REPUBLIC OF TURKEY
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL SCIENCES AND TECHNOLOGIES

THE ISOLATION OF PLASMIDS AND INVESTIGATION OF ANTIBIOTIC RESISTANCE GENES AND TOXINS IN NOVEL BACILLUS CEREUS SENSU LATO STRAINS

Prepared by
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Supervisor
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Master Thesis

August 2022

Kayseri

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August 2022

KAYSERI

COMPLIANCE WITH SCIENTIFIC ETHICS

I, Abdul Aziz Karim, hereby declare that all information provided in this document has been obtained and presented per the academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Abdul Aziz KARİM
Signature:

The study named **"The Isolation of Plasmids and Investigation of Antibiotic Resistance Genes and Toxins in Novel *Bacillus cereus sensu lato* Strains"** an **M. Sc.** thesis has been prepared following Erciyes University's Graduate Thesis Proposal and Thesis Writing Directives.

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August 2022, KAYSERI

YEREL *BACILLUS CEREUS SENSU LATO* SUŞLARINDA PLAZMİDLERİN İZOLASYONU VE ANTİBİYOTİK DİRENÇ GENLERİ VE TOKSİNLERİNİN İNCELENMESİ

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Yüksek Lisans Tezi, Ağustos 2022
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ÖZET

Bacillus cereus grubu olarakta bilinen Bacillus cereus sensu lato, filogenetik olarak yakından ilişkili, birçok ortamda bulunabilen, spor oluşturan, fakültatif anaerob veya aerobik, çubuk şekilli Gram pozitif bakterilerden oluşan bir gruptur. Bu grubun üyeleri, çoğunlukla büyük plazmitleri üzerinde bulunan genler tarafından kodlanan çok çeşitli toksin ve virülans faktörleri nedeniyle gıda kaynaklı ve diğer hastalıklarla ilişkilendirilmiştir. Bu çalışmada Adana, Türkiye'den toprak örneklerinden izole edilmiş olan 15 B. cereus s.l. bakterisi üzerinde Kirby Bauer disk difüzyon yöntemi kullanılarak antibiyotik duyarlılık testleri yapılmıştır. Direnç gösteren 3 suşun 18 plazmit DNA'sı Illumina NovaSeq 6000 platformu kullanılarak dizilenmiştir. Plazmit aracılı antibiyotik direnç genlerinin, toksinlerin ve virülans peptitlerinin taranması in silico araçları kullanılarak yapılmıştır. Tüm suşların siprofloksasin, levofloksasin, kloramfenikol ve meropenem'e tamamen duyarlı olduğu belirlenmiştir. Suşların büyük bir oranı (%80) S/trimetoprim'e, %73'ü de penisilin ve ampisiline karşı direnç göstermiştir. Suşların tetrasiklin (%47), eritromisin (%47), gentamisin (%40), imipenem (%20), vankomisin ve amikasin ile (her biri %13) klindamisin ve linezolide (her biri %7) karşı düşük düzeyde direnç gösterdiği belirlenmiştir. Yedi suş imipenem'e karşı ampisilin kaynaklı β-laktamaz direnci göstermiştir. Suşların MAR indeksleri 0.13 ile 0.53 arasında değişiklik göstermiş olup %66.7'sinin MAR indeksleri 0.2'nin üzerinde bulunmuştur. Ek olarak, PGAP ile yapılan fonksiyonel anotasyon sonucunda Bt SY27.1, Bt SY62.1A ve Bt SY56.3 plazmit dizilerinin analizlerinde antibiyotik direnci, toksinler ve virülans faktörlerini kodlayan genleri taşıdıkları belirlenmiştir. Bu genler çoğunlukla megaplazmitlerde bulunmuştur. Sonuç olarak, bu çalışmada Bacillus cereus s.l.'nin antibiyogram paternleri belirlenmiş ve Bt SY27.1'in virülans ve patojenisitesine katkısı da ortaya çıkarılmıştır. Bt SY27.1, Bt 62,1A, ve Bt 56.3 suşlarının patojenisite potansiyellerinin tam olarak anlaşılabilmesi için tüm genom dizi analizi ve proteomik çalışmaların yapılması önerilmiştir.

Anahtar Kelimeler: *Bacillus cereus sensu lato*, antibiyotik direnci, plazmit, toksin, β-laktamaz indüksiyonu, Virülans.

THE ISOLATION OF PLASMIDS AND INVESTIGATION OF ANTIBIOTIC RESISTANCE GENES AND TOXINS IN NOVEL *BACILLUS CEREUS SENSU LATO* STRAINS

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Erciyes University, Graduate School of Natural and Applied Sciences
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Supervisor: Prof. Dr. Semih YILMAZ

ABSTRACT

Bacillus cereus sensu lato, also known as the *Bacillus cereus group*, is a group of ubiquitous, spore-forming, facultative anaerobe or aerobic, rod-shaped Gram-positive bacteria which are closely related phylogenetically. Members of this group have been implicated in foodborne and other diseases owing to their diverse toxins and virulence factors, mostly encoded by genes located on large plasmids. In this study, we examined the antibiotic susceptibility patterns of 15 *B. cereus s.l.* strains isolated from soil samples collected from Adana, Turkey, using the Kirby Bauer disk diffusion method. Plasmid DNA was extracted from 3 strains and sequenced using Illumina NovaSeq 6000 platform. Screening of plasmid-mediated antibiotic resistance genes, toxins, and virulence peptides was performed using *in silico* tools. It was found that all strains were completely susceptible to ciprofloxacin, levofloxacin, chloramphenicol, and meropenem. The highest percentage (80%) of strains demonstrated resistance towards S/trimethoprim, followed by penicillin and ampicillin (73% each). A low level of resistance to tetracycline (47%), erythromycin (47%), gentamicin (40%), imipenem (20%), vancomycin and amikacin (13% each), and clindamycin and linezolid (7% each) was found. Seven strains displayed ampicillin-induced β-lactamase resistance to imipenem. The MAR indexes of the strains ranged from 0.13 to 0.53 and 66.7% of the strains had MAR indexes above 0.2. In addition, functional annotation with PGAP and computational analyses of the *Bt* SY27.1, *Bt* 62.1A, and *Bt* 56.3 plasmid sequences uncovered genes coding for antibiotic resistance, toxins, and virulence factors. These genes were mostly found on the megaplasmids. In conclusion, this study elucidated the antibiogram patterns of *Bacillus cereus s.l.* strains, and the contributions of plasmids to the virulence and pathogenicity of *Bt* SY27.1. Whole genome sequencing and proteomics studies are recommended to fully understand the pathogenic potential of *Bt* SY27.1.

Keywords: *Bacillus cereus sensu lato*, antibiotic resistance, plasmid, toxin, β-lactamase induction, Virulence.

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ABBREVIATIONS

NRPS	:	Non-Ribosomal Peptide Synthetase
Bt	:	*Bacillus thuringiensis*
WHO	:	World Health Organization
s.l.	:	*Sensu lato*
s.s.	:	*sensu stricto*
cyt	:	cytolytic
MTX	:	Mosquitocidal toxins
Cry	:	Crystal
VIP	:	Vegetative insecticidal protein
NGS	:	Next Generation Sequencing
MAR	:	Multiple Antibiotic Resistance
β	:	Beta
δ	:	Delta
B.	:	Bacillus
NB	:	Nutrient Broth
CLSI	:	Clinical and Laboratory Standard Institute
R	:	Resistant
I	:	Susceptible, increased exposure
S	:	Susceptible
AMP	:	Ampicillin
P	:	Penicillin
TE	:	Tetracycline
CIP	:	Ciprofloxacin
LEV	:	Levofloxacin
GM	:	Gentamicin
AN	:	Amikacin
VA	:	Vancomycin
SXT	:	Sulfamethoxazole/Trimethoprim
E	:	Erythromycin

DA	:	Clindamycin
IPM	:	Imipenem
MEM	:	Meropenem
LZD	:	Linezolid
C	:	Chloramphenicol
DNA	:	Deoxyribonucleic Acid
WB	:	Wash Buffer
PE	:	Paired-End
PGAP	:	Prokaryotic Genome Annotation Pipeline
NCBI	:	National Center for Biotechnology Information
CARD	:	Comprehensive Antibiotic Resistance Database
GC	:	Guanine-Cytosine
Bp	:	Base pair
ABC	:	ATP-binding cassette
GNAT	:	Gcn5-related N-acetyltransferase
Sdp	:	Sporulation Delaying Protein
UV	:	Ultraviolet
MBL	:	Metallo-β-lactamase
DMT	:	Drug/metabolite transporter

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INTRODUCTION

The rise and dissemination of antibiotic resistance mechanisms in pathogenic bacteria, in addition to a declining number of effective antibiotics, has caused health crises globally (Davis et al., 2016). The World Health Organization (WHO) estimates that 10 million deaths could occur each year by 2050 as a result of drug-resistant diseases if action is not taken, and about 24 million people could be forced into extreme poverty due to antimicrobial resistance (WHO, 2020). In the last 2 decades, various bacterial pathogens with multidrug resistance have risen to a pandemic level, resulting in difficult-to-treat and/or sometimes untreatable bacterial infections using conventional antibiotics (Akova, 2016). Genes responsible for bacteria antibiotic resistance are mostly encoded by extrachromosomal elements called plasmids. Since most *Bacillus* species dwell in the same ecosystem as streptomyces and other antibiotics producers, they may have developed resistance to antibiotics produced under natural conditions (Bernhard et al., 1978).

Some members belonging to the *Bacillus cereus sensu lato* family, especially *Bacillus thuringiensis (Bt)* species, can produce a variety of toxins such as delta endotoxins (Cry), Cytolytic (Cyt) toxins, Mosquitocidal toxins (MTX), β-exotoxin, and vegetative insecticidal proteins (Vip). These toxins demonstrate specific pathogenicity against target insects, nematodes, mites, protozoa, etc. (Health Canada, 2018). Other non-insecticidal *Bt* strains produce parasporal inclusions that exhibit cytocidal activities against human cancer cells without exerting a hemolytic effect on normal cells (Ekino et al., 2014). Cry and Cyt proteins produced by *Bt* are selective, therefore using these organisms as pest control agents helps to eliminate only larvae of target insects and makes them suitable alternative for ecofriendly biocontrol of insect pests. There are various other known substances produced by *Bt* bacteria, that may help with further elimination of pests and promote plant growth. Furthermore, attempts are being made to use *Bt* strains in

bioremediation of contaminated sites as well as in medicine, especially in combating human and animal pathogens, or cancer cells (Gęsicka et al., 2020).

Bacillus cereus sensu stricto causes a diarrheal type of food poisoning by producing complex enterotoxins in the small intestines during their vegetative growth (Per Einar Granum & Lund, 1997). *Bacillus thuringiensis* (*Bt*), a member of this family, produces δ-endotoxin which provides an alternative to chemical insecticides for controlling many species of pest insects (McGaughey & Whalon, 1992).

