularly likely in view of the observation that the inhibition of the synthesis of a high

molecular weight saccharide covalently links to the S-layer protein cause a change in cell shapes from rods to spheres (182, 197).

3.6.4 Bacteriophage receptor

The S-layer of some bacteria contain glycoprotein such as *B. stearothermophilus* (149, 198) *C. thermohydrosulfuricam* (199), *B. sphaericus* (154), *H. fetus* (200); therefore, it is speculated that S-layer might act as cell receptor in some microorganisms. This potential role has been revealed by Howard and Tipper (164). There appeared that the wild type *B. sphaericus*, which possess tetragonally array of S-layer, was sensitive to phage M, but the mutant strain of *B. sphaericus*, which possess the lower molecular weight protein than the S-layer, was resistant to phage M (164).

3.6.5 DNA uptake

The S-layer of *Azotobacter vinelandii* has a role in competing for DNA uptake (201). The strains that do not produce the S-layer and the strains grown with S-layer protein present on the surface but not assembled into a periodic structure (calcium deficient media), are non-competitive. Bavoil et al. (202) found that one of the proteins in the *Chlamydia* S-layer is a porin, that played a role in the channel-forming activity.

According to 3-dimensional analysis of various S-layers in both structure and dimensions of the channel forming parts, Baumiester and Hegerl proposed that

S-layers might mediate cell-cell interactions by regular surface assemblies which allow temporary cell-cell contacts and communication, and named this phenomenon as "Connexon" (203).

Furthermore, they hypothesized that if connexons are formed *in vivo*, the biological function might be the transfer of genetic material in the conjugational process. Because the diameter of the channel seems sufficient for DNA to passthrough, and the tight fitting of the connexon would provide protection during DNA transfer. In addition, S-layers tend to be lost on prolonged cultivation in laboratory and agreed with the hypothesis of connexon-mediated gene transfer. Genetic variability conferred by exchange of genetic materials has no advantage under laboratory conditions.

3.7 Molecular cloning and sequencing of S-layer protein gene(s)

The morphological properties of S-layers have been extensively characterized for a wide range of microorganisms as previously described, whereas the genes for S-layer protein(s) have been isolated from only a few microorganisms, such as *B. brevis* 47 (204-207), *B. brevis* HPD31 (208), *D. radiodurans* (209, 210), *B. licheniformis* NM 105 (211), *C. cresentus* (212), *A. salmonicida* (213), *H. halobium* (214), *Acetogenium kivui* (215) and *Haloferax volcanii* (216). Various cloned S-layer protein genes have been shown in Table 6. The S-layer protein(s) gene(s) of

Table 6. List of various cloned bacterial S-layer protein genes.

Organism	Location	S-layer protein gene (Kb)	Protein (kDa) authentic	cloned	Reference
B.brevis 47 MWP	chr	3.0	115	115	204
OWP	chr	3.0	104	104	204
B. brevis HPD31 HWP	chr	3.9	135,150		183
B. licheniformis NM105	chr	4.5	98	50	186
B. salmonicida A450	chr	4.0	49	49	188
D. radiodurans sark	chr	2.95	100	98	184
A. kivui	chr	1.95	82	71	190
C. cresentus	chr	4.4	130,74,20	130	187
H. halobium	chr	3.1	200	86.5	189

these microorganisms can be expressed in *E. coli* under it own promoter or *lac Z* promoter of the cloning vectors (209).

The genes for two S-layer proteins, named the outer wall protein (OWP) and middle wall protein (MWP) of *B. brevis* 47 constitute a cotranscriptional unit and are transcribed from several tandem promoters located upstream of the MWP gene. The MWP gene contains two translation initiation sites (144). The complex structure of the 5 region of the cotranscription operon has been suggested to play an important role in differential regulation of the gene expression (144).

B. brevis HPD31, HPD52 and HPD33 contain one S-layer protein, designated HWP, and its structure is different from S-layer protein of *B. brevis* 47 (208). Immunological analysis indicated that HWP of *B. brevis* HPD31 is closely related to MWP of *B. brevis* 47. The HWP gene of *B. brevis* HPD31 was cloned and sequenced. The HWP and MWP genes showed highly homologous sequences (217). The 5 region of HWP was found to have five tandem promoters, two Shine-Dalgarno sequences, two translation initiation codons, TTG and ATG while TGA as stop codon (218). The N-terminal amino acid sequence of HWP of *B. brevis* HPD31 indicated that it is synthesized as precursor protein with a signal peptide of 53 or 23 amino acid residues (using first or second initiation codon) (217).

The S-layer protein gene of *B. licheniformis* NM105 was cloned in lamda phage EMBL3 and expressed in *E. coli* NM 539 infected with recombinant phage (153). However, the protein expressed in *E. coli* had a lower molecular mass (50 kDa) than the purified authentic S layer protein (98 kDa) (211). The gene was subcloned in *B. subtilis* MI112 by using the shuttle plasmid pMK4, which demonstrated that the gene encoded the 98-kDa protein (211).

Belland and Trust (213) have cloned S-layer protein genes of fish pathogen (named A protein), *A. salmonicida*, and found that it was a single copy on the chromosome and was conserved among a wide range of *A. salmonicida* strains. However, certain region of the gene has been shown to be variant resulted in different phenotypic characteristics. Some variation, the expression of the gene was found to result from genetic deletion at the N-terminal sequence (213).

The amino acid residues at N-terminal sequence of various S-layer protein is shown in Table 7.

Table 7. Amino acid sequences at the N-terminal region of S-layer proteins from various bacteria.

Organism	N-terminal sequence	Reference
B. thuringiensis 4045	?GKTFPDV?P	157
A. salmonicida A450	DVVIGPNDNTTNSLASVTKQLSF	151
A400	DVVISPNDNTTT?LASVTKQLSDFSTEQNT	213
A461	DVVIGPNDNTTT?LASVTKQLDF???QQNL	213
A. hydrophila TF7	VNLDTGAGVSFKASGIKVDGAAGTTLGGXA	157
B. brevis 47 MWP	AEEAATTTAPKMDADMEKTVKRLEALGLVA	142
OWP	APKDGIYIGGNIKKYYSYDVFEMTPQAKAT	146
D. radiodurans Sark	MKKNIALMALTGVLTLASCGQNGNTPTADT	148
H. halobium	ANASDLNDYQRFNENTNYTYSTASEDGKTE	147
C. fetus VC119	MISKSEVSELFIVLFGRP	159
H. volcanii	TKLKDQTRAILLATLMVTSVFAGAIAFTGS	153
A. kivui	KNLKKLIAVVSTFALVFSAMAVGFAATTPF	152

CHAPTER III

MATERIALS AND METHODS

1. Microorganisms and maintenance conditions

Bacterial strains and plasmids used in this study are listed in Table 8. Most strains of various subspecies of *B. thuringiensis (B.t.)* were provided by Dr. H. De Barjac (WHO culture collection number as appeared in Table 8) through Prof. Somsak Pantuwatana (Department of Microbiology, Faculty of Science, Mahidol University). *Bacillus thuringiensis* subsp. *israelensis (B.t.i.)* strain 4Q272 originated from Bacillus Genetic Stock Center, Ohio, USA, was kindly given by Dr. Sakol Panyim (Department of Biochemistry, Faculty of Science, Mahidol University. *B.t.i.* strain 4Q2, and c4Q272 were given by Dr. D.H. Dean (Bacillus Genetic Stock Center, Ohio, USA).

Spontaneous rifampicin resistant (Rif^r), streptomycin resistant (Str^r) and nalidixic acid resistant (Nal^r) mutants were isolated by growing appropriate strains in 20 ml LB-broth at 30°C with continuous shaking until reaching exponential phase. One-tenth milliliter of cultures were spread on nutrient agar supplemented with rifampicin (50 ug/ml) or streptomycin (50 ug/ml) or nalidixic acid (40 ug/ml) respectively. The plates were incubated at 30°C overnight, the isolated colonies were restreaked on nutrient agar containing appropriate antibiotics, and incubated at 30°C overnight.

Table 8 Bacterial strains and their relevant properties.

Strain	Strain number[a]	Flagella serotype	Phenotype[c]	Remark
B.t.i. A084-16-194	TO14001	14	Cam^r (pC194) Tet^r (pBC16) Str^r	Ref.16
B.t. thuringiensis	TO1001	1	Pen^r Rif^r	This study[b]
B.t. finitimus	TO2001	2	Pen^r Rif^r	This study
B.t. finitimus	TO2001	2	Pen^r Str^r	This study
B.t. kurstaki	TO3A001	3a3b	Pen^r Rif^r	This study
B.t. dendrolimus	TO4A001	4a4b	Pen^r Rif^r	This study
B.t. sotto	TO4001	4a4b	Pen^r Rif^r	This study
B.t. sotto	TO4001	4a4b	Pen^r Str^r	This study
B.t. kenyae	TO4B001	4a4b	Pen^r Rif^r	This study
B.t. galleriae	TO5001	5a5b	Pen^r Rif^r	This study
B.t. entomocidus	TO6001	6	Pen^r Rif^r	This study
B.t. subtoxicus	TO6A001	6	Pen^r Rif^r	This study
B.t. ostrinae	TO8A001	8a8b	Pen^r Rif^r	This study
B.t. ostrinae	TO8A001	8a8b	Pen^r Str^r	This study
B.t. morrisoni	TO8001	8a8b	Pen^r Rif^r	This study
B.t. tolworthi	TO9001	9	Pen^r Rif^r	This study
B.t. caucasicus	T10007	10a	Pen^r Rif^r	This study
B.t. toumanoffi	T11001	11	Pen^r Rif^r	This study
B.t. toumanoffi	T11001	11	Pen^r Str^r	This study
B.t. kyushuensis	T11A001	11a11c	Pen^r Rif^r	This study
B.t. thompsoni	T12001	12	Pen^r Rif^r	This study
B.t. dakota	T15001	15	Pen^r Rif^r	This study
B.t. indiana	T16001	16	Pen^r Rif^r	This study
B.t. tohokuensis	T17001	17	Pen^r Rif^r	This study
B.t. kumamotoensis	T18001	18	Pen^r Rif^r	This study
B.t. tochigiensis	T19001	19	Pen^r Rif^r	This study
B.t. darmstadiensis	T10001	10	Pen^r Kan^r Rif^r	This study
B.t. pakistani	T13001	13	Pen^r Rif^r	This study
B.t. subage yunnanensis	T20001	20a20b	Pen^r Rif^r	This study
B.t. wuhanensis	TX1001	-	Pen^r Rif^r	This study
B.t.i. 4Q2		14	Pen^r	Ref. 46
B.t.i. 4Q2-Nal^r		14	Nal^r Pen^r	This study
B.t.i. 4Q2-16		14	Nal^r Pen^r Tet^r	This study
B.t.i. 4Q2-72		14	Pen^s	Ref. 46
B.t.i. 4Q2-72		14	Pen^s Rif^r	This study
B.t.i. c4Q272		14	Pen^s	Ref. 46
B.t.i. c4Q272		14	Pen^s Rif^r	This study
B.t.i. c4Q272-16		14	Pen^s Rif^r, Tet^r	This study
B. cereus GP7			Tet^r	Ref. 16
E. coli DH5α			Amp^r (pBluescriptKS)	Stratagene
E. coli DH5α			Amp^r (pBluescriptSK)	Stratagene
E. coli DH5α			Amp^r (pUC12)	Stock culture
E. coli DH5α			-	BRL

[a] Original strain number (IEBC No.) provided by WHO center through Prof. H. de Barjac.
[b] The strains were originally obtained from indicated sources and subsequently subjected to selection for Rif^r, Str^r Kan^r, Nal^r phenotypes.
[c] Antibiotic susceptibility was performed by Disk diffusion method (219).

A streptomycin resistant strain of *B.t.i.* A084-16-194 (16) harboring plasmids pBC16 from *B. cereus* GP7 and pC194 from *B. subtilis* HVS62 which conferred tetracycline resistance (Tet^r) and chloramphenicol resistance (Cam^r) respectively was used as donor strains unless indicated otherwise.

All *B.thuringiensis* cultures were maintained on nutrient agar slants and grown in LB-broth medium for the mating procedure.

Escherichia coli DH5α F' φ80d *lac*Z ΔM15 Δ(*lac* ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, M_k^+) *sup*E44 λ^- *thi*-1 *gyr*A *rel*A1 (BRL, USA) was used as host in DNA transformation experiment. Pure culture of *E. coli* was kept as stock cultures in 15% glycerol at -70°C.

Plasmid pBluescriptKS (-) and pBluescriptSK (-) (Stratagene, USA) as shown in Fig. 7 was kindly given by Dr. Skorn Mongkolsuk (Department of Biotechnology, Faculty of Science, Mahidol University) and Dr. T. Seki (International Center of Cooperative Research in Biotechnology, Faculty of Engineering, Osaka University, Japan) respectively. Plasmid pUC12 was kindly provided by Dr. Watanalai Panbangred (Department of Biotechnology, Faculty of Science, Mahidol University).

2. Chemicals and reagents

All microbiological media used in this study were obtained from Difco Lab. Detroit, USA. Agarose was

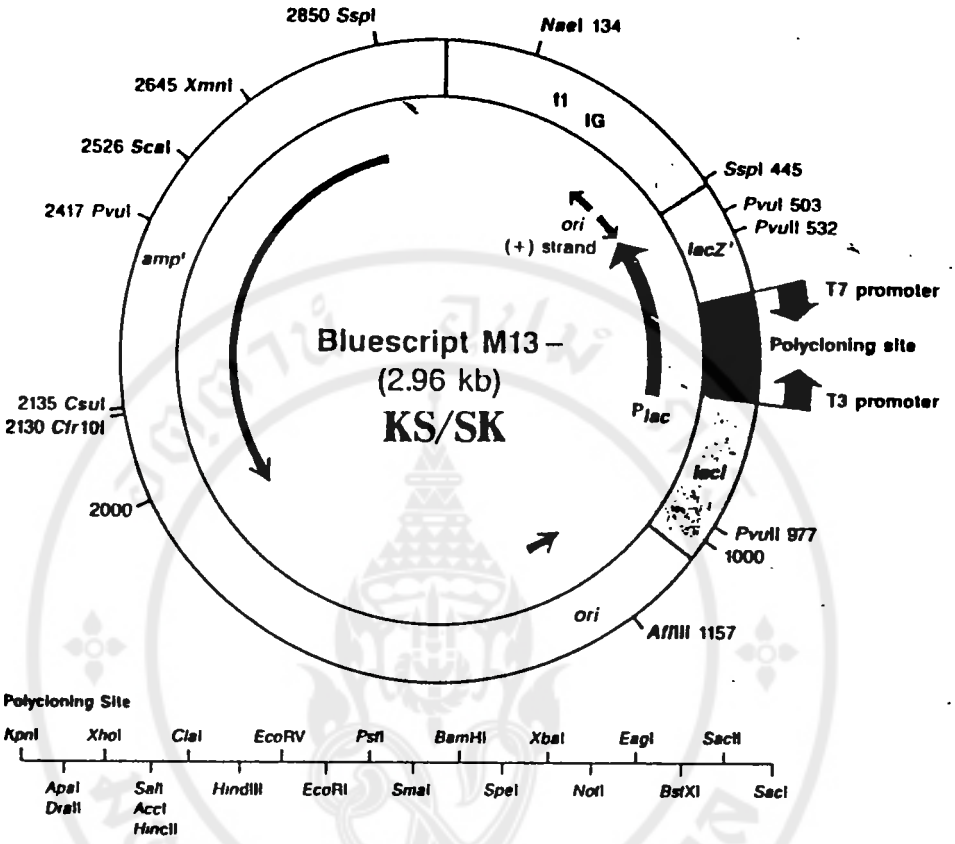


Fig. 7 Restriciton map of pBluescript KS/SK. The vector contains the bacteriophage M13 origin of DNA replication inserted in opposite orientations into a vector derived from a pUC plasmid that contains bacteriophage T3 and T7 promoters

In pBluescript SK (M13-), the *Sac*I site lies immediately downstream from the T3 promoter and the *Kpn*I site lies immediately downstream from the T7 promoter.

In pBluescript KS (M13-), the polycloningsite is in the opposite orientation.

Restriction and modifying enzymes were obtained either from BRL (USA), Toyobo (Japan), Takara (Japan), or Promega (USA). Antibiotics were obtained from Sigma Co., Ltd. (USA) and molecular weight markers were purchased from Sigma and Pharmacia (Sweden). Fluorescien isothiocyanate conjugated swine anti-rabbit immunoglobulins were obtained from Darkopatts (Denmark). Goat anti-rabbit IgG alkaline phosphatase conjugate was obtained from Sigma. ^{35}S-dATP was obtained from Amersham (USA). Sequenase kit ver. 2.0 was obtained from USB (USA). X-ray films were obtained from Kodak (USA). Nick translation kit and BluGENE kit were purchased from BRL. All other chemicals were obtained from Sigma Co., Ltd. (USA).

3. The procedure for conjugation-like gene transfer process

Donor and recipient cells were grown separately in 1.5x15 mm test tubes containing 4 ml of LB-broth. They were incubated at 37°C with shaking at 200 rpm for 14 hr. Then, each culture was separately transferred (1% inoculum) to a fresh batch of the same medium and further incubated under the same conditions for an additional 3 to 4 hr, to assure exponential growth. Mating mixtures were prepared by mixing 2 ml of donor cells with 2 ml of recipient cells in 1.5x10 mm test tubes containing 4 ml of LB-broth medium. Control tubes contained 4 ml of LB-broth and 2 ml of either donor or recipient cells.

For mating experiments, mixtures were incubated at 37°C with slow shaking for 8 hr unless indicated otherwise. Samples were removed and plated on appropriate selective media for determining the number of donors, recipients and transconjugants. Dilutions were made in 0.05 M phosphate buffer pH 7.0. Plates were incubated at 37°C, and colonies were scored after 24 to 48 hr. The frequency of transfer was calculated by dividing the number of transconjugants by the lesser number of the mating pair. All data for frequency of transfer reported were the average of the three independent experiments.

For studying the effect of various chemicals on conjugation-like gene transfer process, the donor and recipient cells were harvested by centrifugation at 6,500 rpm for 10 min at 4°C (Sorvall RC-5B, rotor SS34), the supernatants were discarded, the pellets were washed twice with 0.05 M phosphate buffer pH 7.0, and then, cells were resuspended in the same buffer to the original volume. The washed cells were used for mating experiments. Equal volumes of donor and recipient (2 ml, each) were mixed together, and then, added with a certain volume of stock solutions of various chemicals, i.e. 0.2 M EDTA, 4 M $MgCl_2$, 4 M $MgSO_4$, 0.1 M $CaCl_2$ to make the various final concentrations as shown in Tables 13 through 16. The mixtures were incubated at 30°C for 6 hr and then the numbers of donors, recipients and transconjugants were determined as described previously.

For demonstration of antibody inhibition experiment, the mating mixtures were prepared by using 200 ul of washed donor cells which were treated with 200 ul of anti-S-layer protein antibody in the 1.5 ml Eppendorf tube at room temperature for 2 hr. and then mixed with 200 ul of washed recipient cells. The mixtures were incubated at 30°C with continuous shaking at 180 rpm for 6 hr. Samples were removed and plated on nutrient agar containing appropriate antibiotics and the frequency of transfers were calculated as described above. The control experiments were performed accordingly except that either buffer or preimmunized rabbit serum was used instead of the anti-S-layer protein antibody.

4. Determination of penicillin susceptibility

Penicillin susceptibility of *B.t.i.* was performed by Kirby-Bauer disk diffusion method (219) and then the minimal inhibition concentration (MIC) was determined by broth dilutions technique (220).

4.1 Disk diffusion method

Overnight cultures of *B.t.i.* strain 4Q2, 4Q272 and c4Q272 were subcultured in Mueller Hinton medium (Difco), incubated at 37°C for 3 hr. The cultures were diluted to obtain the cells density of 10^3 to 10^4 cells/ml. The cultures were spread on Mueller Hinton agar plate with a sterile cotton swab, the penicillin G disks (10 units/disk, Difco) were placed on culture

plates, and incubated at 37°C overnight. The zone of inhibition was examined and measured.

4.2 Broth dilution method

Various concentrations of penicillin G were prepared by serial dilution in 0.05 M phosphate buffer (100,000 ug/ml to 0.004 ug/ml). The culture preparations of *B.t.i.*, as previously described, were inoculated into the serial dilution of penicillin G to give the final dilution 50,000 ug/ml to 0.002 ug/ml. The mixtures were incubated at 37°C overnight, the growth of cultures were indicated by its turbidity, and the MIC of penicillin to each strain was recorded.

5. Determination of clumping

Different strains of *B.t.i.* were separately grown in either LB-broth, brain heart infusion broth, nutrient broth or tryptic soy broth in the same conditions as described for the conjugation-like procedure except that either normal saline or phosphate buffer was added.

When washed cell suspensions were employed, the cultures of *B.t.i.* were also centrifuged at 6,500 rpm for 10 min at 4°C (Sorvall RC-5B, rotor SS34), the supernatants were transferred to the fresh tubes, the cell pellets were washed twice in 0.05 M phosphate buffer pH 7.0, and then resuspended in the same buffer to the original volume. The mixture of the two strains of *B.t.i.* was placed at room temperature for 2-5 min, and

the cell clumping was observed by macroscopic examination.

Various supernatants and cell suspensions were used to examine clumping by mixing an equal volume of the cell suspensions or supernatants or cell suspension and supernatant together, placed at room temperature for 2-5 min, and then clumping was observed.

The extent of clumping was determined as 4+ when the clumping occurred immediately with large aggregates, or as 3+ when the clumping occurred immediately with small aggregates, or as 2+ when the clumping occurred within 1-2 min with large aggregates or as 1+ when the clumping occurred after 3 min with small aggregates, or as - when there was no aggregate at all when the suspension was left standing over 10 min.

6. Preparation of S-layer protein

6.1 Isolation of S-layer protein

B.t.i. strain 4Q2, 4Q272 and c4Q272 which were examined for the presence of S-layer protein were grown separately at 30°C in Erlenmyer flasks (180 rpm) of 5 ml NYSM medium (2% Difco nutrient broth, 0.05% yeast extract, $5x10^{-5}$ M $MnCl_2$, $7x10^{-4}$ M $CaCl_2$, $1x10^{-3}$ M $MgCl_2$). Two ml of overnight culture was used as inoculum which was transferred into 200 ml of NYSM medium (500 ml flask), incubated at 30°C with continuous shaking at 180 rpm for 12-14 hr.

Four liters of cultures were harvested by centrifugation (Beckman) at 4200 rpm at 4°C for 30 min and the pellets were thoroughly washed twice by cold, sterile distilled water. Cells were resuspended in 40 ml of unbuffered 6 M urea, or 5 M Guanidine hydrochloride or 0.05 M Tris-HCl pH 8.0 and incubated at 37°C with continuous shaking at 300 rpm for 4 hr. After extraction, the cells were removed by centrifugation at 10,000 rpm for 20 min (Kubota KR 2,000, rotor RA-3), and the supernatant was dialyzed overnight at 4°C against three changes of 3 liters each of 0.01 M Tris-HCl, pH 8.0. The dialyzed supernatant designated as crude extract was concentrated by lyophilization and stored at -20°C.

6.2 Purification of S-layer protein

S-layer protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (221). Crude extract prepared as described previously was mixed with sample buffer for SDS-PAGE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2.5% SDS, 5% B-mercaptoethanol) in equal volume, boiled for 5 min before loading into SDS-PAGE slab gel. Gels were run by using the discontinuous buffer system (25 mM Tris, 192 mM glycine, 0.1% SDS) with 1 mm thick gel consisting of 6.5% acrylamide separating gel and 3% acrylamide stacking gel by using vertical slab gel (Bio-Rad Mini protean II apparatus) for 30 min at 200 V. The gels were stained

approximately 45 min at room temperature with 0.1% Coomassie blue R-250 in fixative (40% methanol, 10% glacial acetic acid). Subsequently, the gels were destained with destaining solution (15% methanol, 7% glacial acetic acid), visualized and the bands of interest were cut out with a stainless steel blade.

The protein was electro-eluted from the gel slices by using electro-eluter (Bio-Rad, Model 422) with protein elution buffer (50 mM ammonium bicarbonate and 0.05% SDS). Electroelution was carried out for 5 hr at 10 mA per glass tube. Then, carefully removed the eluate from the membrane cap, and was frozen at -20°C. The eluate was concentrated by lyophilization and tested for its purity by SDS-PAGE.

6.3 Molecular weight determination

Molecular weight was determined by running the protein extract and molecular weight markers on 6.5% separating gels as described by Laemmli (221). The samples were treated with 2.5% SDS and 5% mercaptoethanol and boiled for 5 min. before applying to the gel. After the samples were run for 30 min at 200 V in the resolving gel, the gels were stained with coomassie blue and destained by 15% methanol and 7% acetic acid (221). The molecular weight was determined with reference to the standard from Pharmacia AB (Uppsala, Sweden) and Sigma (USA). The mean molecular weight of the S-layer protein was compared statistically with the known molecular weight protein markers.

