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MOUKTAR ABDI BARKAD
A THESIS SUBMITTED FOR THE DEGREE OF
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GEBZE
2021

T.C.

GEBZE TEKNİK ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

BACİLLUS THURİNGİENSİS'TE LON PROTEAZ YÜKSEK İFADESİNİN ENDOTOKSİN ÜRETİMİ VE STRES DİRENCİ ÜZERİNDEKİ ETKİSİ

MOUKTAR ABDI BARKAD

DOKTORA TEZİ

MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

TEZ DANIŞMANI

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GEBZE

2021

DOKTORA JÜRİ ONAY FORMU

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SUMMARY

Lon protease, an intracellular protease, plays a key role in cell homeostasis in bacteria and is involved in numerous physiological processes. In this work, we aimed to study the impact of Lon on the production of endotoxins and stress response in *Bacillus thuringiensis*, which is an important bioinsecticide alternative for toxic chemicals. For this purpose, *lon* gene was cloned both into a multi-copy vector and into an integrative vector with its original promoter and transcriptional terminator and expressed in *B. thuringiensis* serovar *israelensis* ATCC 35646. Our results showed that the recombinant *lon* gene transcribed and translated efficiently and the resulting protein was active. Although the sporulation efficiency of the recombinant strain was found to be reduced and its mobility impaired, overexpression of the *lon* gene triggered the production of endotoxin. Together with increased biofilm formation, recombinant strain exhibited significantly better adaptation to osmotic and heat shock stresses and UV exposure compared to wild type and the control strain with empty plasmid. To our knowledge, this is the first study focused on understanding the possible link between Lon protease and the production of insecticide and stress response in *B. thuringiensis*.

Key Words: Lon protease, *lon* overexpression, *Bacillus thuringiensis*, endotoxin production, stress response, UV tolerance.

ÖZET

Bir hücre içi proteaz olan Lon proteaz, bakterilerde hücre homeostazında anahtar bir rol oynar ve çok sayıda fizyolojik süreçte yer alır. Bu çalışmada, *Bacillus thuringiensis*’de Lon’un toksik kimyasallar için önemli bir biyoinsektisit alternatifi olan endotoksinlerin üretimine ve stres cevabına etkisini çalışmayı amaçladık. Bu amaçla, *lon* geni orjinal promotorü ve transkripsiyonel terminatörü ile birlikte hem çok kopyalı bir vektöre hem de integratif bir vektöre klonlandı ve *B. thuringiensis* serovar *israelensis* ATCC 35646’de ifade edildi. Sonuçlarımız rekombinant *lon* geninin verimli bir şekilde ifade edildiğini ve üretilen proteinin aktif olduğunu gösterdi. Rekombinant suşun sporulasyon etkinliğinde ve hareketliliğinde azalma tespit edilmesine rağmen, *lon* geninin aşırı ekspresyonunun endotoksin üretimini tetiklediği görüldü. Artan biyofilm üretimi ile birlikte rekombinant suş, ozmotik ve ısı şoku stresleri ile UV stresine yaban tip ve boş plazmit içeren kontrol şusuna kıyasla daha iyi adapte oldu. Bildiğimiz kadarıyla bu çalışma, *B. thuringiensis*'te Lon proteaz ile böcek ilacı üretimi ve stres cevabı arasındaki olası bağlantıyı anlamaya odaklanan ilk çalışmadır.

Anahtar Kelimeler: Lon proteaz, *lon* aşırı ifadesi, *Bacillus thuringiensis,* endotoksin üretimi, stress cevabı, UV toleransı.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms **Explanations**

α : Alfa

σ : Sigma

µL : Microliter

µM : Micromolar

Apr : Apramycin

APS : Ammonium Persulfate

ATP : Adenosine triphosphate

BamHI : *Bacillus amyloliquefaciens* H endonuclease

Bp : Base pair

$CaCl_2$: Calcium chloride

$CaNO_3$: Calcium nitrate

CLSM : Confocal Laser Scanning Microscopy

DSM : Difco Sporulating Media

EcoRI : *Escherichia coli* endonuclease I

EcoRV : *Escherichia coli* endonuclease V

EDTA : Ethylenediaminetetraacetic acid

$FeSO_4$: Iron sulfate

h : Hour

kb : kilobase

KCl : Potassium chloride

K_2HPO_4 : Dipotassium hydrogen phosphate

KH_2PO_4 : Potassium phosphate monobasic

KOAc : Potassium acetate

KpnI : *Klebsiella pneumoniae* endonuclease I

HindIII : *Haemophilus influenzae* endonuclease III
L : Liter
LB : Luria-Bertani
M : Molar
Mg : Milligram
Mg^{+2} : Magnesium ion
$MgSO_4$: Magnesium sulfate
min : Minute
mL : Milliliter
MM : Minimal Media
mM : Millimolar
$MnCl_2$: Manganese chloride
NaCl : Sodium chloride
NaOH : Sodium hydroxide
ng : Nanogram
nM : Nanomolar
OD : Optical Density
PBS : Phosphate Buffer Saline
PCR : Polymerase Chain Reaction
PI : Propidium Iodide
RT : Room Temperature
s : Second
SDS : Sodium Dodecyl Sulfate
SmaI : *Serratia marcescens* endonuclease I
UV : Ultraviolet

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1. INTRODUCTION

1.1. History of *Bacillus thuringiensis*

The use of bacteria as a pesticide is a very ancient method, the writings of ancestral Egyptians in prehistoric times mention that they used to use a collection of microorganisms to destroy the insects that invade their plantations [1]. The history of this bacteria initiated when a Japanese scientist called Shigetane Ishiwata isolated the bacteria, which we recognize today as *Bacillus thuringiensis*, from silkworm larvae (Bombyx mori) when he was investigating a disease called 'sotto disease’. The responsible bacteria named as Bacillus sotto in 1901 by Ishiwata [2]. Ten years later, German bacteriologist Ernst Berliner isolated the same strain from dead Mediterranean flour moths in Thuringia, and the currently known scientific name ‘’*Bacillus thuringiensis’’* was given by that author, it should be noted that “thuringiensis” came from the German province's name ‘’ Thuringia’’. Ernst Berliner studied more deeply about *B. thuringiensis* and discovered for the first time inclusion bodies alongside the endospores [3]. In 1953 Hannay Christopher et al. named those inclusion bodies as parasporal crystals and demonstrated that they are responsible for the insecticidal activities of *B. thuringiensis* [4]. In 1938, the first *B. thuringiensis* commercial biopesticide named ‘’sporeine’’ was produced in France to control flour moths just before the outbreak of the Second World War. Later on, the Environmental Protection Agency (EPA) recognized *B. thuringiensis* as a biopesticide in the United States of America around 1961 as an alternative to chemical insecticide. At that time, the market for *B. thuringiensis* bio-insecticides was designed specifically to combat Lepidoptera insect pests which were the most common in agriculture. However, the discoveries of two new strains in the late 1970s and early 1980s increased the effectiveness of *B. thuringiensis*. These new discovered strains were: “*Bacillus thuringiensis serovar* subspecies *israelensis*” which is toxic to mosquitoes and black flies, and the second was the *“Bacillus thuringiensis serovar* subspecies *tenebrionis*” subspecies which is toxic to beetles [5],[6]. However, the spectrum of action of *B. thuringiensis* continually expanded further due to the evolution of biotechnology in 1989 after the discovery of a large number of various strains of *B. thuringiensis* which were effective against different insects other than known ones. The expansion of recombinant DNA allowed transferring of the insecticidal gene into plants to get insect-resistant plants [7], [8]. Nowadays, *B. thuringiensis* pesticides account for 90 % of the bioinsecticides produced worldwide.

B. thuringiensis is a common Gram+ spore-forming bacterium of the genus *Bacillus*, motile through peritrichous flagella, and a facultative anaerobic organism. In the vegetative state, *B. thuringiensis* is rod-shaped bacteria that vary between 3 and 5 µm in length and 1 µm in width. This bacteria is ubiquitous and can be isolated worldwide from a great diversity of ecosystems including soil, phylloplane [9], from insect or bird feces [10], through dead insect [11], from rhizosphere [12], and also from aquatic environments [13].

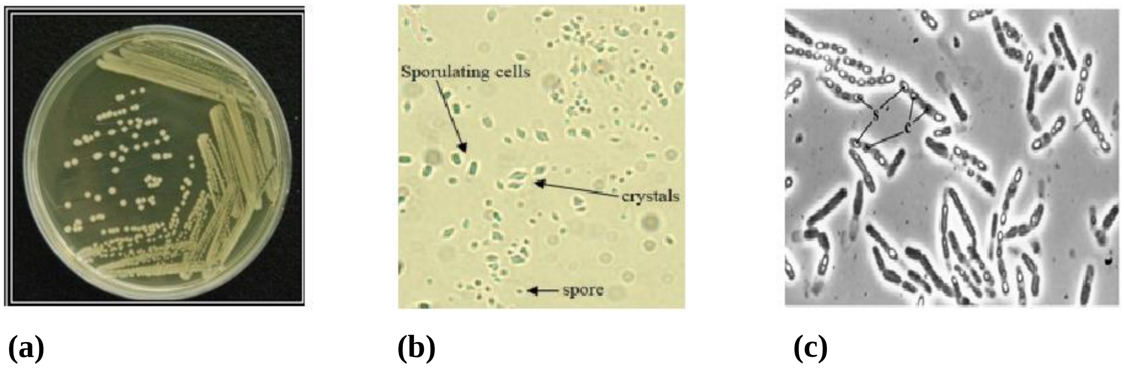
1.2. Classification and Morphological Properties of *B. thuringiensis*

One possible classification of *B. thuringiensis* is based on the antigenic properties of the flagellar antigen (H number) [14] even if it is not related to pathogenicity. By H serotyping, *B. thuringiensis* can be subdivided into more than 70 subspecies based on the type of flagellar antigens in a given a serotype H number (based on immunoassays). Every new isolate with a form of flagellar antigen that varies from others by immunoassays is given a new H antigen sero-variety number and a new sub-specific name. For example, we can give the H number of the four major subspecies of *Bacillus thuringiensis* used as bio-insecticides (Table 1.1): *B. thuringiensis* subsp *kurstaki* is known as H3a3b3c, *B. thuringiensis* subsp. *aizawai* as H7, *B. thuringiensis* subsp. *morrisoni* as H8a8b and the strain used in this thesis *B. thuringiensis* subsp. *israelensis* is classified as H14.

The colonies of *B. thuringiensis* appear round, white, and viscous with smooth edges on solid media (Figure 1.1a). The bacterial colonies that form on the first day are always very small and almost flat; on the second day, the bacteria grow much bigger and on the third day, the bacteria grow so big that the colonies join with each other and became indistinguishable. The spore of this bacterium is characterized by its ellipsoidal shape (mostly cylindrical) present in the central or paracentral region of the mother cell, refractive or shiny shape [12]. Parasporal crystal inclusions and spores shown under the microscope are presented in Figures 1.1b and 1.1c.

Table 1.1: *B. thuringiensis* subspecies used as bio-insecticides and their H number.

Subspecies	H-Antigen	Major Endotoxin Proteins	Insect spectrum
Bt kurstaki	H3a3b3c	Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa	Lepidoptera
Bt aizawai	H7	Cry1Aa, Cry1Ab, Cry1CA, Cry1Da	Lepidoptera
Bt morrisoni	H8a8b	Cry3Aa, Cry3Bb	Coleoptera
Bt israelensis	H14	Cry4Aa, Cry4Ab, Cry11Aa, Cyt1Aa	Diptera



(a) **(b)** **(c)**

Figure 1.1: Morphological properties of *B. thuringiensis*. (a) *B. thuringiensis* colonies on agar (b) Microscopic examination of *B. thuringiensis* showing the presence of crystal proteins and spores (c) Morphology of *B. thuringiensis israelensis* during the sporulation phase in phase contrast microscope. S: spore and C: Crystal protein.

B. thuringiensis is genetically so similar to *Bacillus cereus* that some studies have concluded the two bacteria should be considered as one genus. The only characterization available so far is the production of endotoxins during its sporulation phase by *B. thuringiensis*. Moreover, acrystalliferous *B. thuringiensis* strains are thought to be indistinguishable from *B. cereus. B. thuringiensis* has a genome size of 2.4 to 5.7 million base pairs (Mbp) and particularly, Bti strain ATCC 35646 has a genome of 5.88 Mbp encoding 6132 proteins. It has a guanine-cytosine (GC) content of 34.5 percent to 35.6 percent [15].

Besides, *B. thuringiensis* possess circular and/or linear extra chromosomal plasmids varying between 1 to 17 plasmids of 2 to 80 MDa [16]. The plasmids of *B. thuringiensis* can be classified into two, megaplasmids of size greater than 30 Mbp with a low number of copies and can include important genes such as among others the cry genes, and small plasmids of size less than 30 Mbp, constituting multiple copies without specific roles. Genes found in plasmids carry parasporal inclusions, some of which are self-transmissible during conjugation.

B. thuringiensis also contains many transposable elements that aid in the bacterial cell's cry gene amplification. The transposable elements are thought to trigger the transfer of plasmids between self-conjugative plasmids and chromosomal DNA or non-conjugative plasmids.

1.3. Life Cycle of *B. thuringiensis*

Vegetative cell division and sporulation are the two phases that characterize the life cycle of *B. thuringiensis* [17]. These involve changes in both the morphology and the biochemistry of the cells. During the vegetative phase, *B. thuringiensis* or almost all bacteria divide into two identical daughter cells by forming a septum in the middle of the plasma membrane (Figure 1.2a).

However, when living conditions are extremely harsh, *B. thuringiensis* produces spores and crystalline inclusions, this is called the sporulation phase. In this case contrary to the vegetative cell division, asymmetric cell division is implicated, which is marked by seven stages, which include: (a) axial filament formation; (b) formation of a pre-spore septum; (c) the first appearance of parasporal crystals and formation of a pre-spore; (d) formation of exospore, primordial cell wall, and nucleoid transformation of spores; and ultimately (e) cells lysis and spore maturation (Figure 1.2b). The body's endospores of *B. thuringiensis*, like other sporulating species, are more resistant than vegetative cells to heat, drying, disinfection, and other chemical destructive agents and can be viable for several years. Indeed, once again the nutrients are sufficient, and the environmental conditions are suitable for growth, the germination of the spore to produce vegetative cells occurs, a process called the germination phase.

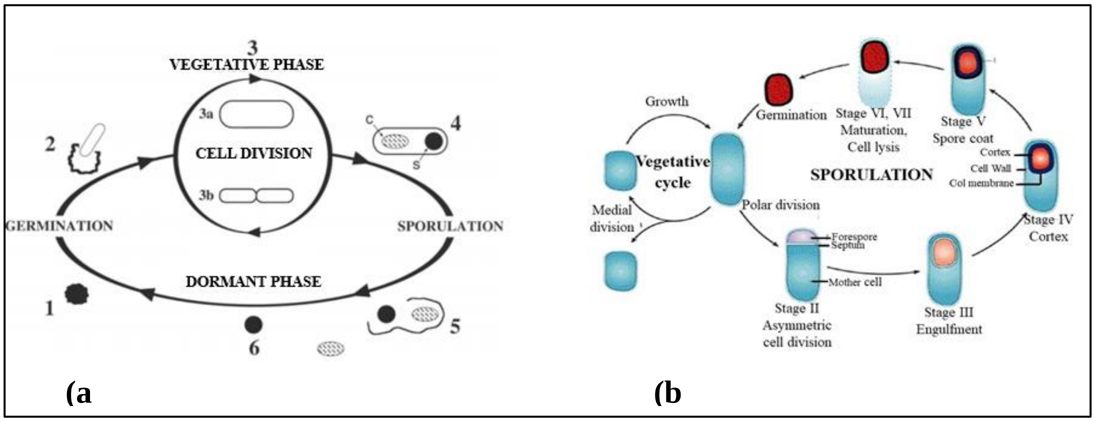


Figure 1.2: Life cycle of *B. thuringiensis* and sporulation cycle. **(a)** The life cycle of *B. thuringiensis* 1: germination (rehydration of the spore), 2: germination (production of the vegetative cell), 3: growth and division of vegetative cells, 4: sporulation (formation of the spore (s), and crystalline inclusion (c)), 5: lysis (release of the spore and crystal), 6: the period of dormancy (the spore resists to unfavourable conditions) **(b)** Key stages of the sporulation cycle in *B. thuringiensis*.

2. Endotoxin Production by *B. thuringiensis*

2.1.1. Types of Parasporal Bodies and Crystal Inclusions

As discussed above, the key difference between *B. thuringiensis* and its relative spore-forming bacilli strains is the production of parasporal inclusion during the sporulation phase of its growth cycles. And through this production of insecticidal toxins, *B. thuringiensis* has attracted the interest of the scientific community and has been able to collect more than one hundred thousand (100 000) isolates of *B. thuringiensis* during the last forty (40) years by researchers in industrial, and university laboratories around the world. Indeed, Cry (abbreviated for crystal) and Cyt (abbreviated for cytolytic) proteins are the two types of insecticidal crystal proteins secreted by *B. thuringiensis*, with variants on each. Different types of parasporal bodies have been demonstrated in these latest decades, some containing one or more Cry proteins (Cry and cyt)[14]. *B. thuringiensis* has a spherical parasporal body that ranges between 0.7 µm and 1.2 µm in diameter (Figure 1.3). Parasporal inclusions can differ in number, shape, location, and size between subspecies. Besides, bipyramidal, cuboid, rhomboid, oval, circular, or even no definite shape crystals are examples of *B. thuringiensis* inclusion bodies.

The parasporal body can be made up of three different types of protein inclusions that are connected by a laminated, net-like envelope whose composition is still unknown. Each inclusion is covered by one or more layers of the envelope's material. The parasporal body appears to be round and composed of only one or two inclusions: one large inclusion with low electron density and another very high electron density with a smaller size. Later, a third type of inclusion, smaller than the two others with a moderate electron density was discovered. In short, the three distinct types of protein inclusions found in parasporal bodies are distinguished by their shape, size, and electron density, among other factors. For instance, at least two parasporal inclusions are secreted by *B. thuringiensis* subsp. *finitimus* with different locations within the sporulating cell [18], [19].

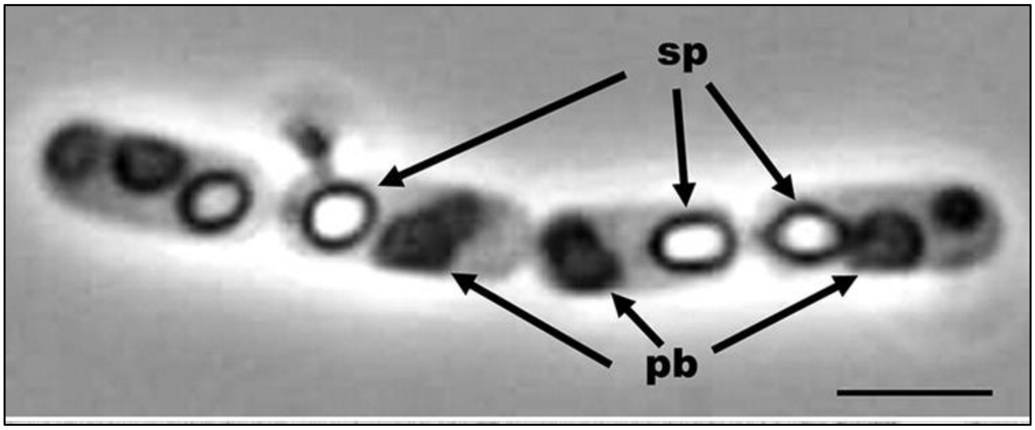


Figure 1.3: Phase-contrast microscopy of mature sporangia from Bt. Sp: spore; pb: parasporal body. Bars, 2 µm.