Plasmids play a crucial role in the evolution of bacteria by providing new capabilities and important functions to the bacteria. Many genes that code for antibiotic resistance, toxins, and virulence factors are located on megaplasmids. Megaplasmids are extrachromosomal replicons with sizes of 100 kb and larger (Schwartz, 2009).

The detection of resistance phenotypes through the traditional culture-based method has a weakness as several organisms are difficult to grow. Molecular methods such as high-throughput detection of biomarkers have over the years improved remarkably, but these methods usually have limitations on the number of markers that can be detected (Kumarasamy et al., 2010; Y. Y. Liu et al., 2016). Because of this, an alternative approach that can accurately determine antibiotic resistance, toxins, and virulence factors are required.

The decreasing cost of Next Generation Sequencing (NGS) technologies provides the best alternative to sequence bacterial genomes and plasmids. NGS, coupled with bioinformatics tools and computational approaches paves the way to accurately examine the functional genes encoded by bacteria. This will allow for timely and precise identification of a specific antibiotic resistance gene encoded by a particular bacterium and the necessary action to be taken to prevent an outbreak. This project, therefore, seeks to isolate plasmids from local strains of *Bacillus cereus* sensu *lato* and *in silico* search for antibiotic resistance genes, toxins, and virulence genes encoded in the plasmids using NGS technology and bioinformatics tools.

CHAPTER 1

GENERAL INFORMATION AND LITERATURE REVIEW

1.1 Background

The group, *Bacillus cereus sensu lato*, are ubiquitous, rod-shaped, Gram (+) bacteria that currently consist of eleven closely related species: *B. thuringiensis, B. anthracis, B. mycoides, B. weihenstephanensis, B. cereus (sensu stricto), B. pseudomycoides, B. toyonensis, B. cytotoxicus* (Glenwright et al., 2017; Guinebretière et al., 2013; Jiménez et al., 2013), *B.gaemokensis, B. manliponensis, and B. bingmayongensis* (Zhuang et al., 2019). Members from this group are considered to have originated from a common ancestor, and their slight differences are a result of a series of genetic arrangements caused, among other factors, by mobile genetic elements (insertion sequences, transposons, and phages), in addition to other mechanisms of horizontal gene transfer (transductions, transformation, and conjugation) which accounts for the acquisition of virulent genes (Van der Auwera et al., 2007).

1.1.1 *Bacillus Cereus* group

The microorganisms making up *Bacillus cereus sensu lato* constitute a group of gram-positive, aerobic, facultatively anaerobic, rod-shaped, spore-forming bacteria belonging to the phylum Firmicutes.

Some members belonging to the *Bacillus cereus* (sensu lato) group exhibit pathological effects on their hosts (Amadio et al., 2009). *Bacillus cereus sensu stricto* is considered an opportunistic pathogen associated with various illnesses and food poisonings such as emetic and diarrheal syndromes (Schoeni & Wong, 2005), owing to the production of a non-ribosomal peptide synthetase toxin (NRPS) or enterotoxins (Ehling-Schulz, Guinebretiere, et al., 2006).

Bacillus thuringiensis (Bt) is a gram-positive bacterium typical of spore formation and production of crystal inclusion during the sporulation stage (Yilmaz et al., 2012). Together with the spores, the crystal inclusion acts as a potent intestinal toxin against specific insect hosts (Vidyarthi et al., 2002).

Bacillus anthracis, like all Bacillus species, is a gram-positive spore-forming bacteria that commonly dwell in the soil (Koehler, 2009). This bacterium is capable of capsule formation and lethal toxin production that cause an acute fatal human and animal disease called anthrax (Rasko et al., 2004; Vilas-Bôas et al., 2007).

The other group members, such as *B. pseudomycoides, B. weihenstephanensis, and B. mycoides,* are considered harmless (Van der Auwera et al., 2007).

1.1.2 Plasmids of *Bacillus cereus s.l.*

Bacteria plasmids have contributed immensely to the evolution of bacterial genomes and play a significant role in the functional metabolism of the host cell. Plasmids usually give a distinct advantage to the bacteria host or code for traits that support their preservation and survival (Andrup et al., 2003).

Members belonging to the *Bacillus cereus* group are known to be closely related genetically. They harbour a peculiar gene pool of plasmids encoding various functions such as self-transfer capabilities and virulence factors (Van der Auwera et al., 2007). These plasmids are essential in characterizing the phenotypic traits associated with ecology and pathogenesis of members of this group (Rasko et al., 2007). The virulence of *Bacillus thuringiensis* towards pest insects is mainly due to the production of lethal proteins encoded usually on plasmids (Amadio et al., 2009; Schnepf et al., 1998). Small plasmids of the *Bacillus cereus s.l.* group generally code for genes responsible for replication and mobilization and they are referred to as cryptic plasmids with few exceptions (Fico & Mahillon, 2006; Loeza-Lara et al., 2005). Pathogenicity and other distinct phenotypic differences are often associated with bigger plasmids; however, several functions of their genes remain a mystery (Van der Auwera et al., 2007).

1.1.3 Antimicrobial resistance of *Bacillus cereus* group

Bacteria antimicrobial resistance is one of the major global health concerns, posing a threat to the effective control and treatment of infectious diseases. The *Bacillus cereus* group is of great significance to the agriculture and the food industry, due to its association with food spoilage which lead to deterioration of food quality and safety, thereby posing a threat to public health and the economy (Fiedler et al., 2019a).

Antibiotics play a pivotal role in treating foodborne illnesses and infections associated with the *Bacillus cereus* group. However, the rise and dissemination of antibiotic resistance mechanisms in pathogenic bacteria, in addition to a declining number of effective antibiotics, has caused health crises globally (Davis et al., 2016).

Bacillus cereus s.l. isolates are generally resistant to β-lactam antibiotics such as penicillin, amoxicillin, oxacillin, and cefepime (Owusu-Kwarteng et al., 2017), while antibiotics such as clindamycin, vancomycin, ciprofloxacin, chloramphenicol, amikacin, imipenem, and gentamicin appear useful in the treatment of *Bacillus* infections (Fiedler et al., 2019b; Sliman et al., 1987). A study conducted by Owusu-Kwarteng et al. (2017), found that *Bacillus cereus s.l.* strains isolated from cattle grazing field were resistant to ampicillin (98%), penicillin (100%), oxacillin (92%), cefepime (100), and amoxicillin (100%). A study conducted by Gdoura-Ben Amor et al. (2019), found that all *Bacillus cereus s.l.* strains were fully susceptible to chloramphenicol, rifampicin, gentamicin, and ciprofloxacin, while resistant to ampicillin and novobiocin. High incidence of resistance to ampicillin and sulfamethoxazole/trimethoprim by the *Bacillus cereus* group has been reported (Hsu et al., 2021).

For effective and timely treatment of resistant strains and to reduce the spread of multidrug resistance traits among *Bacillus cereus* s.l., the determination of antibiotic susceptibility and resistance mechanisms of isolates is crucial. Previous studies have demonstrated the relevance of molecular techniques in screening for antibiotic resistance genes, toxins, and virulence factors (Bennedsen et al., 2011; Feldgarden et al., 2021; Mason et al., 2018; Nahar et al., 2017).

1.1.4. Toxins and virulence factors of the *Bacillus cereus* group

Pathogenic strains of the *Bacillus cereus* group are implicated in foodborne illnesses and other gastrointestinal and no-gastrointestinal tract infections due to the diverse toxins and virulence factors they produce (Dréan et al., 2015).

Bacillus cereus s.s. produces toxins such as enterotoxins, hemolysins, phospholipases, and emetic toxins (P. E. Granum, 1994). These toxins when present in food can cause diarrhea and other serious health complications when ingested (Notermans & Batt, 1998). Among the members of the *Bacillus cereus* group, *B. cereus s.s.* is next in significance to only *B. anthracis* as a human and animal pathogen, with reported infections in immunocompromised and immunocompetent individuals (Logan, 2012).

B. anthracis, in its vegetative form, produces exotoxins and capsule formation responsible for the synthesis of several virulence factors (Friebe et al., 2016). Virulent strains harbor two large plasmids, pXO1 and pXO2, that encode the major virulence factors (Brossier & Mock, 2001).

Bt, on the other hand, produces crystalline inclusions comprising of Cry, Vip and Cyt proteins, with toxic activity against nematodes, insects, cancer cells (Palma et al., 2014). According to Jisha et al. (2013), during the stationary phase of growth, Bt produces δ-endotoxins with virulent activity against coleopteran, lepidopteran, and dipteran insects as well as nematodes, mites, flukes, and protozoa. Strains producing these toxins have been used worldwide for the control of insects and other soil dwelling pests (Liao et al., 2022).

1.2. Problem Statement

Members of the *Bacillus cereus* group have been implicated in foodborne and other diseases owing to their diverse toxins and virulence factors, mostly encoded by genes located on large plasmids. Bacteria of this group are mostly transmitted to humans after the consumption of contaminated food. *Bacillus cereus s.l.* is believed to dwell in the soil as spores, and activate and grow when they come in contact with organic matter, insects, or mammal hosts (Stenfors Arnesen et al., 2008). Disease symptoms often occur 8 to 24 hours after ingesting food contaminated with spores or vegetative cells of *Bacillus cereus*

s.l (BfR Opinion, 2020). Antibiotics play a pivotal role in treating foodborne illnesses and infections associated with the *Bacillus cereus* group. However, the rise and dissemination of antibiotic resistance mechanisms in pathogenic bacteria, in addition to a declining number of effective antibiotics, has caused health crises globally (Davis et al., 2016). For effective and timely treatment of resistant strains and to reduce the spread of multidrug resistance traits among *Bacillus cereus* s.l., the determination of antibiotic susceptibility and resistance mechanisms of isolates is crucial.

1.3. Objective of study

This study seeks to elucidate the antibiotic susceptibility patterns of *Bacillus cereus s.l.* strains isolated from soil samples in Adana, Turkey using phenotypic analysis. In addition, plasmid DNA extracted from three strains strain was screened for the presence of genes associated with antibiotic resistance, toxins, and virulence factors of the *Bacillus cereus* group using plasmid DNA sequencing and in silico tools. This is done to understand the contribution of plasmid-encoded genes to the virulence and pathogenicity of the *Bacillus cereus* s.l. isolates.

CHAPTER 2

MATERIALS AND METHODS

2.1. Culturing of Bacterial Strains

Strains (SY18.2, SY16.2, SY49.1, SY49.1, SY28.1, SY6.4, SY13.3, SY27.3, SY56.3, SY33.3, SY62.1A, SY27.1, SY29.1, SY58.5, SY6.1, and SY6.2) taken from stock cultures were grown in a 20 ml Nutrient Broth (NB) medium via incubation in a shaking incubator at 200 rpm overnight at 35 – 37°C. The cultures were then centrifuged at 10,000 rpm for 10 minutes at 4 °C, pellets were collected and used in the plasmid extraction process.