6.4 Determination of glycoprotein

Glycoprotein was determined by Periodic Acid-Schiff (PAS) staining (222). The samples and ovalabumin (MW 45,000) standard were run on SDS-polyacrylamide gel by using slab gel electrophoresis (Hoefer Scientific Instrument Model SE400) at constant power 25 mA for 3 hr. The electrophoresed gel was fix overnight in 100 ml of PAS fixative solution (40% ethanol, 5% glacial acetic acid, 55% distilled water). Subsequently gel was treated with the periodic acid solution (0.7% periodic acid in 5% acetic acid) for 2-3 hr, followed by treatment with sodium metabisulfite solution (0.2% sodium metabisulfite in 5% acetic acid) for 2-3 hr with one solution change after 30 min. The gel was soaked in Schiff reagent (222), color developed in 12-17 hr at room temperature. The presence of glycoprotein was determined by appearance of pink color band on the gel.

7. Preparation of antiserum

7.1 Quantitation of protein to be used as immunogen

The protein content of S-layer protein was measured by Bradford method (223) using bovine serum albumin (BSA) as a standard. The protein sample was dissolved in 50 ul of distilled water. The dye solution (Bio-Rad, USA) was diluted as indicated by manufacturer, 950 ul was added to all the tubes, the tubes were left at room temperature for about 5 min and absorbancy was recorded at 595 nm (Unicam SP 1800 UV spectrophotometer,

USA) against the blank containing 50 ul of distilled water and 950 ul of dye solution. Protein concentrations were determined from appropriate standard curve.

7.2 Immunization

Preimmunized rabbit serum was collected and stored at -20°C for use as control. Antiserum was raised against S-layer protein in New Zealand white rabbits (2 kg each). Rabbits were immunized subcutaneously with 100 ug of S-layer protein which was suspended in 0.5 ml of sterile, distilled water and mixed with 0.5 ml of Freunds' complete adjuvant on first day, and mixed with Fruend's incompleted adjuvant on 14th day and 21st day. On 28th day, blood was collected, placed at room temperature for 1 hr, centrifuged at 3,000 rpm, 10 min (Sorvall, rotor SS34), the clear serum was collected, and the antibody titer was determined by Ouchterlony immunodiffusion. The serum was frozen at -20°C for further use.

7.3 Test for titer and specificity of the anti-S-layer antibody

7.3.1 Ouchterlony immunodiffusion technique

The antibody titer was determined by using the Ouchterlony immunodiffusion technique (224). The test was carried out in 1% Special Noble agar (Difco) dissolved in normal saline solution, 4 ml of gel was overlaid on a microscopic glass slide, and the preparation was allowed to solidify at room temperature.

The wells were made by Gel Punch (Gelman Instrument, USA). The antiserum was filled in the central well, and the S-layer protein and crude extracts of S-layer protein solution of *B.t.i.* were filled in the peripheral wells. The gels were incubated at room temperature in a moist chamber and were observed for precipitin bands after 4, and 24 hr, and then the gels were stained by Amido-Schwarz-10 B (Merck, Germany). The gels were soaked in a larger volume of normal saline solution with several changes for 2-3 days, and soaked in a larger volume of distilled water with several changes for 2-3 days as well. The gels were dried at 37°C overnight, stained in Amido Schwarz (0.6 g Amido Schwarz, 4.5% methanol, 1.0% glacial acetic acid) for 5 min, destained by destaining solution (4.5% methanol, 1% glacial acetic acid) and then, air dried.

7.4 Antiserum absorption

The antiserum was titrated against protein extract of *B.t.i.* strain 4Q272 and cell lysate of *E. coli* DH5α harboring plasmid pBluescriptKS or pUC12 which were used as cloning hosts.

The *B.t.i.* strain 4Q272 cells used in absorption were prepared by using the same condition as for protein extraction. The cell pellets were suspended in normal saline (1 g of cell wet weight/ml). Equal volume of cell suspension was added into rabbit antiserum, incubated at 4°C for 2 hr and centrifuged at 12,000 rpm (Microfuge,

Hettich). The clear supernatant was collected, retested against protein extract of *B.t.i.* strain 4Q272 by dot blot and Western blot. The serum must not show any cross reaction. If there was, repeated absorption until there was no cross reaction. The preparation was then stored at -20°C for further experiments.

The *E. coli* DH5α harboring plasmid pBluescriptKS or pUC12 were grown in LB-broth overnight, the cells were harvested by centrifugation at 6,500 rpm (Sorvall, rotor SS34) for 10 min at 4°C, washed twice with normal saline, the pellets were resuspended to original volume, and the suspensions were sonicated (Soniprep 150). These *E. coli* lysates were divided into small aliquots and stored at -20°C for using in immunological screening and Western blotting experiments. The test system must not show any cross reaction with *E. coli* DH5α harboring pBluescript KS or pUC12. The volume of *E. coli* lysate used in the experiment was calibrated and that condition was used for further experiments.

7.5 Purification of rabbit IgG

An equal volume of saturated ammonium sulfate was added to diluted immunized rabbit serum with gentle stirring, and left standing for 30 min at 4°C to precipitate the globulin. The precipitate was separated by centrifugation at 10,000 rpm for 30 min at 4°C. Subsequently, it was dissolved in a small volume of 0.01 M phosphate buffer, pH 8.0. The residual ammonium

sulfate was removed by dialysis against 0.01 M phosphate buffer pH 8.0 at 4°C overnight. This globulin preparation was used for further purification of IgG by DEAE-cellulose column chromatography (225).

The DEAE-cellulose (Whatman DE-52) was equilibrated in 0.01 M phosphate buffer pH 8.0. Column (LKB) was packed with DEAE-cellulose to within 1.5 inches of the top at room temperature, then washed with approximately 200 ml of 0.01 M phosphate buffer, pH 8.0. The serum sample was applied onto the column by tubing through the pump. IgG did not absorb to DEAE-cellulose under this condition, and passed through the column with the starting buffer. Another portion of IgG subclass was eluted with 0.02 M phosphate buffer, pH 8.0. Several fractions of eluent were tested for the presence of IgG by immunodiffusion, the fractions with high titer were pooled together and confirmed by using immunoelectrophoresis, the precipitin line between pooled IgG and anti-IgG was observed in comparison with unpurified anti 4Q2 S-layer protein antibody or normal rabbit serum.

This IgG was used to perform immunofluorescent for locating the S-layer protein on *B.t.i.*

8. Identification of location for S-layer protein by using Immunofluorescent technique

Indirect immunofluorescent technique (226) was performed by using the antiserum prepared against purified S-layer protein. The antiserum was applied on

the bacterial smears which were prepared by spotting vegetative cells on slides, dried and fixed for 10 min in cold acetone at -20°C. The smear was then further incubated for 30 min at room temperature in a humidified chamber. Subsequently, the slides were washed twice for 5 min, each time in phosphate buffer saline solution (PBS) pH 7.2. The second antibody, Fluorescein isothiocyanate conjugated swine anti-rabbit immunoglobulins (Dakopatts, Denmark) was further applied for 30 min in humidified, foil covered chamber at 37°C, then washed twice with PBS, and mounted in PBS: glycerol (1:9) pH 7.8 solution. The preparations were examined under fluorescent microscope (Leitz Laborlux 12, Germany). The control was performed in a similar way except using preimmunized rabbit serum instead of immunized rabbit serum.

9. Immunoelectrophoresis

Immunoelectrophoresis was carried out as described by Hudson and Hay (227). Slide was prepared as for immunodiffusion, and the well were made according to the pattern as shown in Fig. 18. One well was filled with whole immunized rabbit serum or normal rabbit serum and the other with purified IgG of anti 4Q2 S-layer protein antibody. The prepared slides were electrophoresed at constant power 8 mA per slide for 60 min. The agar trough was removed and filled with anti-whole rabbit serum, and the slide was left to incubate

overnight in a humid chamber at room temperature. The precipitin lines were examined.

10. Preparation of plasmid DNA

10.1 Extraction of plasmid DNA from *B.thuringiensis*

10.1.1 Small scale preparation of plasmids

Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (228).

Cells were grown in 1.5x15 mm test tubes containing 4 ml of LB-broth with or without supplementation with appropriate antibiotics. Cultures were incubated for 12-14 hr at 37°C on a rotary shaker. Cells in 1.5 ml of culture broth were collected by centrifugation at 7,000 rpm in a microcentrifuge (Hettich, Germany) for 1 min at room temperature and suspended in 100 ul E buffer (0.04 M Tris-hydrochloride, 0.002 M EDTA tetra-sodium salt, 15% sucrose, pH 7.9) by gentle vortexing. Cells were lysed by adding 200 ul of lysis solution (3 g of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 15%, wt/vol, sucrose in 0.05 M Tris-hydrochloride). The Eppendorf tubes were rapidly inverted 20 times to mix the cells and lysis solution and were then held in a 60°C water bath for 30 min. The lysate was precipitated with 150 ul 3 M sodium acetate pH 5.0 by inverting and placing the tubes in an ice box for 40 min. The plasmid DNA was obtained by centrifugation at 10,000 rpm for 10 min. The supernatant was removed to a new Eppendorf tube, and the DNA preparations were

concentrated by adding 2.5 volume of 95% ethanol followed by treatment at -20°C for one hour. The plasmid DNA was separated by centrifugation at 12,000 rpm for 10 min, washed twice with 70% cold ethanol, and dried. The precipitate was dissolved in 10 ul TE, pH 8.0.

10.1.2 Large scale preparation of plasmid DNA

Large scale preparation of plasmid DNA from *B. thuringiensis* was carried out by the same procedure as small scale preparation, except the volume of cell cultures and reagents were increased proportionally, and RNA was removed by adding RNAase (10 ug/ml) before further purification by cesium chloride-ethidium bromide gradient.

10.2 Preparation of plasmid DNA from *E. coli*

Plasmid DNA (pBluescriptKS, pBluescriptSK and pUC12) and recombinant plasmids (pAC1, pAC2, pAC3, pAC11, pAC111) from *E. coli* cells was extracted by alkaline lysis method (229).

10.2.1 Small scale preparation of plasmid DNA

The bacterial culture was grown in 5 ml LB-broth with an appropriate antibiotic at 37°C with shaking overnight and 1.5 ml of the culture was used for plasmid extraction as described by Birnboim (229). The cell pellet was harvested by centrifugation at 8,000 rpm (Hettich, Germany) for 1 min and then thoroughly suspended in 100 ul of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing lysozyme 2 mg/ml

and incubated at 37°C for 20 min, then added 200 ul of freshly prepared Solution II (1% SDS, 0.2 N NaOH) and mixed immediately. The mixture was subsequently neutralized by adding 150 ul of 3 M sodium acetate pH 5.0 and maintained at 0°C for 30 to 60 min. The supernatant was collected by centrifugation at 10,000 rpm for 10 min., and the 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 hr. The DNA precipitate was collected by centrifugation at 12,000 rpm for 10 min and the pellet was dissolved in 100 ul TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and 50 ul of 7.5 M ammonium acetate was added to make the final concentration of 2.5 M. The plasmid DNA was reprecipi-tated with 2 volumes of cold absolute ethanol at -20°C for 1 hr and centrifuged as previously described. The pellet was washed twice with 70% cold ethanol, dried, and dissolved in 20 ul of TE buffer. RNA was removed by adding 1 ul of RNAse A (4 ug/ml) and incubated at 37°C for 30 min.

10.2.2 Large-scale preparation of plasmid DNA

The method used was also the alkaline lysis method but the volume of cell cultures and reagents was increased proportionally, and RNA was removed by adding RNAase A before further purification by cesium chloride-ethidium bromide gradient.

10.2.3 Rapid disruption of bacterial colonies to test the size of plasmids (230)

This method was used for screening of deletion subclones at various time intervals to select the clones containing appropriate deletions for further analysis.

Ten colonies from each time interval after exonuclease III digestion were picked to make a single 2 inch streak of each on LB-agar plate containing 100 ug/ml ampicillin, and incubated at 37°C overnight. Using a sterile toothpick, the cells were scraped and suspended in 40 ul of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA in a 1.5 ml Eppendorf tube, 40 ul of phenol/chloroform were added and vortexed for 1 min and centrifuged at 10,000 rpm for 1 min. The aqueous phase was transferred to a fresh tube and 1 ug RNAase A was added and incubated at room temperature for 10 min. 10 ul loading buffer was added to mixture (50% sucrose, 0.05% Bromophenol blue), heated at 70°C for 5 min and 10 ul of sample was used for loading onto an 1% agarose gel.

11. Purification of plasmid DNA

Large-scale preparation of plasmid DNA was further purified by cesium chloride-ethidium bromide (CsCl-EtBr) gradient (231). Five grams of CsCl was added to 5 ml of plasmid DNA, the solution was mixed gently until the salt was dissolved, 375 ul of EtBr (10 mg/ml) in water was added, and immediately mixed. Transferred

the solution to a centrifuge tube (Hitachi) for centrifugation in Hitachi fix angle rotor, filled the remainder of the tube with light paraffin oil and capped the tube. The mixture was centrifuged in Hitachi 70P at 54,000 rpm for 18 hr at 18°C. Plasmid band was removed by puncturing through the tube with 24-gauge hypodermic needle. Ethidium bromide was removed by extraction with isoamyl alcohol saturated with water. The DNA solution was dialyzed for 24-48 hr against several changes of TE (pH 8.0).

12. Plasmid analysis

12.1 Restriction endonuclease digestion

Restriction of plasmids DNA with endonucleases were performed by using general buffer as supplied by manufacturers. The conditions of digestion and incubation time were carried out as recommended by the enzyme manufacturers.

12.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to characterize plasmid DNA. The solution of plasmid DNA, 10 ul, was mixed with 3 ul of loading buffer (50% sucrose, 0.05% bromophenol blue, 50 mM EDTA) and the mixture was loaded into slot of horizontal 0.7 to 1% agarose gel in Tris-borate-EDTA buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA). Electrophoresis was run at constant voltage of 50 or 100 volts at room temperature until the tracking dye was near the edge of

gels. The gels were stained in 1 ug/ml of ethidium bromide solution for 10-30 min and destained in distilled water for 30-60 min. DNA was visualized with UV light transilluminator and photographed on Kodak technical pan film. To estimate the molecular weight of plasmid DNA fragments, Lamda DNA digested with *Hin*dIII, or *Pst*I and pBR322 DNA digested with *Hpa*II were used as standard markers to determine unknown fragment sizes graphically, assuming a logarithmic relationship between molecular weight and electrophoretic mobility.

12.3 Quantitation of DNA

The concentration of purified plasmid DNA was quantitated by measuring the OD_{260} of the final solution of DNA, and calculated the concentration as described in Sambrook et al. (232). Alternatively, the amount of DNA was estimated by electrophorese through minigels (232). DNA sample was mixed with loading-buffer (bromophenol blue only) and loaded the solution into a slot in 0.8% agarose minigel containing EtBr (0.5 ug/ml). The standard DNA solution of Lamda DNA at various concentrations (2.5, 5, 10, 20, 40, 50 ug/ml) was mixed with loading buffer, and the samples were loaded into the wells of the gel, and then electrophoresis was carried out until the bromophenol blue had migrated to approximately 1-2 cm from the edge. The gel was destained, photographed by using UV transilluminator, the intensity of fluorescence of the sample DNA was compared with that

of the standards and the quantity of DNA in the sample was estimated.

12.4 Acrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used to characterize the small fragments of DNA (233), in order to determine the restriction map of plasmid pAC11 clones together with agarose gel electrophoresis. The 15 ul of DNA was mixed with 5 ul of loading buffer, loaded onto the well of vertical slab gel (5% polyacrylamide, 16x18 mm, Hoefer Scientific, USA). Running the gel in 1xTBE buffer at 50 V, constant voltage, until the dye marker migrated approximately 12 cm apart from the top of gel, stained, destained and visualized the same way as described for agarose gel electrophoresis.

13. Recovery of DNA fragments from agarose gels

The endonucleases digested DNAs (pAC1, pAC11, pAC111) were separated by electrophoresis through 1% agarose gel (SeaKem GTG agarose, FMC) that contained 0.5 ug/ml EtBr, visualized the band of interest by UV trans-illuminator, cut out the agar containing the DNA to be recovered with a sterile razor blade. The slice of the gel was put in Eppendorf tube, crushed by siliconized glass rod, added equal volume of phenol saturated in TE pH 8.0 (BRL), mixed thoroughly and frozen at -70°C for 10 min. The aqueous phase was transferred to fresh Eppendorf tube after centrifugation at 10,000 rpm for 5

min. Phenol was removed by twice extraction with chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 2 volumes of cold absolute ethanol as previously described in plasmid preparation.

14. Preparation of DNA from *B. thuringiensis* subsp. *isarelensis* for shotgun cloning experiment

The total DNA from *B.t.i.* strain 4Q2-Nal^r was prepared by method described by Ausubel et al. (234). The overnight culture of *B.t.i.* strain 4Q2-Nal^r in 100 ml LB-broth was harvested by centrifugation at 7,000 rpm for 5 min (Kubota KR 2,000, rotor RA-6), and the supernatant was discarded. The cells were resuspended gently in 9.5 ml TE buffer (pH 8.0), added 0.5 ml of 10% SDS and 50 ul of 20 mg/ml proteinase K, mixed thoroughly and incubated 1 hr at 37°C. The mixture was mixed with 1.8 ml of 5 M NaCl, added 1.5 ml CTAB/NaCl solution (4% NaCl, 10% hexadecyl-trimethyl ammonium bromide), and incubated 20 min at 65°C. The mixture was further added with an equal volume of chloroform/isoamyl alcohol, mixed thoroughly and centrifuged at 7,000 rpm for 10 min (Kubota, rotor RA-3) at room temperature. The aqueous phase was transferred to a fresh tube, 0.6 volume of isopropanol was added, mixed and kept at -20°C for 1 hr. The DNA pellet was precipitated by centrifugation at 10,000 rpm (Kubota KR 2000, rotor RA-3) for 20 min, supernatant was removed, the pellet was washed twice with 70% ethanol, dried, and resuspended in 4 ml TE buffer (pH 8.0). RNA

was removed by incubating with RNAase (20 ug/ml) at 37°C for 30 min. Four ml of 1.6 M NaCl containing 13% polyethylene glycol 8,000 was added into DNA solution. DNA was recovered by centrifugation at 12,000 rpm for 15 min at 4°C (Kubota, Rotor RA-3). Supernatant was discarded. DNA pellet was dissolved in 2 ml TE (pH 8.0), and extracted once with phenol: chloroform, and twice with chloroform. The aqueous phase was transferred to a fresh tube. DNA was reprecipitated with cold isopropanol as previously described.

15. Cloning of S-layer protein gene

15.1 Restriction endonuclease digestion

A purified DNA of *B.t.i.* strain 4Q2 Nal^r was partially digested with various restriction enzymes, *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I and *Sau* 3A by varying concentration of enzymes to 2, 4, 6, 8, 10 units per 1 ug of DNA. Plasmid vector, pBluescript KS (-) (2.96 Kb) or pUC12 (2.7 Kb), as shown in Fig. 7 was also digested completely with various enzymes mentioned above. DNA fragments were analyzed in 0.7% agarose gel electrophoresis as previously described.

15.2 Ligation of DNA fragment into plasmid vectors

Partially digested DNA of *B.t.i.* 4Q2-Nal^r with various enzymes for 15 min and pBluescriptKS (-) or pUC 12 cut with the same restriction endonuclease, were mixed together using a ratio of *B.t.i.* DNA and vector equal to 10:1. The mixture was heated at 65°C in water bath for

10 min and immediately cooled on ice bath for 5 min. The stock solution of Tris-HCl, pH 7.5, $MgCl_2$, ATP, Dithiothreitol (DTT) and Hexammine cobalt chloride (HCC) were added to make the final concentration of 50, 10, 1, 10, 1 mM, respectively (235). The ligation volume was adjusted with sterile distilled water to make final volume of 20-50 µl and 1-2 units of T4 DNA ligase were added. Each reaction mixture was incubated at 4°C for 20 hr and used for transformation experiments.

15.3 Preparation of competent *E. coli* cells

The method described by Sambrook et al. (236) was followed with some modification. *E. coli* strain DH5α was grown in SOB agar medium, incubated for 16 hr. at 37°C, and then transferred ten well-isolated colonies into 50 ml of SOB containing 20 mM $MgSO_4$, and the cells were grown at 37°C with shaking at 200 rpm until the exponential phase (approximately 3 hr). The culture was transferred into sterile ice-cold 50-ml polypropylene tubes, storing the tubes on ice for 10 min, and then centrifuged at 3,000 rpm for 6 min at 4°C in a CRU 5,000 centrifuge. The pellet was resuspended by gentle vortexing in 20 ml of ice-cold FSB (10 mM Potassium acetate pH 7.5, 10 mM $MnCl_2$, 100 mM $CaCl_2$ 3 mM KCl, 3 mM Hexammine cobalt chloride, and 10% glycerol, pH 6.4, the solution was sterilized by filtration through Millipore membrane (0.45 u). The cell suspensions were stored on ice for 10 min, and subsequently, the cells were

recovered by centrifugation at 3,000 rpm for 6 min at 4°C. Subsequently, the buffer was decanted from the cell pellet, and the pellet was resuspended by gentle vortexing in 4 ml of ice-cold FSB. Added 140 µl of DMSO per 4 ml of resuspended cells, mixed gently by swirling, and the suspension was stored on ice for 15 min., an additional 140 ul of DMSO was added, mixed, and the suspension was quickly stored on ice bath. Aliquots of the suspensions were dispensed into chilled, sterile Eppendorf tubes, subsequently the competent cells were snap-frozen by immersing the closed Eppendorf tube in acetone-dry ice and stored at -70°C until used.

15.4 Transformation of *E. coli* with plasmid DNA

Transformation of competent *E. coli* DH5 α was performed by using method described by Sambrook et al. (236) as follows. The competent cell (200 ul) was mixed with plasmid DNA or ligated DNA, the tubes were gently swirled for thoroughly mixing, the Eppendorf tubes were stored on ice for 30 min., and then the mixtures were transferred to circulating water bath that had been preheated to 42°C, for 90 sec, and rapidly transferred the tubes to chill on ice bath for 2 min. Added 800 µl of SOC medium (SOB broth, 0.02 M glucose) to each tube, incubated the culture at 37°C in shaking incubator for 45 min to allow the *E. coli* to recover and express the antibiotic resistance marker encoded by the plasmid. The mixture was, then, spread on LB-agar plates containing

ampicillin 100 ug/ml, 40 ug/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 5 mM Isopropyl β-D-thiogalactopyranoside (IPTG). The plates were incubated at 37°C overnight. The white colonies on X-gal, ampicillin plate were picked for further analysis.

15.5 Immunological screening for S-layer protein expression clones

The white colonies of *E. coli* were picked and replicated on LB-agar plates containing (100 ug/ml) ampicillin and incubated overnight. The nitrocellulose filter, previously soaked in IPTG, was overlaid on plate bearing *E. coli* colonies, filters were left on plates for 2 hr at 37°C to allow transfer of *E. coli* cells to the filter. The orientation of the filter to the plate was recorded by stabbing a needle through the filter into the agar at 3 asymmetric points around the edge of the plate. The filter was removed slowly from the plate and immerged to chloroform-saturated atmosphere tank for 3-5 min, the filter was washed in phosphate-buffered saline (1.45 M NaCl, 0.75 M Na_2HPO_4, 0.025 M NaH_2PO_4) with 1% Tween 20 (PBST) for 10 min at room temperature. Subsequently that the filter was incubated in PBST containing 3% Bovine serum albumin (BSA) at room temperature for 30 min to remove the bulk of bacteria from the filter. The filter was incubated with the anti-4Q2-S-layer protein antibody diluted 1:100 in PBST containing 3% BSA and *E. coli* lysate at room temperature for 2 hr with continuous

shaking, the membrane was washed with PBST 3 times, 10 min each. The filter was incubated with goat anti-rabbit alkaline phosphatase (Sigma) diluted 1:1000 in PBST containing 3% BSA and *E. coli* lysate for 1 hr at room temperature. The membranes were washed as described above, and substrates were added, the mixture of o-dianisidine tetrazotized (Sigma) and beta-naphthyl phosphate (Sigma) in substrate buffer (0.1 M carbonate buffer containing 1 mM $MgCl_2$). The reactions were stopped by rinsing with water.

The positive clones, designated pAC1, pAC2, and pAC3 were re-tested 3 times by colony-screening method as previously described and were used for further analysis by Western blotting, and Southern blotting.

15.6 Characterization of pAC1

The recombinant plasmid pAC1 was further characterized by digesting with *Pst*I and the 5.2 kb-*Pst* I-*Pst*I fragment was recovered from the agarose gel electrophoresis, purified and religated into pBluescript-KS vector. The new recombinant plasmid was designated as pAC11, and was transformed into *E. coli* DH5α , and its expression was examined by Western blotting analysis (237).