2.1.2. Classification of Insecticidal Toxins of *B. thuringiensis*

B. thuringiensis produces two types of insecticidal crystal proteins named Cry (for crystal) and Cyt (for cytolytic). In 1985 for the first time, the gene encoding endotoxin was sequenced by Schnepf, and to date more than 150 Cry proteins and 12 different types of genes encoding Cyt proteins have been sequenced [20]. The toxins of *B. thuringiensis* were initially classified into five classes based on the insect orders for which they were effective. The five groups consisted: Hemiptera, Diptera, Hymenoptera, Coleoptera, Lepidoptera, Nematode (Figure 1.4a) [21]. For example, Group I toxins (CryI, CryIX) were effective against the insect order of Lepidoptera, group II (CryII) to Lepidoptera and Diptera; group III (CryIII, CryVII, and CryVIII) to beetles; Group IV (CryIV, CryX, CryXI) to Diptera and finally group V (Cry1I) to Lepidoptera and Coleoptera [22]. But later certain types of proteins have shown complex characteristics, for example, CryI-type toxins which were normally specific to Lepidoptera demonstrated dual activity against Lepidoptera and Diptera (CryIAb and CryIC proteins) [23]. So integrating the new toxins into existing groups caused a serious problem and, therefore, other classification methods based on amino acid sequence homology were developed by Crickmore et al. in 1998 [24]. In this nomenclature proposed by Crickmore et al, the roman numerals were replaced by Arabic numerals in the first row (for example, Cry2 instead of CryII) but the nomenclature of "Cyt" has been retained. Several subclasses were later developed (Cry1A, Cry1B, Cry1C, etc.) with sub-families (Cry1Aa, Cry1Ab, Cry1Ac, etc.). The three-dimensional structure of Crystal proteins toxins secreted by *B. thuringiensis* has been determined by X-ray crystallography (Figure 1.4b).

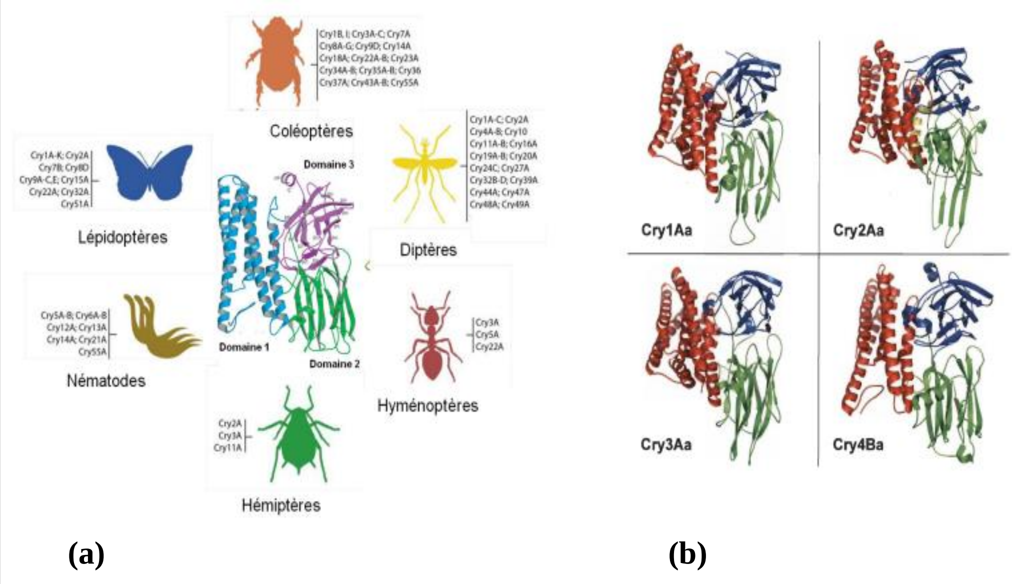


Figure 1.4: Insect orders and 3D structure of Crystal protein. (a) Coleoptera, Diptera, Hymenoptera, Hemiptera, and Lepidoptera are the different orders of insects that Cry toxins are effective (b) 3D structures of insecticidal toxins secreted by *B. thuringiensis*.

2.1.3. *B. thuringiensis* Mode of Action

Cry or Cyt proteins are the major component of the bio-insecticide and are different from each other in terms of amino acid sequence homology and in term of insecticidal activity mode [21]. These proteins are highly specific to their target insects, and are non-toxic to humans and other vertebrates, and can biodegrade easily [25], [26]. The mode of action of toxins starts with the ingestion of crystals proteins by the larvae. After ingestion, the crystals proteins must be dissolved in the alkaline environment of the larvae and release the protoxins which is the nontoxic product of the crystal proteins. Generally, the pH of digestive juices in the lumen of the intestine of most susceptible insects is 8 or higher, and that promotes the solubilization of the protoxins in the larvae's gut. Although, Cry and Cyt protein crystals can dissolve in the highly acidic stomachs of many vertebrates, including humans, but gastric juices quickly break them down into non-toxic peptides, typically within two minutes. This explains why these compounds are non-toxic to many not-targeted organisms, including humans [20]. Next, the protoxins have to be cleaved by the larvae's proteases to be active and effective against susceptible larvae.

This activation involves proteolytic cleavage of both the C-terminus and the N-terminus of the crystal protein (protoxin) which allows for the retaining of the active part of the crystal protein (active toxin). The detail of Cry protoxin structure and protease cleavage sites are shown schematically in Figure 1.5a. To cause toxicity, the toxin must cross the peritrophic membrane and attach to specific protein receptors on the microvilli of the intestine's membrane. There is no binding and therefore no toxicity if the specific receptors are not accessible and that explains the specificity of crystal proteins to insect species. To accentuate the existence of specific receptors on the microvilli of the intestine, we can review two examples: (1) lepidopteran larvae sensitive to Cry1 proteins, are not sensitive to Cry3 proteins because they lack the appropriate receptors. (2) *Heliothis virescens* larvae are extremely sensitive to Cry1A, whereas this particular toxin remains ineffective against Spodoptera. Once the toxins bind to specific receptors in the microvilli of the apical membranes, lytic pores form, cell lysis occurs, and the contents of the cell are released, resulting in the death of the insects at the end [27]. However, this proposed mode of action is not the only plausible one, certain authors believe the existence of other phenomena triggering toxicity but not yet demonstrated yet so far [20].

Besides, the mode of toxicity of Cyt proteins is different from that of the Cry. In the case of Cyt, it has been demonstrated that without the use of a protein receptor, the Cyt proteins bind directly to the lipid region of the microvilli membrane. They then accumulate in the membrane, triggering an osmotic imbalance that leads to cell lysis and ultimately causing the death of the insects.

Even though the toxicity mechanisms of the Cry and Cyt proteins are different, it seems that the two toxins are both pore-forming proteins.

Briefly, the mode of action of Cry proteins can be summarized as (Figure 1.5b):

i. To be toxic, the Crystal protein must be ingested by the larvae;
ii. After ingestion, the protoxins must be dissolved in the alkaline environment of the larvae’s gut (around pH8 or high);
iii. To be active, Cry proteins must be cleaved by midgut proteases;

iv. Once activated, the toxin must attach to specific receptors on the midgut microvillar membrane and then form a lytic pore;

v. Cells swell and release the contents leading to the death of the insects.

Besides the major role of Cry or Cyt in the insecticidal activities, the death of insects seems to be dependent also on a variety of other factors including β exotoxins and Vip. Recently, the works of Donovan et al showed that the suppression of *Vip3* gene from the genome of *B. thuringiensis* caused an important decrease of its insecticidal activity against *Agrotis ipsilon* and *Spodoptera frugiperda* larvae [28]. Other than the effects on insecticidal activities, these other factors also play a role in the protection of toxins from host defenses and allowing for solubilization of toxins in the gut of the insects [28], [29].

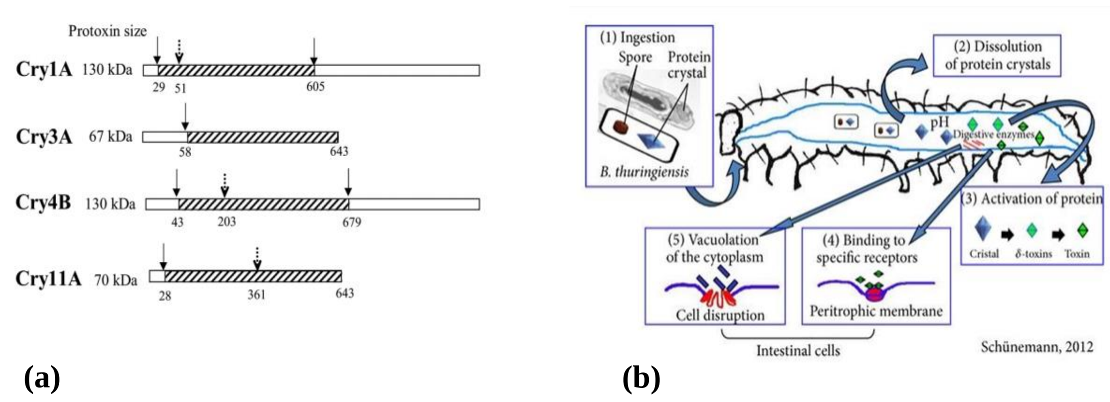


Figure 1.5: Mode of action of *B.thuringiensis* toxins. (a) Examples of the position of protease digestion on Cry protoxins (Cry1A, Cry3A, Cry4B, and Cry11A). The active toxin is illustrated by striped boxes. The amino- and carboxy-terminal cleavage sites of the *Bt* toxins are shown by black arrows whereas the dotted arrows show the intramolecular cleavages. (b) Mode of action of *B. thuringiensis* toxins in an insect larva. Toxins are shown in green while spores are depicted in blue.

2.2. Other Secondary Metabolites Produced by *B. thuringiensis*

In 1985, Kossel described secondary metabolites as compounds that aren't primary metabolites, but some people disagreed, and he received a lot of criticism for it. Later, he explained that primary metabolites are chemical elements of organisms that are essential to their normal functioning, while substances that are dispensable are secondary metabolites. The study of secondary metabolism was unfortunately neglected nowadays because it was considered non-essential and left to industrial and chemical laboratories and academics. Secondary metabolites perform various functions in the organisms, including providing defense against other microorganisms, acting as metal transporters, and serving as symbiotic agents between bacteria and other species [30]. Other than insecticidal crystal proteins discussed in detail in the previous sections, *B. thuringiensis* produces several secondary metabolites including Vegetative insecticidal proteins (Vips), β-exotoxin, and Zwittermicin A which are described in the following paragraphs.

As an example of secondary metabolite production by this bacterium, we can mention the production of vegetative insecticidal proteins (Vips) by *B. thuringiensis*. Unlike crystal proteins secreted during the sporulation, Vips are produced during the vegetative growth stage of *B. thuringiensis* and can be isolated from the culture medium. The structure of Vips is largely different from the Cry proteins and so do the mode of action. Vips are classified into four families (Vip1, Vip2, Vip3, and Vip4 proteins) each of which has shown insecticidal activities [31].

β-exotoxin, also known as ‘’thu’’, is a thermostable exotoxin, secreted by *B. thuringiensis* during vegetative growth, first identified by McConnell and al in 1959 and confirmed later by other authors [32]. Although its insecticidal mechanism is not yet fully elucidated, β-exotoxins are demonstrated to be toxic to several insect orders including Diptera, Coleoptera, Lepidoptera, Hymenoptera, Orthoptera, and Isoptera [33].

Zwittermicin A, an antibiotic of the amino-polyol class, was first found in *B. cereus*, which is genetically very close to *B. thuringiensis*. But subsequent studies discovered also that different strains of *B. thuringiensis* also produce this antibiotic. Zwittermicin A prevents the growth of a variety of procaryotes, lower eucaryotes, and fungi [34]. *Bacillus spp.* can also secrete various antibiotics such as Bacteriocins and Lantibiotics [30]. Figure 1.6 represents the chemical structures of secondary metabolites produced by *B. thuringiensis*.

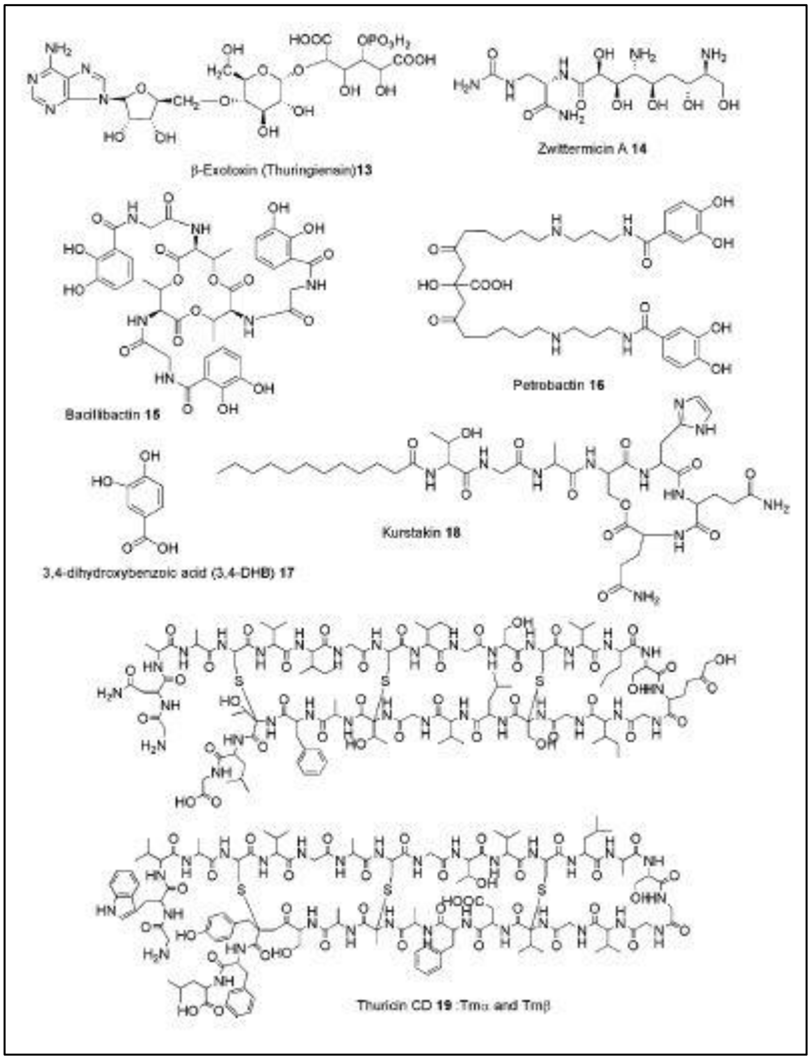


Figure 1.6: Chemical structures of secondary metabolites produced by *B. thuringiensis*.

2.3. Proteases and Their Role in *B. thuringiensis*

It is well understood that proper protein folding is needed for all cells to live and function properly. A protein's tertiary structure, however, cannot be maintained indefinitely, and even the most stable proteins inevitably can lose it naturally or after cellular stress. Conditions such as elevated temperatures, or unsuitable pH, or exposure to oxidative reagents can speed up the incidence of protein misfolding events. Therefore, all cells have molecular mechanisms referred to as "protein quality control" that promote survival under misfolded proteins stress. In all cells, protein quality-control systems have developed to protect from the harmful effects of unfolded or misfolded proteins both in prokaryotes and also in eukaryotes. *B. thuringiensis*, like other eubacteria, use many specific proteases, such as cytosolic Clp proteases (ClpAP, ClpXP, and ClpYQ), FtsH, and Lon to maintain cellular protein homeostasis [35]. All of these ATP-dependent proteases belong to the large AAA+ family proteins (ATPases associated with various cellular activities) [36]. Lon protease is one of the most important components of this degradation machine network [37]. It is an oligomer with identical subunits (~87 kDa) that form a ring-shaped hexamer [38]. Each subunit contains three domains: amino-terminal domain (allowing recognition and binding of substrates), ATPase domain (ATP binding site), and carboxy-terminal domain (proteolytic active site) [39]. Unlike other proteases of the same category, Lon proteases are comprised of the ATPase and the proteolytic sites on the same polypeptide chain [35].

Logically, when a protein is unfolded or misfolded, the cellular mechanism tries to refold it first; if this is ineffective, an ATP-dependent protease degrades it in order to prevent the formation of protein aggregation, which can be harmful to cells. Indeed, the mechanisms that allow the proteases to selectively recognize the abnormal proteins (misfolded or unfolded proteins) are critical for the proper functioning of the cells. However, it should be noted that this identification mechanism is not yet fully understood but it is known that the substrate selection is closely controlled to prevent excessive degradation of cellular proteins.

Previously, many hypotheses have been postulated via various research. Among them, it is advanced that the enzyme-substrate recognition could be based on the presence of specific amino acids which are exposed in the unfolded or misfolded proteins and probably hidden in the native structure of well-folded proteins (normal proteins). For example, short hydrophobic sequences that are visible in misfolded proteins but hidden in native structures were shown interacting with the DnaK and GroEL chaperones of *E. coli* [40]. However, a specific motif present in all the substrates recognized by Lon has not yet been identified, but the presence of certain amino acids or regions of certain substrates has been reported in many studies (e.g., the carboxy-terminal histidine of SulA, the first 24 amino-terminal amino acids of UmuD, a 20-residue sequence from β galactosidase binds to Lon in *E. coli*, the amino-terminal domain of SoxS and MarA). The architecture of the proteins and the positioning of the appropriate targeting sequences allow the degradation of Lon.

In addition to misfolded proteins, Lon degrades several natively folded regulatory proteins, which typically have a degradation tag at their amino or carboxy terminus [41]. Proteins that are tagged at their termini may be recognized by Lon even in the absence of unfolding. As an example, we can cite SulA, a cell division inhibitor, that has a Lon degradation tag (hydrophobic region) at its C-terminal of its sequence. Despite the fact that degradation tags are commonly found at protein termini, Lon can identify also internal tags, as has been demonstrated previously for the related ClpXP, ClpAP, and FtsH proteases [42]. Lon is believed to identify features of misfolded proteins, such as exposed hydrophobic elements, rather than specific sequences, for most of its quality control targets.

It has been established in previous studies that certain proteases produced by *B. thuringiensis* have an important role throughout the cycle of growth of bacteria. There are two types of proteases, intracellular and extracellular, playing distinct roles in the organisms. As is currently understood, cells produced the two types of proteases in two different phases of cellular function. The organisms produce mainly intracellular proteases and extracellular proteases in the sporulation phase and the vegetative phase respectively, and that infers the existence of distinct roles in sporulation and crystal proteins synthesis [43]. About the roles, previous studies suggested that intracellular proteases intervene in the cell lysis and then may be responsible for the release of crystal proteins and spores. While extracellular proteases play a role in the degradation of substrates in the culture permitting the extraction of amino acids necessary for the synthesis of spores and toxins. Moreover, it is demonstrated in previous works that during the early stages of sporulation, several enzymes, mainly proteases, are produced by the cell during the spore formation and became decreased after the sporulation [44], [45]. This implies a role that protease would play during sporulation in *B. thuringiensis*.

Since endotoxin production occurred during the sporulation phase, it is easy to understand that *Bt*'s proteases can play a role in the insecticidal activity of *B. thuringiensis*. Some authors tried to activate the protoxins with the own protease of *B. thuringiensis* and they showed that the proteases were able to activate the protoxins and showed similar toxicity to the ones activated by insects' proteases [46]. Moreover, the use of protease inhibitors exhibited a defect of insecticidal activities in *B. thuringiensis* [43]. These findings demonstrated the existence of roles of proteases in the insecticidal activity of *B. thuringiensis*. Hence, in this work, we aimed to investigate the role of lon protease in the production of endotoxins and stress response in *B. thuringiensis*.

3. Lon ATP Dependent Protease

3.1. Lon ATP Dependent Protease and its Implication in Cellular Processes

Lon protease may be essential in a variety of cellular processes in most bacteria; however, this enzyme may be tightly regulated at the transcriptional, translational, and even post-translational levels. Since overexpression of the *lon* gene has been shown to be harmful to cells in *E. coli* and *Streptomyces lividans*, this protease must be maintained at an appropriate level in certain species to ensure the normal progression of various cellular processes [47]. Nevertheless, under some situations, a higher Lon rate would be necessary. The regulation of Lon proteases is required to avoid unnecessary degradation of cellular proteins. *Lon* gene is under control of Heat shock regulon even if all are not heat-induced (e.g. one of the two *lon* genes of *Mycobacterium xanthus* (*lonV*) and the *lon* gene of *Bacillus brevis*). For example, one of *Bacillus subtilis'* two Lon paralogues (the lonA gene) is a member of HS genes, but besides the heat stress, it can also be induced by a variety of other stresses, including salt, ethanol, H_2O_2, and puromycin [48]. *Lon* genes can be under the control of positive or negative regulators or even sometimes both. For instance, *lon* gene of *S. lividans* is negatively regulated by HspR and positively regulated by ClgR. The σ^{32} factor is responsible for transcriptional activation of the Heat Shock (HS) regulon in *E. coli*. HS genes are usually negatively regulated by specific repressors in most bacterial organisms. The negative regulation of Lon by HspR is needed for normal growth conditions.