2.2. Antibiotic Susceptibility Testing

The susceptibility of the *Bacillus cereus sensu lato* strains to antibiotics was tested using the disk diffusion method by Kirby-Bauer (Bauer et al., 1966). A total of 15 clinically important antibiotics belonging to 11 different classes (Table 2.1) were used. Each antibiotic disc was placed onto a freshly plated bacteria on the Mueller Hinton agar (BD, USA) and incubated at 35 °C for 24 hours. Bacterial susceptibilities were determined by measuring the inhibition zone diameter (in mm) around each disc. The results were interpreted as resistant (R), susceptible (S), or susceptible, increased exposure (I) according to EUCAST (EUCAST, 2022) and Clinical and Laboratory Standards Institute (CLSI) guidelines. Strains exhibiting resistance to two or more different antibiotics were characterized as multiple antibiotic resistance (MAR) and labelled with an asterisk (*).

The MAR index of all strains resistant to more than one antibiotic was determined with the formula: MAR = n/t, where ‘n’ constituted the number of antibiotics a strain was

resistant to, and ‘t’ represented the total number of antibiotics tested against the strain (Krumperman, 1983).

Table 2.1. The different antibiotics used in this study and their mode of action

Antibiotics class	***Mechanism of action*** / ***Spectrum of activity***	***Abbreviation***	***Concentration (µg)***
Penicillins	Inhibit cell wall synthesis		
Ampicillin	Broad-spectrum	AMP	10
Penicillin	Broad Spectrum	P	10
Tetracyclines	Inhibit protein synthesis		
Tetracycline	Broad-spectrum	TE	30
Fluoroquinolones	Interfere with DNA synthesis		
Ciprofloxacin	Broad-spectrum	CIP	5
Levofloxacin	Broad-spectrum	LEV	5
Aminoglycosides	Inhibit protein synthesis		
Gentamicin	Broad-spectrum	GM	10
Amikacin	Broad-spectrum	AN	30
Glycopeptides	Inhibit cell wall synthesis		
Vancomycin	Narrow-spectrum	VA	5
Sulfonamides	Interferes with tetrahydrofolate synthesis pathway		
Sulfamethoxazole/Trimethoprim	Broad-spectrum	SXT	25

Macrolides	Inhibit protein synthesis		
Erythromycin	Narrow-spectrum	E	15
Lincosamides	Inhibit protein synthesis		
Clindamycin	Broad-spectrum	DA	2
Carbapenems	Inhibit cell wall synthesis		
Imipenem	Broad-spectrum	IPM	10
Meropenem	Broad-spectrum	MEM	10
Oxazolidinones	Inhibit protein synthesis		
Linezolid	Broad-spectrum	LZD	10
Amphenicols	Inhibit protein synthesis		
Chloramphenicol	Narrow-spectrum	C	30

2.3. Plasmid extraction

Plasmids were isolated from the centrifugated pellets of bacteria samples using the EasyPure® HiPure Plasmid MiniPrep Kit. This kit is capable of extracting purified plasmid DNA, suitable for a variety of molecular biology applications such as restriction enzyme digestion, transformation, ligation, transfection, and DNA sequencing according to the manufacturer's protocol. Plasmids were extracted according to the protocol as follows:

- 500 µl of Resuspension Buffer (premixed with RNase A) was added to each bacteria pellet sample in an Eppendorf tube and resuspended completely by pipetting.
- 500 µl of Lysis Buffer was added and immediately mixed by inverting the tube 4-6 times.

- 700 µl of Neutralization Buffer was added and mixed by inverting the tube 5-6 times. The lysates were then incubated at room temperature for 2 minutes.
- The solutions were centrifuged at 12,000xg for 5 minutes and the supernatant was gently transferred to a spin column. Samples in the spin column were then centrifuged at 12,000xg for 1 minute and the flow-through was discarded.
- 250 µl Toxin-Out Buffer (TB) was added to each sample and incubated at room temperature for 10 minutes. They were then centrifuged at 12,000xg for 1 minute and the flow-through was discarded.
- 650 µl Wash Buffer (WB) with alcohol was added to each column and centrifuged at 12,000xg for 1 minute. Flow-through was discarded.
- The columns were centrifuged at 12,000xg for 1-2 minutes to completely remove the residual Wash Buffer.
- The spin columns were placed in clean microcentrifuge tubes and 70 µl of Elution Buffer prewarmed at 70 °C were added directly to the center of the column matrix.
- The columns were then centrifuged at 10,000xg for 1 minute to elute plasmid DNA. Isolated plasmids were stored at -20 °C while awaiting electrophoresis analysis.

2.4. Electrophoresis Procedures

Agarose Gel Electrophoresis (0.8% w/v) was performed in Tris Acetate Buffer (TAE) containing 40 mM Tris, 20 mM acetic acid, and 2 mM Na_2EDTA (pH 8.1). 3 µl of loading dye was mixed with a 10 µl plasmid sample and loaded into the wells. Electrophoresis was performed at 80 – 90 Volts (3.6 V/cm) +4 °C for 3-4 hours. Gels were visualized after staining with Biotium GelRed® Nucleic Acid Stain (10,000X in water). After plasmid bands were confirmed by the electrophoresis procedure, the samples were sent for sequencing separately.

2.5. Library Preparation, Genome Sequencing, and Quality Control

Sequence-ready libraries of the plasmid DNA samples were prepared using the Illumina Nextera XT DNA Library Prep Kit (Illumina, San Diego, USA). After the library

preparation, the samples were sequenced using the Illumina NovaSeq platform for a 2 x 150 paired-end (PE) run.

2.6. Plasmid assembly, annotation, and data logging

After sequencing and quality control, raw reads of SY27.1, SY56.3, and SY62.1A plasmids were uploaded into Geneious Prime software® v2022.1.1 (Biomatters, Auckland, New Zealand). *De novo* assembly was performed with Geneious *de novo* assembler by using the default settings with a little modification. Forward and reverse fastq reads were paired by choosing Forward/Reverse (inward pointing) and an Expected Distance/Insert size of 350. An output file containing the paired read was generated. After this, the paired read was trimmed using BBDuk (Bushnell, 2014), to remove adapters and low-quality (<20) reads. In the assembly settings, medium sensitivity/fast, save assembly report, save consensus sequences, and save contigs options were checked and assembly was initiated.

Annotation of plasmid sequences was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Li et al., 2021; Tatusova et al., 2016). Complete annotated plasmid sequences were submitted to the GenBank database and accession numbers were provided.

2.7. In silico detection of genes coding for antibiotic resistance, toxins, and virulence factors

Following functional annotation with PGAP, protein-coding regions were examined for the presence of antibiotic resistance genes, toxins, and virulence factors by comparing annotated features to data in the NCBI Pathogen Detection Microbial Browser for Identification of Genetic and Genomic Elements (MicroBIGG-E) database and other published data. Further screening of plasmid sequences for antibiotic resistance genes and virulence factors was executed using the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013), and ABRicate (Seemann, 2022). Mining of pesticidal Cry, VIP, Mtx, and other toxins were performed using *BtToxin_Digger* (H. Liu et al., 2021).

CHAPTER 3

RESULTS

3.1.0 Antibiotic susceptibility

The isolated *Bacillus* spp. (n=15) were subjected to antibiotic susceptibility test by the disk diffusion method on BD Mueller Hinton II agar for 24 hours to observe their susceptibility patterns to a set of antibiotics. All strains showed one or multiple resistance to the 15 antibiotics used. Resistant (high probability of therapeutic failure), susceptible, standard dosing regimen (high prospect of therapeutic success), or susceptible, increased exposure (high prospect of therapeutic success as a result of an increased adjustment of dosing concentration/regimen at infection site) (Kahlmeter & EUCAST, 2019) strains were determined according to the zone of inhibition diameter produced around each antibiotic disc. An overview of the antibiotic susceptibility profile of the *Bacillus cereus* s.l. strains is displayed in Table 3.1.

Table 3.1. Antibiotic susceptibility of Bacillus isolates used in this study

Strains	**CIP**	**VA**	**LEV**	**TE**	**SXT**	**MEM**	**E**	**GM**	**AN**	**C**	**P**	**DA**	**LZD**	**IPM**	**AM**
SY18.2^{+*}	I	R	I	R	R	S	R	R	S	S	R	S	S	S	R
SY16.2^{+*}	I	S	I	R	R	S	R	R	S	S	R	S	S	R	R
SY49.1	I	S	I	S	R	S	S	S	S	S	S	S	S	S	S
SY28.1^{+*}	I	S	I	R	R	S	R	S	S	S	R	S	S	S	R
SY6.4^{+*}	I	S	I	S	R	S	R	S	S	S	R	S	S	S	R

SY13.3+	I	S	I	S	R	S	S	R	S	S	R	S	S	S	R
SY27.3+	I	S	I	S	R	S	S	S	S	S	R	R	R	S	S
SY33.3+	I	S	I	R	S	S	S	S	S	S	S	S	S	S	R
SY56.3+	I	S	I	R	S	S	S	S	S	S	S	S	S	S	S
SY62.1A+	I	S	I	S	R	S	S	R	S	S	R	S	S	S	R
SY27.1+*	I	S	I	R	R	S	R	R	R	S	R	S	S	R	R
SY29.1b	I	S	I	S	S	S	S	S	S	S	S	S	S	S	S
SY58.5+	I	S	I	S	R	S	S	R	S	S	R	S	S	S	R
SY6.1+*	I	R	I	S	R	S	R	S	R	S	R	S	S	R	R
SY6.2+*	I	S	I	R	R	S	R	S	S	S	R	S	S	S	R

R = Resistant S = Susceptible, standard dosing regimen I = Susceptible, increased exposure

+ = strains with multiple antibiotic resistance

*= strains displaying ampicillin-induced beta-lactamase resistance to imipenem

3.1.1 Bacteria resistance to antibiotics

After the susceptibility test, it was found that no bacteria strain was resistant to ciprofloxacin, levofloxacin, chloramphenicol, and Meropenem. The highest percentage of the bacteria strains was resistant to sulfamethoxazole/trimethoprim 80% (n = 12). Penicillin and ampicillin resistance were found in 73% (n = 11) isolates while resistance to tetracycline and erythromycin was found in 47% (n = 7) isolates. It was shown that 40% (n = 6) of isolates were resistant to gentamicin. A low level of resistance to amikacin 13% (n = 2), vancomycin 13% (n = 2), clindamycin 7% (1), and linezolid 7% (n = 1) was also recorded (Figure 3.1). In this analysis, strains exhibiting ‘susceptible, standard dozing regime’ and ‘susceptible, increased exposure. were considered susceptible.