15.7 Restriction mapping of the S-layer protein gene

Plasmid pAC11 was digested with various restriction enzymes either singly or in combinations. The digested fragments were determined by measuring their

sizes in 1% agarose gel electrophoresis and 5% acrylamide gel electrophoresis and they were compared to those of standard molecular weight markers. The restriction map was then calculated and drawn after analysis of these fragments.

15.8 Subcloning of pAC11 S-layer protein gene with *Hin*dIII

The plasmid pAC11 was completely digested with *Hin*dIII and *Pst*I. The digestion fragments were separately recovered from agarose gel electrophoresis, purified and ligated on to plasmid vector pBluescript KS at *Pst*I/*Hin*dIII sites or *Hin*dIII site. The various sizes of recombinant plasmids were separately transformed into *E. coli* DH5α and transformants were selected by the method as described previously.

The pAC111 containing 1.5 kb *Pst*I-*Hin*dIII fragment was digested with *Pst*I and *Hin*dIII, the fragment was recovered from 1% agarose gel electrophoresis, religated into pBluescriptSK vector at *Pst*I/*Hin*dIII sites and designated as pAC112, then transformed into *E. coli* DH5α and transformants were selected as described previously.

The overall subcloning strategies are shown schematically in Fig. 8.

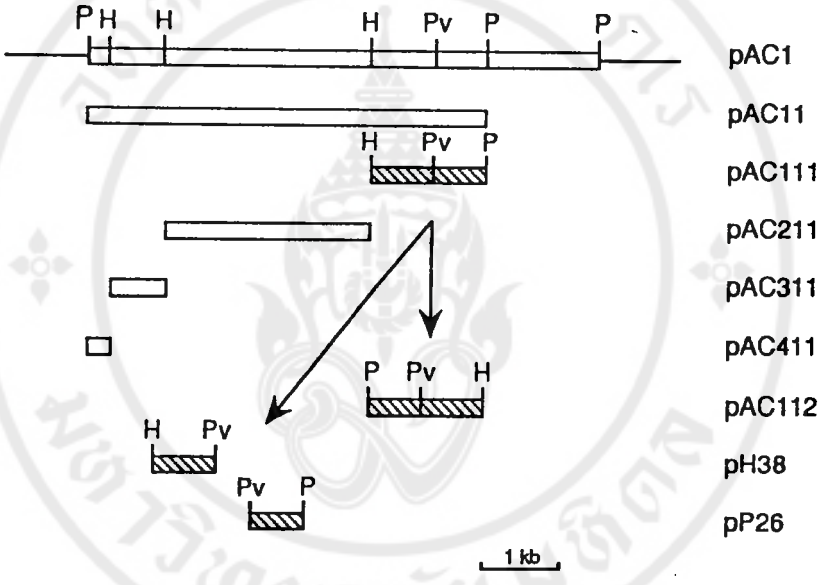


Fig. 8. Strategy for subcloning. Recombinant plasmid pAC1 containing 6.6 Kb *slp* gene was subsequently subcloned to obtain various recombinant plasmids. Stippled bars indicate the cloned gene. Single lines indicate the portion of vector. H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II.

16. Western Blot Analysis

16.1 Preparation of cell extracts from *E. coli*

Cultures of *E. coli* were grown in LB-broth with shaking at 200 rpm, at 37°C. Three ml of each culture was centrifuged in a sterile Eppendorf tube at 8,000 rpm for 1 min, the supernatant was discarded and cells were washed once in solution containing 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA. The cells were resuspended in 85 ul of sterile distilled water, mixed with equal volume of SDS-reducing buffer (62 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) and boiled for 5 min, cooled in ice bath for 5 min and centrifuged for 2 min and 5-10 ul of supernatant was then loaded in SDS-PAGE (238).

16.2 Western Immunoblotting

The protein samples were separated by SDS-PAGE (221) using Mini-Protean II apparatus (Bio-Rad), proteins from gel were transferred to nitrocellulose filter by using Bio-Rad Mini blot apparatus. Porous pads (Scotch-Brite), Whatman 3 MM filter paper, and nitrocellulose membrane filter were immersed in the electroblotting buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 1% SDS, 10% methanol) (237). Three sheets of Whatman 3 MM filter paper were placed on a Scotch-Brite pad, gels were placed on the filter paper and moistened with electroblotting buffer. Air bubbles were removed between gel and filter paper. A piece of nitrocellulose membrane filter was

placed directly onto the anode side of the gel, after all air bubbles were removed. Then three sheets of Whatman 3 MM filter paper were placed on the nitrocellulose membrane, and another Scotch-Brite pad was put on top of filter paper. Finally, they were tightened by another plastic grid. The support containing the sandwich was placed into the electroblotting apparatus in the correct orientation. The proteins were electrophoretically transferred from the gel to nitrocellulose at 100 V constant voltage for 2 hr at 4°C. The filter was then immersed into a large volume of PBST, incubated at 37°C for 30 min. Such prepared filters were then ready for further steps in preparation for immunoenzyme staining.

17. Southern blot DNA-DNA hybridization

17.1 Southern transfer

DNAs from *B.t.i.* strain 4Q2, 4Q272 and c4Q272, were partially digested with *Pst*I, and electrophoresed in 0.7% agarose gel, and photographed. DNA preparations on the gels were denatured by soaking in 1.5 M NaCl and 0.5 M NaOH for 20 min and then neutralized by soaking in 3 M NaCl in 0.5 M Tris-HCl, pH 7.0 for 40 min. The DNA's were transferred from the gel to nitrocellulose filter by Southern blotting techniques (239) overnight, and then the nitrocellulose filter was washed with 2xSSC (1xSSC = 0.15 M NaCl, 0.015 M Sodium citrate, pH 7.0), the dried nitrocellulose filter was placed between two sheets of Whatman filter papers and baked at 80°C for 2 hr.

17.2 Preparation of biotin-labelled DNA probes

One microgram of plasmid DNA (5.2 kb, *Pst*I fragment of pAC11) was labelled with biotin-11-dUTP by nick translation (240) in the presence of unlabelled dCTP, dATP and dGTP (without dTTP) using nick translation kit (BRL). After labelling, the biotin-labelled DNA was separated from free nucleotides by ethanol precipitation and resuspended in 50 ul TE and the probe was tested for intensity of labelling by comparison with the standard biotinylated DNA (200 pg/ul) available in BluGENE kit.

17.3 Prehybridization and hybridization with the biotinylated probe

The baked filter from the previous step (239) was once soaked in 2xSSC until thoroughly hydrated and presoaked in 4xSET (1xSET = 0.15 M NaCl, 0.03 M Tris-HCl, pH 8.0, 0.1 mM EDTA) for 10 min at room temperature. The filter was soaked in the prehybridization solution containing 10x Denhardt's solution (1xDenhardt's solution = 0.02% Ficoll MW 400,000, 0.02% Polyvinyl pyrrolidone MW 360,000, 0.02% Bovine serum albumin), 4xSET, 0.5% SDS, 10 ug/ml heat denatured calf thymus DNA. The filter was incubated at 42°C in the sealed polypropylene bag with the prehybridization solution for 2 to 4 hr.

Subsequently, the prehybridization solution was removed and hybridization solution containing 45% formamide, 2xDenhardt's solution, 4xSET, 0.5% SDS, 10 ug/ml heat denatured calf thymus DNA, and 100 ng/ml heat-

denatured biotinylated DNA probe was added to the nitrocellulose filter. The filter was incubated at 42°C in water bath overnight to achieve maximal hybridization. The filter was washed twice with 250 ml of 2xSSC, 0.1% SDS for 2 min, twice with 250 ml of 0.2xSSC, 0.1% SDS for 3 min at room temperature, twice with 0.16xSSC, 0.1% SDS for 15 min at 50°C with agitation, and finally the filter was rinsed briefly in 2xSSC at room temperature.

The hybridized filter was rehydrated in blocking buffer 1 (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl) for 3 min with agitation and incubated for 1 hr at 65°C in buffer 2 (3% BSA in buffer 1), and then the filter was exposed to BRL Streptavidin-Alkaline Phosphatase (SA-AP) conjugate in buffer 1 for 10 min (7 ul SA-AP conjugated in 7 ml buffer 1/100 cm^2 filter) with agitation at room temperature. Filters were rapidly washed twice with 20-40 fold greater volume of buffer 1 with agitation for 15 min and washed once with buffer 3 (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) at room temperature for 10 min. For color development, filters were incubated in the dye solution, 7.5 ml buffer 3 containing 33 ul nitroblue tetrazolium (NBT, 75 mg/ml) and 25 ul 5-bromo-4-chloro-3-indolylphosphate (BCIP, 50 mg/ml, BRL) within a sealed hybridization bag. Color development took place in the dark for 30 min to 3 hr, after that, filter was washed in 20 mM Tris-HCl pH 7.5, 0.5 mM EDTA solution to terminate

the reaction. The filters containing DNA-DNA hybridization were stored dry and protected from strong light.

18. DNA sequence analysis

18.1 Generation of nested sets of deletion with Exonuclease III (241)

Plasmid pAC111 containing the 1.5 kb *Pst*I-*Hin*dIII fragment to be sequenced was purified by CsCl-EtBr gradient, and 10 ug of this DNA was digested with two pairs of restriction enzymes, *Hin*dIII/*Kpn*I and *Bam*HI/*Sac*I.

The DNA's were digested first with *Hin*dIII or *Bam*HI that generated the recessive 3' terminus. Complete digestion was confirmed by agarose gel electrophoresis, the buffer was adjusted accordingly prior to the addition of the second enzyme, *Kpn*I or *Sac*I. The DNA's were purified by extraction with phenol/chloroform, then precipitated DNA by 2 volumes of cold absolute ethanol, and DNA pellet was washed twice with 70% ethanol, the DNA was dissolved in 40 ul of 1 x exonuclease III buffer (66 mM Tris-HCl pH 8.0, 6.6 mM $MgCl_2$), and stored on ice.

Placed 7.5 ul of S1 reaction mixture (Distilled water 172 ul, 7.4 x S1 buffer 27 ul, nuclease S1 60 units; 7.4 x S1 buffer containing 0.3 M potassium acetate, pH 4.6, 2.5 M NaCl, 10 mM $ZnSO_4$, 50% glycerol). The tubes containing DNA were incubated for 5 min at 37°C, 150 units of exonuclease III was added, then the solution was mixed

as rapidly as possible. Then, 2.5 ul samples were removed at 30 sec. intervals into the S1 reaction mixture tubes on ice, pipetting up and down briefly to mix. After all of the samples had been taken, the Eppendorf tubes were incubated for 30 min at room temperature, and 1 ul of S1 stop buffer (0.3 M Tris base, 50 mM EDTA, pH 8.0) was added and incubated for 10 min at 70°C for inactivating nuclease S1 and any residual exonuclease III.

To determine the extent of digestion, 2 ul samples were removed from each time interval for analysis on a 1% agarose gel. The samples were transferred to 37°C and added 1 ul Klenow mix (30 ul of 1xKlenow buffer, 3 Units of Klenow DNA polymerase; 1x Klenow buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$), incubated for 3 min and then added 1 ul of the dNTP mixture, incubated for 5 min more at 37°C. The samples were moved to room temperature and added 40 ul ligase mix (790 ul deionized water, 100 ul of 10x ligase buffer, 100 ul 50% PEG, 10 ul 100 mM DTT, 5 Units of T4 DNA ligase; 10x ligase buffer containing 500 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 10 mM ATP) to each Eppendorf tube, incubated at 4°C for 20 hr. These samples were transformed into DH5α competent cells as previously described and screened for deletion subclones by using rapid method described in 10.2.3. Further analysis of plasmid DNA was carried out by rapid alkaline lysis, and digested with restriction enzymes to determine the size of inserted DNA in the

clones of interest comparing with the linearized original clone and pBluescriptKS vector.

18.2 Subcloning for sequencing

The plasmid pAC111 was digested completely with restriction enzymes *Pst*I and *Hin*dIII, the vector and 1.5 kb *Pst*I/*Hin*dIII fragment were separated by agarose gel electrophoresis. The 1.5 kb fragment was recovered, purified and digested again with *Pvu*II. The *Pst*I/*Pvu*II and *Pvu*II/*Hin*dIII, digested DNA fragments were recovered from agarose gel electrophoresis and purified. Each fragment was religated into pBluescriptKS vector, at *Pst*I/*Eco*RV digested vector or *Hin*dIII/*Sma*I digested vector, transformed into *E. coli* DH5α, selected for the clones containing the right insert fragment that was characterized by double digestion with restriction enzyme. DNA's from the clones containing, *Pst*I/*Pvu*II fragment and *Pvu*II/*Hin*dIII fragments were further isolated and purified for sequencing.

18.3 Double-stranded DNA template preparation

Plasmid DNAs for sequencing were prepared by large scale alkaline lysis method (229) and further purified by CsCl-EtBr gradient centrifugation (231).

18.4 Denaturation of double-stranded DNA template

Double-stranded DNAs were converted to a single-stranded form prior to sequencing by using alkali denaturation of supercoiled plasmid DNA.

Plasmid DNAs (5 ug) were pipetted to Eppendorf tube and deionized water was added to 18 ul, 2 ul of 2 M NaOH and 2 mM EDTA solution were added and incubated for 30 min at room temperature. The reaction mixtures were neutralized with 3 ul of 3 M sodium acetate pH 5.0, 7 ul of distilled water and 75 ul absolute ethanol were, then, added and the reaction mixtures were kept at -20°C for 1 hr. The DNA pellets were collected by centrifugation at 12,000 rpm for 10 min in a microcentrifuge, the pellets were washed twice with 500 ul cold 70% ethanol, the supernatants were removed, and the pellets were dried. Then, they were resuspended in 7 ul deionized water for sequencing.

18.5 Sequencing reaction (242, 243)

18.5.1 Annealing template and primer

DNA solution (7 ul) was added to 2 ul of reaction buffer (Sequenase, USB) and 1 ul of primer, i.e. M13 primer, or M13 reverse primer (USB) or T3 primer, or T7 primer, or KS primer or SK primer (Stratagene, USA). The mixture was warmed to 65°C for 2 min in heat block, then the temperature of the tube was allowed to cool slowly to room temperature over a period of about 30 min. Once the annealing was completed, the tube was placed on ice.

18.5.2 Labeling reaction

To the annealed template-primer the followings were added: 0.1 M DTT 1 ul, diluted labeling mix (7.5 uM dGTP, 7.5 uM dCTP, 7.5 uM dTTP) 2 ul, [^{35}S] dATP (10 uCi/ul) 0.5 ul, and diluted sequenase (USB) [1 ul sequenase, 7 ul ice-cold enzyme dilution buffer (10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA)] 2 ul. The mixture was mixed thoroughly and incubated for 5 min at room temperature.

18.5.3 Termination reactions

Four Eppendorf tubes were labelled G, A, T, and C, placed 2.5 ul of the ddGTP, ddATP, ddTTP and ddCTP Termination mix (USB) in the tubes labeled G, A, T, and C, respectively. The tubes were prewarmed at 37°C in heating block at least 1 min, removed 3.5 ul mixture from labeling reaction tube to the tube labeled G, mixed and continued incubation at 37°C for 5 min. Similarly, each 3.5 ul of the labeling reaction was transferred to the A, T and C tubes, and continued incubation. Then to each termination reaction was added 4 ul of stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% xylene cyanol FF), mixed and stored on ice until ready to load onto the sequencing gel. The samples were heated to 75-80°C for 2 min and loaded immediately onto the gel.

18.6 Sequencing gel

The sequencing gel containing 6% or 8% acrylamide and 8 M Urea were prepared 2-20 hr prior to use. For 100 ml of gel solution, containing 10 ml of 10xTBE buffer (TBE = 0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA), 40 ml of deionized water, 48 g of urea, 5.7 (or 7.6 g) of acrylamide and 0.3 g (or 0.4 g) bis acrylamide, 500 ul of 10% ammonium persulfate, 50 ul TEMED. The mixture was mixed thoroughly by magnetic stirrer, the solution was filtered through Whatman No. 1 paper, the volume was adjusted with deionized water. Then, this gel solution was poured into assembled glass plates (0.4 mm thick gel, gel dimension 31x38.5 cm (BRL, sequencing gel electrophoresis apparatus model S2), the gel was allowed to polymerize at room temperature. After the gel was assembled on apparatus it was filled with 1xTBE, flushed out the well with TBE buffer to completely remove crystallized urea and unpolymerized acrylamide.

After pre-running the gel for 15-60 min, each sequencing reaction was loaded onto the gel. The gel was run at 1,800 V, constant voltage, for 3 loads, for 3, 7, and 12 hr, respectively. When the electrophoresis was completed, gel was soaked in 5% acetic acid, 15% methanol to removed the urea, for 20 min, carefully aspirated off the acetic acid/methanol solution, and placed a sheet of Whatman paper over the gel, smoothed out any air bubbles, slowly picked up Whatman paper

making sure that the gel adhered to it, dried the gel under vacuum on a gel dryer at 80°C for 2 hr. The dried gel was autoradiographed by exposing to x-ray film (Kodak XAR or Fuji) in metal cassette for 48 hr. at room temperature. The autoradiograph was developed and the sequence of DNA was read at least twice for each reaction.

18.7 DNA sequence editing and analysis

The DNA sequence data were joined, edited and analysed by using computer program, Seqaid II as edited by Rhoads and Roufa (1986).

CHAPTER IV

RESULTS

1. Plasmid patterns of *B. thuringiensis* subspecies

The donor strain, *B. thuringiensis* subsp. *israelensis* A084-16-194 harbored its own plasmids in addition to the 4.25 kb pBC16 plasmid and the 2.91 kb pC194 plasmid which conferred tetracycline and chloramphenicol resistances, respectively. The plasmid pattern of all 25 *B. thuringiensis* subspecies which were used as recipients were also examined (Fig. 9).

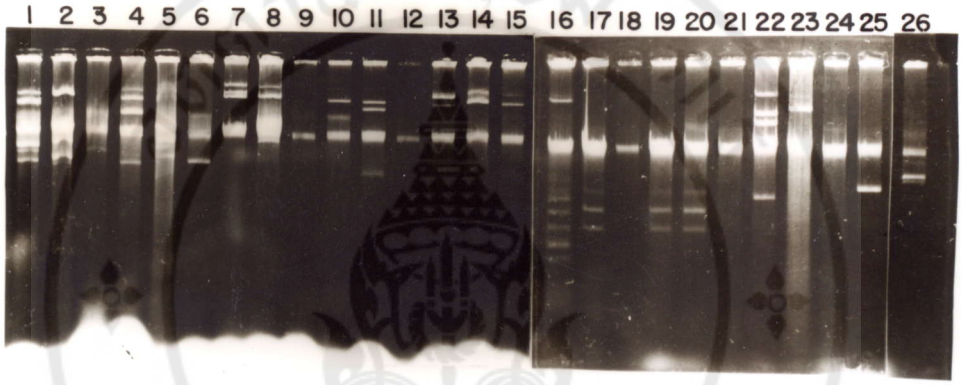
All subspecies except one contained plasmid DNA ranging from 1 to 7 plasmids. Only one subspecies, namely subspecies *entomocidus*, did not harbor any plasmids. Four subspecies, i.e., *finitimus, subage yunnanensis, tolworthi* and a non-motile *wuhanensis* carried only small plasmids which migrated faster than chromosomal DNA. Other subspecies contained ranges of both small and large plasmids.

2. Conjugation-like gene transfer among *B. thuringiensis* subspecies

2.1 Transfer of plasmids pBC16 and pC194

B. thuringiensis subsp. *israelensis* A084-16-194 (Str^r Tet^r Cam^r) was tested for the ability to transfer pBC16 and/or pC194 plasmids into various subspecies of *B. thuringiensis* by the broth mating technique. As a

Fig. 9 Agarose gel electrophoresis of plasmid DNA extract from various subspecies of *Bacillus thuringiensis*. All samples were extracted from 1.5 ml of overnight cultures with A_{600} = 1.2. Conditions used for extraction and for electrophoresis are described in the Materials and Methods. Lane 1, *B.t. thuringiensis*; lane 2, *B.t. kurstaki*; lane 3, *B.t. dendrolimus*; lane 4, *B.t. sotto*; lane 5, *B.t. kenyae*; lane 6, *B.t. galleriae*; lane 7, *B.t. ostrinae*; lane 8, *B.t. tolworthi*; lane 9, *B.t. kyushuensis*; lane 10, *B.t. thompsoni*; lane 11, *B.t. dakota*; lane 12, *B.t.tohokuensis*; lane 13, *B.t. darmstadiensis*; lane 14, *B. pakistani*, lane 15, *B.t.subtoxicus*; lane 16, *B.t.i.* A084-16-194; lane 17, *B.t. finitimus*; lane 18, *B.t. entomocidus*; lane 19, *B.t.subage yunnanensis*; lane 20, *B.t. morrisoni*; lane 21, *B.t.caucasicus*; lane 22; *B.t.indiana*; lane 23, *B.t. kumamotoensis*; lane 24, *B.t. tochigiensis*; lane 25, *B.t. wuhanensis*; lane 26, *B.t. tolworthi*.



selective pressure, a rifampicin resistant marker was used in each of the recipient strains. The transconjugants which acquired plasmids pBC16 were scored on nutrient agar plates containing 20 ug/ml tetracycline and 50 ug/ml rifampicin, whereas the transconjugants with pC194 were selected on nutrient agar plates containing 15 ug/ml chloramphenicol and 50 ug/ml rifampicin. Similarly, the transconjugants which acquired the chromosomal marker were selected on nutrient agar plates containing 40 ug/ml streptomycin and 50 ug/ml rifampicin.

Tables 9 and 10 showed the frequencies of plasmids transfer (average number of three independent experiments) into recipient subspecies by the conjugation-like process. The frequencies of plasmid transfer were found to differ depending on subspecies of recipient. There appeared to be three different categories of recipients based on their ability to acquire plasmids pBC16 and pC194. In group I, subsp. *pakistani* acquired only pBC16 from the donor strain, and there were no transconjugants which contained the plasmid pC194. In group II, subsp. *tochigiensis* and *caucasicus* acquired only pC194 from the donor strain, and there were no transconjugants which contained plasmid pBC16. In group III, there were 22 subspecies which were capable of acquiring both plasmids (i.e., pBC16 and pC194). The frequencies of transfer for pBC16 ranged from 2.1×10^{-9} for transfer into subspecies *pakistani* to 9.8×10^{-5} for transfer into

Table 9. Frequencies of transfer of plasmid pBC16 in various subspecies of *Bacillus thuringiensis*.

Frequency of transfer[a]							
High[b]		Moderate[c]		Low[d]		No transfer[e]	
finitimus	(9.8×10^{-5})	*thuringiensis*	(1.1×10^{-7})	*kenyae*	(6.5×10^{-9})	*tochigiensis*	
ostrinae	(5.5×10^{-5})	*kurstaki*	(1.3×10^{-6})	*kyushuensis*	(2.5×10^{-8})	*caucasicus*	
entomocidus	(1.3×10^{-5})	*dendrolimus*	(1.2×10^{-7})	*dakota*	(6.0×10^{-8})		
		sotto	(1.7×10^{-7})	*tohokuensis*	(2.4×10^{-8})		
		morrisoni	(7.7×10^{-6})	*kumamotoensis*	(7.8×10^{-8})		
		toumanoffi	(2.3×10^{-7})	*Pakistani*	(2.1×10^{-9})		
		thompsoni	(4.8×10^{-6})	*galleriae*	(1.1×10^{-8})		
		subtoxicus	(2.5×10^{-6})	*tolworthi*	(7.7×10^{-8})		
		darmstadiensis	(1.1×10^{-7})	*indiana*	(2.5×10^{-8})		
		subage yunnanensis	(2.2×10^{-7})				
		wuhanensis	(2.2×10^{-7})				

[a] The frequencies of transfer are listed in parentheses after the name of the subspecies. Each number was the averaged from 3 independent experiments.

[b] Frequencies of transfer greater than 1×10^{-5}.

[c] Frequencies of transfer between 1×10^{-6} to 1×10^{-7}.

[d] Frequencies of transfer less than 1×10^{-8}.

[e] No transconjugant was detected when 1×10^{-9} cells of donors and recipients were plated on NA plates containing tetracycline and rifampicin.

Table 10. Frequencies of transfer of plasmid pC194 in various subspecies of *Bacillus thuringiensis*.