As shown above, Lon protease is regulated at the transcriptional level (HS regulon). However, Lon regulation is not only occurred at the transcriptional level but also a post-translational regulation exists in cells. The proteolytic activity of Lon is controlled by a specific inhibitor. Since Lon proteases need to hydrolyze ATP to fulfill its role of protein cleavage, some inhibitors block the possibility of hydrolyzing ATP and therefore the activity of Lon is inhibited. The degradation of misfolded or unfolded proteins by Lon proteases was reduced in phage-infected *E. coli* because of protein T4 (PinA protein) which inhibited the activity of Lon protease [49]. This protein blocks the activity of lon by interfering with the hydrolysis of ATP.

Besides, whether Lon protease is an indispensable gene for the organism is still debated. In general, most authors believe that *lon* gene is not an essential gene for viability and does probably not play a major role in bacteria. However, it is discovered via various research that Lon mutant strains display several cellular defects in different organisms particularly in *E. coli*, among others we can quote:

i. *E. coli* lon mutant displayed first an accumulation of abnormal proteins since Lon is the main protease involved in the degradation of unfolded or misfolded proteins.
ii. Mucoid colonies and long filaments are also shaped in lon deficient *E. coli* strain.
iii. Furthermore, *lon* gene-deficient *E. coli* results in pleiotropic phenotypes, such as increased sensitivity to UV irradiation and SOS-inducing agents.
iv. Likewise, the impossibility of sporulation in *M. xanthus* mutated at LonD were also reported in the literature.
v. Kurada and coworkers showed that *E. coli* lon mutant didn’t adapt to a nutritional downshift [50]. This can be explained by the fact that during nutritional starvation, Lon protease degrades unnecessary proteins to generate amino acids which will be used in the synthesis of proteins or enzymes useful to overcome this particular condition.

Moreover, similar effects have been observed in *lon* mutant strains of eukaryotic species, such as Lon-deficient yeasts, which are unable to live on a non-fermentable carbon source. These different findings suggest the existence of an implication of Lon protease in physiological processes in a variety of species

3.1.1. Impact of Lon on Cell Cycle and Differentiation

Bacteria undergo differentiation, resulted in morphologically and developmentally differentiated cells, each of which is driven by the expression and/or activity of specific regulatory factors at each point. In literature, it is demonstrated that ATP-dependent proteolysis is needed to control the amount and availability of all these regulatory factors. Thus, protein regulation shows oneself as critical for bacterial growth and development. During the stress response, the protease helps the cell to recover from cell cycle depletion and the resulting damage. Indeed, Lon protease is known to degrade unfolded or misfolded protein on the stress condition. Interestingly in *Caulobacter crescentus* both in normal and stress conditions, Lon protease plays a crucial role in the degradation of several important regulators. For instance, in order to complete cell cycle division, the chromosome of *C. crescentus* needs to be methylated by CcrM methylase, yet this enzyme is controlled by Lon protease [51]. Similarly, in *E. coli*, Lon protease controls the DNA methylation, necessary for replication of DNA, via Dam methylase [52]. Therefore, Lon is also implicated in the progression of the cell cycle in bacteria. Moreover, during proteotoxic stress, Jonas et al. revealed that Lon protease causes cell cycle arrest by degrading the AAA+ ATPase DnaA (the initiator of DNA replication) in *C. crescentus* [53]. As shown in Figure 1.7, at normal conditions, DnaK binds to σ^{32}, causing DnaA accumulation and allowing DNA replication to begin in bacteria. But in the case of severe stress, unfolded proteins increase causing the dissociation of DnaK and σ^{32}. Then, as Lon protease production rises, DnaA degradation occurs, causing ultimately cell cycle arrest. However, when the unfolded proteins are well managed by the proteases, the cell cycle may continue.

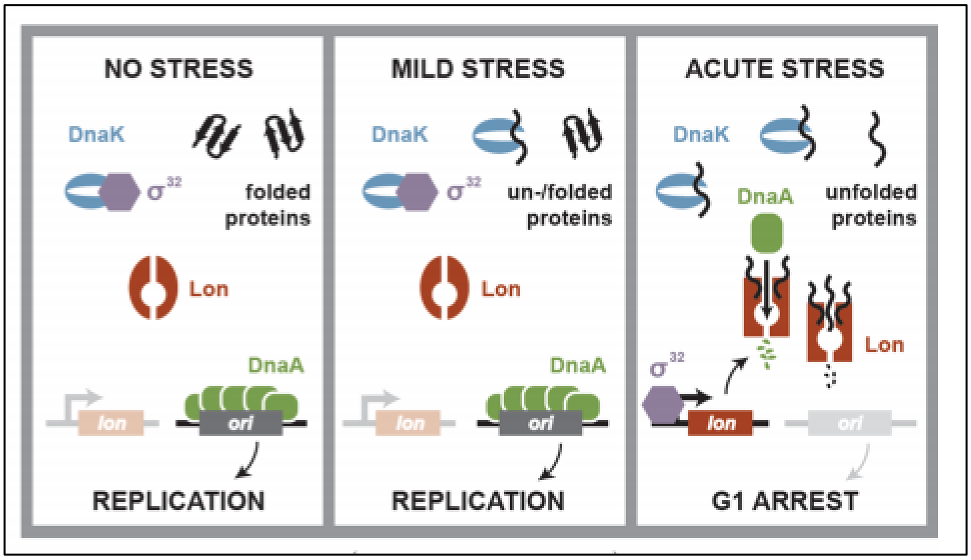


Figure 1.7: Control of DnaA by Lon protease in *C. crescentus*.

3.1.2. Effect of Lon on Pathogenesis and Sporulation

Pathogenesis is known being the capacity of a bacterium to cause disease. It is measured by its virulence which is related to the ability of the bacteria to cause disease despite host defense. Pathogenesis refers to both the process by which the bacteria accomplish infection and to the mechanism by which the disease evolves. The virulence factors allow the organisms to attack the host, run away from the host's defense then leading to disease in the host. Bacteria first eject virulence proteins (or also named effectors) into the host cell. These effectors have the ability to escape the immune system of the host and boost the parasitism. Type Three Secretion Systems (TTSS) is used by some pathogenic bacteria to accomplish this mechanism explained above. In literature, it is acknowledged that Lon regulates the transcription of TTSS and hence influences the virulence of some pathogenic bacteria. Indeed, Lon protease also influences the virulence of *Salmonella enterica* serovar Typhimurium by controlling the transcription of gene encoding TTSS by degrading HilC and HilD which are the transcription activator. Besides, studies realized on mice showed that lon mutant *S. enterica* was unable to escape the phagocytosis of murine macrophage. This suggests that Lon involves in two stages of *S. enterica* that is epithelial invasion and the escape from macrophage phagocytosis.

Essentially, sporulation refers to the formation of spores from vegetative cells induced by nutritional limitation. As such, it may be described as an adaptive response that allows the organism to survive during unfavorable environmental conditions (such as radiation, extreme heat or cold, desiccation, lack of nutrition). The formation of spore starts when some of the vegetative cells go through a series of morphological changes and it is characterized by some level of timed gene expression events and coordination between cells. Indeed, proteolysis by ATP-dependent proteases also play a big role in sporulation since this event needs to degrade the unnecessary proteins or regulators for this condition. *M. xanthus* has two *lon* genes, one is essential for viability (*lon*V) and the other one (*lon*D) for sporulation and fruiting body formation. The expression of *lon*D tends to increase during development. Moreover, it was shown that *M. xanthus lon*D mutant fails to sporulate.

Regards *B. subtilis*, sporulation is triggered by the accumulation of phosphorylated form of transcription factor *SpoA*, regulated itself by the sigma factor σ^H. And Lon protease of *B. subtilis* regulates this sigma factor H (σ^H), controlling the expression of transcription factor of the sporulation.

Figure 1.8 indicates a diagram summarizing the various substrates of Lon and the biological processes influenced by ATP-dependent lon protease.

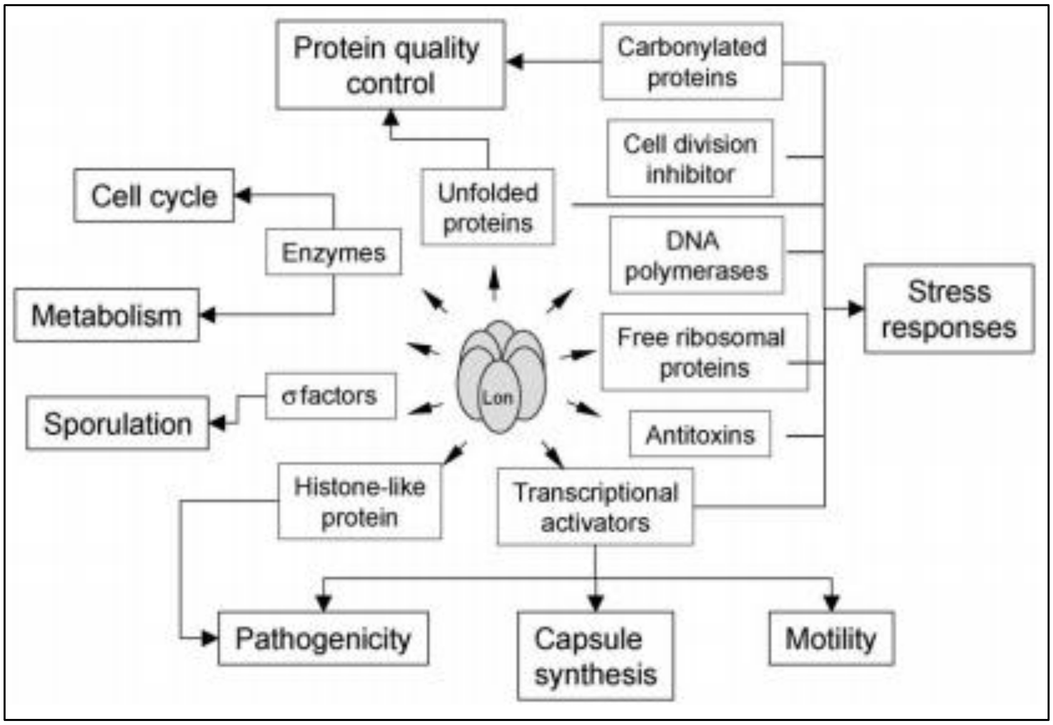


Figure 1.8: Diagram recapping the different substrates of Lon and the biological processes controlled by ATP Lon protease.

3.2. Aim and Contribution of the Thesis

Lon protease is an intracellular serine protease which is known to be involved in a various physiological process. A previous study in our lab showed the impact of Lon protease on the production of secondary metabolites in *Streptomyces coelicolor*. To investigate whether Lon protease would also affect the secondary metabolite production in *B. thuringiensis* was a matter of curiosity and pushed us to provide answers in this present study.

B. thuringiensis produces larvicidal toxins known as insecticidal crystal proteins (ICP) during sporulation, which are now used as biological pesticides all over the world. These bioinsecticides are now used to control insect pests and to substitute chemical insecticides with their undesirable effects. Indeed, to compete with chemical insecticides, the *B. thuringiensis* pesticide industry must produce more endotoxins at a low cost and in a short period. For this purpose, scientists are needed to study in-depth the molecular analysis of crystal proteins in order to boost endotoxin production in *B. thuringiensis*. In our study, we aimed to investigate the effect of Lon protease on endotoxin production in *B. thuringiensis*, and its effects on stress resistance and on other biological processes.

In this present thesis, the construction of a recombinant strain with a high level of *lon* protease expression may result in an increase in the synthesis of endotoxins in *B. thuringiensis* serovar *israelensis*. The *B. thuringiensis* pesticide industries would be interested in this kind of strain for two reasons. First, this recombinant strain would be able to generate a higher level of Crystal proteins than wild strain, and second, this strain would be more Eco-friendly due to its lower spore formation.

In brief, the findings of this thesis may enable the identification of an intracellular protease, Lon, that influences the production of a secondary metabolite of *B. thuringiensis*. This thesis would bring a new insight to the scientific literature by becoming the first to discover a correlation between lon protease and endotoxin production in *B. thuringiensis* serovar *israelensis*.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Media and Solutions

Tables 2.1 and 2.2 list all the media and solutions that were used in this present thesis. Unless otherwise stated, all of the media used here were sterilized in an autoclave at 121 °C for at least twenty minutes.

Table 2.1: Media used in this thesis.

MEDIA
For 500 ml of LB media (Luria Bertani), we weight: - 5 g Sodium Chloride (NaCl) - 5 g Peptone - 4 g Yeast Extract - Add 15% of agar for LB agar
For 500 ml of BHI media (Brain Heart Infusion): - 18.5 g BHI
For 500 ml of DSM (Difco Sporulating Media): - 4 g Nutrient broth - 4.35 g K_2HPO_4 - 3.40 g KH_2PO_4 - 5 ml of 50 % glucose - 1 ml $CaNO_3.4H_2O$ (from stock 118 mg/ml) - 1 ml $MgSO_4.7H_2O$ (from stock 123 mg/ml) - 1 ml $FeSO_4.7H_2O$ (from stock 2.7 mg/ml) - 1 ml $MnCl_2.2H_2O$ (from stock 1.62 mg/ml)

Table 2.2: Solutions used in this thesis.

SOLUTIONS	
Solution I (+4 °C) for Plasmid isolation: - 25 mM Tris (pH: 8.0) - 10 mM EDTA (pH: 8.0) - 50 mM Glucose **Solution II for Plasmid isolation:** - 0.2 N NaOH - 1% SDS Solution II must be prepared freshly and keep at RT. **Solution III (+4 °C)** - 60% 5M KOAc - 11.5% Glacial Acetic Acid **Tris-EDTA (TE) Buffer** - 10 mM Tris - 1 mM EDTA **TfbI Solution (+4 °C)** - 5 M KOAc - 1 M $MnCl_2$ - 1 M KCl - 1 M $CaCl_2$ - 75% Glycerol **TfbII Solution (+4 °C)** - 1 M Na-MOPS - 1 M $CaCl_2$ - 1 M KCl - 75% Glycerol **50x TAE** - 50 mM EDTA - 2 M Tris - 1 M Glacial Acetic Acid **EB** - 0.65 M sucrose - 1 mM $MgCl_2$ **STET (4 °C)** - 8 g Sucrose - 0.5% Triton X-100 - 1 M Tris-HCl - 0.5 M EDTA	**Coomassie Blue (for SDS PAGE)** - 50% Methanol - 0.05% Coomassie - 10% Acetic Acid - Completed the remaining 40% with sterile distilled water **Destaining Solution** - 50% Methanol - 10% Acetic acid Completed the remaining 40% with distilled water **SDS separating gel contents (12%)** - Tris-HCl buffer (pH 8.8) 2500 µl - Acrylamide (40%) 3000 µl - Sterile dH_2O 4300 µl - SDS (10%) 100 µl - APS (10%) 100 µl - TEMED 4 µl **Stacking gel contents** - Tris-HCl (pH 6.8) 500 µl - Acrylamide (40%) 500 µl - Sterile dH_2O 2920 µl - SDS (10%) 40 µl - APS (10%) 40 µl - TEMED 4 µl **1X Running Buffer for SDS (1 L)** - 14.4 g Glycine - 3.02 g Tris - 1 g SDS Completed with dH_2O to 1L

4.1.2. Chemicals

NaCl (Sigma-Aldrich), Peptone (Merck), Yeast Extract (BactoTM), Nutrient broth (Merck), Sucrose (Merck), Brain Heart Infusion (Merck), Agar (Merck), EDTA (Merck), Tris (Merck), Glucose (Merck), KOAc (Merck), Agarose (Sigma Aldrich), $FeSO_4$ (ApplıChem), $CaCl_2$ (Merck), $MgCl_2$ (Merck), KCl (Riedel de Haen), $MgSO_4$ (Merck), $CaNO_3.4H_2O$ (Merck), KH_2PO_4 (Merck), K_2HPO_4 (Merck), K_2SO_4 (Merck), $MnCl_2.2H_2O$ (Merck), NaOH (Merck), SDS (Merck), RedSafe Nucleic Acid Staining Solution (İNtRON Biotechnology), Acetic Acid (Riedel de Haen), Phenol (Sigma Aldrich), Chloroform (Sigma Aldrich), Glycerol (Sigma Aldrich), Triton X 100 (Sigma Aldrich), MOPS (Sigma Aldrich), Isopropanol (Merck), Ethanol (Riedel de Haen), Methanol (Sigma Aldrich), CTAB (Cetyl trimethylammonium bromide) (Merck) were used during this thesis.

4.1.3. Enzymes and Antibiotics

The list of enzymes, as well as the manufactured company, and the final concentration of antibiotic used in this study, are quoted here: Q5 DNA Polymerase (NEB Biolabs), Taq polymerase (Thermo Scientific), Deoxyribonuclease I Dnase (Thermo Scientific), SmaI Restriction Endonuclease (Fermantas), HindIII (Roche), KpnI Restriction Endonuclease (Fermantas), BamHI Restriction Endonuclease (Thermo Scientifıc), EcoRI Restriction Endonuclease (10 U/µL), RNase (Sigma), T4 DNA Ligase (NEB), FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific), Lysozyme (Sigma), EcoRV (NEB), Kanamycin (50 µg/ml) (Vetaş), Apramycin (50 µg/ml) (BİOSYNTH), Erythromycin (25 µg/ml) (Actavis), Ampicillin (100 µg/ml) (Sigma) and Chloramphenicol (50 µg/ml) (Sigma).

4.1.4. DNA Kits and Markers

Plasmids and PCR products were purified from agarose gel by using the PCR gel cleaning and extraction kit (Macherey-Nagel™ MN). The cells were stained with the

LIVE/DEAD Bac-Light Bacterial Viability Kit (L-7012) in order to observe and examine the samples in Confocal Laser Scanning Microscopy (CLSM). In this thesis, we utilized the RNA Isolation ‘’NucleoSpin RNA Plus’’ Kit (Macherey-Nagel, Germany (MN) for RNA isolation and iScript cDNA Synthesis Kit (Bio-rad, USA) for cDNA synthesis. RT-qPCR was used in this research to assess gene expression of certain genes using SYBR Green qPCR Master Mix (Bio-rad, USA). Figure 2.1 illustrates the molecular DNA markers (1 kb and 100 bp ladder Thermo Fisher Scientific) used in this research to examine the sizes of the DNA or RNA band and the protein marker PageRulerTM Plus Prestained Protein Ladder (Invitrogen).

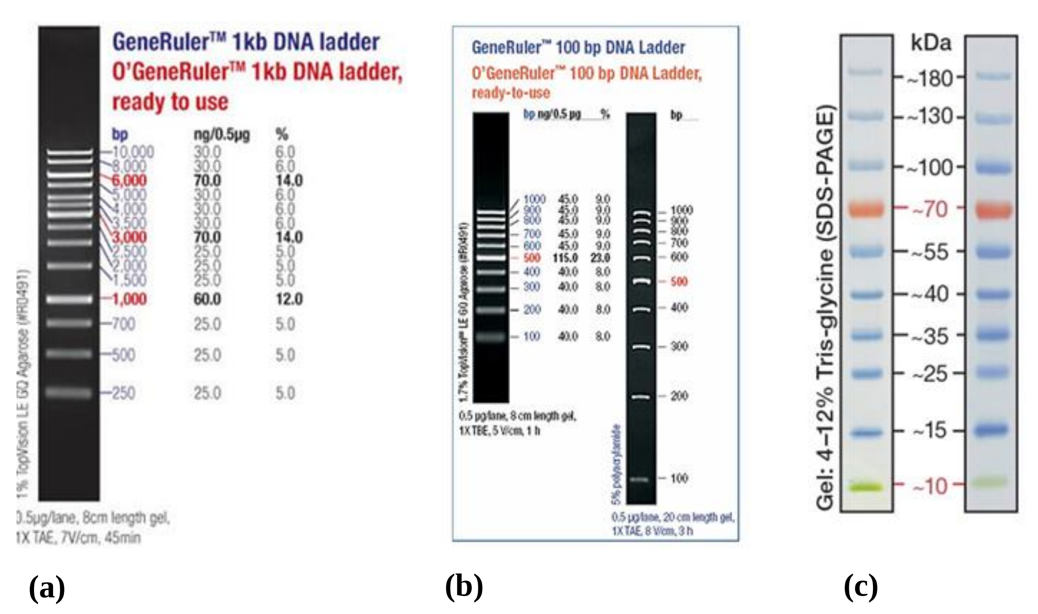


Figure 2.1: Molecular markers used in the study. (a) 1 kb molecular DNA marker (Thermo Scientific); (b) 100 bp molecular DNA marker (Thermo Scientific) and (c) protein marker pre-Stained protein ladder.