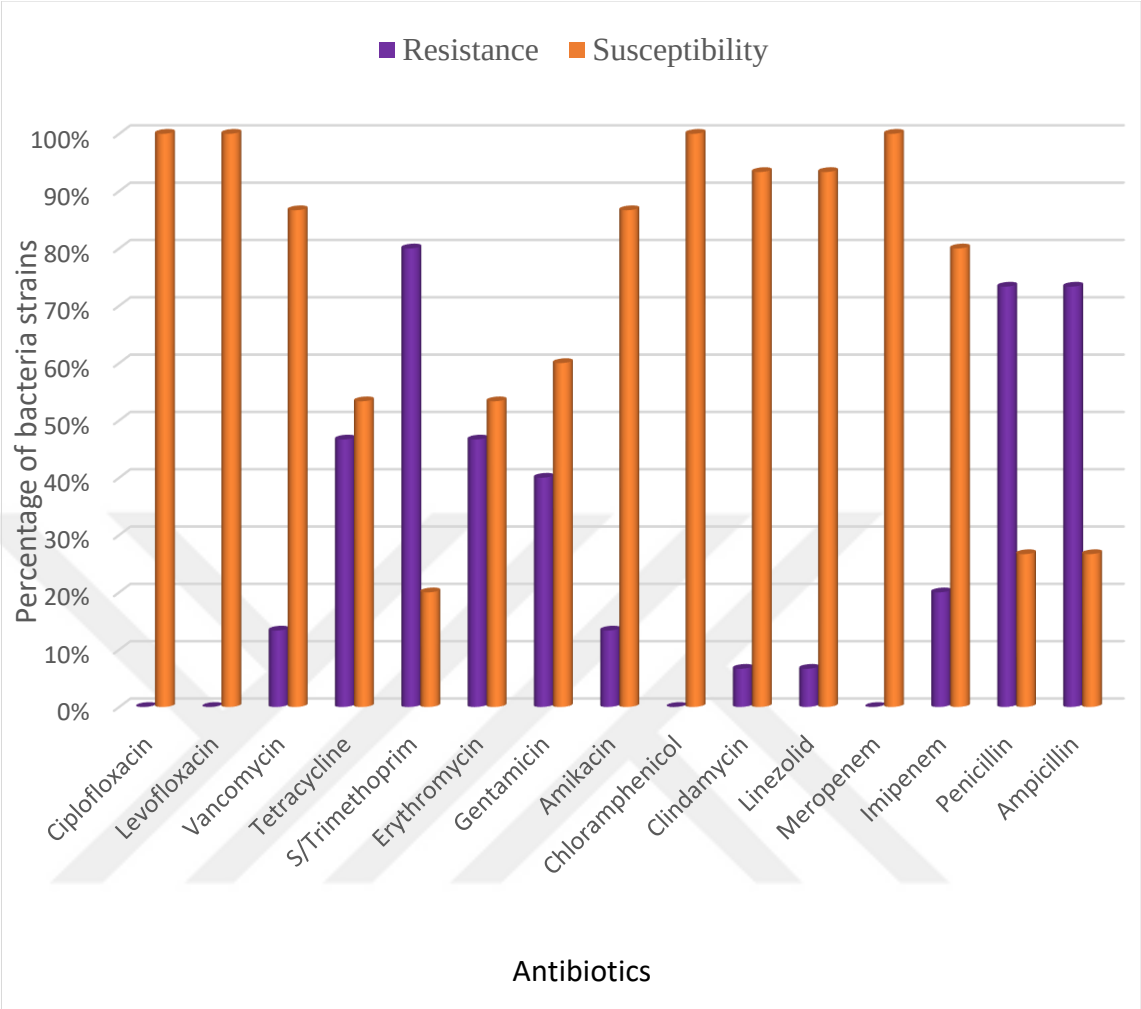


Figure 3.1. Resistance of bacteria isolates to a particular antibiotic

3.1.2. Multiple antibiotic resistance (MAR) index of isolates

MAR analysis revealed that out of the 15 strains, 12 showed multiple antibiotic resistance, representing 80% of the strains. Two strains (SY49.1 and SY56.3) were resistant to only one antibiotic while SY29.1 was completely susceptible to all antibiotics. The strains had MAR indexes ranging from 0.13 to 0.53 and showed resistance to 2 to 8 antibiotics belonging to 2 to 6 antibiotic classes (Figure 3.2). The highest (8) resistance to multiple antibiotics belonging to 6 antibiotic classes and the highest MAR index (0.53) were recorded for SY27.1, while three strains such as SY18.2, SY16.2, and SY6.1 were resistant to 7 antibiotics belonging to 6 antibiotic classes with MAR index of 0.47. Two strains (SY28.1 and SY6.2) were resistant to 5 antibiotics belonging to 4 classes.

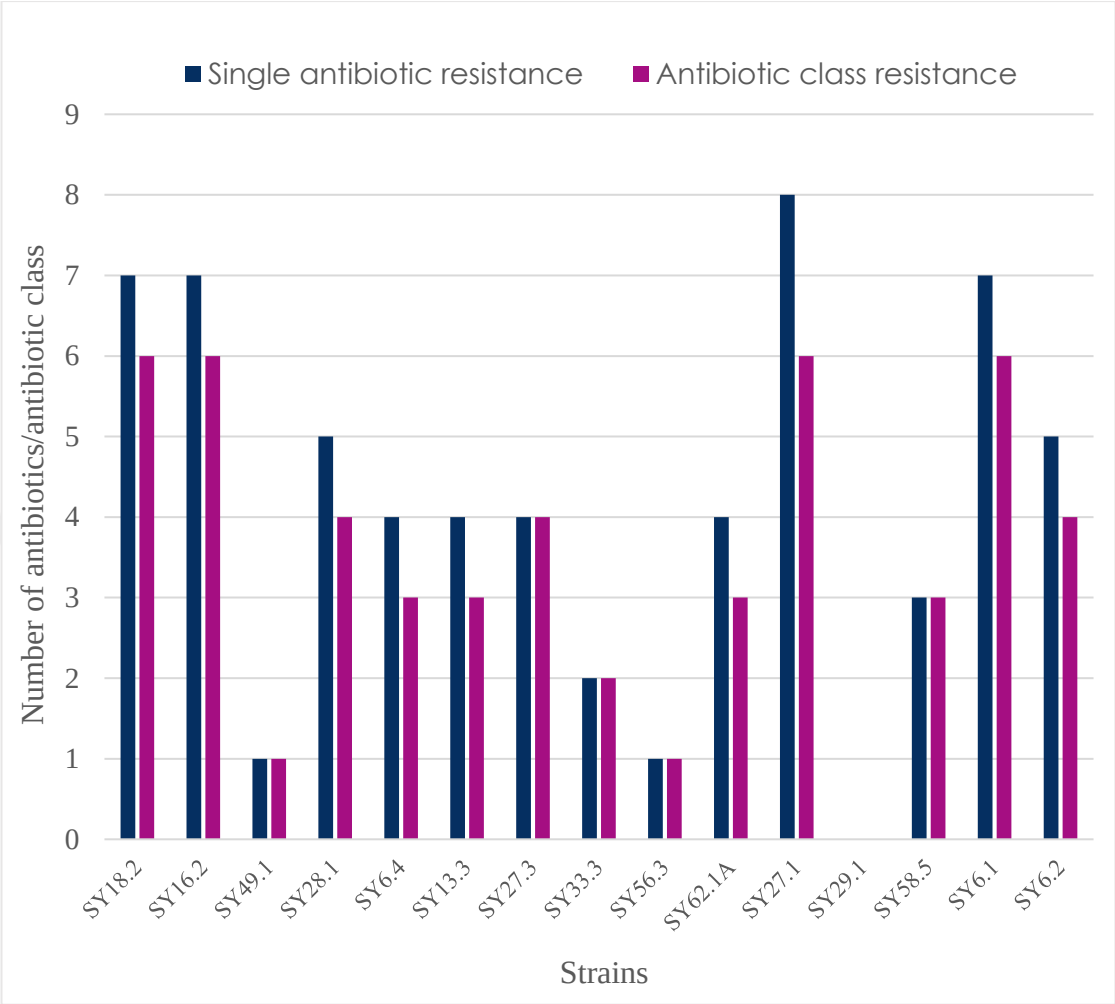


Figure 3.2. Multiple resistance of bacteria strains to antibiotics and antibiotic classes.

3.1.3. Inducible Beta-lactamase

After the antibiotic susceptibility tests, it was found that 7 strains (SY18.2, SY16.2, SY28.1, SY6.4, SY27.1, SY6.1, and SY6.2) expressed inducible beta-lactamase. These strains may have produced high levels of beta-lactamase capable of hydrolyzing imipenem, after exposure to ampicillin. Figure 3.3 displays some of the strains that exhibited ampicillin-induced beta-lactamase resistance to imipenem.