Frequency of transfer[a]				
Moderate		Low		No transfer
thuringiensis	(1.6×10^{-6})	*sotto*	(9.5×10^{-8})	*pakistani*
finitimus	(6.8×10^{-7})	*morrisoni*	(4.6×10^{-8})	
tochigiensis	(1.3×10^{-7})	*toumanoffi*	(1.8×10^{-8})	
kurstaki	(2.3×10^{-7})	*subtoxicus*	(6.2×10^{-9})	
dendrolimus	(2.5×10^{-7})	*dakota*	(4.3×10^{-8})	
kenyae	(1.1×10^{-7})	*tohokuensis*	(1.7×10^{-8})	
ostrinae	(2.6×10^{-7})	*darmstadiensis*	(1.0×10^{-8})	
caucasicus	(4.3×10^{-7})	*indiana*	(4.8×10^{-9})	
kyushuensis	(1.5×10^{-6})			
thompsoni	(6.3×10^{-7})			
entomocidus	(6.0×10^{-7})			
kumamotoensis	(3.8×10^{-7})			
subage yunnanensis	(1.9×10^{-7})			
wuhanensis	(9.0×10^{-7})			
galleriae	(4.0×10^{-7})			
tolworthi	(1.1×10^{-7})			

[a] See details in Table 9.

subspecies *finitimus*. The frequency of transfer for pC194 ranged from 4.8×10^{-9} in subsp. *indiana* to 1.6×10^{-6} in subsp. *thuringiensis*. The members of group III were subsp. *thuringiensis*, *finitimus*, *kurstaki*, *dendrolimus*, *sotto*, *kenyae*, *ostrinae*, *morrisoni*, *caucasicus*, *toumanoffi*, *kyushuensis*, *thompsoni*, *entomocidus*, *subtoxicus*, *dakota*, *tohokuensis*, *kumamotoensis*, *darmstadiensis*, *subage yunnanensis*, *wuhanensis*, *galleriae*, *tolworthi* and *indiana*.

The ability to transfer the relevant plasmids was confirmed by analysis of the plasmid patterns of the various transconjugants. Fig. 10 shows the plasmid profiles of the representative mating pairs of donor strains, recipients, and transconjugants. Plasmid patterns from the randomly selected transconjugants revealed clearly that the plasmids pBC16, and/or pC194 had been transferred to the various recipient subspecies. However, except for subsp. *finitimus* and *ostrinae*, the rates of transfer of plasmids in all the subspecies were relatively low. The frequency of transfer for plasmids between strains within subspecies, i.e., between *B.t.i.* and *B.t.i.*, were found to be 1.6×10^{-4} and 1.5×10^{-5} for pBC16 and pC194, respectively. This was comparable to the transfer between the subsp. *israelensis* and *ostrinae*. Interestingly, the plasmids pBC16 and pC194 were either independently transferred or cotransferred into different subspecies, and large plasmids of recipients did not seem

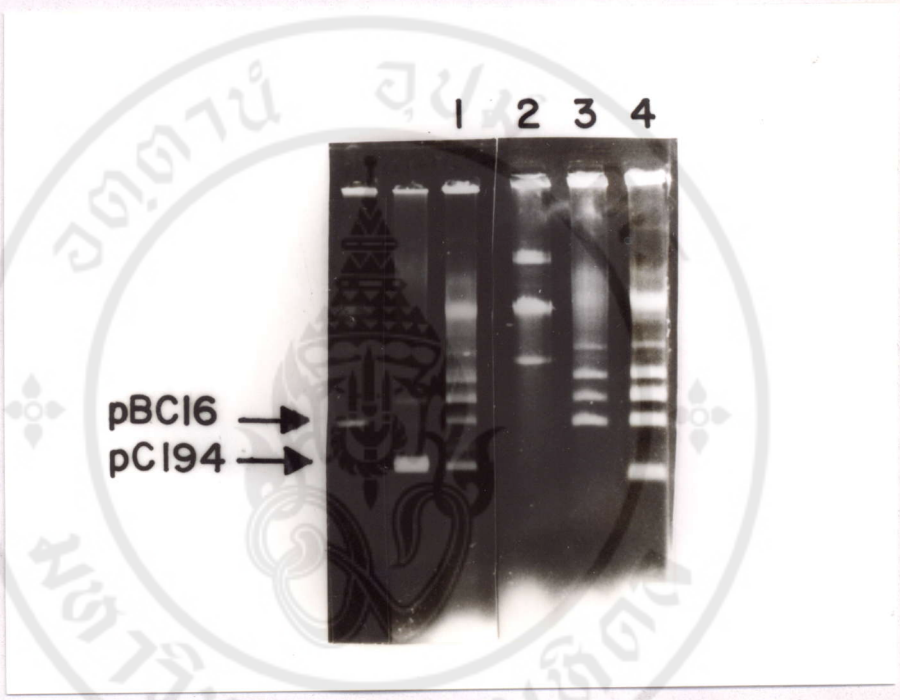


Fig. 10 Agarose gel electrophoresis of plasmid DNA extracts from a donor, *B.t.i.* A084-16-194 (lane 1); a recipient, *B.t. sotto*, (lane 2); a transconjugant that received pBC16 (lane 3), and a transconjugant that received pC194 (lane 4). Bands for pBC16 and pC194 are marked with arrows. The position of pBC16 and pC194 were obtained from extracts of *B. cereus* GP7 and *B. subtilis* HVS62, respectively.

to play an important role in acquisition of these two drug resistant plasmids because recipients harboring either small or large plasmids acquired plasmids pBC16 and/or pC194. Furthermore, *B. thuringiensis* subsp. *entomocidus*, which lacked plasmids, could acquire both pBC16 and pC194 at transfer frequencies of 1.3×10^{-5} and 6.0×10^{-7}, respectively.

On the basis of plasmid aquisition frequency, the strain studied could also be categorized into three groups; namely, those with a high frequency of transfer (greater than 1×10^{-5}), those with a moderate frequency of transfer (between 10^{-6} to 10^{-7}) and those with a low frequency of transfer (less than 1×10^{-8}). Using this classification for acquisition pBC16, the subspecies which fell in the high frequency group included subsp. *finitimus*, *ostrinae*, and *entomocidus*. Those in the moderate frequency group were subsp. *thuringiensis*, *kurstaki*, *dendrolimus sotto*, *morrisoni*, *toumanoffi*, *thompsoni*, *subtoxicus*, *darmstadiensis*, *subage yunnanensis* and *wuhanensis*. Those in the low frequency group were subspecies *kenyae*, *kyushuensis*, *dakota*, *tohokuensis*, *kumamotoensis*, *pakistani*, *galleriae*,*tolworthi* and *indiana*.

The frequency of acquisition plasmid pBC16 was found to be higher than that of pC194 plasmid. There were sixteen subspecies (i.e., *thuringiensis*, *finitimus*, *tochigiensis*, *kurstaki*, *dendrolimus*, *kenyae*, *ostrinae*, *caucasicus*, *kyushuensis*, *thompsoni*, *entomocidus*,

kumantoensis, subage yunnanensis, wuhanensis, galleriae, and tolworthi) which acquired plasmid pC194 at a moderate frequency of transfer, while there were only eight subspecies (*sotto, morrisoni, toumanoffi, subtoxicus, dakota, tohokunensis, darmstadiensis and indiana*) which acquired pC194 at low frequency of transfer.

2.2 Transfer of chromosomal DNA

When attempts were made to detect the transfer of chromosomal markers, it was found that transconjugants acquiring the chromosomal marker Str^r from the donor, *B. thuringiensis* subsp. *israelensis* A084-16-194 could be selected on nutrient agar plates containing streptomycin at 40 ug/ml and rifampicin at 50 ug/ml. Spontaneous mutation was ruled out by the inability to detect similar colonies after plating only donor or recipient cells on the same media.

Table 11 showed that ten *B. thuringiensis* subspecies acquired chromosomal DNA from the donor strain. The frequency of transfer was found to be very low and ranged from 4.3×10^{-9} in subsp. *wuhanensis* to 3.7×10^{-7} in subsp. *kurstaki*. There was no mating pair which was found to transfer solely the chromosomal marker. In all cases where the chromosomal marker was transferred, both plasmids pBC16 and pC194 were also transferred.

Table 11. Transfer of chromosomal marker (streptomycin and rifampicin resistant) in *Bacillus thuringiensis*.

Frequency of transfer[a]				
Moderate		Low		No transfer
thuringiensis	(1.0×10^{-7})	*finitimus*	(9.3×10^{-9})	*tochigiensis*
kurstaki	(3.7×10^{-7})	*sotto*	(1.7×10^{-8})	*dendrolimus*
subage yunnanensis	(2.7×10^{-7})	*toumanoffi*	(4.7×10^{-9})	*kenyae*
		thompsoni	(4.0×10^{-8})	*ostrinae*
		kumamotoensis	(5.5×10^{-8})	*morrisoni*
		darmstadiensis	(1.0×10^{-8})	*caucasicus*
		wuhanensis	(4.3×10^{-9})	*kyushuensis*
				entomocidus
				subtoxicus
				dakota
				tohokuensis
				pakistani
				galleriae
				tolworthi
				indiana

[a] See details in Table 9.

2.3 Transfer of plasmids pBC16 and pC194 between and within subspecies

Eight transconjugants which resulted from mating between *B. thuringiensis* subsp. *israelensis* A084-16-194 and subspecies *finitimus*, *sotto*, *ostrinae*, and *toumanoffi* were selected as representative donor strains. These harbored the plasmids pBC16 or pC194 (Fig. 11), and they were used to investigate the frequency of plasmid transfer between subspecies and within subspecies. In all mating pairs, streptomycin-resistant mutants of the corresponding subspecies strains were selected and employed as recipient cells.

The frequency of transfer within subspecies for these five different subspecies ranged from 2.2×10^{-7} to 1.6×10^{-4} (Table 12). Comparison with the transfer rate between subspecies (Tables 9 to 12) indicated that mating within subspecies gave a higher frequency of transfer than did mating between subspecies. However, there were two exceptions. The frequency of transfer of pBC16 within the subspecies *ostrinae* and *finitimus* was lower than the frequency of transfer between them.

Fig. 11 Agarose gel electrophoresis of plasmid DNA extracts from a recipient, *B.t.finitimus* (lane 1); a donor with pBC16, *B.t.finitimus*-pBC16 (lane 2); a transconjugant that received pBC16 (lane 3); a donor with pC194 (lane 4); a transconjugant that received pC194 (lane 5). The positions of pBC16 and pC194 are marked with arrows.

Table 12. Frequency of transfer of plasmids pBC16 and pC194, among the intrasubspecific mating pairs.

Mating pair	Frequency of transfer pBC16	Frequency of transfer pC194
B.t.fin - pBC16 x *B.t.fin*	2.2×10^{-7}	-
B.t.fin - pBC194 x *B.t.fin*	-	4.0×10^{-6}
B.t.sot - pBC16 x *B.t.sot*	1.8×10^{-5}	-
B.t.sot - pBC194 x *B.t.sot*	-	1.6×10^{-6}
B.t.ost - pBC16 x *B.t.ost*	8.7×10^{-6}	-
B.t.ost - pBC194 x *B.t.ost*	-	2.1×10^{-5}
B.t.tou - pBC16 x *B.t.tou*	9.2×10^{-6}	-
B.t.tou - pBC194 x *B.t.tou*	-	4.0×10^{-6}
B.t.i A084-16-194x *B.t.i*	1.6×10^{-4}	1.5×10^{-5}

3. Effect of various chemicals on conjugation-like gene transfer process between *B. thuringiensis* subsp. *israelensis* strain A084-16-194 and c4Q272.

Earlier studies indicated the high frequency in transferring plasmid pBC16 from A084-16-194 to c4Q272 via conjugation-like procedure in 0.05 M phosphate buffer. Previous experiment by Chapman and Carlton (118) indicated that this transfer required cell to cell contact. Attempts were made to investigate the nature of cell interaction by the addition of wide range of chemicals. Thus, the experiments for studying the effect of various chemicals at various concentrations were performed in 0.05 M phosphate buffer pH 7.0 supplemented with stock solution of each chemical to obtain the desirable concentrations as shown in Tables 13 to 16.

Experiments using EDTA, the metal chelating agent, at concentrations from 0.05 mM to 0.4 mM indicated that EDTA at 0.2 mM could inhibit (frequency of transfer less than 10^{-7}) the transfer of plasmid pBC16 between *B.t.i.* strain A084-16-194 and c4Q272 as shown in Table 13. Furthermore, it was interesting to note that EDTA at concentration 0.2 mM could also reduce the extent of clumping from ++++ to +++ as shown in Table 13.

For other inorganic substances, results from a number of experiments indicated that the acquisition of plasmid pBC16 by c4Q272 were found to be inhibited at 0.25 M of $MgCl_2$ (Table 14), 0.5 M of $MgSO_4$ (Table 15), 0.05 M

Table 13. Effects of EDTA on clumping and transfer of pBC16 plasmid from *B. thuringiensis* subsp. *israelensis* strain A084-16-194 to c4Q272 via conjugation-like process.

Concentration of EDTA	Freq. of transfer	Degree of Clumping
0.05 mM	5.8×10^{-5}	++++
0.1 mM	5.1×10^{-7}	+++
0.2 mM	0 (Less than 1×10^{-7})	+++
0.4 mM	0 (Less than 1×10^{-7})	+++
Control (without EDTA)	2.5×10^{-5}	++++

Table 14. Effects of $MgCl_2$ on clumping and transfer of pBC16 plasmid from *B. thuringiensis* subsp. *israelensis* strain A084-16-194 to c4Q272 via conjugation-like process.

Concentration of $MgCl_2$ (M)	Freq of transfer	Degree of Clumping
0.05	2.0×10^{-4}	++
0.075	1.1×10^{-5}	++
0.1	2.3×10^{-5}	+
0.25	0 (Less than 1×10^{-7})	+
0.5	0 (Less than 1×10^{-7}	+
Control	1.2×10^{-4}	++++

Table 15. Effects of $MgSO_4$ on clumping and transfer of pBC16 plasmid from *B. thuringiensis* subsp. *israelensis* strain A084-16-194 to c4Q272 via conjugation-like process.

Concentration of $MgSO_4$ (M)	Freq. of transfer	Degree of Clumping
0.05	2.2×10^{-3}	++
0.075	1.0×10^{-3}	++
0.10	2.8×10^{-4}	+
0.25	9.5×10^{-6}	+
0.50	0 (less than 1×10^{-7})	-
Control	6.2×10^{-4}	++++

of $CaCl_2$ (Table 16). In addition, 0.25 M of $MgCl_2$ could reduce the extent of clumping to + (Table 14), and 0.5 M $MgSO_4$ could totally inhibit clumping as shown in Table 15. Addition of $CaCl_2$ into the mixture of mating pair at high concentration caused turbidity of the mixtures, therefore we could not determine the clumping. Although high concentrations of these chemicals were found to inhibit the transfer of plasmid, it was noted that the viability of either the donor or recipient were not effected by this high level of salt concentrations. Thus, it appeared that the high level of these chemicals might interfere with the transferring of the plasmids via conjugation-like process.

4. Cell aggregations between various strains of *B. thuringiensis* subsp. *israelensis*

During experiments on conjugation-like gene transfer process, it was observed that when certain pairs of *B.t.i.* were mixed together, cell aggregation was observed (Fig. 12). The nature of cell aggregation could be observed by having the mixed culture stand at room temperature for few minutes. The aggregate of cells precipitated to the bottom leaving clear suspension at the top portion of the cell mixture whereas, the non-aggregate mixture still appeared homogenously turbid. The mutant strains derived from the same wild type strain (4Q2) can be divided into two groups, namely, clumping group I (composed of strains 4Q2) and clumping group II (composed

Table 16. Effect of $CaCl_2$ on the transfer of pBC16 plasmid from *B. thuringiensis* subsp. *israelensis* strain A084-16-194 to c.4Q272 via conjugation-like process.

Concentration of $CaCl_2$ (M)	Freq. of transfer
0.01	2.7×10^{-4}
0.025	1.2×10^{-5}
0.05	0 (Less than 1×10^{-7})
Control	2.9×10^{-4}

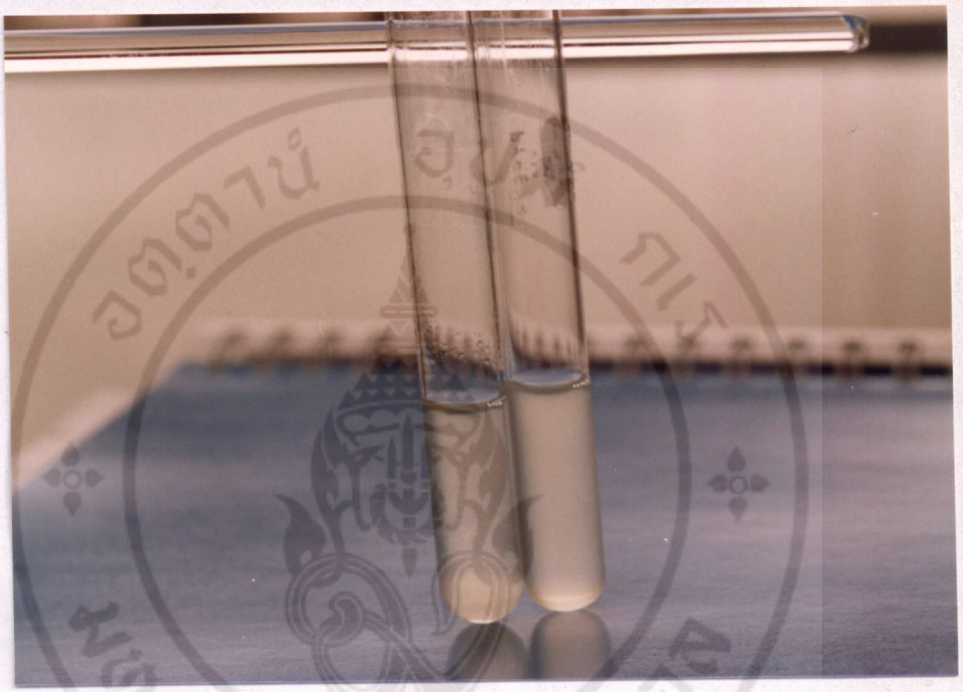


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Fig.12 Clumping between *B. thuringiensis* subsp. *israelensis* strains. Two cultures of appropriate strains; 4Q2 and c4Q272 (Tube 1), 4Q272 and c4Q272 (Tube 2), were mixed in 0.05 M phosphate buffer and left standing for 5 minutes to allow cell clumping to occur.

of strains 4Q2-72 and c4Q272). When *B.t.i.* strains from clumping group I were mixed with those from clumping group II clumping occurred; however, there was no clumping when the strains from the same grouping were mixed together. There was no self clumping in all the strains used in these experiments. The data as summarized in Table 17 indicated that there might be a common factor(s) present (or absent) from strain 4Q2-72 and c4Q2-72 during the curing experiment (46) which might promote the clumping phenomenon.

There was no appearance of clumping when mixing the culture filtrate of strain 4Q2 with cells of strain 4Q272 or c4Q272. It was also found that there was no appearance of clumping when mixing culture filtrate of strain 4Q2 with culture filtrate of strain 4Q272 or c4Q272 or when mixing culture filtrate of strain 4Q272 or c4Q272 with cells of strain 4Q2 or c4Q272 as shown in Table 18. Therefore, there appeared to be requirement(s) for the presence of two different types of cells for initiation of clumping phenomenon and this factor(s) was not present in the culture filtrate. Moreover, the growth of cells in different culture media; LB-broth, TSB, NB and BHI, did not appear to affect the ability of cells to clump (Table 19).

Table 17. Clumping between various strains of *B. thuringiensis* subsp. *israelensis*.

B.t.i. strain	4Q2	4Q2-72	c.4Q2-72
4Q2	-	++++	++++
4Q2-72	++++	-	-
c.4Q2-72	++++	-	-

Table 18. Effect of cell and/or culture fluid on cell clumping between various strains of *B.thuringiensis* subsp.*israelensis*.

Cell	Mixture supernatant	Degree of clumping 0.05 M Phosphate buffer	NSS*
4Q2 and c4Q272		++++	++++
4Q2 and 4Q272		++++	++++
c4Q272 and 4Q272		-	-
	4Q2 and c4Q272	-	-
	4Q2 and 4Q272	-	-
	c4Q272 and 4Q272	-	-
4Q2	c4Q272	-	-
4Q2	4Q272	-	-
c4Q272	4Q272	-	-

* NSS is 0.85% sodium chloride in distilled water.

Table 19. Effect of growth medium on cell clumping between various strains of *B. thuringiensis* subsp. *israelensis*.

Mixture	Growth medium	degree of clumping
4Q2 x 4Q272	LB broth	++++
	BHI	++++
	NB	++++
	TSB	++++
4Q2 x c.4Q272	LB broth	++++
	BHI	++++
	NB	++++
	TSB	++++
c.4Q272 x 4Q272	LB broth	-
	BHI	-
	NB	-
	TSB	-

5. Penicillin susceptibility

It has been previously reported that most wild type strains of *B. thuringiensis* possessed resistances to penicillin (see ref. in Table 8). There were also reports that the presence or absence of S-layer protein lead to the difference in the susceptibility to lysozyme or protease (119, 123, 159, 172). *B.t.i.* strains 4Q2, 4Q272, and c4Q272 were tested for their ability to resist to penicillin. The penicillin susceptibility test using disk diffusion method (219) and broth dilution method (220) demonstrated that the wild type strain 4Q2 was found to be highly resistant to penicillin G, i.e. minimal inhibitory concentration (MIC) of 125,000 ug (Fig. 13 and Table 20). However, strains derived from 4Q2, i.e. strains 4Q2-72, and c4Q2-72 were found to be quite susceptible to penicillin at very low concentration with MIC of 0.06, and 0.03 ug, respectively as illustrated in Fig. 13 and Table 20.

6. Localization of S-layer protein on *B. thuringiensis* subsp. *israelensis*

6.1 Isolation and purification of S-layer protein

The clumping phenomenon as well as the difference in the susceptibility to penicillin G in various strains of *B.t.i.* has lead to the possibility of the presence or absence of S-layer protein(s) of these strains. All these evidences have lead to the possibility of the presence of

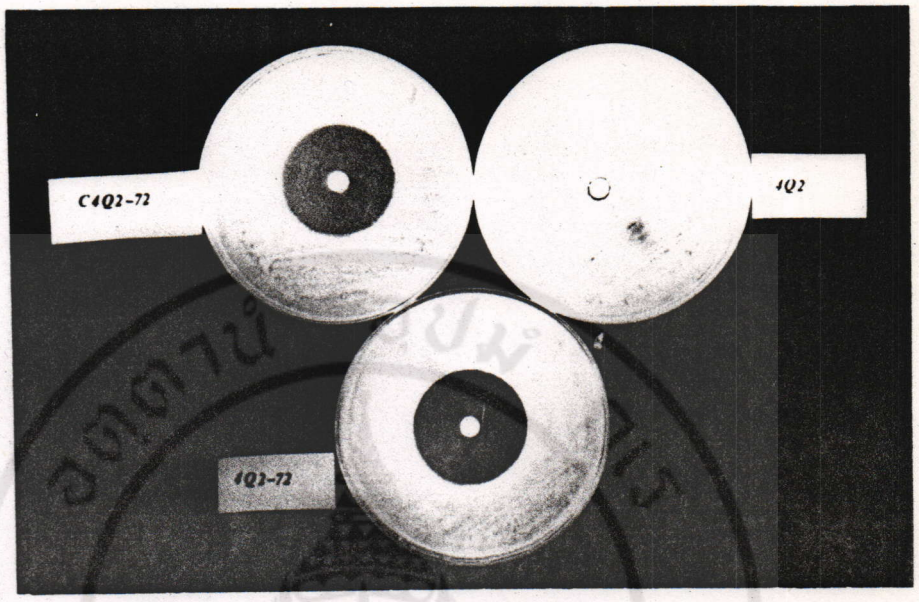

Fig. 13 Penicillin G susceptibility of various strains of *B.thuringiensis* subsp.*israelensis* determined by Disk Diffusion method. The cultures 4Q2, 4Q272, and c4Q272 were spreaded on Mueller-Hinton agar plates, penicillin G disks were placed on the spreaded plates and incubated overnight. This figure showed inhibition zones surrounded strains 4Q272 and c4Q272 (penicillin G susceptible), and no inhibition zone surrounded strain 4Q2 (penicillin G resistant).

Table 20. Minimal inhibition concentration of penicillin G to the various strains of *B. thuringiensis* subsp. *israelensis*.

Strains of *B.t.i.*	MIC (ug)
4Q2	12,500.00
4Q272	0.06
c4Q272	0.03

S-layer protein in *B.t.i.* strain 4Q2. As such, attempts were made to elucidate the presence of S-layer protein in *B.t.i.* strain 4Q2. To determine the appropriate conditions for extraction, 4 liters of culture *B.t.i.* strain 4Q2 or 4Q272 were extracted by various denaturants, i.e. 6 M Urea, 5 M Guanidine hydrochloride and 0.05 M Tris-hydrochloride pH 8.0, the detailed procedures for extraction were as described in Materials and Methods. The crude extracts were subjected to SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). The results demonstrated that, extractions of strain 4Q2 with 6M Urea, 5M Guanidine hydrochloride, and 0.05 mM Tris hydrochloride lead to the presence of high molecular weight protein bands on SDS-PAGE. Whereas, when the same extraction procedures were performed on strain 4Q272, the high molecular weight protein band was absent (Table 21). However, extraction of the strain 4Q2 by 5 M Guanidine hydrochloride or 0.05 M Tris hydrochloride produced lower yields than 6 M urea (estimated from SDS-PAGE; by using the same volume of sample originated from the same volume of culture). Thus, further isolation procedure was achieved by using 6 M urea.