4.2. Methods

4.2.1. Strains and Plasmids

The plasmids and strains used in this present work are resumed in Table 2.3. Figure 2.2. represents the map of the plasmids used in this thesis (pRA and pHT315).

Table 2.3: Strains and Plasmids used in this thesis.

Strains and plasmids	**Comments**	**References**
Plasmids		
pHT315	*E. coli*/*Bacillus* shuttle vector	[54]
pRA	Integrative and conjugative plasmid derived from pSET152 *aac(3)IV*	[55]
pUZ8002	*tra*, *neo*, RP4	[56]
pHT315lon	*lon* gene cloned into pHT315	This work
pRAlon	*lon* gene cloned into pRA	This work
Strains		
E. coli DH5α	F– *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (rK–, mK+), *sup*44, *rel*A1λ-,(σ80 dL*ac*ZAM15), *D(lacZYA-argF*)U169	[57]
E.coli ET12567	dam, dcm, hsdS, cat, tet	[58]
E.coli ET12567/pUZ8002	*dam, dcm, hsdS, cat, tet/ tra, neo,* RP4	[58]
Bacillus thuringiensis	Bti ATCC 35646 wild type	ATCC

Table 2.3: Continue.

Bti pHT315	Bti containing pHT315	[59]
Bti pHT315 lon	Bti containing pHT315lon	This work
Bti pRA	Bti containing pRA	This work
Bti pRAlon	Bti containing pRAlon	This work

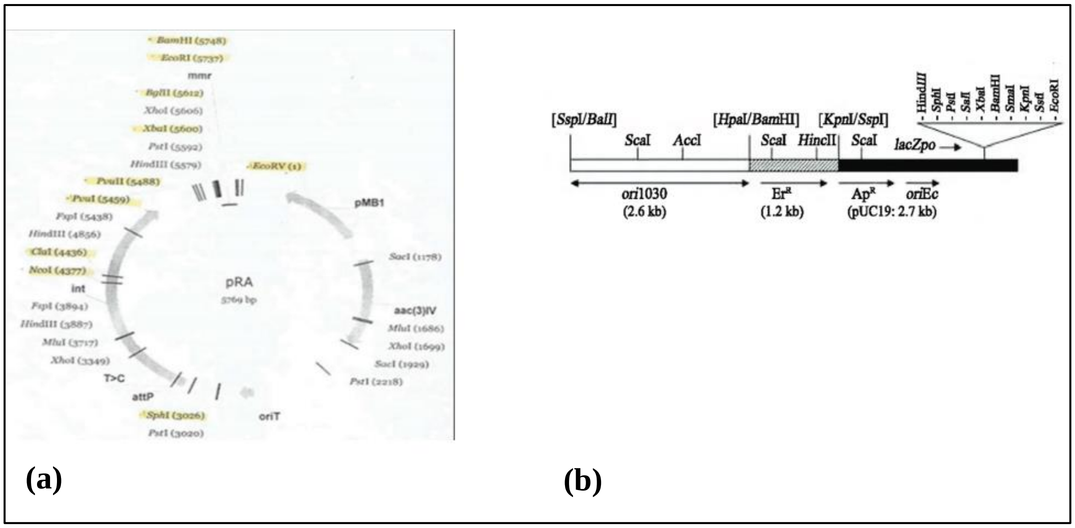


Figure 2.2: Plasmids used in this thesis. (a) pRA shuttle vector (5769 bp), (b) pHT315 *E. coli/*Bacillus shuttle vector (6500 bp).

4.2.2. Chromosomal DNA Isolation

B. thuringiensis genomic DNA was isolated according to the method described by Ausubel et al [60]. Briefly, after centrifugation of the overnight culture cells at 4 ^{o}C for 10 min, the resultant pellet was resuspended in Tris EDTA (TE) buffer and lysozyme was added (final concentration 10 mg/ml) before incubating for two hours at 37 ^{o}C in a water bath. Later 300 µL of 5 M NaCl and 240 µL of CTAB/NaCl solution was added and incubated at 65 ^{o}C for 10 min. Thereafter, an equal volume of sample and chloroform: isoamyl-alcohol (CIA, 23: 1) solution was mixed, cooled at room temperature for few minutes, and then centrifuged at 11.000 rpm for five min. The upper phase was then treated first time with phenol: CIA solution and a second time with an equal volume of CIA. Following each treatment, a centrifugation phase was performed. The last pellet was washed with 70% ethanol, centrifuged, and the supernatant was removed before drying in a laminar hood to evaporate the remaining ethanol and dissolving the pellet with distilled water and stored at -20 ^{o}C for further use.

4.2.3. Construction of Recombinant Plasmids

Before amplification of *lon* endopeptidase sequence gene by PCR, it was inevitable to first design the necessary primers to amplify the desired DNA sequence. For this, we designed the necessary primers for *lon* gene amplification in Bti using Primer3 software. Then, IDT Oligo-Analyzer Tool (Integrated DNA Technologies Inc., IA, USA) was used to analyze the presence of self-dimer, secondary structure, and heterodimer formation. All the nucleotide sequences of oligonucleotide primers used in this work are shown in Table 2.4. The genomic DNA that contains the *lon* endopeptidase coding sequence with its promotor and transcriptional termination sequence was amplified by using bti-lonF and bti-lonR as forward and reverse primers respectively. The whole genome of *B. thuringiensis* was used as a template for the PCR reaction and the High-Fidelity Q5 DNA polymerase as polymerase enzyme. Before the analysis of experimental samples, the optimum annealing temperature for each primer was technically tried to establish. The PCR product was cloned into the multicopy vector pHT315 at the SmaI site of the plasmid resulting in the recombinant vector pHT315lon.

Concerning the cloning of the *lon* gene into the pRA vector, the primers F-lonBti-BamHI (lon primer containing BamHI restriction enzyme site at 5’ end) and R-lonBti-EcoRV (lon primer with EcoRV restriction enzyme site) were used as forward and reverse primers respectively to amplify *lon* gene, with its putative promoter and the transcriptional termination site. The gene fragment was treated with BamHI and EcoRV before purified from agarose gel. The purified fragment was then cloned into integrative pRA vector, which was also cut beforehand with the same restriction enzymes, BamHI and EcoRV. The novel-plasmid designated as pRAlon.

Table 2.4: Nucleotide sequences of oligonucleotide primers.

Primers	Sequence 5’---3’	Usage
bti-lonF	CTT GTT GGT TGG TGA CGT A	PCR
Bti-lonR	GGG ATT GGC GGA TCT AAT G	PCR
F-lonBti-BamHI	CAT GGA TCC GAT AGC TTG GAA GTA AGA A	PCR
R-lonBti-EcoRV	CCG CTT GAT ATC CCT CAT CAC TTG TTG GTT	PCR
qpcr-F-lonBti	GTGCATCTCTTGGTGGTGTG	Q-PCR
qpcr-R-lonBti	GTAATGCCGCTGATGGATCT	Q-PCR
qpcr-F16-srrna	TGGGGAGCAAACAGGATTAG	Q-PCR
qpcr-R-16srrna	CCTTTGAGTTTCAGCCTTGC	Q-PCR
qpcr-F-Cry11a	TACGCCAGATCAAGCAACAG	Q-PCR
qpcr-R-Cry11a	CCAGCAGGTAAGCGATAAGG	Q-PCR

4.2.4. Plasmid Isolation and Restriction Enzyme Digestion

Plasmid isolation was performed by the method of Sambrook et al. (1989) which allowed for the isolation of pRA and pHT315 plasmids from *E. coli*. After incubating the strain containing the interesting plasmid in 50 ml of LB broth for overnight, the cells were centrifuged at 14.000 rpm for 10 min. The pellet was then resuspended in 800 µL of solution I (also known as resuspension buffer), vortexed, then 200 µL of the mixture was dispensed into separate 1.5 ml eppendorf tubes and held at room temperature (RT) for five minutes. 500 µL of solution II (lysis solution) was added to the bacterial suspension and incubated for five minutes on ice. 400 µL of solution III (neutralization solution) was then applied and incubated on ice for ten minutes with gentle mixing every two minutes (by inverting the tubes slowly several times), then centrifuged at 4 ^{o}C at 14.000 rpm for 20 min. The supernatant was transferred to a new 1.5 mL eppendorf tube, mixed then with 600 µl of isopropanol to precipitate the plasmid DNA. After incubation at room temperature for at least 25 min, centrifugation was done at 14.000 rpm for 15 min. The pellet was then dried in a laminar hood for 10-30 min to evaporate the remaining isopropanol and resolved in 20-30 µL of sterilized water. The sample (2 µL of DNA) was then visualized in agarose gel to confirm the presence of the expected plasmid. Finally, RNase was applied and incubated for 30 min at 37 ^{o}C to remove the RNA artifacts.

After the isolation of the plasmids, pHT315 plasmid was treated with blunt-end cutter endonuclease enzyme (SmaI restriction endonuclease) whereas EcoRV and BamHI were used to cut pRA plasmid as recommended by the manufacturer.

4.2.5. Ligation

In this thesis, using the method described by Sambrook et al (1989), the ligation process was carried out. Depending on the concentration and the size of the plasmid on one hand, and on the size of the insert (DNA in kb) on another hand, the concentration of the insert in nanogram (ng) was calculated using the following equation (Figure 2.3):

$$\boxed{\text{insert (ng)} = \frac{\text{vector (ng)} \times \text{insert (kb)}}{\text{vector (kb)}}}$$

Figure 2.3: Used formula for ligation.

The ligation reactions were established so that the ratio of vector: insert was 1:3, 1:5, and 1:7. For that, the reaction of ligation was prepared as the following: vector (plasmid), insert (DNA), 1 µl of T4 DNA ligation buffer, 1 µl of the enzyme (T4 DNA ligase), and completed with distilled water to 10 µL. The reaction was mixed carefully, spinned briefly, and then incubated at 14-16° C for overnight (approximately 16-18 hours). After incubation and before the transformation, inactivation was carried out by heating the reaction at 65 °C for 10 min as mentioned by the manufacturer.

For the blunt end ligation, the vector (pHT315) was treated with fast digest alkaline phosphatase to remove the phosphate group of the vector and therefore allowed to avoid the self-ligation which is the most frequent problem in the blunt end ligation process. The protocol of the treatment of fast digest alkaline phosphatase was:

1 µg cut vector

1 µL buffer
1 µL (1 U) enzyme Alkaline phosphatase
Complete dH_2O to 10 µL

- Mix well and incubate at 37 °C for 10 min
- Heat for 10 min at 75 °C to stop the reaction

4.2.6. Preparation of Competent Cell

In order to prepare *E. coli* DH5α competent cells, a step necessary for transformation, the method developed in the Sanders laboratory (2014) with a slight modification was followed in this present work. Briefly, a single well-isolated colony was selected and inoculated in 10 ml of LB media and cultured for overnight at 37 °C in a moderate shaker incubator (Innova®43). Next, 1% of the overnight culture in 100 ml LB media was incubated at 37 °C until the OD 600 nm reached to 0.4-0.6. After centrifugation at 4 °C at 3.000 rpm for 10 minutes, the resultant pellets were dissolved in 40 ml of TfbI solution. The cell suspension was incubated on ice for 10 minutes, then centrifuged at 3.000 rpm for eight minutes. After discarding the supernatant, the pellets were resuspended in 1 ml of TfbII. The cells were finally divided to 50 µl in well-identified eppendorf tubes and frozen in liquid nitrogen, stored at -80 °C for future use.

4.2.7. Chemical Transformation

The transformation was applied in order to transfer the plasmid DNA to the competent *E. coli* cells. For that, the competent cells were thawed on ice for few minutes. The protocol for transformation can be summarized as follows: 5 µl of ligation product was transferred to the competent *E. coli* cells and let incubated on ice for 25-30 minutes, then another incubation at 42 °C in a water bath for 45 seconds was applied. After this thermal shock, the cells were quickly put again on ice and kept for two minutes. After this procedure, 800 µl of sterile LB media without antibiotic was immediately added to the reaction. The cells were then incubated at 180 rpm for one hour at 37 °C in a shaking incubator for outgrowth. Finally, 200 µl of cells were spread to selective LB media agar (containing selective antibiotic) and left to grow for overnight at 37 °C.

4.2.8. Screening of *E. coli* DH5α Transformants

In order to identify the recombinant plasmids from the transformants *E. coli* DH5α, certain colonies were chosen randomly and grown on a selective medium containing a specific antibiotic. Plasmid isolation was performed using the miniprep method which is a quick and brief method for plasmid isolation.

The isolation of the plasmid by miniprep was carried out by the method proposed by Holmes et al. [61]. In the first step, some colonies were chosen after transformation, were then cultivated in 1 ml of LB + selective antibiotic for overnight at 37 ^{o}C. The following day, the cells were then centrifuged for five minutes at 13.000 rpm at room temperature (around 25 ^{o}C). The supernatants were removed after centrifugation, and the pellets were immediately dissolved by vortexing with 250 µl of STET. The samples were next put in boiling water for around one minute and then centrifuged for 15 minutes at 13.000 rpm at 4 °C. The pellets containing macromolecules (lipids, proteins, or chromosomal DNA) were discarded using sterile toothpicks and the supernatant was mixed with 600 µl isopropanol to precipitate the plasmid DNA. After at least half an hour of incubation at room temperature, the isopropanol was eliminated by centrifugation and the pellets were dried in a laminar hood for few minutes to evaporate the remaining isopropanol before dissolving in 25 µl of sterile dH_2O. The isolated plasmids were then run on agarose gel and compared with the empty vector. The suspected plasmids were cut with adequate restriction enzymes to ascertain the presence of the recombinant plasmid.

4.2.9. Demonstration of the presence of the Recombinant Plasmid

After miniprep, any plasmid whose size was bigger than the original plasmid was suspected of being recombinant plasmid. However, to be certain, the presence of the DNA inserted in the plasmid has to be demonstrated. For this purpose, two methods have been used in our experiments, which involve cutting plasmids with restriction enzymes or making a colony PCR by using specific primers.

4.2.9.1. Colony PCR

Colony PCR was performed by picking a colony with a toothpick and mixing directly with 50 µL of sterile H_2O in a 1.5 ml eppendorf tube. The mixture was then heated to 100 ^{o}C for 10 min to lyse the cells and used as a template in PCR reaction in which Taq polymerase enzyme was used. The preparation of the master mix and the thermocycling conditions for the PCR reaction were shown in Tables 2.5 and 2.6 respectively.

Table 2.5: Reaction setup for colony PCR.

Components	VOLUME (µL)
dH_2O	15.75
10X Taq Buffer	2.5
dNTP	0.5
Forward Primer	1
Reverse Primer	1
$MgCl_2$	2
Taq Polymerase	0.25

Table 2.6: Thermal cycling conditions for colony PCR.

STEP	TEMPERATURE	TIME	
Initial Denaturation	95 ^{o}C	10 min	
Denaturation	95 ^{o}C	30 seconds	30 cycles
Annealing	63 ^{o}C	30 seconds	
Extension	72 ^{o}C	1 min and 30 seconds	
Final Extension	72 ^{o}C	7 min	
Hold	4 ^{o}C	∞	

4.2.9.2. Digestions of recombinant plasmids with restriction enzymes

The second method used to prove the presence of recombinant plasmid consisted of cutting the suspected plasmids and the original plasmid with the same restriction enzymes and compare the size of the plasmids. Logically the size of the recombinant plasmid must be approximatively the sum of the size of the empty plasmid and the size of the insert DNA(the *lon* gene is approximately 2200 bp). Moreover, the plasmids sometimes were cut with double restriction enzymes in order to prove the presence of the insert in the recombinant plasmid.

4.2.10. Electroporation

Isolating the recombinant plasmid (pHT315lo*n* and pRAlon) from methylation deficient *E. coli* strain (ET12567) was necessary for the transformation of *B. thuringiensis*. For that, the recombinant plasmids were first transferred to ET12567 and isolated from that strain and subsequently electroporated to Bti. In this thesis, electroporation was attempted using a variety of methods, including the method mentioned by Macaluso et al [62], but we were unable to obtain the desired results. Later, the method of Shurter et al [63] was tested, and mutant strains of Bti were obtained successfully.

Electrocompetent Bti cells were prepared according to Schurter et al.[63] with little modifications. 1 ml of the overnight culture of Bti grown in BHI broth was inoculated into a fresh 100 ml BHI in a 500 ml Erlenmeyer flask and incubated at 30 °C until the OD 600 nm reached to 0.4. After centrifugation at 4000 rpm for 15 min, the pellet was mixed with 20 mL of cold Electroporation Buffer (EB) (400 mM sucrose, 1 mM $MgCl_2$, 7 mM phosphate buffer, pH 6.0) and recentrifuged. The pellet was then resuspended in 2 ml of EB, mixed well by pipetting up and down. The cells were dispensed into eppendorf tubes (300 µL) and stored at -80 °C. The electroporation was also carried out according to Schurter et al. [63]. The plasmid DNA was mixed with electrocompetent cells and the mixture was kept on ice for 10 min. Using the Gene-Pulser transfection device (Bio-Rad), electroporation was performed with 200 Ω resistance, 25 µF capacitance, and 1.3 kV voltage. Later on, 700 µL of cold LB was added immediately and incubated for two hours at 30 °C on a rotary shaker at 180 rpm. The cells were then plated on LB agar containing 25 µg/ml erythromycin.

4.2.11. Plasmid Isolation from Recombinant *B. thuringiensis*

After electroporation step, the plasmids were isolated from the recombinant *B. thuringiensis* strains to highlight the presence of the recombinant plasmid (pHT315lon). The standard plasmid isolation based on alkaline lysis has been tested but in Bacillus it did not work. Also, other plasmid isolation techniques were attempted and, regrettably, no successful outcomes have occurred by the methods proposed by Reyes-Ramirez et al. [64] et Birnboim [65]. By the procedure described by O'Sullivans et al [66] that the plasmids have been isolated from *B. thuringiensis serovar israelensis* in this present study.

4.2.12. Endotoxin Isolation

A protocol proposed by Donovan et al. [67] was used for endotoxin isolation from Bti. Briefly, equal numbers of *B. thuringiensis* cells were inoculated and incubated at 30 °C for 72 hours in DSM media. The cells were centrifuged at room temperature for 10 min, the resultant pellets were washed twice with 1 M NaCl and once with sterile distilled water. The cells were then suspended in distilled water to have a final concentration of 100 mg wet cell/ml. 50 µL of the cell suspension was mixed with 50 µL of lysis buffer (10 mg/mL lysozyme suspended in Tris EDTA buffer) before incubating at 37 °C for 30 min. 10 µL of 2% sodium dodecyl sulfate (SDS) was added, vortexed briefly, and centrifuged for five minutes. The pellets were next dissolved in 50 µL of 0.2% SDS. The constituent proteins were separated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) in a 12% gel followed by Coomassie blue staining to visualize the crystals proteins using Azure Biosystems (Bioanalytical Imaging System).

4.2.13. Total Protein Isolation and Enzyme Assay

A single colony was grown for overnight in LB broth and incubated in a shaker incubator for 16 hours at 30 °C. After centrifugation at 4.000 rpm for 20 min at 4 ^{o}C, the resultant pellets were washed with sterile LB (without antibiotic) and then suspended with lysis buffer (50 nM NaH_2PO_4, 300 nM NaCl, 10 nM imidazole). Using a Sonifier® S-250D digital sonifier (Branson, Danbury, CT, USA), the cell suspension was sonicated 60 seconds with 40 percent amplitude (20 sec 'on' and 20 sec 'off'). The supernatant was then collected after centrifugation at 4.000 rpm for 30 min at 4 ^{o}C. Once the concentration of proteins was measured using the Bradford method, an equal amount of protein was then run on SDS-PAGE and stained with Coomassie blue to visualize the total proteins in the Azure Biosystems device.