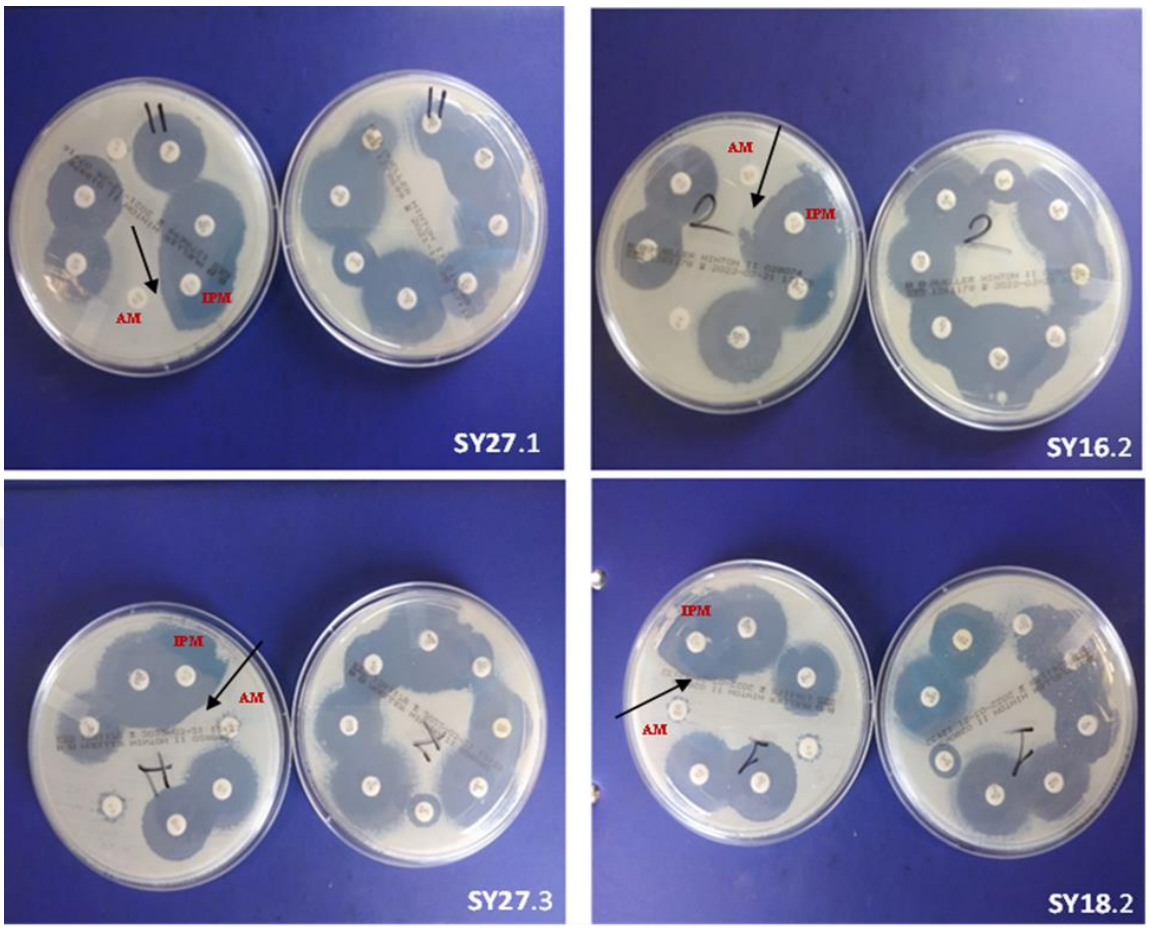


Figure 3.3. Some strains display blunt foggy shape around the imipenem (IPM) antibiotic disc on the side closer to the ampicillin (AM) (indicated with black arrows) owing to ampicillin-induced resistance to imipenem.

3.2. Resistance, virulence, and toxin profiles of plasmids extracted from selected strains

It is commonly known that antibiotic resistance, toxins, and virulence factors in bacteria are mostly carried by mobile genetic elements, especially by plasmids. Plasmids from three strains were sequenced and screened for the presence of these genes. Three strains, including SY27.1, SY56.3, and SY62.1A (Figure 3.4) were selected to further analyze their plasmids analyses. Each of these strains had multiple extrachromosomal DNA of different sizes. Plasmids extracted from these strains were examined for antibiotic resistance genes, toxins, and virulence genes.



Figure 3.4. Bacillus cereus s.l. strains Bt SY27.1, Bt SY56.3, and Bt SY62.1A growing in LB media

3.3. Isolated Plasmids Gel View

Plasmid isolation of *Bacillus cereus lato* strains 27.1, 56.3, and 62.1A was performed using *EasyPure*® HiPure Plasmid MiniPrep Kit (EM111-01). At the end of the isolation, multiple plasmids were detected in each bacterial strain. The plasmid marker used in this study had a range of 2 – 10 kilobases. There were small plasmids (<5 kb), medium (5 - 10 kb), and megaplasmids (100 kb and above) as shown in Figure 3.5.

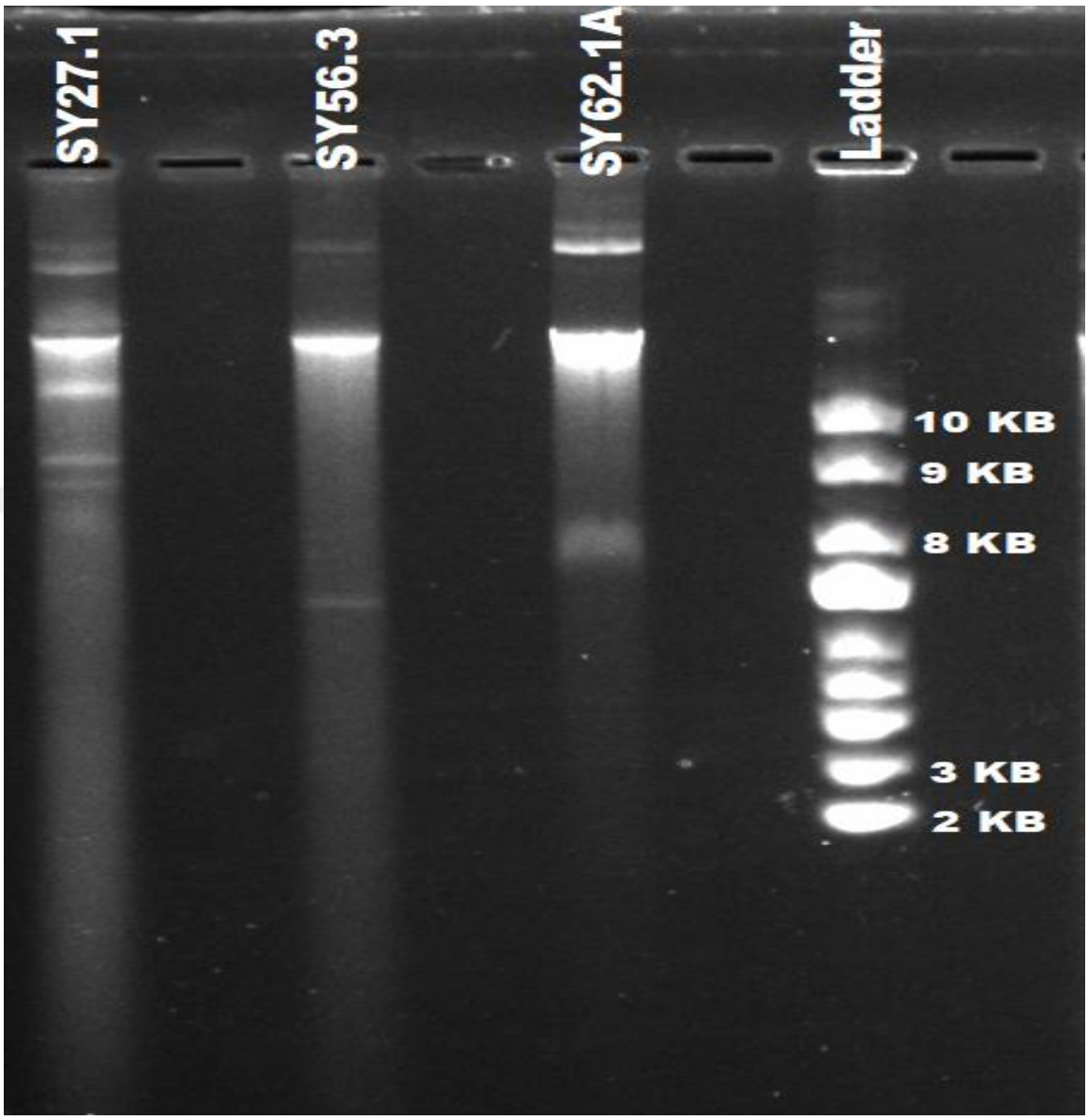


Figure 3.5. Agarose gel electrophoresis view of plasmids isolated from Bacillus cereus sensu lato strains 27.1, 56.3, and 62.1A

3.4. Bacteria Plasmid Genome Sequencing Data

After sequencing, raw Illumina output reads of the plasmids were filtered and cleaned. The raw output data were sequenced and filtered to remove adapter sequences, contamination, and low-quality reads. The statistical results of data produced after sequencing are displayed in Table 3.2. In the read statistic results, sample name, length of raw and clean reads, effective and error rate, quality score, and the guanine-cytosine (GC) content are shown. The results showed that the plasmid sequences of *Bacillus cereus*

sensu lato strains SY27.1, SY56.3, and SY62.1A had clean reads of 3,726,524 bp, 6,216,251 bp, and 5,860,705 bp respectively. All three samples recorded an effective rate of more than 99 %. Sequence from strain 62.1A had the highest (35.41%) GC content, followed by 56.3 and 27.1 with GC contents of 34.41% and 34.10%, respectively.

Table 3.2. Raw sequence data of *Bacillus cereus sensu lato* strains

Sample	**Raw Reads**	**Clean Reads**	**Raw Base(G)**	**Clean Base(G)**	**Effective Rate (%)**	**Error Rate (%)**	**Q20 (%)**	**Q30 (%)**	**GC Content (%)**
SY27.1	3,737,258	3,726,524	1.12	1.12	99.71	0.03	96.84	91.49	34.10
SY56.3	6,244,065	6,216,251	1.87	1.86	99.55	0.03	96.86	91.39	34.41
SY62.1A	5,882,981	5,860,705	1.76	1.76	99.62	0.03	97.46	92.70	35.41