SDS-PAGE patterns of the urea extracted protein from strains 4Q2 and 4Q272 were shown in Figs 14.1 and 14.2 indicated that there were some distinct differences in the protein patterns of these strains. The slowest moving and predominant band was presented only in the

Table 21. Extraction of S-layer protein of *B. thuringiensis* subsp. *israelensis* by various chemical agents.

Chemical agent	*B.t.i.* strain	
	4Q2	4Q272
6.0 M Urea	+	-
5 M Guanidine hydrochloride	+	-
50 mM Tris hydrochloride	+	-

\+ = Presence of S-layer protein in extraction as determined by SDS-PAGE.

\- = Absence of S-layer protein in extraction as determined by SDS-PAGE.

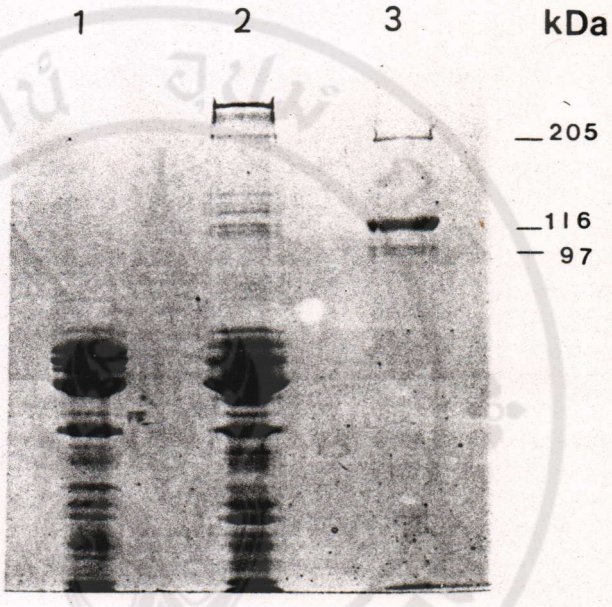

Fig. 14.1 SDS-PAGE patterns of protein extract of *B. thuringiensis* subsp. *israelensis*. The crude protein extracts of strain 4Q272 (lane 1), and the crude protein extract of strain 4Q2 (lane 2) were prepared and subjected to SDS - PAGE. Lane 3 represented as protein molecular weight standards, myosin (205 kDa), β-galactosidase (116 kDa), and phosphorylase b (97 kDa).

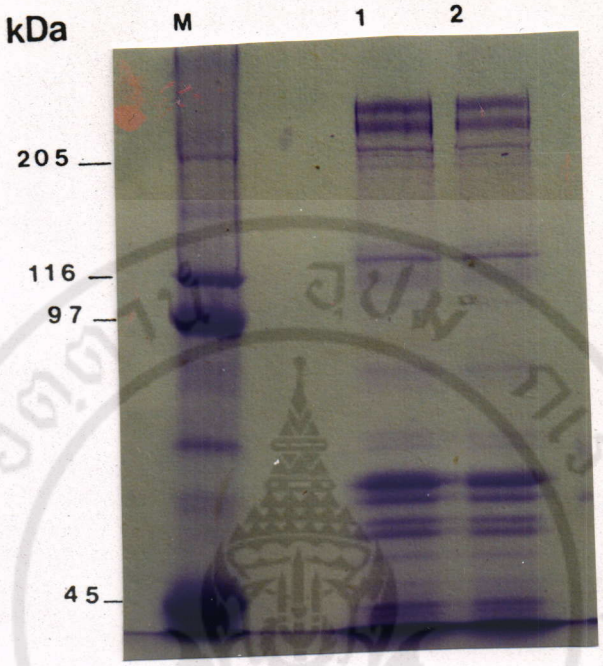

Fig. 14.2 SDS-PAGE patterns of protein extract of *B. thuringiensis* subsp. *israelensis* strain 4Q2. The crude protein extract of strain 4Q2 (lane 1 and lane 2) were prepared and subjected to SDS-PAGE. The lane marked M represented as protein molecular weight standards as myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa),oval albumin (45 kDa).

protein extracted of *B.t.i.* strain 4Q2 which is the wild type strain, but was absent in the protein extract of the strain 4Q272.

The presumed S-layer protein was purified by cutting the band from several gels after staining and destaining, and were collected and eluted by electro-eluter (BioRad) as described in Materials and Methods. The purity was confirmed by another run of SDS-PAGE, and the single protein band on SDS-PAGE was obtained as shown in Fig. 15. This purified S-layer protein was concentrated by lyophilization and used for immunization of New Zealand white rabbits.

6.2 Molecular weight determination

Molecular weight of S-layer protein of *B.t.i.* strain 4Q2 was determined from SDS-PAGE by comparing with standard proteins, namely myosin (205,000), β-galacto-sidase from *E. coli* (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000). These standard markers were obtained from Sigma. The molecular weight of 4Q2 S-layer protein was estimated to be approximately 260,000 (Figs. 14.1 and 14.2). Because the 4Q2-S-layer protein was higher than myosin (205,000), the molecular weight was calculated by extrapolating the standard curve. The method of extrapolation might lead to substantial inaccuracy for determination of the mobility of the high molecular weight proteins. Several attempts were made to compare the 4Q2 S-layer protein with the

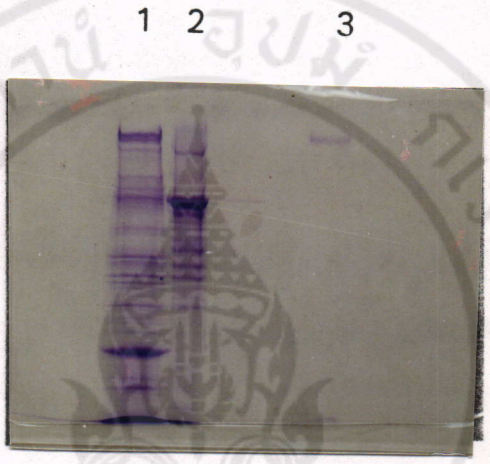

Fig.15 SDS-PAGE patterns of purified S-layer protein from *B. thuringiensis* subsp. *israelensis* strain 4Q2. The crude protein extract of strain 4Q2 (lane 1), and purified S-layer protein from *B. thuringiensis* subsp. *israelensis* strain 4Q2 (lane 3) were prepared and subjected to SDS-PAGE. Lane 2 represented as protein molecular weight standards, myosin (205 kDa), β-galactosidase (116 kDa), and phosphorylase b (97 kDa).

higher molecular weight standards, using the high molecular weight calibration kit (Pharmacia) and cross-linked phosphorylase b (Sigma), but it was not successful. There seems to be several degradative bands of the thyroglobulin (669,000) and ferritin (440,000), so that the mobility of the subunit of the highest molecular weight band (330,000) appeared to migrate to the same mobility of myosin (205,000). Thus, it might be more accurate to extrapolate the standard curve of various molecular weights.

The carbohydrate composition of various S-layer proteins were reported (140-143, 145-149). To determine the carbohydrate composition the S-layer protein of *B.t.i.* strain 4Q2, SDS-PAGE of S-layer protein extract was subjected to PAS staining technique as described in Materials and Methods. The result revealed that S-layer was found to be glycoprotein because a pink color band was observed at the same position of migration with the S-layer protein band (Fig. 16). However, the positive signal was not strong.

6.3 Anti S-layer protein antibody of strain 4Q2

The antibody against the 260 kDa protein was obtained as described in Materials and Methods. The presence of a specific antibody was demonstrated by Ouchterlony immunodiffusion technique. There was the appearance of the precipitin line between the S-layer protein of the strain 4Q2 (purified protein and crude

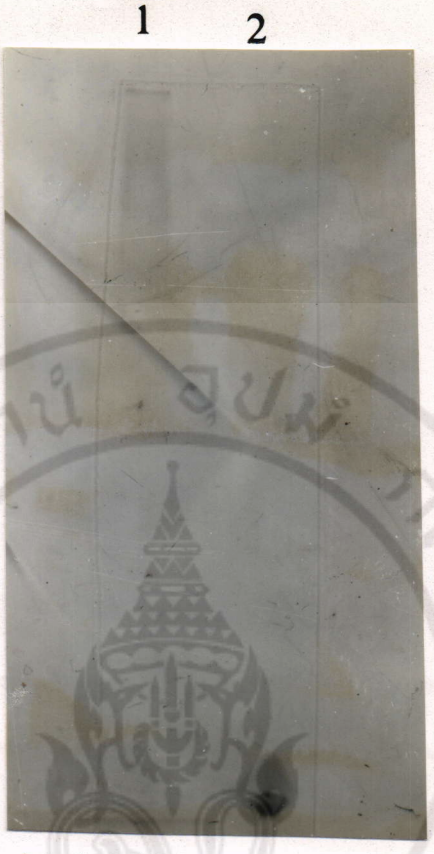

Fig. 16 Periodic Acid-Schiff staining of protein extract of *B.thuringiensis* subsp.*israelensis*. The crude protein extract of strain 4Q2 (lane 1) and oval albumin with M.W. of 45,000 (lane 2) were subjected to SDS-PAGE . Subsequently, the gel was subjected to Periodic Acid-Schiff staining after the protein were subjected to SDS-PAGE by using slab gel electrophoresis (Hoeffer Scientific Instruments) at constant power 25 mA for 3 hr.

extracted with urea) and its anti S-layer protein. There was no reaction between anti-S-layer protein and the protein extract of the strain 4Q272 and c4Q272 as shown in Fig. 17.

The antiserum was purified by precipitation with 50% ammonium sulfate, and DEAE-cellulose column to obtain the immunoglobulin G (IgG). The presence of IgG was demonstrated by immunoelectrophoresis as shown in Fig. 18.

6.4 Identification of S-layer protein on *B. thuringiensis* subsp. *israelensis* cell using immunofluorescent technique.

Antibody toward the 260 kDa protein was used for locating of S-layer protein on *B.t.i.* by using indirect immunofluorescent technique, which demonstrated that the S-layer protein was located on the outer most part of the strain 4Q2 (Fig. 19). The polyclonal antibody, whole serum and purified IgG against S-layer protein of the strain 4Q2 could interact with the antigenic determinant on the cell surface and then interact with Fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulins reveals the fluorescent bacteria under the fluorescent microscope. The control experiment was performed by using the preimmunized rabbit serum instead of the immunized rabbit serum, and there appeared to be no specific reaction with the conjugated antibody as shown in Fig. 19.

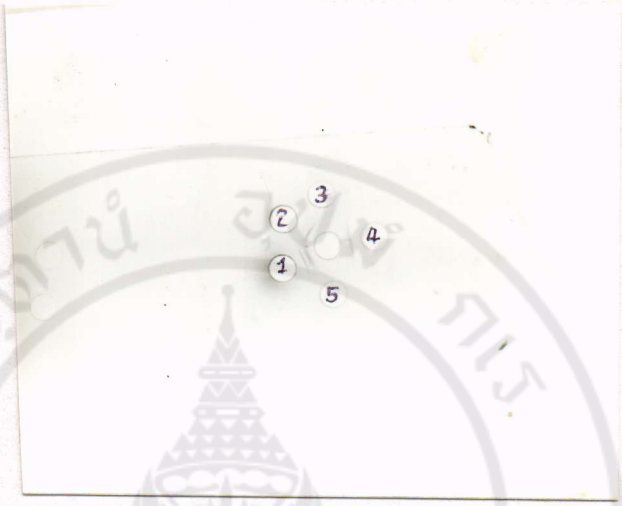

Fig. 17 Immunodiffusion reaction between anti-4Q2-S-layer protein antibody (central well) and protein extracts from *B. thuringiensis* subsp.*israelensis* strains A084 (1), 4Q2 (2), purified S - layer protein of strain 4Q2 (4), crude protein extract of strains 4Q272 (3) and c4Q272 (5). The result showed the precipitin lines between anti- 4Q2-S-layer protein antibody and *B.thuringiensis* subsp.*israelensis* strains A084, crude protein extract of strain 4Q2, purified S-layer protein of strain 4Q2. There was no precicipitin line between anti-4Q2-S-layer protein antibody and protein extract of strains 4Q272 and c4Q272.

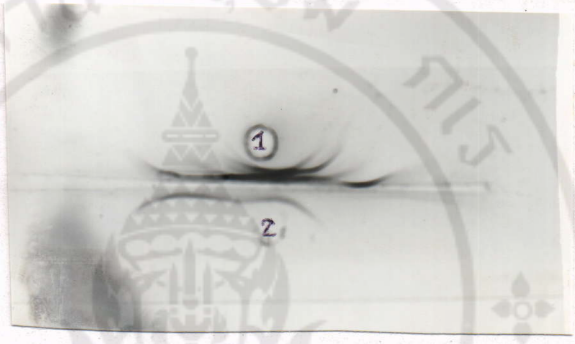

Fig. 18 Immunoelectrophoresis pattern of anti 4Q2-S-layer protein antibody after purified by DEAE-cellulose column. Anti- whole rabbit serum was applied in the trough. The figure showed the precipitin line between anti- whole rabbit serum and purified anti-4Q2 S-layer protein antibody (2) and precipitin lines between anti -whole rabbit serum and normal rabbit serum (1).

A

B

Fig. 19 Fluorescein-isothiocyanate staining of vegetative cell of *B.thuringiensis* subsp.*israelensis* strain 4Q2. The cells were stained with purified anti-4Q2 S-layer protein antibody as primary antibody (A) or preimmunized rabbit serum as primary antibody (B).

6.5 Effect of anti-4Q2-S-layer protein antibody on conjugation-like gene transfer process.

The antibody raised against S-layer protein of *B.t.i.* strain 4Q2 could be shown to inhibit the conjugation-like gene transfer process between *B.t.i.* strain 4Q2-16, harboring plasmid pBC16 and strain c4Q272-Rif^r. This mating experiment was demonstrated by detecting the transconjugants which acquired the plasmid pBC16. The control experiments, there appeared to be no different in the frequency of transfer of plasmid either in LB-broth ($1.6x10^{-6}$) or 0.05 M phosphate buffer ($9.7x10^{-6}$) as shown in Table 22.

Also, the mating mixture which pretreated the donor strain with preimmunized rabbit serum or heated anti-4Q2-S-layer protein antiserum only slightly reduced the transfer of pBC16 plasmid. The frequency of transfer was found to reduce to $1.9x10^{-6}$ and $0.1x10^{-6}$ with the treatment of donor strain with pre-immunized rabbit serum or heated anti-4Q2-S-layer protein antibody, respectively.

In addition, the study on the effect of anti-4Q2-S-layer protein antibody on the conjugation-like gene transfer process between *B.t.i.* strains A084-16-194 and c4Q272-Rif^r was carried out under the same conditions as in the previous experiment. The donor (A084-16-194) of this mating pair originated from different series of wild type than that of 4Q2. This mating experiment was also conducted by detection of the transconjugants which

Table 22. The effect of anti-4Q2 S-layer protein antibody on conjugation-like process between *B. thuringiensis* subsp. *israelensis* strains 4Q2-16 and c4Q2-72.

Condition of conjugation medium	donor	Freq. of transfer[a]
LB-broth	no treatment	1.6×10^{-6}
phosphate buffer	no treatment	9.7×10^{-6}
phosphate buffer	treated with pre-immunized rabbit serum	1.9×10^{-6}
phosphate buffer	treated with anti-4Q2 S-layer protein antibody	less than 10^{-8}
phosphate buffer	treated with heated anti-4Q2 S-layer protein antibody	0.1×10^{-6}

a) average of three independent experiments.

acquired the plasmid pBC16. The result showed that the antibody could reduce the frequency of transfer to the level of $1.2x10^{-6}$. The control experiments, mating in LB-broth or 0.05 M phosphate buffer showed that the plasmid pBC16 could be transferred at a frequency of $2.2x10^{-5}$ or $1.1x10^{-5}$ respectively (Table 23).

Pretreated donor, either with preimmunized rabbit serum or heated-4Q2-S-layer protein antiserum did not effect the frequency of transfer. In this procedure the plasmid pBC16 could be transferred at a frequency of transfer of $2.5x10^{-5}$ and $2.0x10^{-5}$ under the treatment with preimmunized rabbit serum or heated anti-4Q2 S-layer protein antibody, respectively. This frequency of transfer is similar to those of un-treated mating pairs.

6.6 Correlation of S-layer protein, cell clumping and penicillin susceptibility.

According to various experiments on the presence of S-layer protein on *B.t.i.* strain 4Q2 (Fig. 19), cell clumping between certain mating pair (Table 17), and penicillin G susceptibility test (Fig. 13), these evidences supported that there appeared to be some correlation between the presence of S-layer protein and the penicillin G resistance (Table 24). The *B.t.i.* strains 4Q2 possessed S-layer protein and was also resistant to penicillin G. Whereas strain 4Q272 and c4Q272 which did not possess S-layer protein were sensitive to penicillin G. The *B.t.i.* strains which were

Table 23. The effect of anti-4Q2 S-layer protein antibody on conjugation-like process between *B. thuringiensis* subsp. *israelensis* strains A084-16-194 and c4Q272.

Condition of conjugation medium	donor	Freq. of transfer[a]
LB-broth	no treatment	2.2×10^{-5}
phosphate buffer	no treatment	1.1×10^{-5}
phosphate buffer	treated with pre-immunized rabbit serum	4.1×10^{-5}
phosphate buffer	treated with anti-4Q2 S-layer protein antibody	1.2×10^{-6}
phosphate buffer	treated with heated anti-4Q2 S-layer protein antibody	2.0×10^{-5}

a) average of three independent experiments.

categorized into clumping group I namely, the strain 4Q2 also found to possess S-layer protein and could resist penicillin G. The *B.t.i.* strains which were categorized into clumping group II i.e. strains 4Q272, and c4Q272 were missing of S-layer protein. Such correlations were tabulated in Table 24.

7. Molecular cloning of S-layer protein gene *(slp)* from *B. thuringiensis* subsp. *israelensis* strain 4Q2

7.1 Screening of S-layer protein producing *E. coli* clones

Total DNA of *B.t.i.* 4Q2-Nal^r was digested partially by various restriction enzymes as described in Materials and Methods. The concentration of enzymes that could digest DNA fragments into approximately 3-10 kb fragments were selected for preparation the DNA fragments to be cloned (Fig. 20). The partially digested DNA preparations was prepared by adding with 6 units of *Pst*I/ug DNA, 6 units of *Hin*dIII/µg DNA, 10 units of *Eco*RI/ug DNA, 8 units of *Xba*I/µg DNA, 6 units of *Bam*HI/ug DNA or 2 units of *Sau*3A/µg DNA and was followed by incubating at 37°C for 15 min. The partially restricted DNA preparations were then selected and ligated with the vectors, pBluescript KS or pUC12 which were completely cut with the same enzymes. After ligation at 16°C overnight, the ligated products were transformed into *E. coli* DH5α and spread on LB-agar containing 100 ug/ml ampicillin

Table 24. Correlation between S-layer protein, cell clumping and penicillin susceptibility in various strains of *B. thuringiensis* supsp. *israelensis*.

Physiological properties	*B.t.i.* strains 4Q2	4Q2-72	c.4Q2-72
S-layer protein	+	-	-
Cell clumping	Gr. I	Gr. II	Gr. II
Pen G susceptibility	R	S	S

Fig. 20 Agarose gel electrophoresis patterns of partially digested DNAs of *B.thuringiensis* subsp. *israelensis* strain 4Q2-Nal^r. The DNA of *B. thuringiensis* subsp. *israelensis* strain 4Q2-Nal^r were restricted with enzyme *Pst*I (lane 1), *Hin*dIII (lane 2), *Eco*RI (lane 3), *Xba*I (lane 4), *Bam*HI (lane 5), *Sau*3A (lane 6). Lane 7 was Lamda DNA which was digested with *Hin*dIII.

which were previously incorporated with IPTG and X-gal. The white colonies which might contain the relevant inserts were selected for further analysis.

Approximately 20,000 white colonies of potential transformants with appropriate inserts were screened with the anti-4Q2 S-layer protein antibody and alkaline phosphatase conjugated with anti-rabbit-IgG. In order to avoid non specific interaction, the anti-4Q2-S-layer protein antibody was also absorbed with *E. coli* lysate prepared as described in Materials and Methods. Three positive clones designated pAC1 (Fig. 21), pAC2 and pAC3 were obtained from the ligated products of *Pst*I partially digested fragments and pBluescript KS vector (pAC1) or pUC12 vector (pAC2 and pAC3). The presence of S-layer protein cloned gene products were confirmed by Western blot analysis which gave the positive signal as shown in Fig. 22 by reacting with immunoenzyme reaction (anti 4Q2-S-layer protein antibody and alkaline phosphatase conjugated with anti-rabbit-IgG).

7.2 Restriction analysis of plasmid DNA of recombinant clones.

The plasmids designated pAC1, pAC2 and pAC3 were extracted and analysed. Plasmid DNA of pAC1 was completely digested with *Pst*I and yielded three fragments of 2.96 kb (pBluescript KS), 5.2 kb and 1.4 kb of inserted fragments (Fig. 23). Both plasmid DNA of pAC2 and pAC3 were completely digested with *Pst*I yielding two fragments

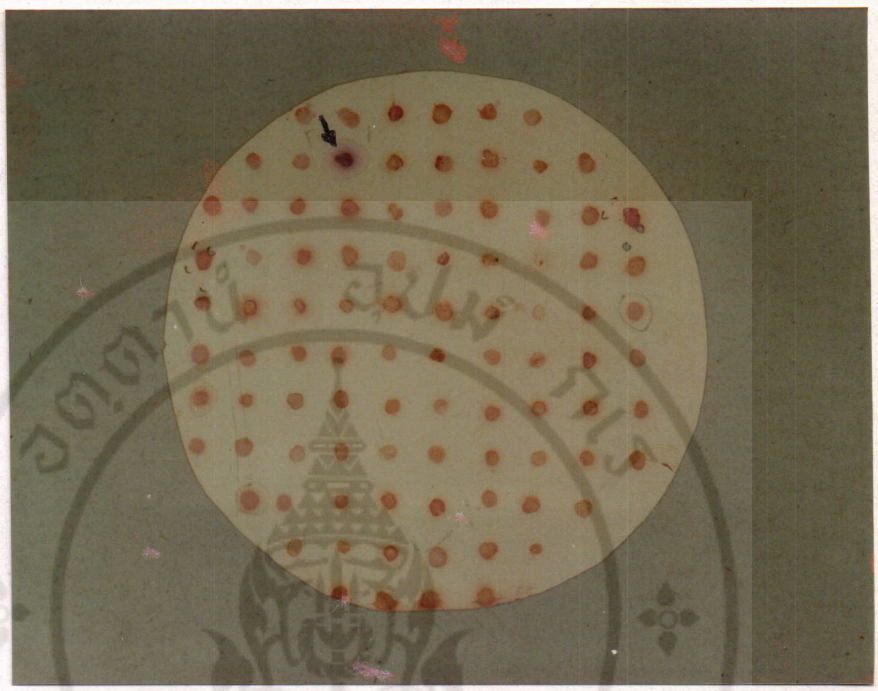

Fig. 21 Immunoscreening of S-layer protein producing in *E.coli* transformants . The transformants were detected by using anti-4Q2 S-layer protein antibody as primary antibody. The arrow indicated the positive clone, namely pAC1 containing 6.6 kb *Pst*I fragment of S-layer protein gene from *B. thuringiensis* subsp.*israelensis* strain 4Q2.

Fig. 22 Western blotting of protein extracts from *E. coli* DH5α transformants. Protein bands from *E. coli* harboring pUC12 vector (lanes 1 and 2), *E. coli* harboring pAC2 (lanes 3 and 4), *E.coli* harboring pAC3 (lanes 5 and 6), *E. coli* harboring pBluescript KS vector (lanes 7 and 8), and *E.coli* harboring pAC1 (lanes 9 and 10) were transferred onto nitrocellulose after electrophoresis on SDS-PAGE. And then detection of positive band was carried out by using anti - 4Q2 S-layer protein antibody as primary antibody.

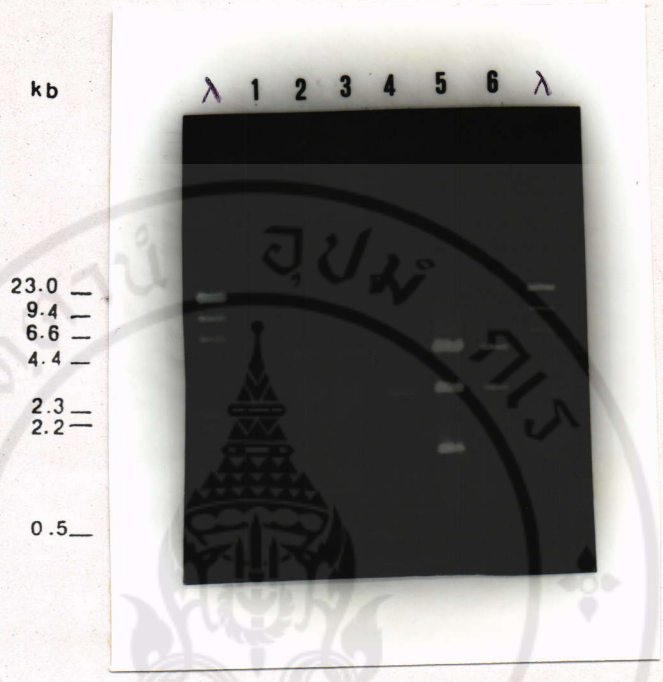

Fig. 23 Agarose gel electrophoresis patterns of various recombinant plasmids. Plasmids; pUC12 (lane 1), pAC2 (lane 2), pAC3 (lane 3), pBluescript KS (lane 4), pAC1 (lane 5), and pAC11 (lane 6) were digested with *Pst*I. The lanes marked λ . represented lamda DNA which was digested with *Hind*III.

of 2.7 kb (pUC12) and 5.2 kb of the inserted fragment (Fig. 23).