As for the enzyme activity, it was determined as described by Thys et al [68]. Using azocasein as substrate, Lon protease activity was determined and the protocol was as follows: a reaction mixture composed of 120 µl of enzyme extract, 480 µl of azocasein suspended in 50 mmol Tris Buffer, 1 mM ATP, and 7.5 mM $MgCl_2$ was incubated at 30 ^{o}C for 30 min. 1 % of Trichloroacetic acid (TCA) was then added to the reaction to stop the reaction and centrifuged then for 5 minutes at 13.000 rpm. To measure the enzymatic activity of the protease, the absorbance at 420 nm was calculated after applying 800 µl of the supernatant to 200 µl of 1.8 N NaOH. One unit of enzyme activity was taken as the amount of enzyme that resulted in a change of absorbance of 0.01 at 420 nm in 30 min at 30 °C.

4.2.14. RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR Analysis

B. thuringiensis strains were harvested at the exponential growth phase (after 6 hours of growth) to analyze the gene expression of *lon* and *Cry11a*. Total RNA was extracted from *B. thuringiensis* strains using the following procedure: 5 ml of culture was centrifuged at 12.000 rpm for 10 minutes at 4 °C before being washed twice with TE buffer (pH 8.0). The pellet was resolved in 200 µL TE and 75 µL of lysozyme (10 mg/ml) and incubated at 37 °C for at least one hour and a half. Using NucleoSpin RNA Plus kit (Macherey–Nagel, Germany), total RNA isolation was effectuated by following adequately the manufacturer's instruction. RNAs were then run on agarose gel to control the quality of the isolated RNA. The quality and the quantity of RNA were then calculated using nanodrop (Lite Spectrophotometer; Thermo Fisher Scientific, MA, USA). Total RNA was treated with DNase I (Thermo Scientific, USA) and later the absence of DNA was confirmed by PCR. The protocol used for DNA elimination can be seen in Table 2.7. RNA (300 ng) was reverse transcribed into cDNA using Iscript Advanced cDNA Synthesis Kit (Bio-Rad Laboratories, USA), and cDNA presence was confirmed using conventional PCR. Real-time PCR was carried out using cDNA as a template and SYBR Green as a master mix (Bio-Rad Laboratories, USA) in StepOnePlus™ Real-time PCR (Applied Biosystems, CA, USA). The condition of qPCR reaction was as follows: heat at 95 °C for 2 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. 16S rRNA of *B. thuringiensis* was used to normalize RNA samples. The gene expression analysis was determined using the $2^{\wedge-\Delta\Delta Ct}$ formula [69].

Table 2.7: Deoxyribonuclease I reaction

Reaction Components	Volume
RNA	300 ng
10X $MgCl_2$ Buffer	1 µL
Deoxyribonuclease I	1 µL
RNase free water	completed with to a total volume of 10 µl

4.2.15. Sporulation Efficiency

After incubated the *B. thuringiensis* cells in Difco Sporulating Medium (DSM) at 30 °C for three days (72 hours) in an incubator with shaking at 180 rpm,spore count was determined as follows: 100 µL of cells were incubated at 80 °C for 20 min to kill vegetative cells and keep only heat resistant spore cells. Then, dilution was made before plating in nutrient agar. After one day of incubation at 30 °C, sporulation efficiency was calculated by counting the number of colonies on the plates and expressed as spore per ml (spore/ml).

4.2.16. UV Tolerance

In order to check the UV tolerance of *B. thuringiensis* strains, two methods were applied in our experiments. One was a protocol proposed by Whistler et al. [70]. 1 % of the overnight culture in LB media was grown at 30 ^{o}C for 4 hours (OD_{600}= 0.4-0.5). Cells were diluted before spreading on LB agar plate without cover and exposed to UV irradiation for different durations (15 s, 30 s, and 45 seconds). The plates were then covered with aluminum foil and incubated at 30 °C for counting the surviving *B. thuringiensis* after 24 hours of growth.

In the second method 5 ml of liquid culture poured in a petri dish and exposed to UV irradiation for five minutes, after serial dilutions samples speaded onto LB agar. We next determined the number of surviving cells by counting the colonies after 24 hours of growth.

4.2.17. Motility and Biofilm

Using LB solidified with 0.3% agar, the motility of strains was examined. Briefly, 5µL of an overnight culture was put gently as a single drop on LB agar, dried in the laminar hood for few minutes before incubating at 30 ^{o}C for 12 hours. The diameter of the migration zone was then measured in millimeters.

Regarding biofilm formation, overnight cultures were washed twice with Phosphate Buffered Saline, pH 7.2 (PBS), and centrifuged at room temperature for 20 minutes. The last pellet was resolved with PBS then a serial dilution was done until an OD of 1.0 was reached. A mixture of 100 µl of sterile LB and 100 µl of bacterial cells ($1x10^{6}$ CFU) was prepared in 96-well polystyrene plates. A blank reaction containing only the LB broth media was also dispensed and used as control. Then the polystyrene plate was incubated at 30 ^{o}C for three days (72 hours) to let grow the bacteria. To determine the biofilm formation of *B. thuringiensis* strains, the cells were stained with crystal violet (1%) for 10 min. Then, after washing the microtiter plates with sterile PBS and drying well (about one hour at RT), the crystal violet was extracted with 33% acetic acid and the absorbance was measured at A_{570} to calculate the biofilm formation of Bti cells using FLUOstar® Omega Plate Reader (BMG LABTECH).

4.2.18. Growth Curve Analysis

We analyzed the growth of different *B. thuringiensis* strains (BtiWT, BtipHT315 and BtipHT315lon) in both normal conditions and stressful conditions in this thesis. For this purpose, the strains were grown in LB, DSM, and minimal media according to Demain [71], at different temperatures, at different pH to compare the growth of the strains.

Using a spectrophotometer (UV-1900 UV-Vis Spectrophotometer, Shimadzu, Kyoto, Japan), the growth curves of *B. thuringiensis* strains were prepared by measuring the optical density at 600 nm.

4.2.19. Confocal Analysis

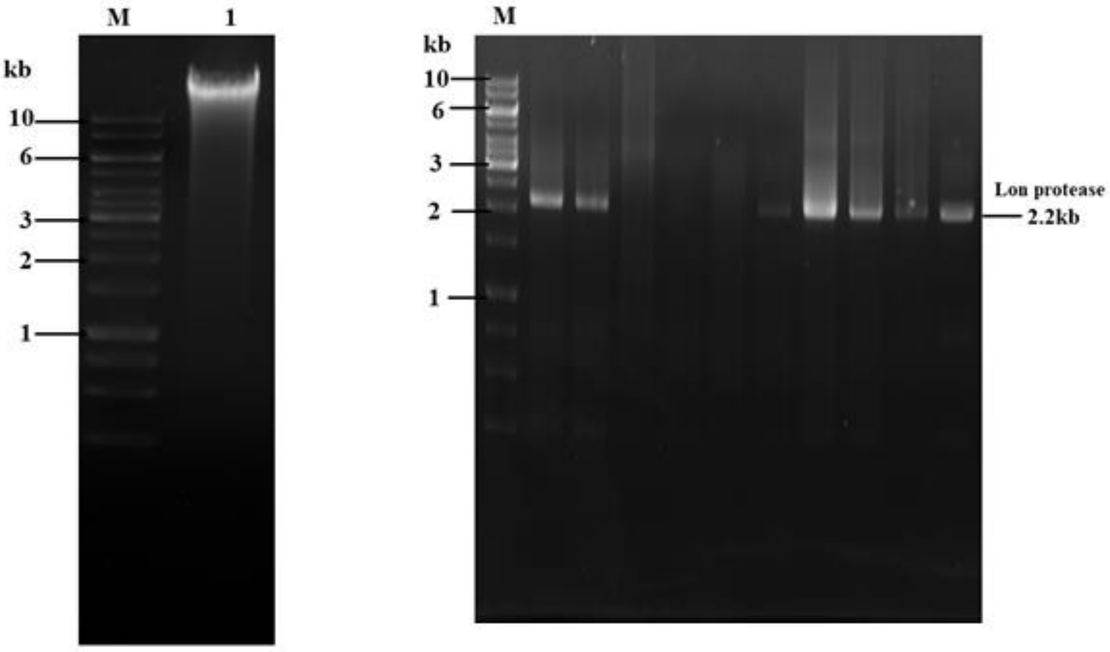
The cells of *B. thuringiensis* strains (BtiWT, BtipHT315, and BtipHT315lon) were stained for confocal microscopy using the LIVE/DEAD Bac-Light Bacterial Viability Kit (L-7012). For that, four hours grown cells (2 ml) were centrifuged for five minutes at 13.000 rpm. Then, a dye mix composed of 1.5 µl of each fluorescent nucleic acid stains (SYTO 9-green and propidium iodide (PI)-red) in 1 ml of H_2O was prepared. The resultant pellets were resolved with a dye mixture and incubated for at least 10 minutes in the dark. The *B. thuringiensis* cells were explored using a confocal microscope at wavelengths of 488 and 568 excitation and 530 (green) or 630 (red) emission. The non-permeable nucleic acid stain, PI, labels dead cells in red whereas permeable SYTO 9 fluorescent nucleic acid dye labels live cells in green.

3. RESULTS

3.1. Cloning of *lon* gene in *E. coli* DH5α

3.1.1. Amplification of *lon* gene by PCR

Using compatible forward and reverse primers (Table 2.4) designed based on the *B. thuringiensis* genome sequence, *lon* gene with its promoter and transcriptional terminator was amplified by PCR using *B. thuringiensis* genomic DNA as a template (Figure 3.1).



(a) **(b)**

Figure 3.1: Amplification of *lon* gene from genomic DNA of *B. thuringiensis* serovar *israelensis* ATCC 35646 **(a)** Genomic DNA of *B. thuringiensis*. Lane 1 genomic DNA; **(b)** Optimization of *lon* gene amplification by changing the annealing temperature (53°C, 55°C, 58°C, 60°C, 63°C and 65°C) and master mix in different PCR reactions. Lane M is a 1 kb DNA ladder, and the other lanes were different PCR reactions performed to optimize the amplification.

As a preliminary experiment, the PCR primers were tested in different binding temperatures, and concentrations of the master mix components were optimized (particularly magnesium, primers, and template). Phusion enzyme "High Fidelity DNA Polymerase" was used as an enzyme for the PCR reaction, which has a much lower error rate than *Taq* DNA polymerase. DNA fragment was shown to be *lon* protease gene by DNA sequencing (Figure 3.2).

GTGAAACGTTCAATGGAGCGCACGCAAAAAGAATATTTCTTACGTGAGCAAATGAAGGCGATTCAAACTGAACTTGGCGACAAAG
AAGGTAAGGGCGGAGAAGTAGAAGAACTTCGTGAAAAAATTGAACAGTCAGGAATGCCTGAAGAAACAATGAAGGCTGCGCTGAA
AGAATTAGATCGTTATGAAAAGTTACCAGCAAGTTCTGCGGAGAGTGGTGTTATTCGCAATTATATTGATTGGTTATTAGCGCTTCC
GTGGACAGAGGCAACAGAAGATATAATTGATCTTGCTCATTCAGAAGAGATTTTAAATAACGATCATTACGGTCTTGAAAAAGTGA
AAGAGCGTGTACTTGAATATTTAGCTGTACAGAAGTTAACGAATTCATTAAAAGGACCTATCCTTTGTTTAGTAGGCCCTCCTGGGG
TCGGCAAAACTTCGTTAGCGCGTTCAATTGCAACATCATTGAATCGTAAATTTGTCCGTGCATCTCTTGGTGGTGTGCGTGATGAATC
TGAAATTCGTGGTCACCGCCGTACATATGTTGGAGCAATGCCAGGACGCATTATTCAAGGTATGAAAAAGGCGAAAACAGTTAATC
CAGTCTTCTTATTAGATGAGATTGATAAAATGTCTAACGATTTCCGTGGAGATCCATCAGCGGCATTACTTGAAGTATTAGATCCAG
AACAAAACCATAACTTCAGTGATCATTACATTGAAGAACCATATGATCTATCGAAAGTTATGTTTGTAGCAACCGCTAATACACTTT
CAAGTATTCCAGGTCCATTACTTGACCGTATGGAAATCATTTCGATTGCTGGCTATACAGAACTTGAAAAAGTACACATTGCTCGTG
AGCATTTATTGCCGAAACAATTAAAAGAGCATGGCTTACGAAAAGGTAATTTACAAGTGCGTGATGAGGCGCTTCTTGAAATTATTC
GTTATTATACACGTGAGGCTGGTGTTCGTACGCTAGAACGCCAAATTGCAAAAGTTTGCCGTAAAGTAGCGAAAATTATCGTTACAG
CAGAACGTAAACGTATCGTTGTGACAGAGAAAAAGATTGTTGATTTACTTGGTAAACACATATTCCGTTATGGGCAAGCTGAAAAA
ACAGACCAAGTTGGTATGGCAACAGGTCTAGCTTACACAGCAGCAGGCGGCGATACACTTGCAATTGAAGTTTCTGTAGCGCCAGG
AAAAGGGAAACTAATTTTAACGGGGAAACTTGGGGATGTTATGAAAGAATCAGCACAAGCTGCGTTTAGTTATATACGCTCTCGTG
CAGAAGAGCTTCAGATTGACCCGAATTTCCATGAGAAAAATGATATTCATATTCATGTTCCAGAAGGAGCAGTTCCAAGAGATGGA
CCGTCAGCAGGTATTACGATGGCAACGGCACTTATTTCTGCACTAACAGGTATTCCTGTAAGTAAAGAAGTAGGTATGACAGGTGA
AATTACACTTCGTGGTCGTGTATTACCAATTGGTGGCTTAAAAGAAAAAACATTAAGTGCTCACCGCGCAGGCTTAACAAAAATTAT
TTTGCCAGCAGAAAACGAGAAAGATTTAGATGATATTCCAGAGAGCGTAAAAGAAAACCTTACGTTTGTGCTTGCATCTCATTTAGA
TGAAGTATTGGAGCACGCATTAGTAGGAGTGAAACAA**TGA**

Figure 3.2: DNA sequence of *Bacillus thuringiensis* serovar *israelensis* ATCC 35646 *lon* gene

3.1.2. Cloning of *lon* gene into pHT315 and pRA plasmids

The 2200 bp DNA fragment of the *lon* gene with its promoter and the transcriptional terminator was gel extracted (Figure 3.3a) and used in ligation.

An integrative vector pRA (5.6 kb) was used to insert an extra single copy of *lon* gene into *B. thuringiensis* serovar *israelensis* genome, and a *Bacillus*/*E.coli* shuttle pHT315 (6.5 kb) vector to overexpress the *lon* gene. The pHT315 plasmid was cut with SmaI endonuclease enzyme (blunt end cutter) whereas pRA plasmid was cut with dual enzymes EcoRV and BamHI. They were then both purified from the gel (Figure 3.3b). The ligation reaction between the plasmid (pHT315 or pRA) vectors and the *lon* gene was carried out as described in Section 2.2.5 and the ligation products were transferred to *E. coli* DH5α cells.

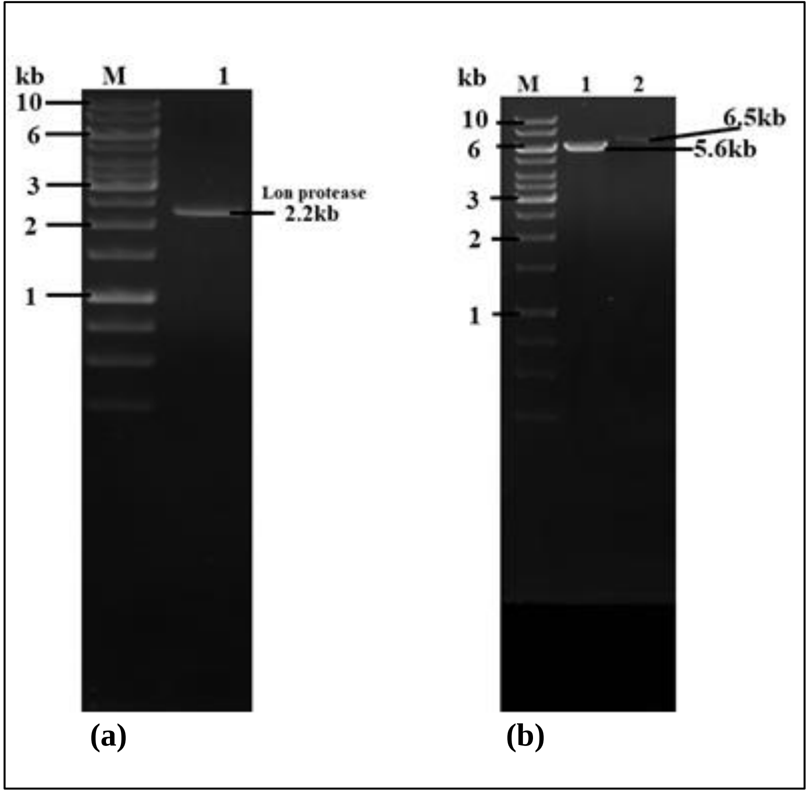


Figure 3.3: *lon* protease gene and isolation of pRA and pHT315 plasmids from *E.coli* Dh5α. (a) *lon* protease gene (2.2 kb) amplification. Lane M is a 1 kb DNA ladder (Thermo Fisher Scientific). (b) The plasmids were cut with BamHI. Lane 1 pRA and lane 2 pHT315. Lane M is a 1 kb DNA ladder (Thermo Fisher Scientific).

Plasmids from transformants have been isolated and run on the agarose gel together with empty plasmids (Figure 3.4). The red arrows indicate the selected plasmids which were next deeply studied for confirmation. The strains were confirmed on one hand with the restriction enzyme digestions and on the other hand with colony PCR.

The presence of *lon* protease gene (2.2 kb) in the recombinant pHT315 vector was proved by cutting the pHT315 plasmids with dual restriction enzymes, HindIII and KpnI (Figure 3.5a). After the double restriction enzyme cuts, the existence of two bands of 6.5 kb and 2.2 kb, corresponding to pHT315 and the protease *lon* gene, respectively, were the expected bands (Table 3.1). Besides that, colony PCR of the recombinant plasmids was performed and presence of *lon* gene in the recombinant plasmid was reconfirmed (Figure 3.5b).

Similarly, the restriction endonuclease enzymes HindIII and EcoRI were used to confirm the recombinant pRA plasmids. Table 3.2 indicated the expected bands both in the presence (+) and absence (-) of the insert (*lon* protease gene) in pRA plasmid. The presence of recombinant pRA plasmid (pRAlon) was verified after HindIII and EcoRI cuts and the colony PCR reconfirmed the results (Figure 3.6). In this thesis, "pRAlon" denoted a pRA plasmid containing *lon* protease gene, while "pHT315lon" denoted a pHT315 comprising *lon* protease gene.