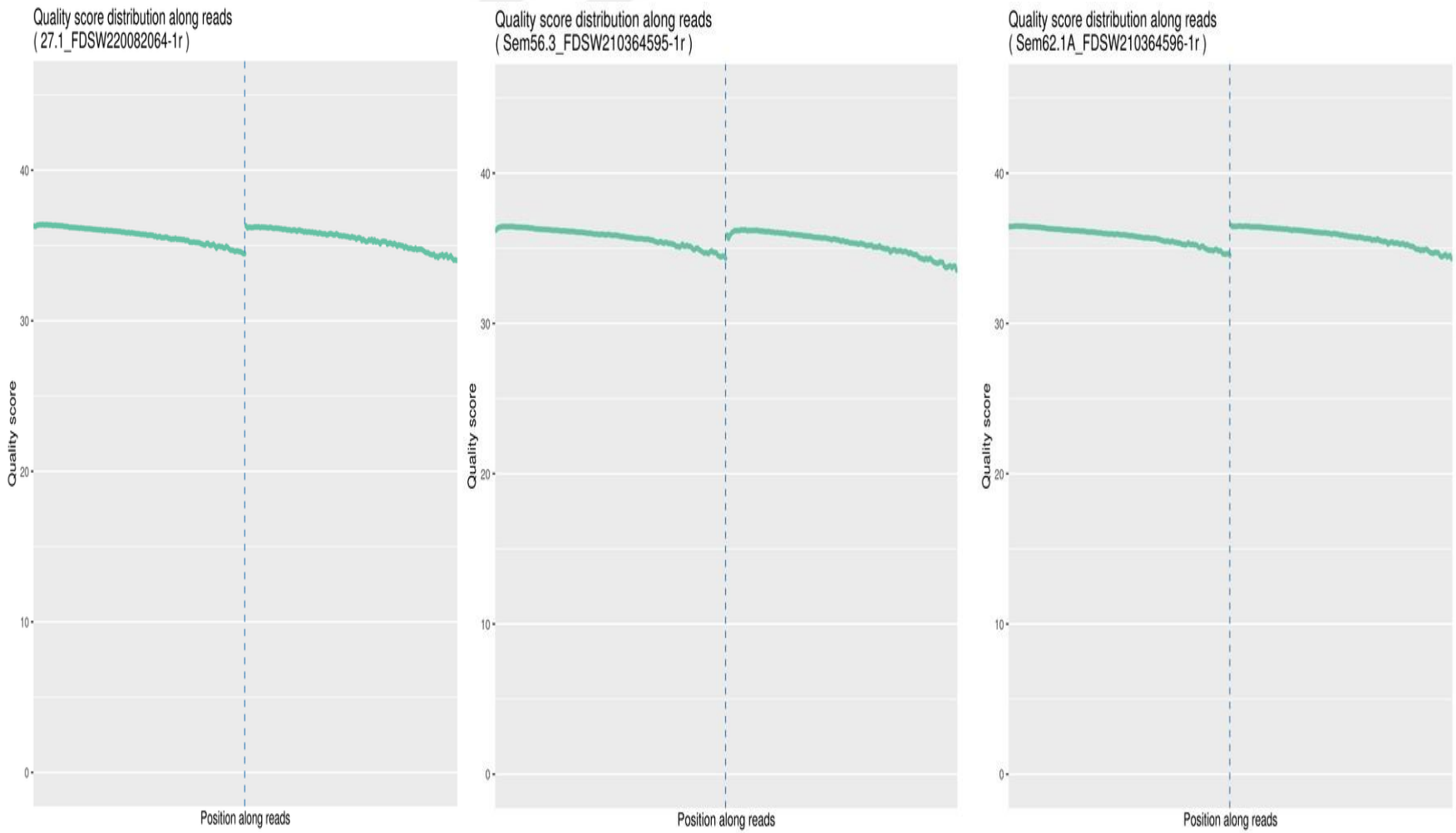


Figure 3.6. Sequence Quality of raw reads after sequencing

3.5. Sequence Features of Plasmids

3.5.1. SY27.1 Plasmid Sequence Features

The *Bacillus cereus sensu lato* strain SY27.1 is found to contain 12 extrachromosomal replicons, named pSY27.1_1 (7,635 bp), pSY27.1_2 (8,240 bp), pSY27.1_3 (8,513 bp), pSY27.1_4 (15, 258 bp), pSY27.1_5 (17,742 bp), pSY27.1_6 (53,366 bp), pSY27.1_7 (54,745 bp), pSY27.1_8 (66,972 bp), pSY27.1_9 (67,483 bp), pSY27.1_10 (77,288 bp), pSY27.1_11 (293,237 bp), and pSY27.1_12 (411,434 bp) (Table 3.3). These extrachromosomal replicons had G + C contents ranging from 29.7% to 40.0% for pSY27.1_2 and pSY27.1_4, respectively, and did not show significant deviation from that of the raw sequence (34.1%). This strain carries two megaplasmids, pSY27.1_11 and pSY27.1_12, which contain 262 and 345 coding DNA sequences (CDSs), respectively. The remaining 10 replicons, pSY27.1_1, pSY27.1_2, pSY27.1_3, pSY27.1_4, pSY27.1_5, pSY27.1_6, pSY27.1_7, pSY27.1_8, pSY27.1_9, and pSY27.1_10, have 9, 10, 10, 28, 15, 79, 54, 63, 61, and 69 CDSs, respectively.

Table 3.3. Sequence features of 12 plasmids from Bacillus thuringiensis strain *Bt* SY27.1

Plasmid Name	Length (bp)	G + C content (%)	No. of CDSs (forward/reverse)	Total length of CDSs (bp)	% of Coding sequences	Average length of CDSs (range) (bp)	GenBank accession no.
pSY27.1_1	7,635	32.2	9 (5/4)	4,188	54.85	465.33 (213-1,182)	NZ_ON470175
pSY27.1_2	8,240	29.7	10 (1/9)	5,356	65.00	535.80 (141-2,373)	NZ_ON470176
pSY27.1_3	8,513	30.8	10 (9/1)	5,082	59.70	508.20 (147-1,182)	NZ_ON470177
pSY27.1_4	15,258	40.0	28 (28/0)	13,671	89.60	488.25 (141-2,190)	NZ_ON470178
pSY27.1_5	17,742	31.5	15 (8/7)	12,351	69.61	823.40 (144-3,072)	NZ_ON470179
pSY27.1_6	53,366	35.7	79 (13/66)	46,416	86.98	587.54 (141-4,929)	NZ_ON470180
pSY27.1_7	54,745	34.3	54 (42/12)	46,395	84.75	859.17 (144-3,594)	NZ_ON470181
pSY27.1_8	66,972	34.3	63 (52/11)	56,103	83.78	890.52 (147-3,594)	NZ_ON470182
pSY27.1_9	67,483	34.5	61 (50/11)	56,136	83.19	920.26 (147-3,594)	NZ_ON470183
pSY27.1_10	77,288	34.7	69 (52/17)	64,629	83.62	936.65 (147-3594)	NZ_ON470184
pSY27.1_11	293,237	33.2	262 (142/120)	219,084	74.71	836.20 (138-4,020)	NZ_ON470185
pSY27.1_12	411,434	32.6	345 (148/197)	307,750	74.80	892.03 (63-10,017)	NZ_ON470186

3.5.2. Plasmid Sequence Features of Bt SY56.3

The assembled sequence of *Bacillus cereus sensu lato* strain 56.3 consisted of 2 replicons with one being a megaplasmid. These two circular replicons, named pSY56.3_1 and pSY56.3_2 have lengths of 50,408 bp and 224,612 bp, and G + C contents of 36.7% and 33.2% respectively (Table 3.4). The plasmids pSY56.3_1 and pSY56.3_2 contain 79 and 195 CDSs respectively. The CDSs in plasmid pSY56.3_1 cover 83.87% of the entire sequence, whilst that of pSY56.3_2 make up 76.10% of the entire sequence.

Table 3.4. Sequence features of 2 plasmids from Bacillus thuringiensis strain *Bt* SY56.3

Plasmid Name	Length (bp)	G + C content (%)	No. of CDSs (forward/reverse)	Total length of CDSs (bp)	% of Coding sequences	Average length of CDSs (range) (bp)	GenBank accession no.
pSY56.3_1	50,408	36.7	79 (12/67)	42,276	83.87	535.14 (138-3,150)	ON470187
pSY56.3_2	224,612	33.2	195 (88/107)	170,931	76.10	876.57 (99-10,455)	ON470188

3.5.3. Plasmid Sequence Features of SY62.1A

The *Bacillus cereus sensu lato* SY62.1A harbors four plasmids (pSY62.1A_1, pSY62.1A_2, pSY62.1A_3, and pSY62.1A_4) (

Table 3.5). These four circular plasmids have lengths of 14,519 bp, 46,634 bp, 52,228, and 64,433 bp, with G + C contents of 38.7%, 35.4%, 35.7%, and 34.7%, respectively. These plasmids each contained 21, 67, 76, and 59 protein-coding genes. The percent of coding regions in the entire sequence ranges from 84.81% to 87.42% for pSY62.1A_4 and pSY62.1A_1, respectively.

Table 3.5. Sequence features of 4 plasmids from Bacillus thuringiensis strain *Bt* 62.1A

Plasmid Name	Length (bp)	G + C content (%)	No. of CDSs (forward/reverse)	Total length of CDSs (bp)	% of Coding sequences	Average length of CDSs (range) (bp)	GenBank accession no.
pSY62.1A_1	14,519	38.7	21 (1/20)	12,693	87.42	604.43 (141-2,697)	ON470189
pSY62.1A_2	46,634	35.4	67 (55/12)	39,666	85.06	592.03 (177-4,710)	ON470190
pSY62.1A_3	52,228	35.7	76 (63/13)	45,495	87.11	598.62 (141-4,866)	ON470191
pSY62.1A_4	64,433	34.7	59 (12/47)	54,648	84.81	926.24 (153-3,594)	ON470192

3.6.1. Proteins involved in antibacterial compounds and resistance to antibiotics

In this study, one antibacterial peptide (M23 family metallopeptidase) involved in bacterial cell wall lysis and two phage holin proteins commonly understood to mediate bacterial cell wall degradation were found in plasmids carried by SY27.1 and SY62.1A, respectively (Table 3.6). Six proteins encoded by SY56.3 plasmids, one peptide by SY62.1A, and 8 proteins found in plasmids carried by SY27.1 were associated with antibiotic resistance. In SY56.3 plasmids, proteins coding for antibiotic resistance mostly comprised of ABC-F ribosomal protein protection, beta-lactamase family, GNAT family N-acetyltransferase, families of MarR and TetR/AcrR transcriptional regulators, and a transpose of the Tn3 family. The above resistance proteins, in addition to azaleucine resistance protein AzlC, SdpA family antimicrobial peptide system protein, HEPN domain-containing protein, and an MBL fold metallo-hydrolase which are implicated in beta-lactamase resistance, multidrug resistance, efflux pumps, and other forms of antibiotic resistance were detected in plasmids carried SY27.1. A Tn3 family transposase involved in antibiotic resistance and two phage holin peptides associated with antibacterial activity were found in SY62.1A's plasmids.

Additional proteins corresponded to compounds involved in other important bacterial resistance and tolerance such as heavy metal resistance, drug transporters, conjugal protein transfer, heat/UV resistance, etc. In addition to the major resistance proteins, these additional resistance peptides facilitate the persistence of bacteria in an environment.

Table 3.6. Proteins corresponding to antibacterial compounds, antibiotic resistance, and additional resistance and tolerance.

Function	**SY56.3**	**SY27.1**	**SY62.1A**
Antibacterial	NA	M23 family metallopeptidase bacteriocin immunity protein	phage holin Phage holin family protein
Antibiotic resistance genes	ABC-F type ribosomal protection protein Beta-lactamase family protein GNAT family N-acetyltransferase MarR family transcriptional regulator TetR/AcrR family transcriptional regulator Tn3 family transposase	Azaleucine resistance protein AzlC GNAT family N-acetyltransferase HEPN domain-containing protein MarR family transcriptional regulator MBL fold metallo-hydrolase SdpA family antimicrobial peptide system protein TetR/AcrR family transcriptional regulator Tn3 family transposase	Tn3 family transposase
Additional Resistance and Tolerance	AbrB/MazE/SpoVT family DNA-binding domain-containing protein MerR family transcriptional regulator	AbrB/MazE/SpoVT family DNA-binding domain-containing protein Alpha/beta-type small acid-soluble spore protein ArsR family transcriptional regulator Condensation domain-containing protein Conjugal transfer protein TraG CPBP family intramembrane metalloprotease DMT family transporter MerR family transcriptional regulator	AbrB/MazE/SpoVT family DNA-binding domain-containing protein

3.6.2. Proteins involved in the toxicity of Bacillus cereus group

In this study, a total of 13 peptides were identified as proteins that play a role in *Bacillus cereus sensu lato* toxicity. These peptides were largely toxins that have activity against pest insects and nematodes, an emetic toxin, and other toxins exerting different functions. The pesticidal peptides consisted of insecticidal delta-endotoxin Cry8Ea1 protein, pesticidal Cry2Ab protein, vegetative insecticidal protein Vip3A family protein, and NPP1 (necrosis-inducing Phytophthora protein) family protein. Most of the pesticidal proteins (multiple copies of different Cry proteins, one VIP toxin, and one Mtx-like toxin) were found in plasmids carried by SY27.1 while only one pesticidal toxin was carried by 62.1A's plasmid (Table 3.7)

Table 3.7. Proteins corresponding to *Bacillus cereus sensu lato* toxins.