7.3 Subcloning of pAC1 with *PstI*.

There were two *PstI* fragments of the inserted DNA, 5.2 kb and 1.4 kb in the pAC1 recombinant plasmid. Therefore, the 5.2 kb fragment was recovered from agarose gel electrophoresis, purified, re-ligated into pBluescript KS at *PstI* site, and transformed into *E. coli* DH5α host. The clones were selected on LB-agar containing ampicillin 100 ug/ml which was previously spreaded with IPTG and X-gal. The white transformants were selected for further analysis. Plasmid DNAs of 10 white transformants were extracted, digested with *PstI*, and subjected to agarose gel electrophoresis. The clone containing 5.2 kb insert was selected and designated pAC11. The expression of the pAC11 gene product was confirmed by Western blot analysis as shown in Fig. 24. It demonstrated that, this gene encoded protein which was similar to the original clone (pAC1) with reference to the identical position on SDS-PAGE.

In addition, when the clones were grown in LB-broth without induction by IPTG, it could be demonstrated that the clones still produced the gene product which could react with our test system (Fig. 24).

However, the molecular weight of protein of the pAC11 gene product producing in *E. coli*, was lower than the original S-layer protein in *B.t.i.* strain 4Q2. The

Fig. 24 Western blotting of protein extracts of *E. coli* DH5$^{\alpha}$ harboring various recombinant plasmids. Protein extracts of *E. coli* harboring pBluescript KS vector (lane 1), *E.coli* harboring pAC1 (lanes 2 and 3), *E. coli* harboring pAC11 which was induced with IPTG (lanes 4 and 5), and *E. coli* harboring pAC11 which was not induced with IPTG (lanes 6 and 7) were blotted onto nitrocellulose and then detection for positive band was carried out by using anti- 4Q2 S-layer protein antibody as primary antibody.

molecular weight of gene product in *E. coli* was approximately 160 kDa (Figs. 22, 24, 25). Using dot blot and Western blot technique, it could be shown that the protein encoding by the recombinant clone reacted with anti-4Q2-S-layer protein antibody. The antibody was found to be specific to react to the high molecular weight protein in strain 4Q2, since this antibody did not react with any of the protein band of strain 4Q272 as shown in Figs. 26.1 and 26.2.

7.4 Restriction analysis of plasmid pAC11

In order to determine restriction map of plasmid pAC11, various enzymes were used to digest the plasmid and the restriction fragments were characterized by agarose gel electrophoresis. The plasmid had one cutting site located on multicloning sites with enzymes *Apa*I, *Xho*I, *Not*I, *Bam*HI and *Kpn*I, but contain multiple cutting sites for enzymes *Eco*RI, *Hin*dIII, *Pvu*II, *Hinc*II and *Pst*I. Various combinations of enzyme digestion were used, and restriction patterns resulting from single and double enzymes digestion are shown in Fig. 27. Molecular size of each fragment was determined by comparing to known molecular weight DNA fragments of Lamda DNA digested with *Hin*dIII or *Pst*I. The restriction map of plasmid pAC11 was shown in Fig. 28.

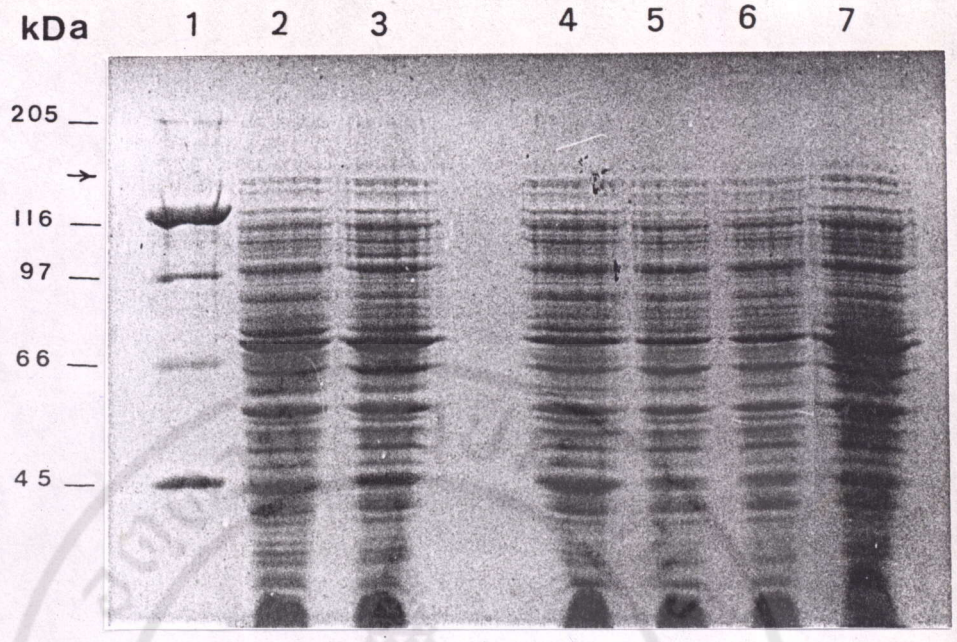

Fig. 25 SDS-PAGE patterns of protein extracts of *E. coli* DH5$^{\alpha}$ harboring various plasmids. The protein extracts of *E. coli* harboring pBluescript KS (lane 2), *E. coli* harboring pAC1 (lane 3), *E. coli* harboring pUC12 vector (lanes 4 and 7), *E.coli* harboring pAC2 (lane 5), and *E.coli* harboring pAC3 (lane 6) were prepared and subjected to 10% separating gel of SDS-PAGE. Lane 1 represented as protein molecular weight standards, myosin (205 kDa), B-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66), and oval albumin (45 kDa). The position marked by the arrow represented the position for S-layer protein determined by Western blot analysis.

Fig. 26.1 Immuno-dot blot reaction of *B. thuringiensis* subsp. *israelensis*. The single colony of overnight culture of strain 4Q2 (1), and strain 4Q272 (2) were blotted onto nitrocellulose and then detection for positive reaction was carried out by using anti-S-layer protein antibody as primary antibody.

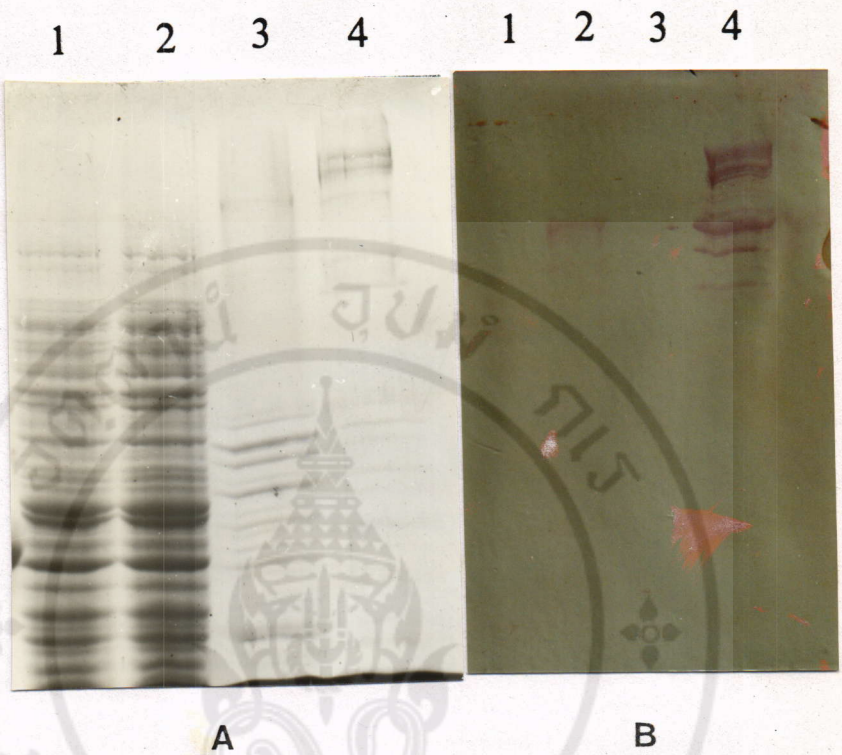

Fig. 26.2A SDS-PAGE pattern of various protein extracts. The protein extracts of *E. coli* harboring pBluescript KS (lane 1), *E.coli* harboring pAC11 (lane 2), *B.t.i.* strain 4Q272 (lane 3), and strain 4Q2 (lane 4) were prepared and subjected to SDS-PAGE.

Fig. 26.2B. Western blotting of various protein extracts resulted from the transfer of proteins in the gel from A to nitrocellulose and detection for positive band was carried out by using anti -S-layer protein antibody as primary antibody.

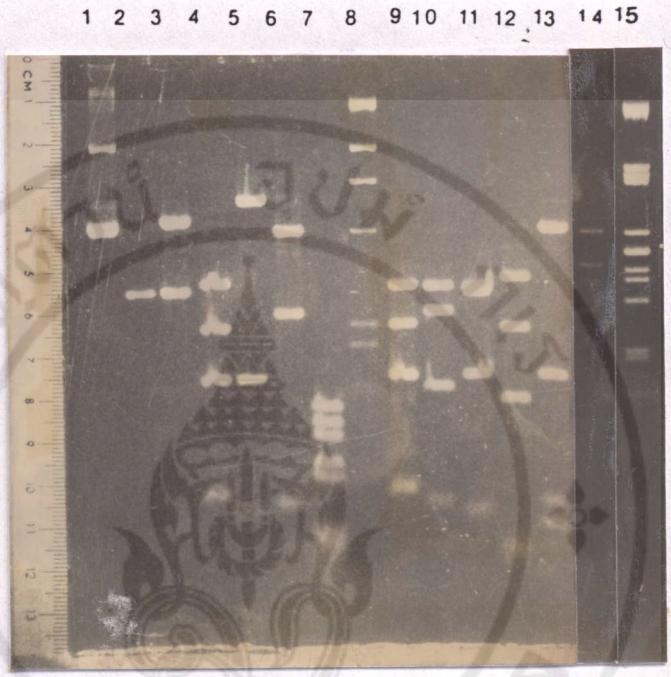

Fig.27. Agarose gel electrophoresis of recombinant plasmid pAC11 digested with various restriction endonucleases. The DNA of pAC11 was restricted with *Pst*I (lane 3), *Eco*RI (lane 4), *Hinc*II (lane 5), *Hin*dIII (lane 6), *Sau*3A (lane 7), *Pst*I/*Eco*RI (lane 9), *Pst*I/*Hin*dIII (lane 10), *Pst*I/*Hinc*II (lane 11), *Eco*RI/*Hin*dIII (lane 12), *Hin*dIII/*Hinc*II (lane13) and *Eco*RI/*Pst*I (lane 14). The other lanes were loaded by uncut-pAC11 (lane 1), the pBluescript KS which was digested with *Pst*I (lane 2), *Hin*dIII- digested lamda DNA (lane 8), and *Pst*I -digested lamda DNA (lane 15).

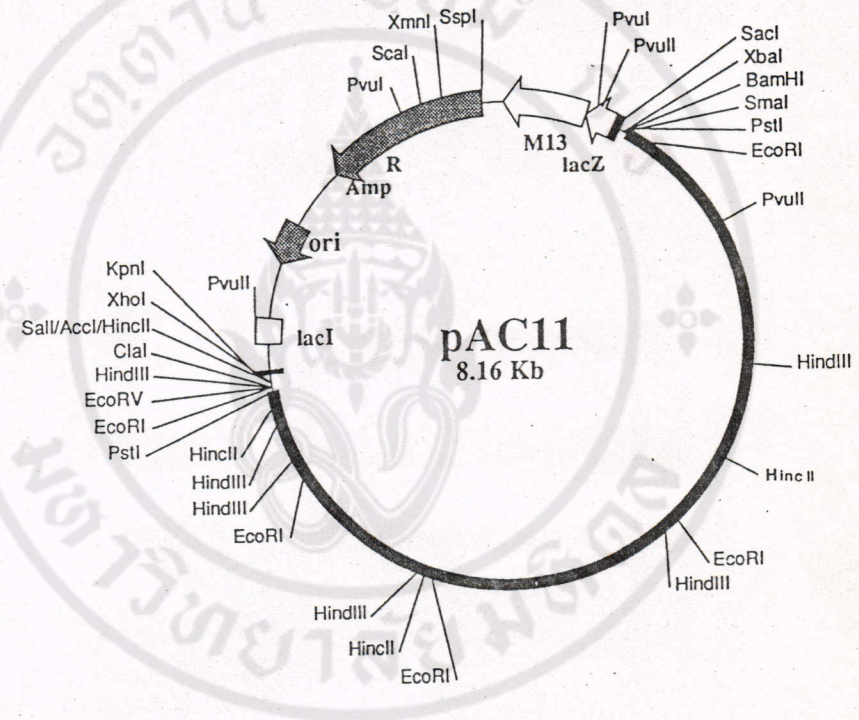

Fig. 28 Restriction map of recombinant plasmid pAC11 carrying the 5.2 kb fragment of S-layer protein gene from *B. thuringiensis* subsp. *israelensis* strain 4Q2. The thick line indicated 4Q2-S-layer protein gene *slp*, and the thin line indicated plasmid vector, pBluescript KS.

7.5 Subcloning of plasmid pAC11 with *Hin*dIII

Plasmid pAC11 which was digested with restriction enzymes *Pst*I and *Hin*dIII yielded 5 fragments of 2.96, 2.7, 1.5, 0.7 and 0.3 kb. If it was digested with *Hin*dIII, it yielded 4 fragments of 4.5, 2.7, 0.7 and 0.3 kb (Fig. 27). Therefore, it was decided to subclone the plasmid pAC11 with *Hin*dIII. Each fragment was recovered from agarose gel. Because the fragment 4.5 kb was already contained all essential parts of plasmid vector therefore, it could be religated by itself and transformed into *E. coli* DH5 α. The other three fragments were recovered and ligated into *Hin*dIII cut pBluescript KS vector and also transformed into *E. coli* DH5 α The clones were selected on LB-agar containing ampicillin, IPTG and X-gal as previously described. Five to ten clones of white transformants from each fragment were selected for further analysis.

Plasmids extracted from five to ten clones of each recovered fragment were digested with *Hin*dIII in order to determine the size of inserts after subcloning as shown in Fig. 29. Three clones of each inserted fragment were selected to study for the expression of S-layer protein using Western blot analysis. The capability of expression the gene product of each clone with different inserted fragments was determined by Western blot analysis. The clone containing 1.5 kb inserted fragment designated pAC111 could express its gene product which gave positive result as shown in Fig. 30, the clones

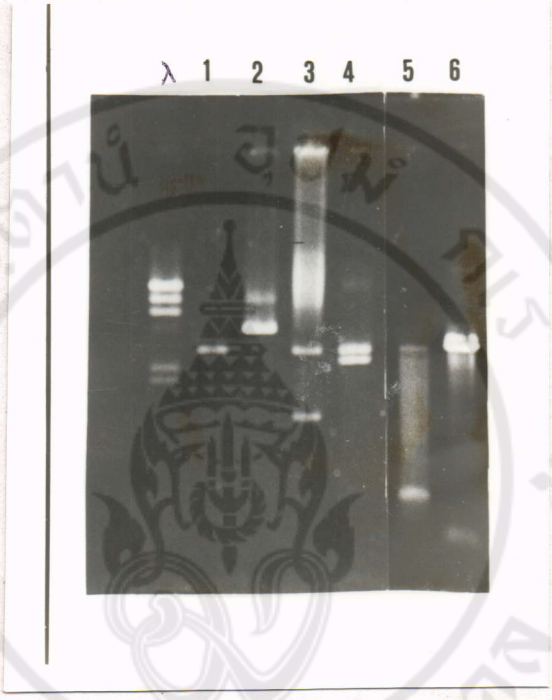

Fig. 29 Agarose gel electrophoresis patterns of various plasmid DNAs. The DNAs of pBluescript KS (lane 1), pAC111 (lane 2) , pAC211 (lane 4), pAC311 (lane 5), and pAC411 (lane 6) were restricted with *Hin*dIII. The DNA of pAC111 was restricted with *Hin*dIII/*Pst*I (lane 3). The lane marked λ represented as lamda DNA which was digested with *Hin*dIII.

Fig. 30 Western blotting of protein extract of *E. coli* DH5α harboring various recombinant plasmids. The protein bands of *E.coli* harboring pBluescript KS (lane 1), *E.coli* harboring pAC11 (lane 2), *E.coli* harboring pAC111 (lane 3), pAC211 (lane 4), *E. coli* harboring pAC311 (lane 5), and *E. coli* harboring pAC411 (lane 6) were transferred onto nitrocellulose after electrophoresis on SDS-PAGE. And then detection for positive band was carried out by using anti- 4Q2 S-layer protein antibody as primary antibody.

containing the other fragments could not express the gene product and resulted in a negative result by Western blot analysis as shown in Fig. 30. However, the clone containing pAC111 expressed gene product which was degraded as shown as smear in Fig. 30.

7.6 Subcloning of plasmid pAC111 with *Pst*I

In pBluescript KS, the *Kpn*I site lies down stream from the T3 promoter which is in the same orientation with *lac Z* promoter, and the *Sac*I site lies down stream from the T7 promoter. In pBluescript SK, the polycloning site is in the opposite orientation (Fig. 7). The 1.5 kb fragment of S-layer protein gene has been inserted into pBluescriptKS. In order to detect the expression of the 1.5 kb fragment in opposite orientation, therefore, it might be inserted into pBluescriptSK vector.

The plasmid pAC111 was extracted and digested with *Pst*I which yielded two fragments of different sizes, 2.96 kb (vector) and 1.5 kb of inserted DNA. The 1.5 kb fragment was recovered from agarose gel, purified and ligated (at 16°C overnight) into doubly cut pBluescript SK vector with *Pst*I and *Hind*III transformed into *E. coli* DH5α and spread on LB-agar containing ampicillin, IPTG and X-gal as previously described. Five colonies of white transformants were selected for further analysis.

Plasmid DNAs from white transformants were extracted and digested with *Pst*I. The digested products were characterized by agarose gel electrophoresis (Fig.

31.1). Three clones containing 2.96 kb (vector) and 1.5 kb of inserted DNA were selected, and determined the ability to express the S-layer protein by Western blot analysis. The results showed that they could express the gene product which yield a positive result with our test system and also with degradation (Fig. 31.2). The positive clone was designated pAC112. Thus, this study indicated that the 1.5 kb fragment of S-layer protein gene could express the gene product in both pBluescriptKS and pBluescriptSK.

8. Location of S-layer protein gene

In order to locate the original location of the S-layer protein gene of *B.t.i.* strain 4Q2, the 5.2 kb fragment was recovered from agarose gel for using as DNA probe. Colony hybridization of *B.t.i.* strains 4Q2, 4Q272 and c4Q272 with biotinylated DNA probe was performed, and the positive results with all of the three strains were obtained. Whole DNA of strains 4Q2, 4Q272, chromosomal DNA of strains 4Q2, 4Q272, and c4Q272 were used as tested DNA. The result of hybridization showed that the probe could hybridize to chromosomal DNA of strains 4Q2, 4Q272 and c4Q272 both intact chromosome and partially digested with *Pst*I, but could not be shown to hybridize with any plasmid DNA of strain 4Q2 or 4Q272 (Figs. 32.1 and 32.2).

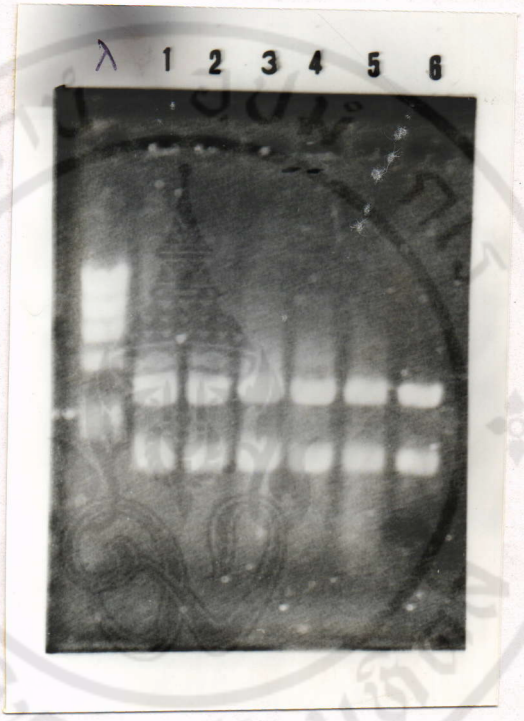

Fig. 31.1 Agarose gel electrophoresis patterns of pAC112 which was restricted with *Hin*dIII/*Pst*I (lanes 1 through 6). The lane marked λ represented as lamda DNA which was digested with *Hin*dIII.

Fig. 31.2 Western blotting of protein extracts from *E. coli* DH5α harboring various recombinant plasmids. Protein bands of *E. coli* harboring pAC112 (lanes 1 through 4), *E. coli* harboring pAC111 (lanes 5 and 6), and *E. coli* harboring pBluescript SK vector (lane 7) were transferred onto nitrocellulose after electrophoresis on SDS-PAGE. And then ,detection for positive band was carried out by using anti-4Q2 S-layer protein antibody as primary antibody.

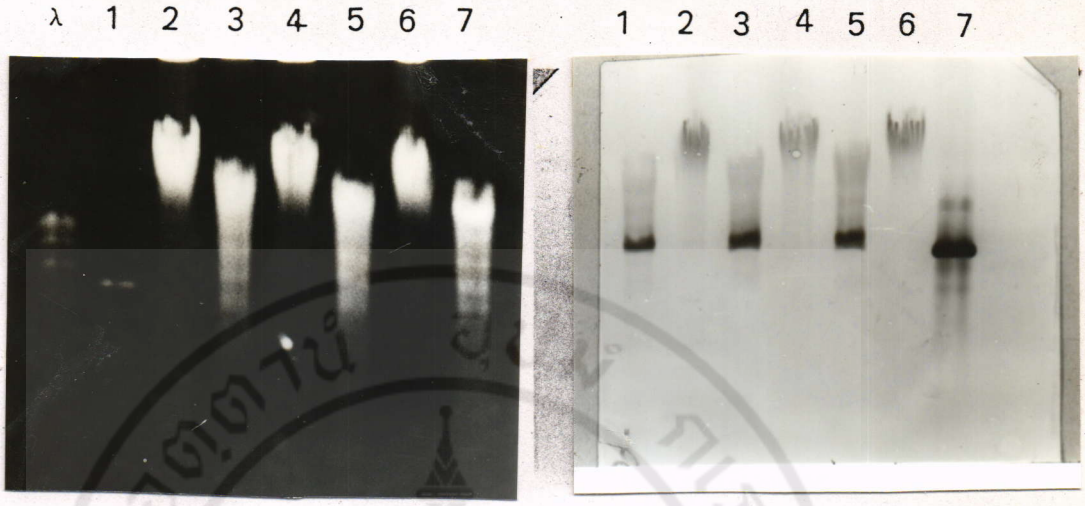

Fig. 32.1 Southern hybridization of DNAs from *B. thuringiensis* subsp.*israelensis* strain 4Q2, 4Q272, and c4Q272 with biotin-11-dUTP labelled 5.2 kb S-layer protein gene probe. The figure A was the photograph of an agarose gel of DNA from *E. coli* harboring pAC11 (lane 1),*B.t.i.* strain 4Q2 (lane 2),*B.t.i.* strain 4Q272 (lane 4), and *B.t.i.* strain c4Q272 (lane 6). The DNAs of *B.t.i.* strain 4Q2 (lane 3), strain 4Q272 (lane 5), strain c4Q272 (lane 7) were partially digested with *Pst*I. The lane marked λ represented lamda DNA which was digested with *Hind*III. The figure B was Southern hybridization resulting from the transfer of the DNAs in the gel from A. to nitrocellulose and hybridized with S-layer protein gene probe.