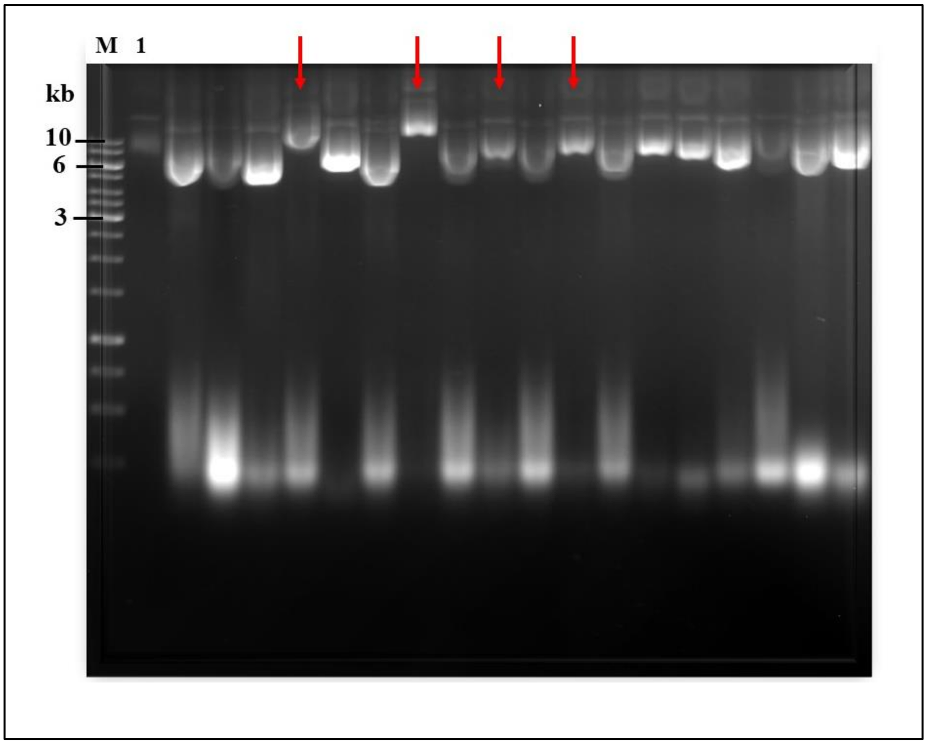
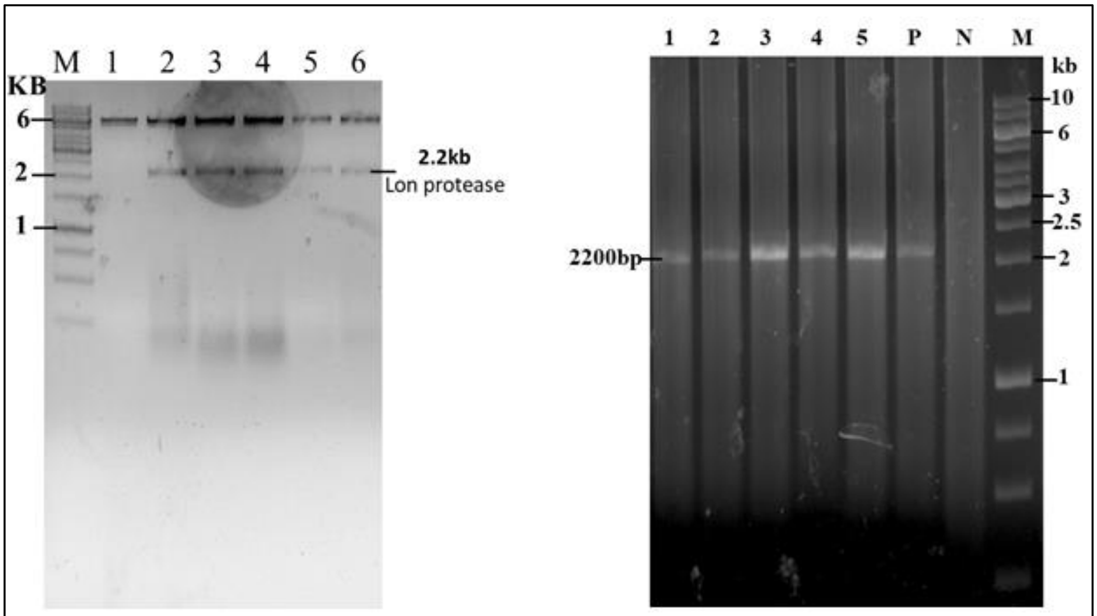


Figure 3.4: Isolation of pHT315 plasmids from transformants. Lane 1 was an empty plasmid used as control. Red arrows indicated some suspected plasmids after transformation. Lane M is a 1 kb DNA ladder (Thermo Fisher Scientific).

Table 3.1: Expected bands after dual digestion of pHT315lon with HindIII and KpnI (8700 bp).

pHT315+lon (8.7 kb)
Enzymes: KpnI + HindIII
6.5 kb **2.2 kb**



(a) **(b)**

Figure 3.5: Confirmation of recombinant pHT315 plasmid. (a) pHT315 plasmids were cut with HindIII and KpnI to show the presence of *lon* protease gene (2.2 kb) in the recombinant plasmid. Lane 1 was an empty plasmid and lanes 2-6 were recombinant plasmids. (b) A colony PCR reaction was performed in order to confirm the presence of *lon* gene (2.2 kb) in the recombinant plasmids. Lanes 1-5 were recombinant plasmids; lanes P and N were positive and negative controls respectively. Lane M is a 1 kb DNA ladder (Thermo Fisher Scientific).

Table 3.2: Expected bands after EcoRI and HindIII restriction enzyme cut of pRAlon (7800bp). (+) indicated the true recombinant plasmid (presence of *lon* gene) while (-) shows the absence of *lon* gene (2.2kb) in the plasmid.

pRAlon (7.8 kb)			
EcoRI		**HindIII**	
(+)	**(-)**	**(+)**	**(-)**
600	**5748**	**6108**	**4056**
7200		**969**	**969**
		723	**723**

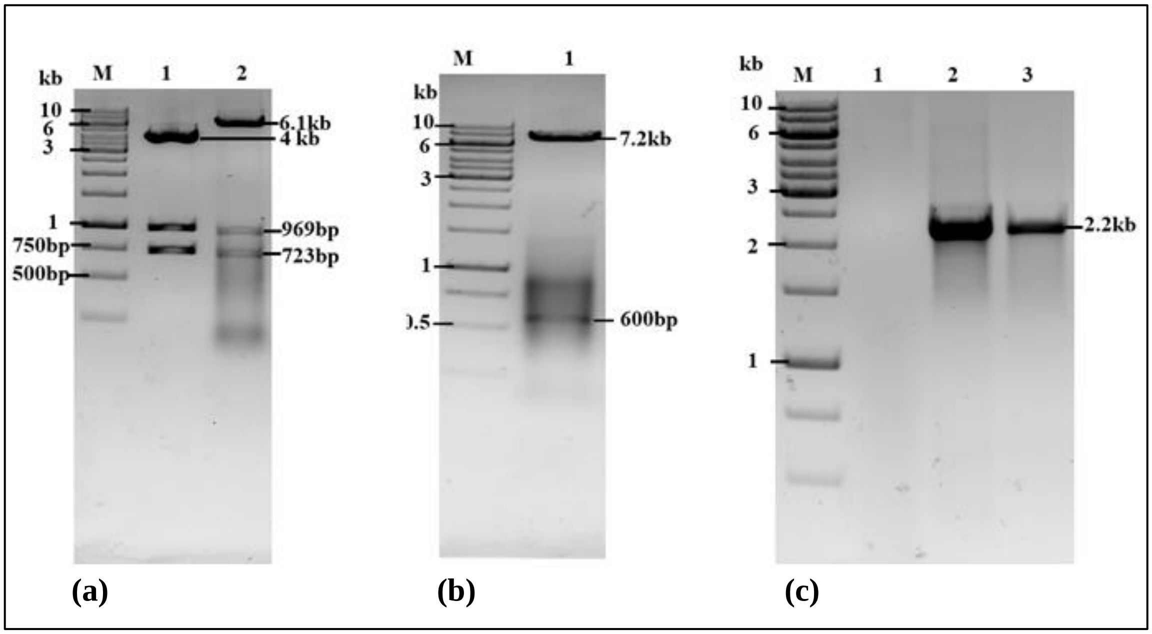


Figure 3.6: Confirmation of recombinant pRA plasmid. **(a)** pRA plasmids were cut with HindIII to confirm the presence of *lon* protease gene (2.2 kb) in the recombinant pRA plasmid. Lane 1 was an empty plasmid and lane 2 was one of the recombinant pRA plasmids. **(b)** recombinant pRA plasmid cut with EcoRI to demonstrate the presence of *lon* protease gene (2.2 kb) in the recombinant pRA plasmid. Lane 1 was a recombinant pRA plasmid. **(c)** The colony PCR reaction was carried out in order to verify the presence of *lon* protease gene (2.2 kb) in the recombinant pRA plasmid. Lane 1 was an empty plasmid, Lane 2 was a recombinant plasmid, lane 3 was used as a positive control. Lane M is a 1 kb DNA ladder (Thermo Fisher Scientific).

3.2. Expression of *lon* gene in *B. thuringiensis*

3.2.1. Transfer of recombinant plasmids to *B. thuringiensis*

After confirmation of the recombinant plasmids pHT315lon and pRAlon, they were transferred into *B. thuringiensis* by electroporation. Since the plasmids pHT315 and pRA carry erythromycin and apramycin resistance genes respectively, resistance to erythromycin and apramycin was the primary selection factor for recombinant BtipHT315lon and BtipRAlon.

The existence of recombinant pHT315lon plasmids was then confirmed by isolating plasmids from the suspected recombinant *B. thuringiensis*. The isolated plasmids were cut with HindIII to distinguish native plasmids from recombinant plasmids for confirmation. Bti contains native plasmids in its genome which is why we can constate also bands in the Bti wild type. A band with a size of 8.7 kb was expected in the event of a positive result, which is the case as shown in Figure 3.7. From the plasmids isolated from BtipHT315 (containing an empty plasmid), a band of 6.5 kb was observed.

For confirmation of BtipRAlon, we compared the relative expression of *lon* gene between the recombinant strain and controls strains, which showed a very net increase in recombinant BtipRAlon compared to BtipRA and Btiwt (Figure 3.9a). The details of this result can be found in the next section.

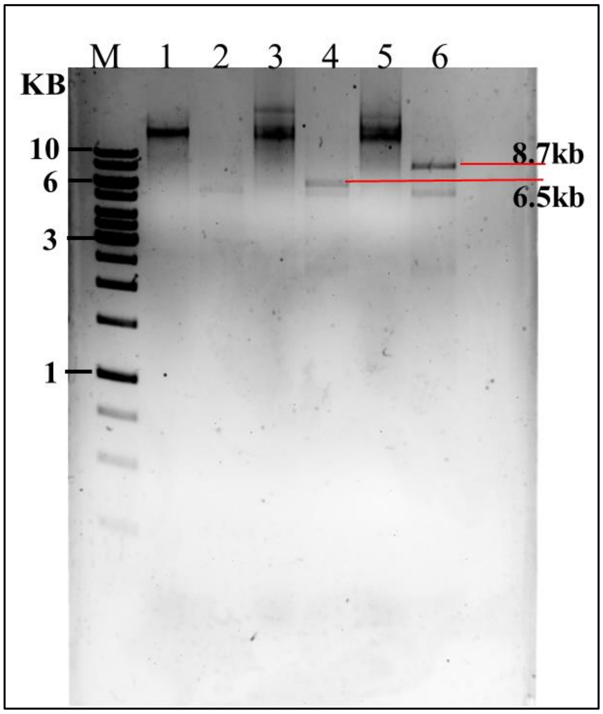


Figure 3.7: Demonstration of recombinant *B. thuringiensis* strain. To prove the presence of pHT315lon plasmid in *B. thuringiensis*, plasmids from BtiWT, BtipHT315, and BtipHT315lon were cut with HindIII. Lane 1: uncut Btiwt's plasmid/s, lane 2: cut BtiWT's plasmid/s, lane 3: uncut BtipHT315's plasmid/s, lane 4: cut BtipHT315's plasmid/s, lane 5: uncut BtipHT315lon's plasmid/s and lane 6: cut BtipHT315lon's plasmid/s. The expected size of pHT315lon is 8.7 kb. Lane M is a 1 kb DNA ladder (Thermo Fisher Scientific).

3.2.2. Determination of the level of the transcriptional expression of *lon* gene in *B. thuringiensis* by Real-time PCR

The total RNA was isolated at the 6th hour after *B. thuringiensis* strains inoculation (BtiWT, BtipRA, BtipRAlon, BtipHT315, and BtipHT315lon) on LB medium at 37 °C with 180 rpm shaking. The isolated RNAs were then tested in a 1.2% agarose gel and found to be successfully isolated (Figure 3.8a).

To eliminate possible DNA contamination, the isolated RNAs were treated with DNase I and confirmed its total absence by conventional PCR using specific primers for the housekeeping 16SrRNA gene of *B. thuringiensis*, a gene that is always expressed in the cell. The PCR results were used to check if there was any contamination of the genomic DNA in the treated RNA samples, and no DNA contamination was reported in the RNA samples (Figure 3.8b).

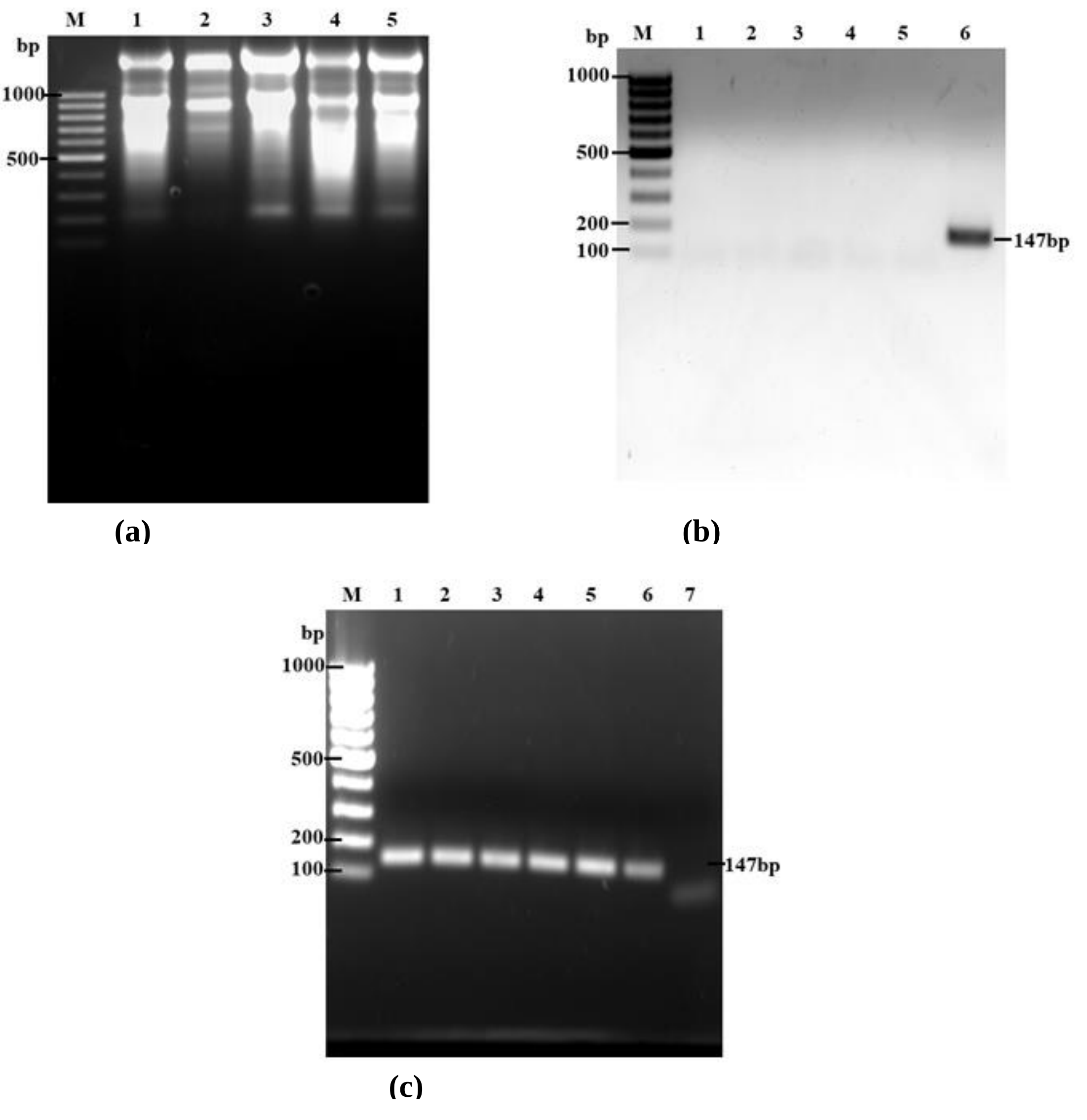
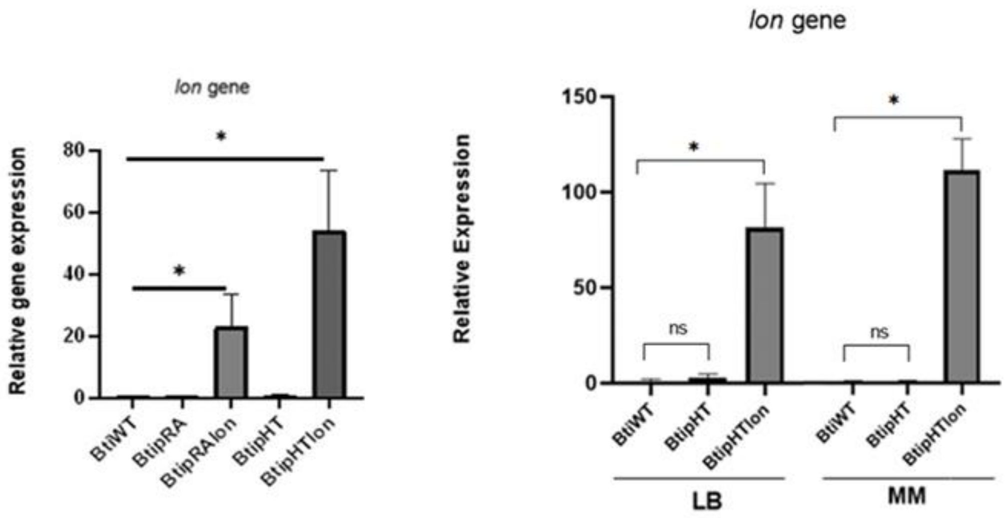


Figure 3.8: RNA isolation, DNA decontamination, and cDNA synthesis. (a) Image of the isolated RNAs from *B. thuringiensis* samples on an agarose gel electrophoresis. Lane1: BtiWT, lane 2: BtipRA, lane3: BtipRAlon, lane 4: BtipHT315 and lane 5: BtipHT315lon. (b) Control of genomic DNA contamination after DNase treatment using PCR. Lanes 1-5: RNA samples and lane 6: Bti genomic DNA used as a positive control. (c) cDNA synthesis from the isolated RNA sample using reverse transcriptase and verified by PCR. Lanes 1-5: RNA samples, lane 6: genomic DNA used as a positive control, and lane 7: NTC (Non-template control). Lane M is a 100 bp DNA ladder (Thermo Fisher Scientific).

300 ng of RNA samples were synthesized into cDNAs by Reverse Transcriptase-PCR after being treated with Deoxyribonuclease I. The cDNA synthesis was confirmed to be successful as shown in Figure 3.8c.

Transcriptional analysis of the *lon* protease gene was carried out using cDNAs synthesized from RNA extracted from BtiWT, BtipRA, BtipRAlon, BtipHT315, and BtipHT315lon strains as templates.

Quantitative PCR was used to examine the gene expression of the *lon* protease gene, and a significant increase was found in the recombinant strains BtipRAlon and BtipHT315lon (Figure 3.9). In addition, as expected the relative expression of *lon* gene was elevated in BtipHT315lon than BtipRAlon. Particularly, the relative expression of *lon* protease gene of BtiWT, BtipHT315, and BtipHT315lon was analyzed in LB and minimal media. The recombinant strain (BtipHT315lon) strain was found augmented at a rate of approximately 80 and 117 times, in LB and minimal media respectively, than the wild type and BtipHT315.



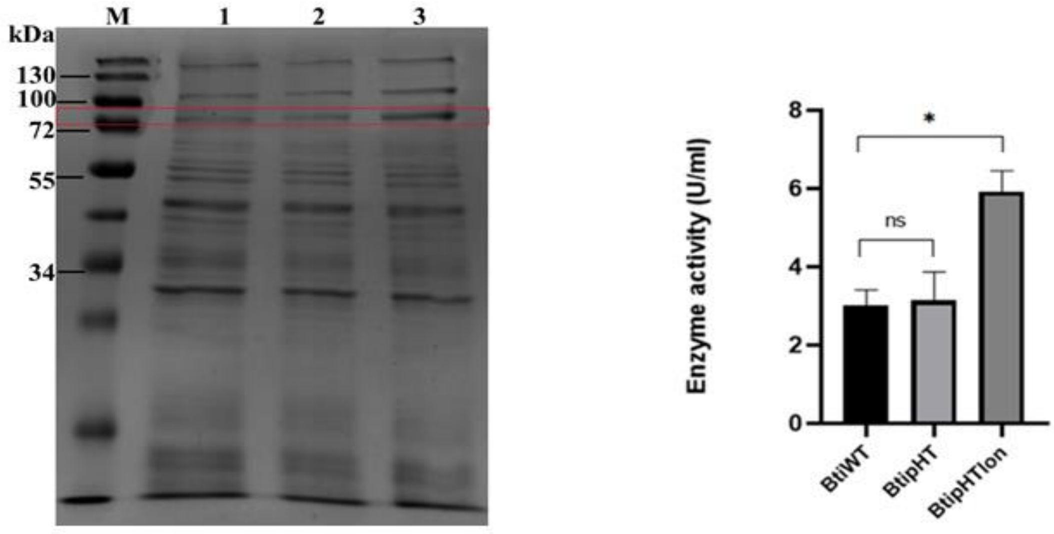
(a) **(b)**

Figure 3.9: Relative expression of *lon* gene. (a) *lon* protease gene expression of BtiWT, BtipRA, BtipRAlon, BtipHT, BtipHTlon grew in LB under aerobic conditions at 30 °C. (b) Comparing relative gene expression of *lon* of BtiWT, BtipHT, and BtipHTlon in LB and Minimal media (MM).