Function	**56.3**	**27.1**	**62.1A**
Pesticidal toxins	NA	Insecticidal delta-endotoxin Cry8Ea1 family protein Pesticidal protein Cry2Ab [2 copies] Vegetative insecticidal protein Vip3A family protein	Insecticidal delta-endotoxin Cry8Ea1 family protein
Other toxins	Cereulide biosynthesis alpha/beta hydrolase CesH	Type II toxin-antitoxin system death-on-curing family toxin HBL/NHE enterotoxin family protein Pre-toxin TG domain-containing protein Sporulation delaying protein family toxin Zeta toxin family protein	NA

A peptide of cereulide biosynthesis alpha/beta hydrolase CesH, responsible for *Bacillus cereus* group emetic food-borne sickness was identified in SY56.3. Other toxin peptides characterized in SY27.1 corresponded to type II toxin-antitoxin system death-on-curing family toxin, pre-toxin TG domain-containing protein, HBL/NHE enterotoxin family protein, sporulating delaying protein family, and zeta toxin family protein.

3.6.3. Proteins associated with virulence factors

Several peptides that play important role in bacterial virulence mechanisms against target hosts were registered in these three strains. These peptides corresponded to DDE-type integrase/transposase/recombinase, IS4 family transposase, Transposase, IS200/IS605 family transposase, Ger(x)C family spore germination protein, and Endospore germination permease in plasmids found in SY56.3 (Table 3.8).

Table 3.8. Proteins associated with virulence factors of *Bacillus cereus s.l.*

Function	**SY56.3**	**SY27.1**	**SY62.1A**
Virulence genes	DDE-type integrase/transposase/ recombinase	Holliday junction resolvase RecU	Holliday junction resolvase RecU
	Endospore germination permease	ATP-binding cassette domain-containing protein	Hemolysin XhlA family protein
	Ger(x)C family spore germination protein	Hemolysin XhlA family protein	Holliday junction resolvase RecU
	IS200/IS605 family transposase		Type IV secretion system DNA-binding domain-containing protein
	Transposase		TcpD family membrane protein
			AHH domain-containing protein
			Non-ribosomal peptide synthetase

In SY27.1, plasmids carried virulence peptides such as Holliday junction resolvase RecU, ATP-binding cassette domain-containing protein, DMT family transporter, and Hemolysin XhlA family protein. This study also identified Type IV secretion system

DNA-binding domain-containing protein, TcpD family membrane protein, AHH domain-containing protein, and non-ribosomal peptide synthetase in SY62.1A that play a crucial role in Bacillus cereus group pathogenicity.

3.6.4. Proteins coding for transporters associated with virulence factors, host colonization, and immune evasion

Several transporters of the ABC-type that may be responsible for the movement of peptides such as those that confer resistance to macrolides and fluoroquinolones, bacitracin, multidrug ABC efflux, siderophores etc. were detected in SY56.3 and SY27.1. In addition, other genes coding for proteins that facilitate the colonization of host bacteria and allow the evasion of the host defense mechanism were also determined (Table 3.9).

Table 3.9. Proteins associated with transporters, immune evasion and host colonization

Function	**SY56.3**	**SY27.1**	**SY62.1A**
ABC transporters	cyclic peptide export ABC transporter peptide ABC transporter ATP-binding protein ABC-F type ribosomal protection protein	ABC transporter ATP-binding protein ABC transporter ATP-binding protein/permease ABC transporter permease ABC-2 transporter permease	
Host colonization and immune evasion	N-acetylmuramoyl-L-alanine amidase peptidoglycan DD-metalloendopeptidase family protein	N-acetylmuramoyl-L-alanine amidase peptidoglycan DD-metalloendopeptidase family protein LysR family transcriptional regulator LysR substrate-binding domain-containing protein D-alanyl-D-alanine carboxypeptidase family protein	N-acetylmuramoyl-L-alanine amidase peptidoglycan DD-metalloendopeptidase family protein

CHAPTER 4

DISCUSSION – CONCLUSION AND RECOMMENDATIONS

4.1. Discussion

This study aimed to understand the antibiotic resistance patterns of local *Bacillus cereus sensu lato* strains used in this study and to screen for and determine plasmid encoded genes responsible for virulence factors, toxins, and antibiotic resistance using NGS and bioinformatic approaches. The first stage of the present study was to perform antibiotic susceptibility on selected bacteria strains, and then three strains (SY27.1, SY56.3, and SY62.1A) were selected for further plasmid analyses. Plasmids from the three strains were extracted and sequenced. In silico mining of plasmid-mediated antibiotic resistance, toxins, and virulence peptides were performed by analyzing the plasmid sequences using bioinformatics tools.

The antibiotic susceptibility test revealed that 80% of the bacterial strains were resistant to sulfamethoxazole/trimethoprim while 73% of the strains were resistant to penicillin and ampicillin (Figure 3.1). The percentage of resistance to sulfamethoxazole/trimethoprim was much higher in this study compared to previous studies which found only 52.4% (Fiedler et al., 2019b) and 70% (Camilo Farfán Esquivel et al., 2014) among strains of *Bacillus cereus* group isolated from vegetables. Despite 73% of the strains being resistant to Ampicillin, it was below the expected percentage as *Bacillus cereus* group is documented to confer greater resistance to these antibiotics (Aina et al., 2022; Fiedler et al., 2019b; Gao et al., 2018). Gao et al. (2018), recorded 99% ampicillin and penicillin resistance by *Bacillus cereus* strains while complete resistance to ampicillin was reported by Amor et al. (2018). High susceptibility of *Bacillus* isolates to tetracycline (93%) and erythromycin (80%) has been reported (Aslim et al., 2002).

However, in this study, 47% of the strains showed resistance to tetracycline and erythromycin. The fact that resistance to these antibiotics by members of the *Bacillus cereus* group was at this level is a major concern. Also, previous studies found that *Bacillus cereus* isolates were highly susceptible to gentamicin (Aina et al., 2022; Fiedler et al., 2019b; Gdoura-Ben Amor et al., 2019). Whong & Kwaga (2007) even recommended gentamicin as one of the most effective antibiotic agents for treating infections originating from *Bacillus cereus* isolates. However, the findings in this study highly oppose these previous studies, as there is a high likelihood of 40% failure in treating isolates used in this study with gentamicin. There is also a risk of these isolates developing complete resistance to gentamicin if they are exposed to this drug for a long period.

The *Bacillus cereus* group is widely considered to be highly susceptible to imipenem (Ikeda et al., 2015), and in fact, few bacteria species including *Pseudomonas aeruginosa* (Ikeda et al., 2015), *Staphylococcus epidermidis*, *Bacillus pumilus*, *Bacillus haynesii* (Çağlayan, 2021), *Xanthomonas maltophilia* (Quinn, 1994), are notoriously reported to confer resistance to imipenem. Even though resistance to imipenem was reported in the above-mentioned studies, the levels of resistance were not up to the level recorded in this study. It is surprising to witness resistance to imipenem by *Bacillus cereus s.l.* members, what is not surprising is that strains SY16.2, SY27.1, and SY6.1 that were resistant to imipenem had complete resistance against other β-lactam class (ampicillin and penicillin) antibiotics tested. Carbapenems, a sub-class of β-lactams, are considered the last line of defense against multidrug-resistant gram-negative bacteria (Hansen, 2021), however, there is high hope as all the strains in this study were highly sensitive to an alternative carbapenem (meropenem), amphenicol (chloramphenicol), and the fluoroquinolones (ciprofloxacin and levofloxacin) classes of antibiotics. Resistance to the imipenem could have been due to the activities of efflux pump systems which facilitate the expulsion of drugs and foreign substances out of the bacteria cell (Meletis, 2016). A more logical explanation for this would be these strains carried genes coding for a beta-lactamase-mediated imipenem resistance which inactivated the imipenem antibiotic. This kind of resistance is facilitated by the increased production of β-lactamases which hydrolyze other β-lactam classes of antibiotics. This explains why an induced imipenem resistance (Figure 3.3) was observed in some of the strains. Imipenem resistance was witnessed in

certain strains when two β-lactams (ampicillin and imipenem) were placed next to each other. The ampicillin-resistant strains around the ampicillin antibiotic may be carrying imipenem-hydrolyzing genes that were triggered after they were exposed to the ampicillin. This allowed the strains to synthesize high levels of beta-lactamase enzymes that were able to inactivate imipenem and consequently led to imipenem resistance.

The MAR index range (0.13 to 0.53) of strains in this study conformed with the findings (0.15 to 0.50) of Kong et al. (2021) for *Bacillus* isolates from meat products but higher than the range of 0.08 to 0.38 documented by György et al. (2021) for *Bacillus* species isolated from spices. According to (Thenmozhi et al., 2014), isolates with MAR indices above 0.2 indicate these isolates originated from potentially high-risk contaminated sources where antibiotics are frequently used. In this study, 66.7% (10) of strains had MAR indexes greater than 0.2 (Figure 4.1), indicating that these strains originated from areas where antibiotics are routinely used.