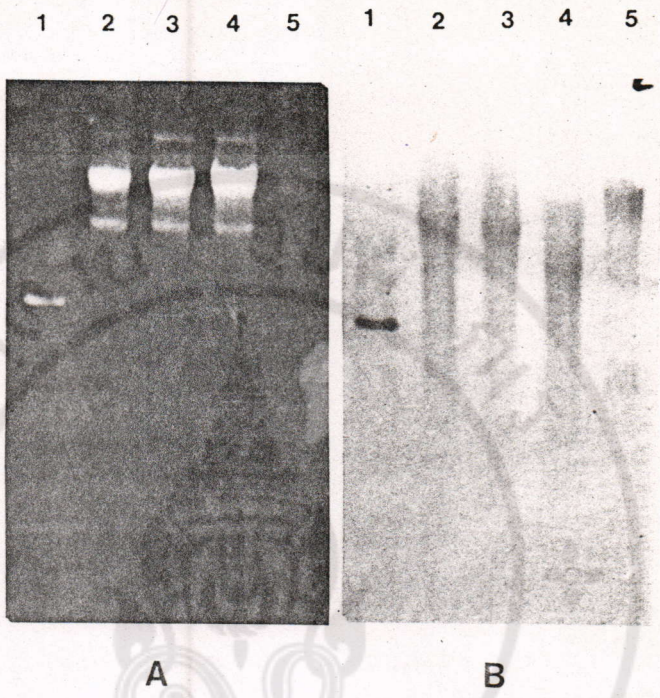

Fig. 32.2 Southern hybridization of DNAs from *B. thuringiensis* subsp.*israelensis* with biotin-11-dUTP labelled 5.2 kb S-layer protein gene probe. The figure A. was photograph of an agarose gel of plasmid DNA preparations from *E. coli* harboring pAC11 (lane 1), *B.t.i.* strain 4Q2-Nal^r (lanes 2,3, and 4), *B.t.i.* strain 4Q272 (lane 5). The figure B. was Southern hybridization resulting from the transfer of DNAs in the gel from A. to nitrocellulose and hybridized with S-layer protein gene probe.

9. Sequencing the pAC111 clone

9.1 Generation of unidirection deletion

The pAC111 clone containing 1.5 kb fragment was doubly cut by *Kpn*I and *Hin*dIII or *Sac*I and *Bam*HI to obtain linearized plasmid with 3' protruding and 5' protruding end. The digested plasmids were deleted by Exonuclease III as described by Henikoff (241) ligated and transformed into *E. coli* DH5α. Ten to twenty deletion subclones from each time intervals were screened to select those containing deletions appropriate for further analysis by rapid screening methods to estimate the plasmid size. The deletion subclones containing various size of recombinant plasmids were selected as shown in Fig. 33.1. Subsequently, the recombinant plasmids were extracted by rapid alkaline lysis (229), digested with *Pvu*II to determine the size of inserted DNA from each deletion subclone by comparing with the pAC111 and pBluescript KS digesting with the same enzyme. The *Pvu*II digested deletion subclones were shown in Fig. 33.2.

9.2 Subcloning of pAC111 with *Pvu*II

Plasmid pAC111 was doubly digested with *Pst*I and *Hin*dIII, and separated by agarose gel electrophoresis. The 1.5 kb fragment was recovered, purified and digested with *Pvu*II, and separated by agarose gel electrophoresis. Two fragments of digested products, approximately 0.7 and 0.8 kb (Fig. 34) were separately recovered and purified, designated P26 and H38 respectively. P26 fragment was

Fig. 33.1 Agarose gel electrophoresis pattern of various deletion clones of pAC111. The recombinant plasmid pAC111 was deleted with exonuclease III at various time intervals, ligated, and transformed into *E. coli* DH5 α . The figure showed the various plasmid preparations from *E. coli* transformants at various time intervals.

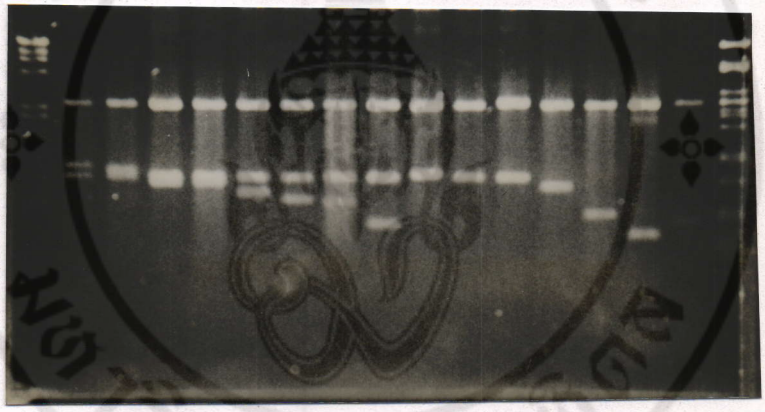

Fig. 33.2 Agarose gel electrophoresis patterns of various deletion clones of pAC111. The DNA of *E. coli* transformants at various time intervals were digested with *Pvu*II.

Fig. 34 Agarose gel electrophoresis patterns of various recombinant plasmids. The DNA preparation of pBluescript KS was restricted with *Hin*dIII (lane 1), pAC111 was restricted with *Hin*dIII/*Pst*I/*Pvu*II (lane 2), pP26 was restricted with *Pst*I/*Hin*dIII (lane 3), pH38 was restricted with *Hin*dIII/*Bam*HI (lane 4). The lane marked λ represented as lamda DNA which was digested with *Hin*dIII.

ligated into pBluescriptKS doubly digested with *Pst*I and *Eco*RV, and H38 fragment was ligated into pBluescript KS, doubly digested with *Hind*III and *Sma*I, each ligated product was transformed into *E. coli* DH5α. Ten white transformants from each ligated product were selected on LB-agar containing ampicillin, IPTG and X-gal as described in Materials and Methods. The recombinant plasmids were extracted, doubly cut with *Pst*I/*Cla*I or *Hind*III/*Bam*HI, and separated on agarose gel electrophoresis, the clones containing inserted fragments similar to P26 or H38 fragments were selected and designated pP26 and pH38 respectively (Fig. 34).

9.3 Strategy for sequencing

Six recombinant plasmid DNAs containing various sizes of inserted DNA that were deleted from *Hind*III site were annealed with M13 reverse primer or T3 primer, and determined the nucleotide sequence by chain-terminating dideoxy method (243) for one strand.

Six recombinant plasmid DNAs containing various size of inserted DNA that were sequentially deleted from *Pst*I site (doubly digested with *Sac*I and *Bam*HI) were annealed with M13 primer or T7 primer, and the nucleotide sequence for complementary strand was determined. Furthermore, the nucleotide sequences were also determined from pP26 and pH38 by anealing with M13 or M13 reverse primer. The strategy for sequencing was shown in Fig. 35.

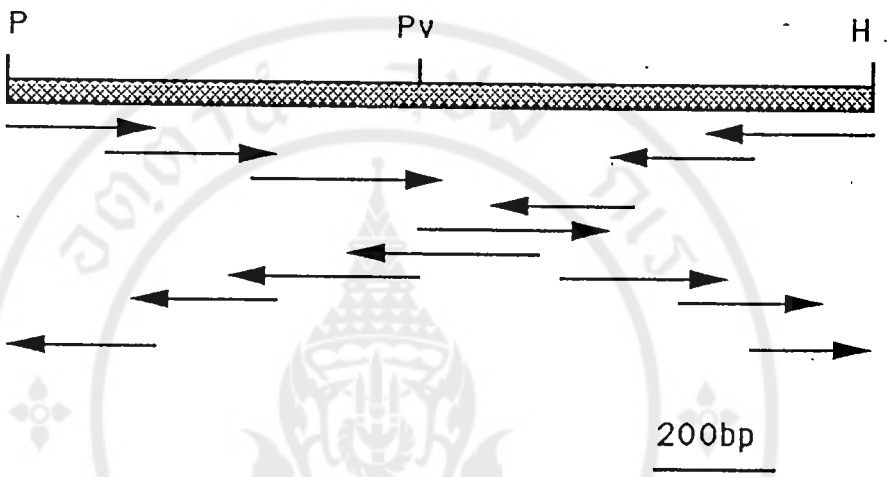

Fig. 35 Strategy for sequencing pAC111. The recombinant plasmid pAC111 which was unidirectionally deleted to be various deletion clones,and also the pAC111 which was subcloned to be pP26 and pH38 were subjected to be sequenced by dideoxy chain-termination method. Each arrow represented individual clone that were subjected for sequencing in the same direction as indicated by arrows. P, *Pst*I; H, *Hin*dIII; Pv, *Pvu*II

9.4 Nucleotide sequence of pAC111

Each nucleotide sequence from various deletion clones was joined by computer program, SEQAID. A sequence of 1,486 nucleotides of *Pst*I-*Hin*dIII fragment of S-layer protein gene was shown in Fig. 36.

By computer analysis, the location of the initiation codons (ATG) in three possible reading frame of each strand of DNA sequence indicated a single open reading frame (ORF) for the S-layer protein gene, which extended from the ATG start codon at nucleotide 307 (Fig. 36), and read through all sequenced nucleotides. It was interesting to note that, DNA sequence was found *Pvu*II cutting site at nucleotide 702. Therefore, this finding was in agreement with the previous experiment which the pAC111 could cleave with *Pvu*II. Agarose gel electrophoresis pattern of the digested products were shown in Fig. 34.

The region upstream from the putative ATG was shown to contain the sequence TTGTCC (at nucleotide 187-192) or sequence TCTTCA (at nucleotide 197-202) which was similar to the -35 consensus sequence (TTGACA), and also contained the sequence TATTAAT (at nucleotide 220-226) which was similar to the -10 concensus sequence (TATAAT) as shown in Fig. 37.

The putative shine-Dalgarno (SD) sequence preceeded the putative ATG was TAATGCAGAAGCA, (nucleotide 276-288) which could form base pair with sequence at 3'

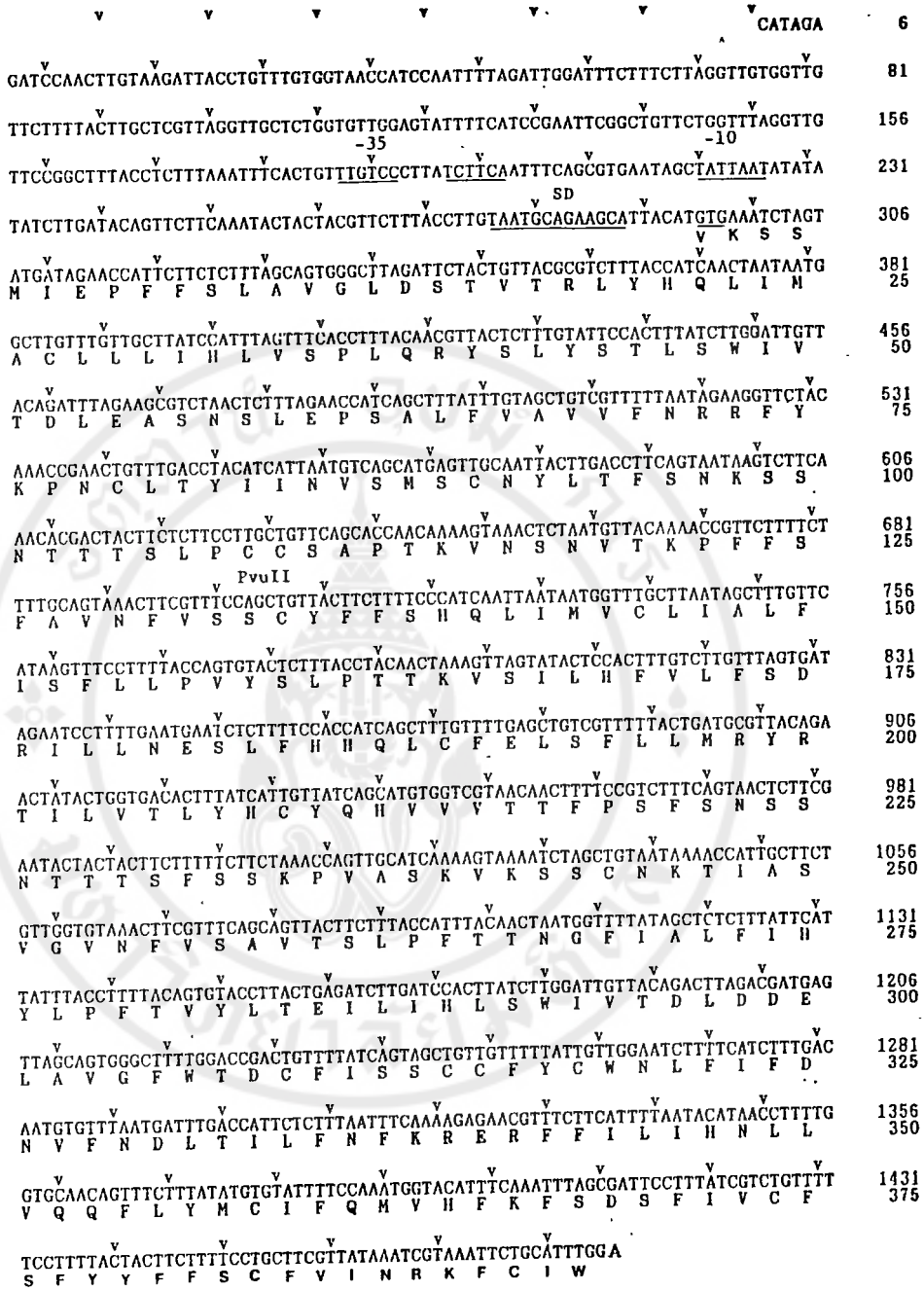

Fig. 36 Nucleotide sequence of S-layer protein gene from pAC111. The predicted amino acid sequence was shown as one letter code under the nucleotide sequence. The putative -35, -10, Shine-Dalgarno sequence,and initiation codon were underlined, respectively. The *Pvu*II site was indicated at nucleotide 702.

Bti/slp	CACTGTTTGTCCCTTATCTTCAATTTCAGCGTGAATAGCTATTAATATATATATCTTGAT
Bti	TTGCACTTTTGGTCTTTTTTAAATCGTATGAATTCAAAATAGTTTATATCAATCTTGTTA
BT88-3	GnCCTACGCTTCTACTACAAATTTACAATTTCCATACATCAATTGACGGAAGACTATT
PBI	GCATCTTT..... CATAGAAT
tycA	ATCCCTATTTTTTAATCGACTTCCAATTTTTCTCTGCTATAATGAGTTTCAGCGTCAGTA
P1	GGAAAAAGGTTCAGTCGTGACAGCCCGCCATATGTCCCCTATAATACGGATTGTGGCGGA
P2	TTTTTTTCGAAGGCGCCGCAACTTTTGATTCGCTCAGGCGTTTAATAGGATGTCACACGA
*P2**	AGGCGCCGCAACTTTTGATTCGCTCAGGCGTTTAATAGGATGTCACACGAAAAACGGGGA
P3	GAATTGTGTAAAAAAGATTCACGAATTCTAGCAGTTGTGTTACACTAGTGATTGTTGCAT
P4	GATTGTTGCATTTTACACAATACTGAATATACTAGAGATTTTTAACACAAAAAGCGAGGC
P5	ACACAATACTGAATATACTAGAGATTTTTAACACAAAAAGCGAGGCTTTCCTGCGAAAGG
Bs2362	TAAGAAAATTTGCTTTAGTTTTGAATTATTCGAGAAAGCTATAATACATAAATTTAGGCA
Bta	AATTTGTTACGTTTTTTGTATTTTTTCATAAGATGTGTCATATGTATTAAATCGTGGTAA
Btk	TTAGTAAAATTAGTTGCACTTTGTGCATTTTTTCATAAGATGAGTCATATGTTTTAAATT
Btte	ATCATACATATTTTCTATTGGAATGATTAAGATTCCAATAGAATAGTGTATAAATTATTT
VEGI/II	TAATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTACTGACCTGG
VEG II	GTCAAAATAATTTTATTGTCAACGTCTTATTAACGTTGATATAATTTGCAAGCTTGCAA
spaA	TTAAGCAATCGTTTGCATTGACAATCACTAGATAAGTGTTATTATAGATAGTATTGTAAC
consensus sequence	TTGACA TATAAT

Fig.37. Comparision of the putative promoter sequence of *B.thuringiensis* subsp.*isrelensis* strain 4Q2 S-layer protein gene with various known promoter sequences. Possible -35 and -10 regions of *B.t.i.* 4Q2 *slp* gene and various known sequences were underlined.*Bti/slp*, putative promoter of *B.t.i.*4Q2 S-layer protein gene;*Bti.*,promoter of *B.t.i.*delta-endotoxin gene (252);*PBT88-3*, putative promoter of *B.thuringiensis berliner* crystal gene (253);*PBI*, promoter of *B.t.i.* delta-endotoxin gene (254);*tycA*, promoter of *B.brevis* (218); *P1,P2,P3, P4*, promoter of *B.brevis* cell wall protein gene (218).*P2*,P5*, putative promoter of *B.brevis* cell wall protein gene (218); *Bs2362*,promoter of *B.sphaericus* 2362 S-layer protein gene (255); *Bta*,promoter of *B.thuringiensis aizawai* crystal toxin gene (252);*Btk*,promoter of *B.thuringiensis kurstaki HD-1* crystal toxin gene (144); *Btte*, promoter of *B.thuringiensis tenebrionis* crystal toxin gene (256);*VegII* and *VegI/II*, promoter of *B.subtilis* (218);*spaA*, putative promoter of *S.sobrinus* surface protein antigen gene (257).

terminal of 16S rRNA of *Bacillus* spp. Whereas sequence at 3' terminal of 16S rRNA of *B. subtilis* and *B.stearothermophilus* are 3' UCUUUCYZZZZZZZG 5' and 3' (A)UCUUUCCUCCACUAG 5', respectively (262). However, considering to the sequence AGAA of putative SD sequence, whereas 4 nucleotides could form base pair with sequence at 3' terminal of 16S rRNA of *Bacillus* spp.

The distance between putative SD sequence and the first ATG is 20 nucleotides which is somewhat longer than other known distance (244). Considering for alternative initiation codon, there might be a GTG which preceeded the first ATG, and also in the same reading frame. Therefore, if the initiation codon is GTG (at nucleotide 295) instead of ATG the distance between putative SD sequence and initiation codon is 8 nucleotides which is similar to other known distance between the SD sequence and the initiation codon (244).

CHAPTER V

DISCUSSION

1. Nature of conjugation-like gene transfer process among *B. thuringiensis*

This study on the ability of *B.t.i.* strain A084-16-194 to transfer its plasmids and chromosomal marker genes to various subspecies recipients required an initial examinaiton of the plasmid patterns of the various subspecies used. As has been reported by numerous investigators (2, 5, 7), the different subspecies possessed different plasmid patterns. In full agreement with Carlton and Gonzalez (7), it was shown that the subspecies *ostrinae* possessed 3 plasmids. The plasmids of *israelensis, kurstaki, sotto*, and *subtoxicus* also showed very similar patterns to those reported by Lereclus et al. (36).

When transfer of plasmids pBC16 and pC194 from *B.t.i.* A084-16-194 was attempted with various subspecies of *B. thuringiensis* using the conjugation-like mating process, it was interesting that not all subspecies were able to successfully receive both plasmids. For example, all subspecies except *pakistani* were capable of acquiring and maintaining the pBC16 plasmid as demonstrated by tetracycline resistance and the presence of the plasmid on agarose gels. Likewise, all subspecies, except *tochigiensis* and *caucasicus*, were capable of acquiring and

maintaining the pC194 plasmid. It is not known whether the lack of success depended upon an inability to transfer or an inability to retain the relevant plasmid after transfer or both. However, since a large number of the subspecies did acquire both pBC16 and pC194, it is unlikely that the inability to transfer the plasmids was the reason for lack of success in transferring of plasmids in only a few subspecies. Rather, it is likely that the inability to acquire certain plasmids depended upon the ability of a particular strain to retain a particular plasmid. This contention is further supported by the fact that all successful transfer of drug resistance plasmids in recipient strain, resulted in the co-transfer and maintenance of other *B.t.i.* plasmids to the recipients. Again, this demonstrated that plasmids could be transferred from one subspecies of *B. thuringiensis* to another freely via the conjugation-like process.

In this study, there was no correlation between the plasmid patterns of the recipient *B. thurigiensis* subspecies and their ability to maintain the plasmids pBC16 and pC194. The subspecies *thuringiensis, dakota, indiana, sotto, toumanoffi, thompsoni,* and *subtoxicus*, which contained large plasmids, readily accepted plasmids pBC16 and pC194 at the same rate as subspecies *galleriae, finitimus*, and *wuhanensis*, which contained only small plasmids, and at the same rate as subspecies *entomocidus*, which did not possess any detectable plasmid at all.

B.t.i. strain A084-16-194, used as the donor in most of this study, contained large plasmids, but subspecies *finitimus*, which acted as the donor in an intrasubspecific transfer test, did not possess any large plasmids (Figs. 9 and 11). Thus, there was also no correlation between plasmid pattern and ability to donate plasmids via the conjugation-like process. This finding contrasts to that of Battisti et al. (17) who reported that two plasmids, pXO11 and pXO12, found in *B. thuringiensis* subsp. *thuringiensis* were responsible for plasmid mobilization via conjugation-like process among *B. thuringiensis, B. anthracis* and *B. cereus*. The discrepancy may arise from differences in the strains employed, in the method of conjugation employed or in the level of detection of transconjugants.

The frequency of plasmid transfer from *B.t.i.* A084-16-194 to various recipients varied greatly depending upon the subspecies of the recipient. The frequencies varied from 2.1×10^{-9} in subspecies *pakistani* to 9.8×10^{-5} in subspecies *finitimus*. These rates of transfer were similar to those reported by Fisher et al. (9). Even so, the rate of gene transfer via the conjugation-like process varied greatly from one report to another (9, 14-17, 19). Nonetheless, the transfer frequency obtained in this study was well within the range of figures being reported elsewhere (9, 14, 16).

Using the same plasmids, and the same conjugation-like conditions, the frequency of transfer within

subspecies was much higher than the frequency between subspecies. The result was to be as expected, since the restriction/modification of foreign plasmids may play a significant role in successful plasmid transfer. Furthermore, the transfer of genes within a subspecies may be supported by better conditions for "pairing" between two conjugants than those found with transfer between subspecies. A specific study on the nature of "pairing" between two cells may shed more light onto the mechanism of this poorly understood conjugation-like process.

With very limited data, it was possible to demonstrate chromosomal transfer from subsp. *israelensis* to subspecies *thuringiensis, finitimus, kurstaki,* and *sotto* using streptomycin and rifampicin resistant markers. Although, chromosomal transfer of these resistance phenotypes was attempted in all the conjugation experiments involving 25 subspecies, only 10 successful mating pairs could be demonstrated. However, it is possible that chromosomal transfer occurs at such a low rate that the methods used in this study were not sensitive enough to allow for its detection. If the conjugation-like gene transfer process were to be performed using the membrane filter technique (16), perhaps more chromosomal transfer could be detected (217). Nonetheless, demonstration of chromosomal transfer opens the way for further optimization of the process. Perhaps it could be used to obtain a better understanding of the

genetic organization of this microorganism and to obtain genetically improved strains of *B. thuringiensis*.

2. The effect of chemicals on conjugation-like gene transfer.

The high concentrations of various chemicals were found to inhibit the transfer of plasmid pBC16. The level of the high concentration of chemicals employed in this experiment did not affect the viability of either donor or recipient, therefore, it could be assummed that the stability of cell to cell contact was inhibited because the conjugation-like gene transfer process needs cell to cell interaction at a certain period of time. In case of *E. coli*, the previous report demonstrated that F pili plays a role on stabilization between the donor and recipient cell for a certain period, but does not need cell to cell direct contact (251). There has been no evident to date on the presence of "sex pili" in *B. thuringiensis*. Therefore, the direct contact between the donor and recipient might be very important in establishing successful gene transfer via conjugation-like process. The high concentration of chemicals was not flavorable on conjugation-like gene transfer process. The high concentration of chemicals may react with some component(s) on cell surface and lead to inhibition of gene transfer process. This phenomenon might be similar to the surface exclusion which has previously been elucidated in *S. faecalis* (98).

In addition, the high concentration of chemicals also behaves as an interferring substance on cell clumping (Tables 13-16). It was interesting to note that 0.5 M $MgSO_4$ could inhibit both the clumping phenomena and the transfer of pBC16 plasmid from strain A084-16-194 to strain c4Q272. EDTA at 0.2 mM, $MgSO_4$ at 0.5 M, was found to inhibit transfer of pBC16 plasmid from *B.t.i.* strain A084-16-194 to strain c4Q272. At these concentrations the inhibition on cell clumping by EDTA, and $MgCl_2$, appeared to be reduced from ++++ clumping to +++ and from ++++ to +, respectively.

The clumping between certain mating mixtures has been previously found to relate to high frequency of plasmid transfer as discovered in *S.faecalis* (98, 100) and *Lactococcus lactis* (247-249). It has been suggested that clumping may have a role in promoting close cell-to-cell contact prior to DNA transfer. However, the exception to this relationship has been observed. When *L. lactis* subsp. *diacetylactis* 176 or 18-16S, possessed lactose plasmid which conferred high frequency of transfer and cell aggregation phenotypes were used as donors in mating with a *L. lactis* 712, high frequency of transfer was still observed but did not exhibited cell aggregation (247, 250). Therefore, gene transfer in *L. lactis* 712 did not appear to relate to cell clumping.