3.2.3. Lon protease activity

We proved in the previous section that the expression of *lon* protease was elevated in the recombinant strains at the transcriptional level. However, it is well proved in the literature that translation and transcription might not be proportionally coupled. The elevated transcription level of a particular protein is not always followed by an elevated protein level in the organism. For this reason, *lon* protease expression at the translational level was controlled in *B. thuringiensis* strains. For that, the total proteins from Bti strains were extracted and analyzed by electrophoresis in SDS-PAGE.

When an equal amount of total protein was loaded into the SDS page gel, nearly 1.5 times increase (calculated by using the image processing program, ImageJ) in Lon specific band (around 87 kDa) in the recombinant strain was seen compared to the BtiWT (Figure 3.10a).



(a) **(b)**

Figure 3.10: Total protein and enzyme activity (a) total protein profile of *B. thuringiensis* strains on SDS gel. Lane 1: BtiWT, lane 2: BtipHT315, lane 3: BtipHT315lon, and lane M: PageRuler Pre-stained Protein Ladder (Thermo Scientific). Possible Lon protease bands (around 87kDa) are framed by a red rectangle (b) Enzyme activity of Bti strains using Azocasein as substrate. Asterisks (*) represent a p-value of less than 0.05 which was considered statistically significant. Vertical bars indicate standard deviation from the mean value. “ns” is not significant.

The activity of the Lon protease enzyme was measured by using crude extracts of Bti strains and it was found that the recombinant enzyme activity was 2.4 times higher than the control strains (Figure 3.10b).

3.3. Comparison of endotoxin production between recombinant *B. thuringiensis* and control strains

To elucidate the effect of overexpression of the *lon* gene in the production of endotoxins, the strains were grown for 72 hours in DSM medium at 30 °C, and toxins were isolated using an equivalent wet weight of each organism. The toxins of the different Bti strains (BtiWT, BtipRA, and BtipRAlon, BtipHT315, BtipHT315lon) were isolated successfully. As illustrated in Figure 3.11a, typical toxin bands were visible at 128 kDa, 70 kDa, and 27 kDa, and the recombinant strains were able to produce more endotoxins as compared to both control strains. BtipHT315lon was able to produce more toxins than the wild type and BtipHT315 whereas the endotoxin production of BtipRAlon was slightly higher than BtipRA and wild type.

We hypothesized that since there was an increase in endotoxin production between the recombinant strain and the controls, the expression of crystal protein genes would be affected as well. To test this hypothesis, quantitative PCR was used to examine the transcript level of one major crystal protein gene (*Cry11a*). An increase in toxin production in the recombinant strain was also shown at the transcriptional level by comparing the expression level of *Cry11a* (Figure 3.11b). However, we revealed that *Cry11a*'s relative gene expression in BtipRAlon (Bti with one extra copy of lon protease gene) was comparable to other strains. This study suggested that *lon* protease overexpression promotes crystal protein synthesis at the transcriptional level.

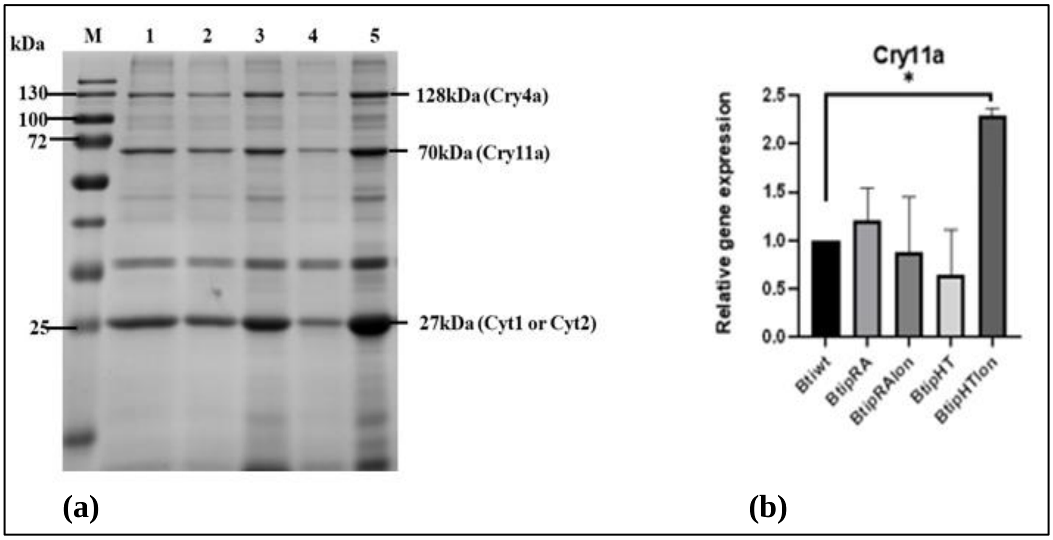


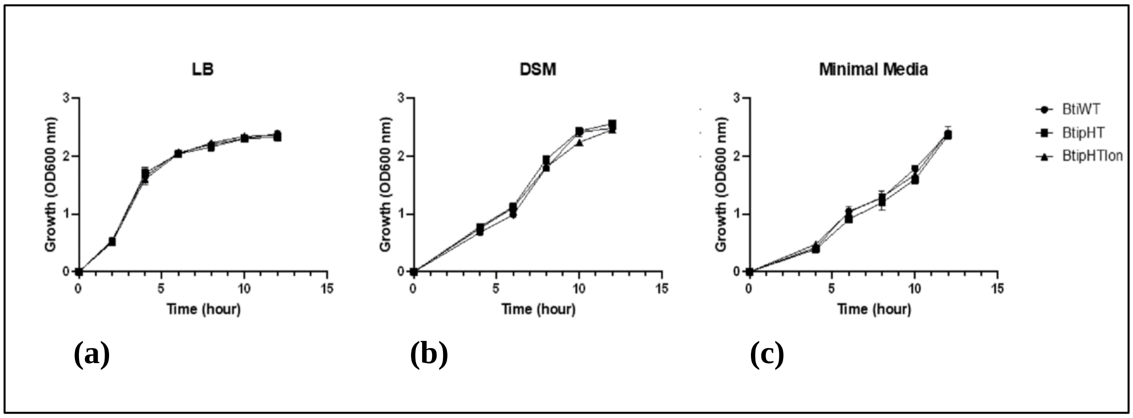
Figure 3.11: Analysis of endotoxin production. (a) Production of δ-endotoxin of *B. thuringiensis* strain grown in DSM medium. 1: BtiWT, 2: BtipRA, 3: BtipRAlon, 4: Bti pHT315, 5: Bti pHT315lon and lane M: PageRuler Pre-stained Protein Ladder (Thermo Scientific). (b) Relative gene expression of *Cry11a*.

3.4. Effect of Overexpression of *lon* protease on cellular functions

According to the literature, Lon protease has a major impact on a variety of cellular functions. Furthermore, several studies reported many cellular function defects in lon deficient strains such as *E. coli*, *Vibrio cholerae,* and *Pseudomonas aeruginosa*. But few are the number of studies reporting the effect of overexpression of Lon protease on cellular and molecular functions in the organisms and less in Bti. In this sense, we decided to look at how overexpression of Lon protease affects cellular functions including stress adaptation, biofilm formation, sporulation, and motility.

3.4.1. Comparison of Growth of Bti strains

Growth of BtiWT, BtipHT315, and BtipHT315lon was found to be similar in LB, DSM, and minimal medium at 30 ^{o}C (Figure 3.12). All the strains entered the stationary phase earlier in the LB medium compared to the other media. The growth rate of all three strains was slower in minimal medium in contrast to LB and DSM.



(a) (b) (c)

Figure 3.12: Growth of BtiWT, BtipHT315, and BtipHT315lon in (a) LB, (b) DSM and (c) minimal media incubated at 30 ^{o}C for 12 hours.

3.4.2. Comparison of Sporulation, Motility and Biofilm formation of *B. thuringiensis* strains

B. thuringiensis produces endotoxins mostly during sporulation [72]. Here, as higher production of endotoxin was noted in the recombinant strain, we sought to know whether it was because *lon* overexpression increases the spore count. Therefore, to determine the effect of overexpressing lon on sporulation, we examined the sporulation efficiency between the recombinant strain and the control. As result, the sporulation efficiencies were significantly higher in BtiWT and BtipHT315 than in BtipHT315lon (Figure 3.12a), BtipHT315 exhibiting comparable sporulation efficiency with the wild type. The overexpression of *lon* protease likely affected the sporulation in *B. thuringiensis* and it results in a decrease of thermo-resistant CFU (spores) in the recombinant strain compared to wild type and BtipHT315. These observations suggest that overexpression of *lon* negatively influenced sporulation. When compared to BtiWT and BtipHT315, the sporulation efficiency of the recombinant strain (BtipHT315lon) was found to be nearly 5 times lower (Figure 3.13a).

The effect of *lon* protease on motility has been well studies previously by many authors mostly in *Proteus mirabilis* [73] and *Pseudomonas aeruginosa* [74]. Most studies stated that the *lon* mutant showed abnormalities in the motility in these species. We aimed in this research to investigate the effect of overexpressed *lon* on the motility in *B.*

thuringiensis since it is not well understood in *B. thuringiensis*. In conclusion, the diameter of the migration zone of recombinant strain is reduced around 1.6-fold compared to the controls. Thus, this finding stated that the overexpressing *lon* may hinder the motility in *B. thuringiensis* serovar *israelensis* (Figure 3.13b).

Several studies have demonstrated that the formation of biofilm involves intracellular *lon* protease [74]–[77]. Even though several studies have shown that the absence of *lon* protease is accompanied by a deficit in biofilms in some organisms [74], [77]. However, the effect of high *lon* protease expression on biofilm formation is not well known, particularly for *B. thuringiensis* serovar *israelensis*. Therefore, in this study, we investigated the effect overexpressing of *lon* might have on the formation of biofilm in Bti. Contrary to sporulation and motility, overexpressing *lon* had a positive effect on the biofilm formation in *B. thuringiensis*. The recombinant strain formed approximately 1.5 times more biofilms than BtiWT and BtipHT315 (Figure 3.13c).

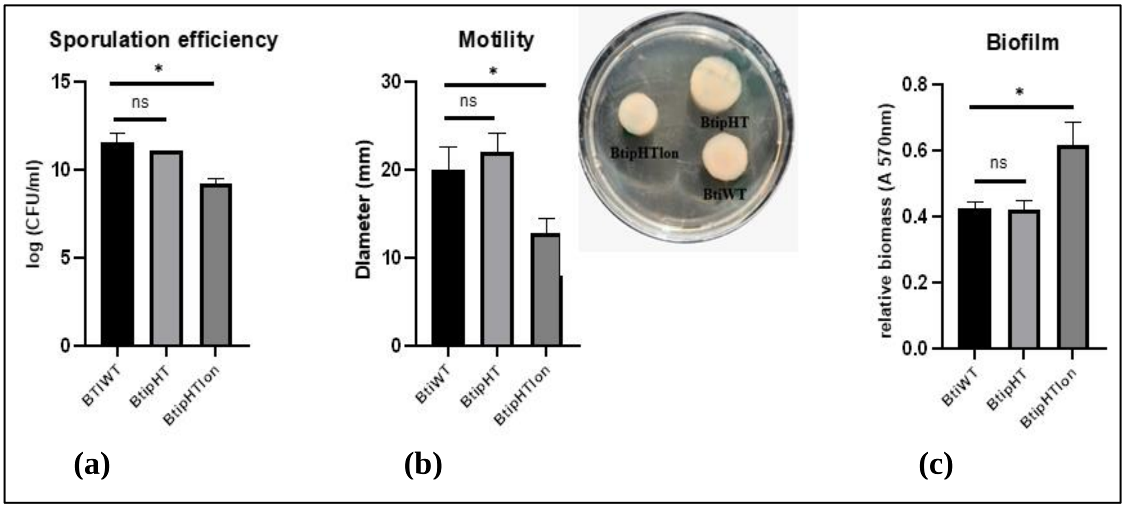


Figure 3.13: Spore count, Motility, and Biofilm Assay of *B. thuringiensis* strains. **(a)** Spore count after 72 hours of growth in DSM media **(b)** Strains were grown on LB solidified with 0.3 % agar at 30 °C for 12 hours before migration zones were measured **(c)** Biofilm formation by BtiWT, BtipHT315, and BtipHT315lon. *Student's t-test with $p<0.05$ was considered to be significant. The error bars indicate the standard deviation from the mean value. "ns" is not significant.

3.4.3. Measuring tolerance of *B. thuringiensis* strains to osmotic stress, high temperature and UV light

The impact of *lon* overexpression on the growth of *B. thuringiensis* serovar *israelensis* under stressful conditions was also investigated. For this, the growth of the cells under ionic and non-ionic osmotic stress, heat, and UV stresses were studied:

3.4.3.1. Osmotic stress

Compared to the normal condition, the growth of all strains was reduced significantly. The effect of overexpression of *lon* on osmotic stress sensitivity was examined using different concentrations of NaCl and sucrose both in liquid and on solid media. The recombinant strain grew faster and seemed to be more tolerant than BtiWT and BtipHT315 to increasing concentrations of NaCl and sucrose separately (Figures 3.14 and 3.15). The difference was much more significant at the higher concentrations. As an example, we can observe that after 12 hours of incubation at 30 ^{o}C, BtipHT315lon had an optical density (600 nm) greater than 0.3 when grown in LB medium supplemented with 1.2 M NaCl or 1.2 M sucrose, while the other strains had an OD (600 nm) less than 0.2.

Similarly, the recombinant strain was found to form healthy colonies on LB agar containing sucrose or extra NaCl, although at the same dilutions the control strains struggled or did not grow at all (Figure 3.16).

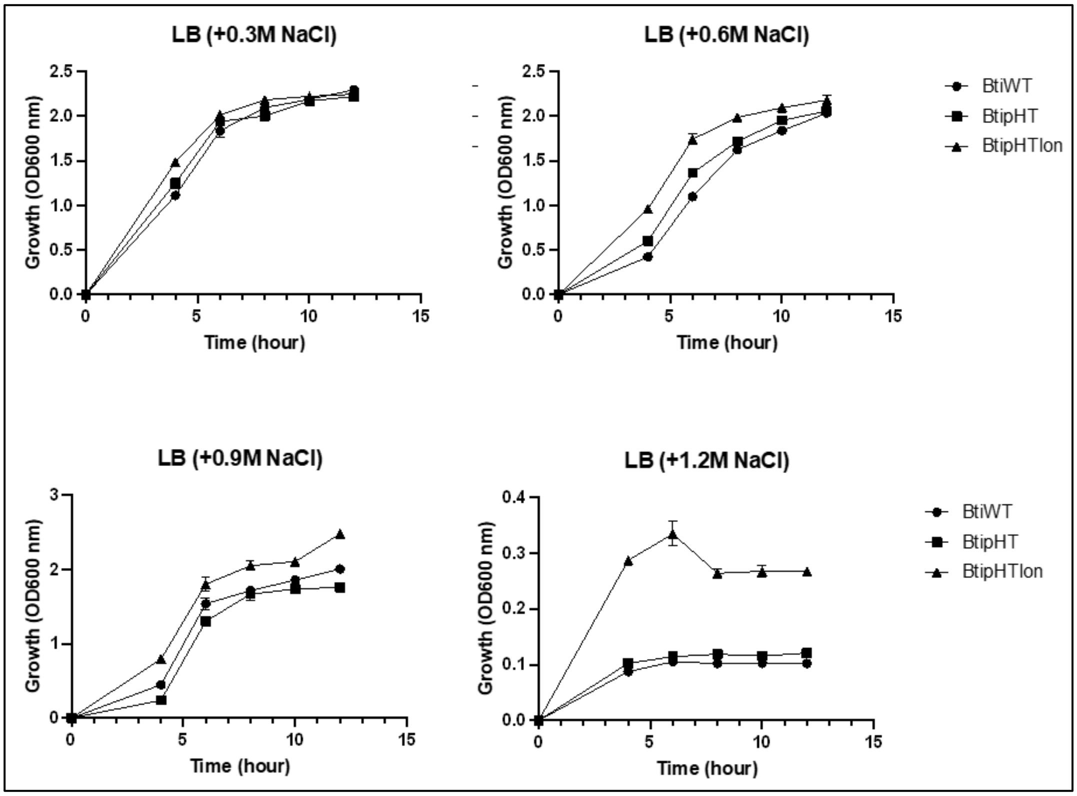


Figure 3.14: Growth of BtiWT, BtipHT315, and BtipHT315lon under ionic osmotic stress at 30 ^{o}C, LB broth was supplemented with different concentrations of NaCl.

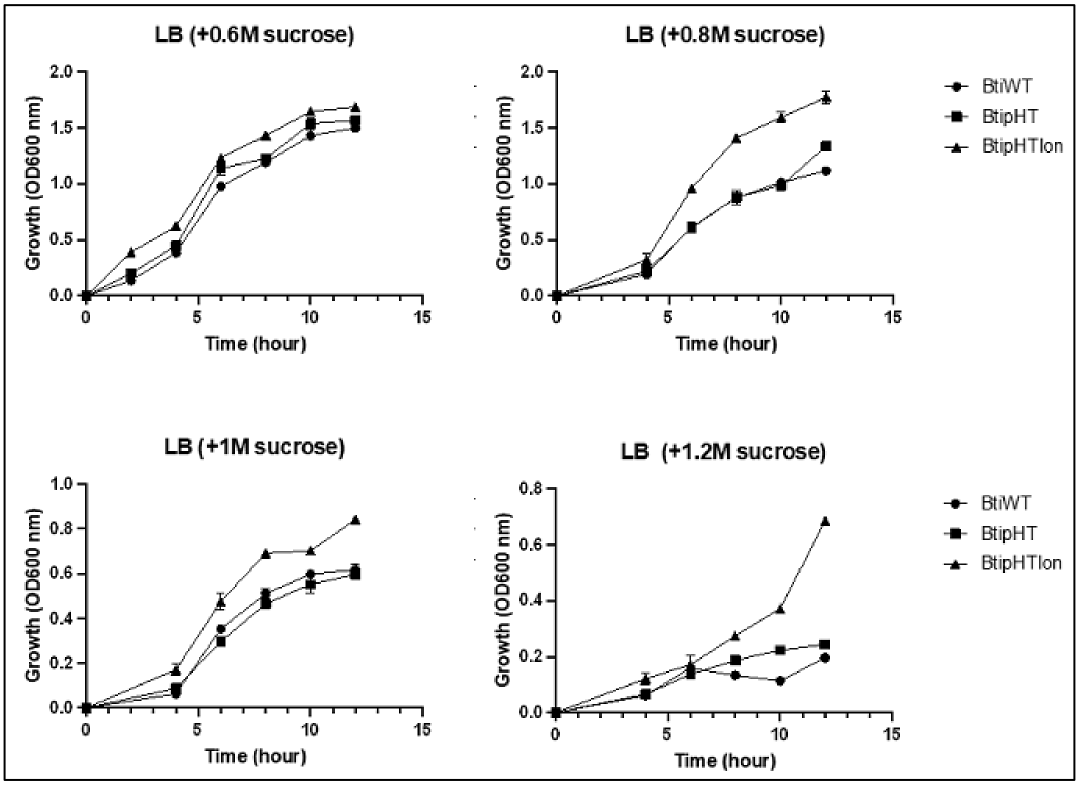


Figure 3.15: Growth of *B. thuringiensis* strains under non-ionic osmotic stress at 30 ^{o}C, LB medium was supplemented with different concentrations of Sucrose.

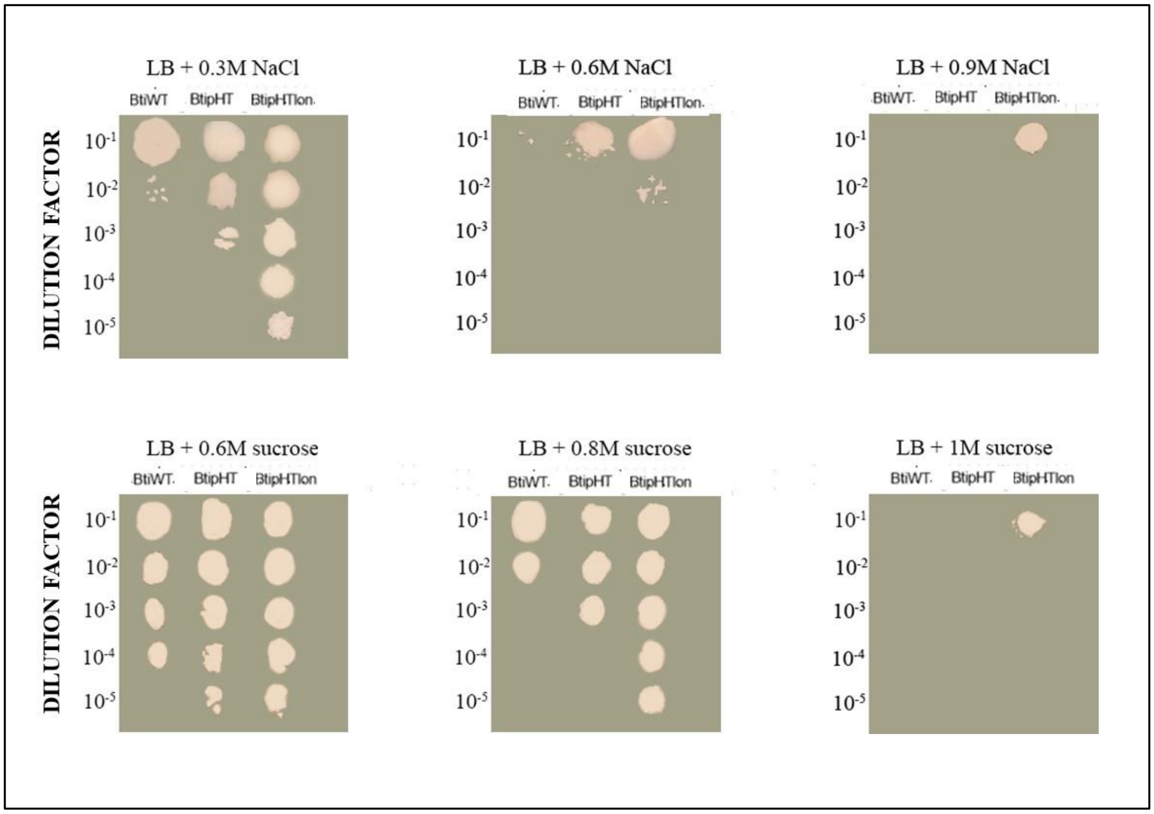


Figure 3.16: Growth of *B. thuringiensis* strains on LB agar under stress conditions. Overnight grown cultures were serially diluted and spotted on the LB agar plates supplemented with the specified concentration of NaCl or sucrose before incubation at 30 ^{o}C for 24 hours.

3.4.3.2. Heat stress

In previous studies, Lon protease was shown to be part of HAIR/HspR stress-response regulon [47]. Furthermore, the expression of *lon* was upregulated in heat shock conditions in *B. subtilis* [48]. Therefore, we sought to determine whether the overexpression of *lon* gene may improve bacterial growth at elevated temperatures (40^{o}C and 45^{o}C).

Incubation of *B. thuringiensis* serovar *israelensis* cells at 40 ^{o}C displayed comparable growth rates, whereas, at 45 ^{o}C, the recombinant strain showed more tolerance to heat stress as compared to BtiWT and BtipHT315 (Figure 3.17).

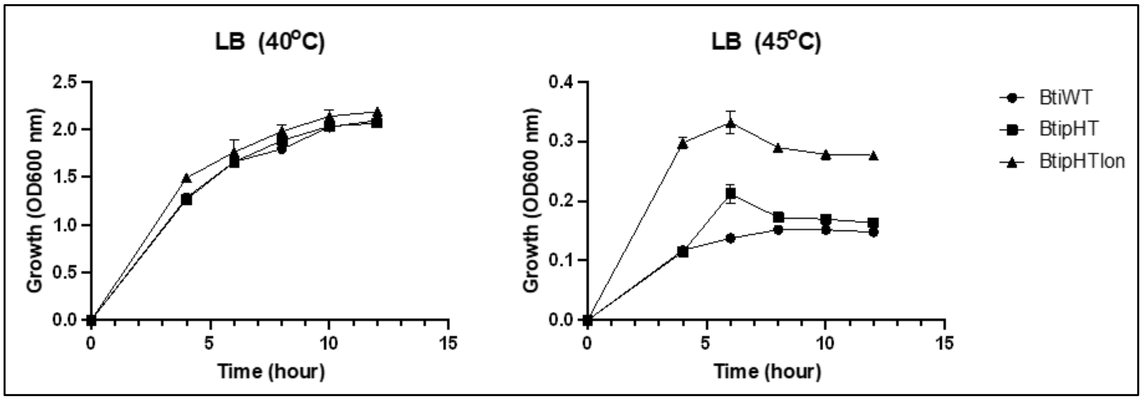


Figure 3.17: Growth of *B. thuringiensis* strains at 40°C and 45°C for 12 hours. The error bars indicate the standard deviation from the mean value.

3.4.3.3. UV tolerance

We tested *B. thuringiensis* strains (Btiwt, BtipHT315, and BtipHT315lon) for their capabilities to survive upon UV exposure because it has been previously reported that *lon* deficient strains are more sensitive to UV irradiation including *E.coli, P. aeruginosa* [13], [38].

Exponentially growing cells spread on LB agar plates (without cover) were treated with UV (254 nm) radiation at a dose rate of 8 J/m^2/s. A remarkable resistance of the recombinant strain to UV irradiation was found. Wild-type strain and BtipHT315 strain showed a survival ratio of 9% and 10.5% respectively while the recombinant strain recorded 32.1% survival (Figure 3.18a).

At the same time, the liquid cultures were treated with UV (254 nm) radiation at a dose of 2400 J/m^2 (8 J/m^2 for 5 min), and survival ratios of 96% in BtipHT315lon, 64% for BtiWT, and 61.9% for BtipHT315 were obtained (Figure 3.18b).

The cells were then examined under the confocal microscope to confirm these results. We found that after 5 min exposure to UV light ratio of the dead cells was higher in the control strains compared to the recombinant strain (Figure 3.19). These results showed that overexpression of lon reduces the sensitivity of *B. thuringiensis* to UV damage.

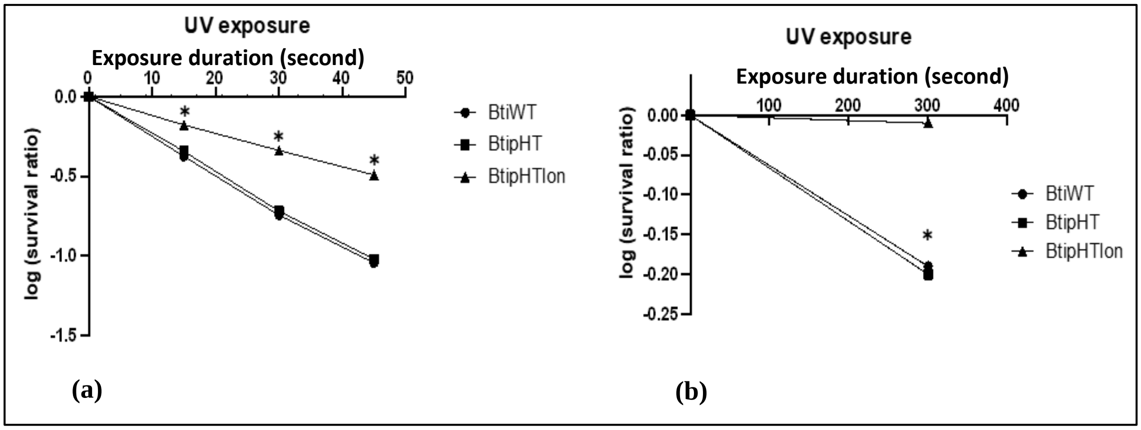


Figure 3.18: The sensitivity of *B. thuringiensis* strains to UV irradiation. **(a)** Cells exposed to UV radiation (254 nm) at a dose rate of 8 J/m^2/sec after spreading onto the LB agar **(b)** Cells exposed to UV radiation (254 nm) at a dose rate of 8 J/m^2/sec before spreading onto the LB agar. The surviving cells were counted and converted as a log survival ratio. Asterisks (*) represents p-value of less than 0.05 which was considered statistically significant. Vertical bars indicate standard deviation from the mean value.

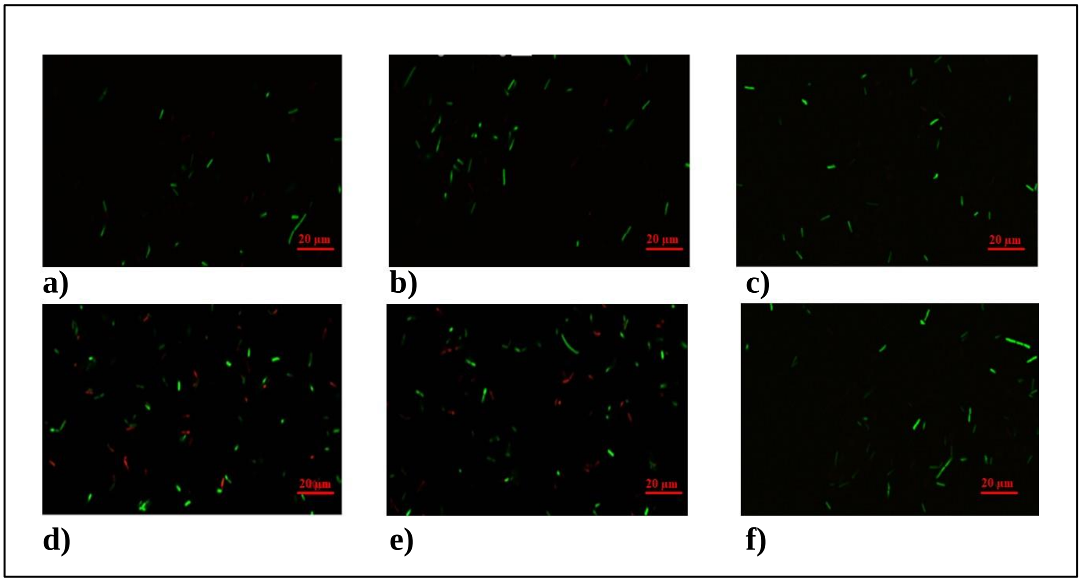


Figure 3.19: Confocal analysis of (a) BtiWT, (b) BtipHT315 and (c) BtipHT315lon before UV exposure and confocal analysis of (d) BtiWT, (e) BtipHT315 and (f) BtipHT315lon after UV exposure. The red cells represent dead cells whereas the live cells are shown in green color.

4. DISCUSSION

δ-Endotoxins (Cry and Cyt toxins) are produced by *B. thuringiensis* serovar *israelensis* during the sporulation phase of their growth and are larvicidal against various insect species by targeting their gut [39]. Previously, the improvement of bioinsecticide production by *B. thuringiensis* strains was accomplished through mutation, selection, or genetic recombination [78], [79]. Other studies have also aimed at increasing *B.thuringiensis* 's larvicidal activity by cloning crystal protein genes or adjusting culture media [80], [81]. Likewise, a previous study by our team showed a significant increase in endotoxin production in *B. thuringiensis* serovar *israelensis* that overexpress polyphosphate kinase gene (*ppk*) [82]. Recently we have shown that an extra copy of the *lon* protease gene in the *Streptomyces coelicolor* genome caused an important increase in antibiotic synthesis [83]. Thus, we wanted to investigate whether this triggering effect of Lon on secondary metabolite production also applies to *B. thuringiensis* serovar *israelensis*. For this purpose, we cloned the *B. thuringiensis lon* gene with its promoter and transcriptional terminator into both a high copy number plasmid (pHT315) and an integrative plasmid (pRA) and determined their effects on growth, sporulation, motility, biofilm formation, endotoxin production, and tolerance to several stress conditions.

The relative expression of the *lon* gene was shown to be around 80 and 117 times higher in the recombinant *B. thuringiensis* serovar *israelensis* (BtipHT315lon) compared to control strains in LB and minimal media respectively. The result of quantitative PCR showed that the relative expression of *lon* protease gene was higher in BtipHT315lon and BtipRAlon. Particularly, as expected the relative gene expression of *lon* was higher in BtipHT315lon than BtipRAlon.

However, the amount of mRNA was found not directly proportional to the level of protein. Since after extracting total proteins, it was found that the recombinant strain displayed just a slightly higher amount of Lon protease than the wild type and BtipHT315, as well as relatively increased enzyme activity. It is safe to hypothesize that *lon* expression may be translationally controlled.

Although we planned to work with both BtipRAlon and BtipHT315lon recombinant strains, unfortunately, most of the analyses were performed by using the recombinant strain BtipHT315lon. Since it was observed that the effect of one copy of the *lon* gene was negligible on *B. thuringiensis* serovar *israelensis*.

We showed that overexpression of *lon* did not affect the growth of the bacteria in different media (LB, DSM, and minimal media). Previous studies suggested that the high expression of *lon* protease may be detrimental to certain organisms. For example, Goff et al. found that *lon* overexpression negatively affected the growth of *E. coli* and cause loss of cell viability in the stationary phase of its growth cycle [84]. Subsequent work revealed that the overproduction of Lon prevents mRNA translation via the toxin-antitoxin mechanism of the chromosomal *yefM-yoeB* in *E. coli* [85]. Although we have shown in our study that *lon* overexpression does not negatively affect Bti growth, this is not the case for its effects on sporulation and motility. We found that overexpressing the *lon* protease gene caused motility defect and reduced sporulation in *B. thuringiensis* serovar *israelensis*. In contrast to controls, the motility of the recombinant strain was reduced by about 40 percent. Previously, the role of Lon protease on motility has been well described in different organisms like *Proteus mirabilis* and *Pseudomonas aeruginosa* [73], [74], and the negative effect of *lon* deficiency on motility was shown in these organisms. On the other hand, in agreement with our observations, the absence of *lon* protease in *B. subtilis* was found to increase the swarming motility [86]. Thus, we propose that overexpression of *lon* may increase the degradation of the activator protein of genes responsible for motility in *B. thuringiensis* and this might lead to diminished motility. Further studies are necessary to confirm this hypothesis.

Contrary to findings on motility, *lon* gene overexpression positively affected the formation of biofilm in *B. thuringiensis*. It has been shown in *P. aeruginosa* [74], *Acinetobacter baumannii* [76], and *V. cholerae* [77] that Lon protease is necessary for biofilm formation. Although it is still difficult today to precisely define the link between biofilm and motility, there are pieces of evidence that they may behave in opposite to each other depending on certain mutations in the regulatory genes [87]. Motility activation and biofilm inhibition, for example, have been reported after decreasing the amount of cyclic di-guanosine monophosphate (c-di-GMP) and vice versa in certain species like *S. typhimurium* [88]. Blair and coworkers also noted that *EpsE* operon, which promotes the formation of biofilm, has an inhibitory effect on motility in *Bacillus subtilis* [89].

Similarly, our results showed that *lon* overexpression had an inverse effect on biofilm and motility in *B. thuringiensis* serovar *israelensis*.

As previously demonstrated, it is during sporulation that *B. thuringiensis* produce delta-endotoxins that are responsible for insecticidal activity [72]. Although the recombinant strain showed decreased sporulation, it produced more endotoxin compared to the wild type. There are studies with similar conclusions that endotoxin production and spore formation may not be parallel to each other. For example, Zouari et al. (2002) found that adequate ventilation caused an increase in delta-endotoxin concentration while causing a decrease in the final spore count in *B. thuringiensis* subsp *kurstaki* [90]. Another study proposed that extensive sporulation may impede toxin synthesis in *B. thuringiensis* [91].

In the present work, by increasing *lon* protease expression, we report an enhancement in the yield of intracellular toxins in *B. thuringiensis* serovar *israelensis*. This suggests a positive correlation between *lon* expression and endotoxin production. Works by Zouari et al (2002) and Ennouri et al (2013) concluded that increased protease production was associated with low endotoxin synthesis [90], [92]. However, in these studies, the effect of extracellular proteases on toxin yield is mentioned. According to the best of our knowledge, our study is the first one showing the effect of Lon protease on endotoxin production. Indeed, our recombinant strain exhibits an increase in endotoxin production, with a decrease in spore count. Thus, we can deduct that by discharging a small amount of spore to the environment compared to the wild type the recombinant strain may be a more eco-friendly strain.

We assessed whether the high amount of protease in *B. thuringiensis* serovar *israelensis* could provide an advantage under stressful conditions. The recombinant strain was found to be better adapted to osmotic and heat stress conditions than the control bacteria. As BtipHT315 is much less resistant to stress compared to BtipHT315lon, the better survival properties can be attributed to the overexpression of the *lon* gene. The role of Lon in bacterial viability under heat and osmotic stress has been previously studied by other groups. For instance, it was shown that upon exposure to osmotic stress and heat shock *lon* expression increased in *E. coli* cells via sigma 32 [39], [93]. In another study, the expression of *lon* was found to be induced by salt and oxidative stress in *B. subtilis*

[48]. The importance of Lon in stress response was also shown in *Dickeya solani* [75], *Pseudomonas aeruginosa* [74], *Acinetobacter baumannii* [76]. Therefore, the high *lon* expression led our recombinant strain to overcome the osmotic and heat stresses. Nevertheless, BtipHT315 resisted better than BtiWT in osmotic stress, this can be explained by the fact that BtipHT315 comprises more genes than BtiWT such as resistance to erythromycin which would likely allow it to cope better to stress conditions. These observations were also advanced previously by Tugrul et al [82], and particularly by Tounsi who suggested that erythromycin resistance affects sporulation and production of endotoxins in *B. thuringiensis* and *B. subtilis* [94]. However, Lon protease is not the only protease that participates in stress tolerance. The importance of Clp protease, which belongs to the same AAA+ superfamily as Lon protease, on stress tolerance, virulence, and biofilm has also been shown by several studies in different species like *Enterococcus faecalis* [95], *Porphyromonas gingivalis* [96], *Staphylococcus aureus* [97]. The effect of Clp protease on stress tolerance and toxin production of *B. thuringiensis* serovar *israelensis* is also a matter of curiosity and can be elucidated by future studies.

Finally, previous studies have shown that the *lon* mutation increased the sensitivity of various organisms to UV radiation [70] [76]. In the present study, the effect of overexpression of *lon* on the UV sensitivity of *B. thuringiensis* cells was analyzed. The results proved that the recombinant strain shows a better resistance to UV treatments compared to the control strains. We noted the survival ratio after UV exposure as 9%, 10.5%, and 32% in Btiwt, BtipHT315, and BtipHT315lon respectively. It has been determined that the UV sensitivity of *lon* deficient *E. coli* mutant is related to cell division inhibitor SulA [98]. As a substrate of Lon protease, SulA accumulates in *lon* mutants and when the cells are exposed to UV radiation, cell division is arrested which causes cell death. We can propose that the increased resistance of recombinant cells to UV stress, maybe because of the increased SulA degradation. This hypothesis needs to be proved with further research.

5. CONCLUSION AND FUTURE PERSPECTIVES

This study is the preliminary study for the elucidation of the role of increased production of Lon protease in several biological functions including endotoxin production, biofilm formation, motility, and sporulation in *B. thuringiensis* serovar *israelensis*. The following points can summarize our findings:

i. Overexpressing the *lon* protease gene increases the crystal protein synthesis in *Bacillus thuringiensis* serovar *israelensis.*
ii. The recombinant strain was found to be able to deal better with stringent environmental conditions than the wild type.
iii. Overexpression of Lon protease negatively affects motility and sporulation coupled with a positive effect on biofilm formation, stress resistance (osmotic, heat, UV), and endotoxin production.

Our data revealed, a relationship between Lon protease and endotoxin production and several other biological functions in *B. thuringiensis* serovar *israelensis*. But the exact molecular mechanisms were not studied in this thesis. For future perspectives, below topics can be studied:

i. the impact of Lon protease on SulA upon UV exposure,
ii. bioassay results by using different insect larva,
iii. the effect of *lon* overexpression in *ppk* overproducing strain,
iv. the link between Lon protease and the activator of flagella or other regulators involved in motility,
v. the effect of Clp protease on stress tolerance and toxin production of *B. thuringiensis* serovar *israelensis*.

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