3. The S-layer protein of *B. thuringiensis* subsp. *israelensis* strain 4Q2

This study has shown that there seemed to be some correlation between the presence and absence of a high molecular weight protein, i.e. S-layer protein with penicillin G susceptibility. The wild type strain, 4Q2, possesses high molecular weight S-layer protein and it is found to be highly resistant to penicillin G, at MIC approximately 12.5 mg. Both of the cured strains 4Q2-72 which harbors only one plasmid (110 kb) and the plasmidless strain c4Q272 which do not possess the S-layer protein are very sensitive to penicillin G at MIC 0.06 and 0.03 ug respectively. This phenomena seemed to suggest that some factors might be lost (or gained) during the curing process, and these factors might be involved in the changes in susceptibility to penicillin G among strains of *B. thuringiensis* subsp. *israelensis*. This evidence was supported by some previous studies which have been reported that several mesophilic Bacillaceae possessing S-layer are resistant to lysozyme (119, 159, 172) and also S-layer on intact cells are frequently rather resistant to a wide spectrum of proteases (123). However, the penicillin G resistant phenotype might not correlate to the presence of S-layer protein, the disappearance of both properties might be coincidence during the process of plasmid curing. Therefore, it needs further experiments to clarify these phenomena. For example, attempts may be

made to introduce the S-layer protein gene into the penicillin G sensitive strains, 4Q272 or c4Q272 and then the ability to resist penicillin G can be investigated in the transformants.

Due to the cell clumping between various strains of *B. thuringiensis* sub. *israelensis* which we categorized into clumping group I and clumping group II, there appeared to be some common factors within the same group, namely, the presence of S-layer protein of clumping group I and the absence of S-layer protein of clumping group II. Interestingly, the clumping occurred when cell of group I was mixed with cell of group II. It might be interpreted that the clumping only resulted from the interaction between S-layer protein on cell surface and uncoated cell. This phenomenon might be similar to the presence of S-layer protein on *Aeromonas salmonicida* which decreases surface hydrophobicity and thus enhances the ability for autoagglutination and for association with phagocytic monocytes (188, 245). However, this clumping phenomenon might not depend only on the presence of S-layer protein based on similar argument in the case of correlation between penicillin G resistance and S-layer protein. Therefore, it needs further experiments to clarify this relationship, perhaps, by transferring the S-layer protein gene into the clumping group II i.e. strains 4Q272 or c4Q272 and observing for the clumping phenomena in the transformants.

Using the antibody raised against S-layer protein of *B.t.i.* strain 4Q2, it could be shown to inhibit the conjugation-like process between *B. t.i.* strain 4Q2-16 and c4Q2-72 at the detectable level of transconjugant by broth mating technique. This experiment demonstrated that S-layer protein might play a role on conjugation-like gene transfer process. Therefore, in the presence of anti S-layer protein antibody, the antibody blocked the gene transfer via conjugation-like process. There have been some good evidences from 3-dimensional reconstructions as well as from higher resolution projection data (246), that S-layer proteins may cause "connexon" channel with an average diameter of about 2.5 nm (203) which may be sufficient for DNA transfer via conjugation-like process. Therefore, the antigen-antibody interaction between S-layer and anti-S-layer as reported in this study may block or shield this channel and then inhibited DNA transfer.

The anti 4Q2 S-layer protein antibody could slightly reduce the frequency of conjugation-like gene transfer process between *B.t.i.* strain A084-16-194 and c4Q272. The lower degree of inhibition in strains A084-16-194 as compared to the strain 4Q2 might be related to the diversity or variation of S-layer protein in a subspecies. The differences of the S-layer proteins in different strains of the same species have been found in other microorganisms for example variation in antigenicity

of *C. fetus*'s S-layer protein (191). Therefore, the less specificity between anti-4Q2 S-layer protein antibody and the strain A084-16-194 could not completely block or shield the gene transfer channel between the two mating pair. Thus, the DNA transfer could be carried out but with reduction in the frequency of plasmid transfer.

4. Molecular cloning of S-layer protein gene from *B. thuringiensis* subsp. *israelensis* strains 4Q2

In this study, the S-layer protein gene has been cloned from genomic DNA of *B. thuringiensis* subsp. *israelensis* strain 4Q2 Nal^r, constructed in phagemid vector pBluescript KS and plasmid vector pUC12,transformed into *E. coli* DH5α , and screening for positive clones with anti-4Q2 S-layer protein antibody probe. Three positive clones containing 6.6 kb and 5.2 kb *Pst*I insert fragments were obtained. The cloned gene products were confirmed by Western blot analysis and it could be shown that all the inserted fragment produced proteins which reacted positively with antibody of S-layer protein of *B.t.i.* However, the molecular weight of the cloned gene products in *E. coli* were found to be lower than gene products of the strain 4Q2. However, the molecular weight as determined by SDS-PAGE, and the mobility of S-layer protein might be interfered by the presence of carbohydrate and/or lipid moiety attached to the protein molecule. Therefore, it might lead to the difficulty and the accuracy in the determination of true molecular weight

of the S-layer protein. Also, the proteolytic cleavage in *E. coli* might lead to the reduction of the molecular size of S-layer protein in *E. coli* host.

The carbohydrate moiety of S-layer protein in *B.t.i.* was detected by Periodic acid Schiff stain and concanavalin A conjugated with Horse radish peroxidase. However, the reaction was found to be weakly positive. In the attempts to remove the speculated lipid moiety from the S-layer protein by dissolving the protein in various concentrations of EDTA (ranging from 1 mM to 5 mM), various concentration of SDS (ranging from 5% to 20%) and also 10% $CaCl_2$, but the mobility on SDS-PAGE was found to be unaltered (data were not shown).

There have been several reports on cloning of S-layer protein genes from various other microorganisms (204, 208, 209, 211-215). All of S-layer protein genes could be expressed in *E. coli* host. Also the DNA fragment in transformed *E. coli* coded for S-layer protein of *B. licheniformis* NM105 had been found to result in a lower molecular mass than the authentic S-layer protein (211) which was suggested to be due to protease degradation. This phenomenon was also found in immunoblotting analysis of S-layer protein of *B. brevis* gene which was cloned in *B. subtilis* (206). The mobility of the authentic S-layer proteins and the cloned S-layer proteins produced by *E. coli* were also found to be different than those appeared in the original hosts *B. licheniformis* (211), and *A. kivui* (215).

Using Southern blot analysis, it was proven that the *B.t.i.* 4Q2 S-layer protein gene was located on chromosomal DNA. This finding was in agreement with all reports concerning the location of various S-layer protein genes of various bacteria, archaebacteria and eubacteria (204, 208, 209, 211, 213, 214). It was further noted that by using labeled S-layer protein gene 4Q2 as probes, the gene was also found in strain 4Q272 and c4Q272 which did not produced S-layer protein as determined by SDS-PAGE. The reason for the absence of the S-layer protein gene in *B.t.i.* strains 4Q272 and c4Q272 might be due to some regulatory mutation or repression in the expression of this gene which might occur during the curing experiments of the wild type strain, 4Q2 (46).

5. Comparison of the nucleotide sequence at putative promoter region.

The nucleotide sequence upstream of putative initiation codon of *B.t.i.* 4Q2 S-layer protein gene was investigated for putative promoter sequence and compared with various known promoter sequences as shown in Fig. 37. It appeared that the putative promoter of *B.t.i.* 4Q2 S-layer protein gene (*B.t.i./slp*) at -10 (TATTAAT) was closely related to -10 sequence of *S.sobrinus* surface protein antigen (TATTATA), i.e. five out of seven nucleotides were identicle. It was also related to -10 sequences of *B.subtilis VEGII* (TATAAT), *tycA* (TATAAT), *B.brevis P1* (TATAATA) and *B. sphaericus* 2362, (TATAAT),

i.e. four nucleotides were identicle. These sequences were identicle to the consensus sequence. The *B.t.i./slp* sequence at -10 also similar to the -10 sequences of *B.t.i.* crystal toxin genes *BT88-3* (TACAAT), *PBI* (CATAGAAT), S-layer protein gene sequences of *B.brevis P2* (TTTAATA), *P2** (TTTAATA) and promoter sequence of *B.thuringiensis* subsp. *aizawai* (CATATGTATT),i.e. three nucleotides were identicle. However, the -10 sequence of *B.t.i./slp* was less similar to *B.t.i.*delta-endotoxin gene putative -10 sequence (CGTATGAAT), *B. brevis P2* (TTTAATA), *P3* (TACACTA), *P4* (ATTT) and *B.thuringiensis* subsp. *tenebrionis* (GTGTATAA) because only two nucleotides were identicle.

Considering to the position of putative -35 sequence of *B.t.i./slp* gene, this might be represented by sequence TTGTCC or TCTTCA (Fig. 37) . The sequence TTGTCC was more similar to the consensus -35 sequence (TTGACA) than the TCTTCA sequence. The sequence TTGTCC of *B.t.i./slp* gene was similar to the -35 sequences of *B.brevis* S-layer protein gene *P2** (TTGAT), putative -35 sequence of *S. sobrinus* surface antigen (TTGACA), and *B.subtilis VEGII* (TTGTCA). The TCTTCA sequence of *B.t.i./slp* gene was similar to *B.subtilis VEG II* (TTGTCA), and *B.brevis* S-layer protein gene *P4* (TACACA).

However, if the sequence TTGTCC was presumed to be the -35 region the distance between the putative sequences, -35 and -10 regions of *B.t.i./slp* gene was

rather long (27 nucleotides) in comparision with the other known promoters as shown in Fig. 38, which ranged from 12 to 19 nucleotides. If the sequence TCTTCA was presumed to be -35 for *B.t.i./slp* gene, the distance between the putative -35 and -10 sequences was 17 nucleotides which was in agreement with other known promoter sequences (Fig. 38).

The nucleotide sequence between -35 and -10 region of *B.t.i.* 4Q2 S-layer protein gene also demonstrated the A-T rich as well as the other well known promoter sequences as shown in Fig. 37. In order to clarify the promoter region of *slp* gene, experiment on S1 mapping and primer extension should be performed to study the transcription start site of the *slp* gene.

Considering to the position of Shine-Dalgarno sequence (SD) of *slp* gene, this might be represented by sequence TAATGCAGAAGCA (Fig. 36 and Fig. 38). Almost all ribosome binding sites have a sequence such as GGAGG centered of 8 to 13 nucleotides upstream from the initiation codon such as SD sequences of *B.t.i.* endotoxin gene, *B.brevis* S-layer protein genes, *B.sphaericus* 2362 S-layer protein gene, *B. thuringiensis* subsp.*aizawai* and *B. thuringiensis* subsp.*kurstaki* endotoxin genes, *S. sobrinus* S-layer protein gene as shown in Fig 38. This sequences could form base pairs with complementary residues in pyrimidine - rich sequence at the 3' end of 16S rRNA chain of *Bacillus* spp.,3' (A)UCUUUCCUCCACUAG 5' (*B.*

E.coli	3' AUUCCUCCACUAG 5'	16S rRNA (262)
B.stearothermophilus(A)	UCUUUCCUCCACUAG	16S rRNA (262)
B.subtilis	UCUUUCY*ZZZZZZZ**G	16S rRNA (262)
B.t.i./slp	TAATGCAGAAGCA	
B.t.i.	TATGGGAGGAATA	δ- endotoxin gene(252)
BT88-3 berliner	TAATCAGGGTAAG	δ- endotoxin gene(253)
B.brevis SD1	GAAAGGAGGTGA	S-layer protein gene (218)
SD2	TAGAGGAGGAGA	S-layer protein gene (218)
B.sphaericus.2362	TAGGGAGGAATA	S-layer protein gene (255)
B.t.aizawai	AGATGGAGGTAA	δ- endotoxin gene(252)
*B.t.kurstaki*HD1	AGATGGAGGTAA	δ- endotoxin gene(144)
S.sobrinus	AATTGGAGGGAA	S-layer protein gene (257)
B.alcalophilus	AAAAATGAGGAGG	alkaline protease gene (258)
P.stutzeri	AAGTGGAAATTCC	outer membrane protein gene (259)
R.meliloti exo	CGGAAAGAAACTG	exopolysaccharide gene (260)
B.subtilis ansB	AGAAAGAAGGTTA	aspatase gene (261)

Fig. 38 The putative Shine-Dalgarno sequence of *slp* gene of *B.thuringiensis* subsp. *israelensis* strain 4Q2 (*B.t.i./slp*) and putative SD sequences of various bacteria. The putative SD sequences of each organism were underlined.

*Y might be pyrimidine

**Z might be any nucleotides

stearothermophilus). Considering to the 3' end of 16S rRNA of *B. subtilis* , 3' UCUUUCYZZZZZZZG 5', whereas Y might be pyrimidine and Z might be any nucleotides (262). It appeared that sequence UCUUUC could be responsible to form base pair with SD sequence. Therefore, the putative SD sequence of *slp* gene, the sequence AGAAG might be strong enough to form base pairs with 16S rRNA chain at UCUUUC region because four nucleotides of *slp* gene could form base pairs with 16S rRNA. And also, there have been reported for other bacteria which possessed SD sequences differring from the consensus sequence such as *B. thuringiensis berliner (BT88-3)* (TAATCAGGGTA),*Pseudomonas stutzeri* outer membrane protein gene (GAA), *Rizobium meliloti exo* exopoly-saccharide gene (GAAAGAAA), and *B. subtilis ansB* aspatase gene (GAAAGAAGGT) as shown in Fig 38.

6. Comparison of N-terminal amino-acid sequences of various S-layer proteins

The deduction of amino acid sequence from nucleotide sequence of 4Q2 S-layer protein gene was aligned in Fig. 36. Amino acid sequences from N-terminal sequence of various S-layer proteins *(B.t.* 4045, *B. brevis 47, A. hydrophila, D. radioduran, H. halobium, C. fetus, B. sphaericus)* were compared to the nucleotide derived amino acid sequence of 4Q2 S-layer protein as shown in Table 25. There appeared to be no significant homology among them. However, there appeared to be similarity in

Table 25 N-terminal sequences of the S-layer protein of *B. thuringiensis* subsp. *israelensis* strain 4Q2 and other S-layer proteins.

Bacterium	N-terminal sequence	% similarity of amino acid content at N-terminal sequence	Reference
B.t.i. 4Q2	VKSSMIEPFFSLAVGLDSTVTRLYHQLIMA	100.0	this study
B.t. 4045	?GKTFPDV?P	40.0	157
B. brevis 47 MWP	AEEAATTTAPKMDADMEKTVKRLEALGLVA	46.7	142
OWP	APKDGIYIGGNIKKYYSYDVFEMTPQAKAT	56.7	146
A. hydrophila TF7	VNLDTGAGVSFKASGIKVDGAAGTTLGGXA	46.7	157
D. radiodurans Sark	MKKNIALMALTGVLTLASCGQNGNTPTADT	56.7	148
H. halobium	ANASDLNDYQRFNENTNYTYSTASEDGKTE	46.7	147
C. fetus VC119	MISKSEVSELFIVLFGRP	72.2	159
B. sphaericus 2362	AKQNKGRKFFAASATAALVASAIVPVASAA	50.0	255

terms of amino acid composition of 10 to 30 amino acid residues at N-terminal ranging from 40% to 70% similarity as shown in Table 25. This percentage similarity was in agreement with the other comparisons. For example, the similarity of amino acid content between MWP and OWP of *B. brevis* was 46.7% or the similarity between OWP of *B. brevis* and S-layer protein *B. sphaericus* 2362 was 50% (calculated from the data shown in Table 25).

7. Future work

The conjugation-like process frequently uses as a mean for genetic manipulation in various subspecies of *B. thuringiensis*. This technique results in rather high frequency of plasmid transfer as well as more reproducible than other techniques. Furthermore, for transferring gene into *B. thuringiensis* the conjugation-like process is very convenient technique to perform when comparing to other gene transfer techniques such as protoplast transformation or transduction. However, since Gonzalez et al. (14) discovered this conjugation-like process in 1982, there has been no report concerning the mechanism of this type of gene transfer process. Therefore, it might be very difficult to increase the frequency of gene transfer via conjugation-like process due to the lack of knowledge regarding the mechanism of gene transfer in *B. thuringiensis*. Although, this study can not clarify the detailed mechanism of this type of gene transfer process, various findings reported in this study might lead to

further experiments concerning detailed mechanism of conjugation-like process in *B.thuringiensis*.

The S-layer protein may play crucial role in conjugation-like gene transfer process but also there might be other factor(s) involved. The S-layer protein gene of *B.t.i.* has already been cloned and partially sequenced in this study. For future work the whole gene, 5.2 kb fragment, should be sequenced and characterized. Also, since the S-layer protein gene of *B.t.i.* strain 4Q2, 4Q272 and c4Q272 was found to locate on the chromosome. However, S-layer protein gene of strain 4Q272 and c4Q272 do not express. Therefore, future work should be attempted to clarify the unability of strain 4Q272 and c4Q272 to express the *slp* gene. Therefore, introducing S-layer protein gene into the penicillin sensitive strains, 4Q272 and c4Q272 will be necessary to clarify the relationship between the penicillin sensitive phenomena and the possessing of S-layer protein. Moreover, it is possible that the penicillin resistance in strain 4Q2 there might require S-layer protein together with other protein(s) encoded by cryptic plasmid(s) of *B.t.i.* To clarify these phenomena, future experiments on introducing each plasmid of strain 4Q2 into strain c4Q272 and then observed its expression and ability to resist penicillin might be very interesting.

Considering to the molecular weight of the S-layer protein, the authentic S-layer protein extract from strain

4Q2 (260 kDa) was much higher than the S-layer protein which encoded by the cloned *slp* gene in *E.coli* (160 kDa). The different molecular weight might be due to factor(s) affecting the mobility of S-layer protein from strain 4Q2. And also, there might be resulted from some factor(s) in *E. coli* such as protease degradation and/or lack of glycosylation. The accurate molecular weight of S-layer protein should be clarified by transferring the cloned *slp* gene into *B.t.i.* strain 4Q272 or c4Q272.

Concerning cell clumping, there appeared to be clear that it need two different cell types to allow clumping to occur. One factor on strain 4Q2, clarified by this experiments was S-layer protein. Factor(s) on strains 4Q272 or c4Q272 which play(s) role on clumping phenomena should be investigated further.

When the mechanism of conjugation-like gene transfer process could be clarified, it will hopefully lead to more efficient gene transfer technique in *B.t.i.* The high frequency of gene transfer will subsequently lead to ability to construct a novel strain of *B.thuringiensis* with higher toxicity and/or broader host range and resulted in better efficacy for use as more effective biopesticide.

CONCLUSION

Twenty-five subspecies of *B. thuringiensis* employed in this study possess various plasmid patterns. Only one subspecies, namely *entomocidus*, did not harbor any plasmid. Four subspecies i.e., *finitimus, subage yunnanensis, tolworthi* and *wuhanensis* carried only small plasmids which migrated faster than chromosomal DNA. Other subspecies contained ranges of both small and large plasmids.

B. thuringiensis subsp. *israelensis* strain A084-194 was tested for the ability to transfer pBC16 and/or pC194 plasmids into various subspecies of *B. thuringiensis* by the broth mating technique. This study revealed that the frequencies of plasmid transfer were found to differ depending on the recipient subspecies. There appeared to be three different categories of recipients based on their ability to acquire plasmids pBC16 and pC194. Group I, subsp. *pakistani*, acquired only pBC16 from the donor strain. Group II, subsp. *tochigiensis* and *caucasicus* acquired only pC194 from the donor strain. Group III, there were 22 subspecies which were capable of acquiring both pBC16 and pC194. The frequencies of transfer for pBC16 ranged from 2.1×10^{-9} for transfer into subsp. *pakistani* to 9.8×10^{-5} for transfer into subsp. *finitimus*. The frequencies of transfer for pC194 ranged from 4.8×10^{-9} in subsp. *indiana* to 1.6×10^{-6} in subsp. *thuringiensis*.

The plasmids pBC16 and pC194 were either independently transferred or cotransferred into different subspecies. Neither plasmid pattern nor the flagella type of the recipient did not appear to play any important role in acquisition of these two plasmids. The acquiring of plasmids pBC16 and/or pC194 by the broth mating technique, did not appear to correlate with the differences in the plasmid pattern or in the flagella types in the various subspecies used as recipient.

Furthermore, ten subspecies of *B. thuringiensis* acquired chromosomal DNA from the donor strain eventhough the frequency of transfer was found to be very low, ranged from 4.3×10^{-9} in subsp. *wuhanensis* to 3.7×10^{-7} in subsp. *kurstaki*.

Experiments on intrasubspecific transfer i.e. subsp. *finitimus, sotto, ostrinae, toumanoffi* and *israelensis*, gave the frequency of transfer ranged from 2.2×10^{-7} to 1.6×10^{-4}. Comparison with the transfer rate between subspecies indicated that mating within subspecies (i.e. intrasubspecific transfer between subspecies *sotto*-pBC16 or *sotto*-pC194 and *sotto*) gave a higher frequency of transfer than did mating between subspecies (i.e. intersubspecific transfer between *israelensis* A084-16-194 and *sotto*).

Experiments on effect of various chemicals on conjugation-like gene transfer between *B.t.i.* strains A084-16-194 and c4Q272 indicated that the acquisition of

plasmid pBC16 by c4Q272 were found to be inhibited with 0.2 mM EDTA, 0.25 M $MgCl_2$, 0.5 M $MgSO_4$ and 0.05 M $CaCl_2$. These chemicals also caused reduction or inhibition of cell clumping at various concentrations as shown in Table 13 through 16. EDTA at concentration of 0.1 mM, reduced the degree of clumping from ++++ to +++, 0.1 to 0.5 M $MgCl_2$ reduced the degree of clumping from ++++ to +, and 0.5 M $MgSO_4$ could inhibit clumping as well as inhibit the plasmid transfer via conjugation-like process at the detectable level. To investigate cell clumping between certain strains of *B.t.i.*, the results indicated that the clumping occurred when mixing each strain from clumping group I (4Q2) with the ones from clumping group II (4Q272 and c4Q272).

Previous reports had established the highly resistant nature of many *B. thuringiensis* to penicillin G. This study found that *B.t.i.* strain 4Q2 was highly resistant to penicillin G (MIC = 12.5 mg), but strains 4Q272, and c4Q272 were found to be quite susceptible to penicillin G with MIC of 0.06, and 0.03, respectively.

Extraction of S-layer protein by treatment with 6M Urea indicated that there appeared to be extra-high molecular weight protein in the extracts obtained from *B.t.i.* strain 4Q2. The protein band could not be detected in extracts obtained from strains 4Q272 and c4Q272. S-layer protein was purified by collecting S-layer protein band from several gels and then was electroeluted by

electroeluter. The antibody toward this purified protein was prepared and used for locating of S-layer protein on *B.t.i.* by using indirect immunofluorescent technique. Immunodiffusion reaction and western blot analysis confirmed the specificity of the anti-S-layer protein antibody.

It was found that the antibody against S-layer protein inhibited the plasmid transfer via conjugation-like process between *B.t.i.* strains 4Q2-16 and c4Q272. The frequency of transfer of pBC16 plasmid was found to be 9.7×10^{-6} and less than 1×10^{-8} in the absence and presence of anti-S-layer protein antibody, respectively. Furthermore, the antibody against 4Q2-S-layer protein could reduce the frequency of pBC16 plasmid transfer between *B.t.i.* strain A084-16-194 and c4Q272 from 2.2×10^{-5} to 1.2×10^{-6}.

Using antibody detection system, S-layer protein gene from *B.t.i.* strain 4Q2 was cloned in pBluescript KS and pUC12 of *E. coli* DH5α. Three positive clones containing the gene encoding for the S-layer protein namely pAC1, pAC2 and pAC3 were obtained. pAC2 and pAC3 had the insert of 5.2 kb, and pAC1 had an insert of 6.6 kb. The expression of the S-layer protein gene (*slp* gene) was confirmed by Western blot analysis.

The 5.2 kb fragment from pAC1 was subsequently subcloned in pBluescriptKS and designated as pAC11 and 1.5 kb fragment from pAC11 was further subcloned in

pBluescriptKS as well and designated as pAC111. The pAC111 containing 1.5 kb fragment which could express in both orientations was subjected to nucleotide sequence technique by using dideoxy chain-termination method. Analysis of the nucleotide sequence of the 1.5 kb fragment indicated that the presumed initiation codon (GTG) was at nucleotide 295, and read through all sequenced nucleotides. The putative -10, TATTAAT at nucleotides 220 to 226, or the putative -35, TTGTCC at nucleotides 187 to 192 was similar to the -10 and -35 concensus sequences.

Colony hybridization and Southern blot analysis of *B.t.i.* strain 4Q2, 4Q272 and c4Q272 with biotinylated DNA probe (5.2 kb fragment from pAC11) were performed. The result indicated that the S-layer protein gene could be located on chromosome of *B. thuringiensis* subsp. *israelensis*.

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