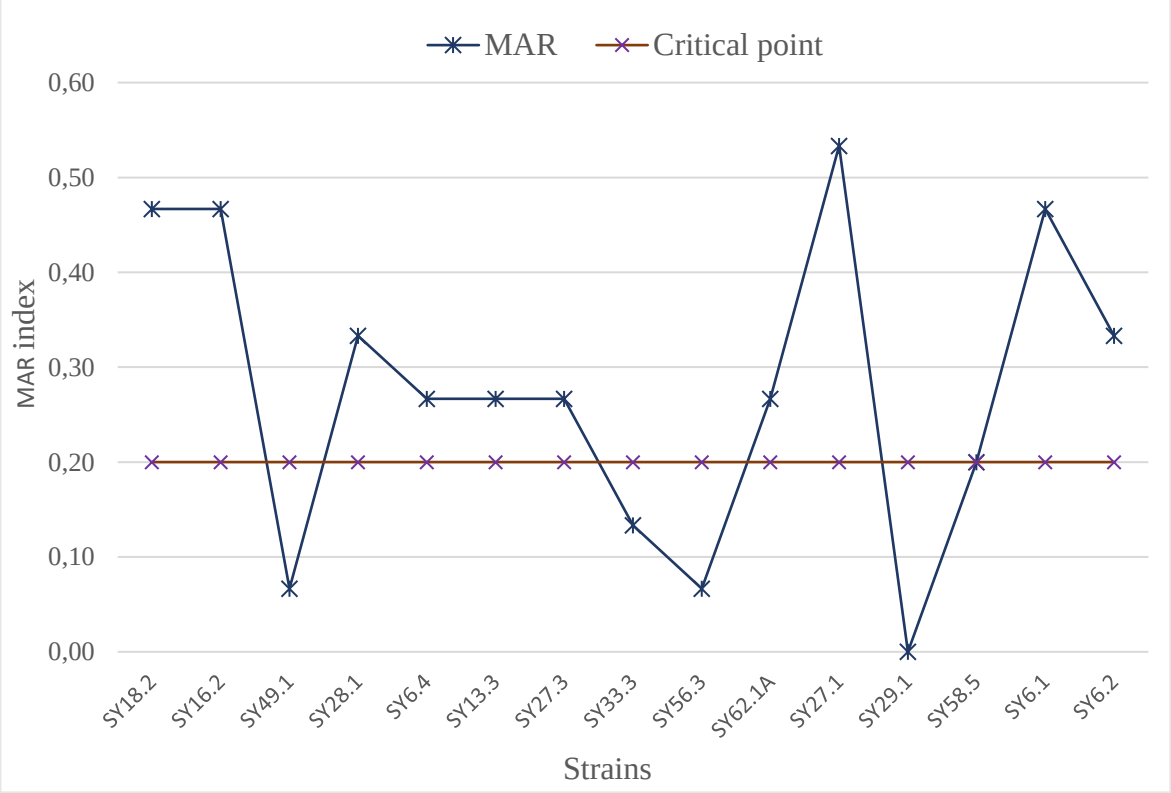


Figure 4.1. MAR indices of *Bacillus* strains used in this study

To determine whether genes conferring resistance to these antibiotics are plasmid-encoded or are facilitated by other factors such as chromosomally encoded resistance, three of the strains were selected for plasmid analysis. Plasmids carry genes coding for specific biological functions including antibiotic resistance and virulence factors that allow bacteria to survive and thrive in the host's hostile environment and resist antibiotic treatment (Ramirez et al., 2014). Plasmids facilitate horizontal dissemination of antibiotic resistance genes, thereby promoting the evolution of resistant strains (San Millan, 2018). Plasmids extracted from strains SY56.3, SY27.1, and SY62.1A were subjected to sequencing using the Illumina NovaSeq 6000 platform with 2 x 150 paired-end (PE) reads. The assembly of complete plasmid DNA sequences revealed that these strains carried megaplasmids (>100,000 bp), medium-sized plasmids (50,000–100,000 bp), and small plasmids (<50,000 bp) with unique features as listed in (Table 3.3, Table 3.4, and

Table 3.5). Functional annotation and analyses of the plasmid sequences with computational and bioinformatics tools uncovered genes harbored by these plasmids coding for antibiotic resistance, toxins, and virulence factors.

The three selected strains for plasmid analysis contained several peptides having antibacterial activities, antibiotic resistance, and additional resistance and tolerance. This study identified four genes coding for antibacterial peptides from the plasmids of SY27.1 and SY62.1A (Table 3.6). One of the proteins is an endopeptidase (M23 family metallopeptidase) that functions to break down bacterial cell wall peptidoglycans. This peptide is similar to the L-Ala--D-Glu endopeptidase of *Bacillus subtilis* (Levdikov et al., 2012). Also, bacteriocin immunity protein, capable of protecting colicin-producing bacteria by binding and inactivating colicin nucleases (Chak et al., 1996), was found in a megaplasmid (pSY27.1_11) carried by SY27.1. There were two *Bacillus* phage holins found in SY62.1A, holins are agents of cell lysis by bacteriophages (Sauder et al., 2014).

In the present study, several genes coding for proteins involved in bacterial resistance to antibiotics, efflux pump systems, and other resistance were identified. All the strains carried a common Tn3 family transposase peptide, which is notorious for its major role in the spread of antibiotic resistance and multi-drug resistant bacteria worldwide (Nicolas et al., 2017). Members of the Tn3 family transposase are notably involved in the increasing prevalence of carbapenem resistance. Other proteins such as the Multiple antibiotic resistance Repressor (MarR) family transcriptional regulator, which is responsible for the regulation of vital bacterial functions including the expression of multi-drug resistant efflux transporters, stress responses, and resistance to organic solvents (Beggs et al., 2020; Gong et al., 2019). Another transcriptional regulator that was found in SY27.1 and SY56.3 was the TetR/AcrR family transcriptional regulators. This protein plays a crucial role in controlling genes associated with a variety of biological processes such as multidrug resistance, efflux pump expression, antibiotic synthesis, and osmotic stress response (Blanco et al., 1985; Ma et al., 1996). One peptide was characterized as belonging to the β-lactamase family such as the metallo-β-lactamase (MBL) fold metallo-hydrolase in SY27.1. The MBL fold metallo-hydrolase belongs to a superfamily of mainly hydrolytic enzymes which perform diverse biological functions. This enzyme is found in the class B metallo beta-lactamases (MBLs) which are capable of hydrolyzing a wide range of β-lactam antibiotics (Garau et al., 2005; Karsisiotis et al.,

2013). According to Bahr et al. (2021), all MBLs, except only 3% of a particular subclass, can thoroughly hydrolyze carbapenems, penicillins, and cephalosporins. This represents a critical challenge for the clinical use of these antibiotics in treating MBL-producing bacteria strains. This indicates that MBL fold metallo-hydrolase with accession number WP_016090640.1 was highly responsible for the beta-lactamase induction which led to imipenem resistance in SY27.1 (Figure 3.3), as this enzyme can inactivate imipenem and other beta-lactam antibiotics. The fasta protein sequence of this peptide is displayed below:

>WP_016090640.1 MULTISPECIES: MBL fold metallo-hydrolase [Bacillus cereus group]

MDKEFFSKHFSLEKVSEGIYVAIAREGSGAVANAGFIDLGDKTIVFDTFNTQQAAEDLK
YIAEKITNRPVTWVVNSHWHGDHIRGNQAFKESNIISSQVTYEKMKDIHPSRINKQKND
IQGLRNYIHSLKDQINQTNDPKLKHQINFLSELEVSLPTLKLVLPQKTFCDAITFRGIERS
AQLFTLGGGHSFCDAVLYIPEEKVIFMGDLLFVDCHPTFFEESNPENWIRILEKVSDMD
VEIAIPGHGSIGTKIRILELIDYITDLTVIAREKDHIEDIKVPNNYKNWGSPEVYQQNFKM
LKGILEC

Several other peptides of biological significance conferring various forms of resistance were also detected as listed in Table 3.6

Another way bacteria can survive antibiotic treatment is a phenomenon called bacterial tolerance. Bacterial tolerance refers to the ability of bacteria to survive temporal exposure to an increased concentration of antibiotics without developing resistance (Brauner et al., 2016). Tolerance can originate from mutations as well as environmental conditions that slow down vital bacterial processes which protect bacteria from the destructive action of antibiotics (Brauner et al., 2016; Windels et al., 2019). The present study revealed peptides with crucial roles in other resistance and bacterial tolerance-associated proteins. These peptides were identified as MerR family transcriptional regulator, which regulates the transcription of multidrug efflux transporters, heavy metal, metal ion transporters, and oxidative stress response genes (Baranova et al., 1999; Brown et al., 2003), DMT(drug/metabolite transporter) family transporter, involved in the transport of drugs and metabolites (Jack et al., 2001), and other peptides (Table 3.6).

The present study identified multiple genes coding for peptides involved in *Bacillus cereus* group toxicity. These peptides consisted of insecticidal toxins, emetic toxins, enterotoxins, and others. Insecticidal toxins were found in SY27.1 and SY62.1A. The Insecticidal delta-endotoxin Cry8Ea1 family protein was found in both strains. This family of Cry protein is reportedly toxic to adult beetles, *Anomala corpulenta* larvae, scarab beetles, and the underground larvae of *Holotrichia parallela*, which are problematic pests that cause severe economic damage in agriculture, horticulture, and forestry (Guo et al., 2009, 2011). Additional pesticidal toxins with unique functions were found in SY27.1. This consisted of pesticidal Cry2Ab protein, which notably has potent insecticidal activity against dipteran and lepidopteran insects (Le Thu et al., 2002), and Vegetative insecticidal protein Vip3A family protein, representing a class of *Bacillus thuringiensis* insecticidal protein with activity against the larvae of lepidopteran insects such as tobacco budworm (*Heliothis virescens*), beet armyworm (*Spodoptera exigua*), fall armyworm (*Spodoptera frugiperda*), black cutworm (*Agrotis ipsilon*), and corn earworm (*Helicoverpa zea*) (Estruch et al., 1996). The accession numbers and protein sequences of these toxins are displayed below:

>WP_000369823.1 MULTISPECIES: insecticidal delta-endotoxin Cry8Ea1 family protein [Bacillus cereus group]

MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGLV
DIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEADPT
NPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
RWGFDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRREL
TLTVLDIVALFSNYDSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHL
MDILNSITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPPVLVSLTGLG
IFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPSTIYRQRGTVDSLDVIPPQD
NSVPPRAGFSHRLSHVTMLSQAAGAVYTLRAPTFSWQHRSAEFNNIIPSSQITQIPLTKS
TNLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTS
IDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYID
RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVECLSDEFC
LDEKQELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDDVFKEN
YVTLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIRYNAKHETVNV
PGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSHHFSLDIDVG
CTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREK

LEWETNIVYKEAKESVDALFVNSQYDQLQADTNIAMIHAADKRVHSIREAYLPELSVIP
GVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNQRSVLV
VPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEIY
PNNTVTCNDYTVNQEEYGGAYTSRNRGYNEAPSVPADYASVYEEKSYTDGRRENPCE
FNRGYRDYTPLPVGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE

>WP_000769223.1 MULTISPECIES: insecticidal delta-endotoxin Cry8Ea1 family protein [Bacillus cereus group]

MKLKNQDKHQSFSSNAKVDKISTDSLKNETDIELQNINHEDCLKMSEYENVEPFVSAST
IQTGIGIAGKILGTLGVPFAGQVASLYSFILGELWPKGKNQWEIFMEHVEEIINQKISTYA
RNKALTDLKGLGDALAVYHDSLESWVGNRNNTRARSVVKSQYIALELMFVQKLPSFA
VSGEEVPLLPIYAQAANLHLLLLRDASIFGKEWGLSSSEISTFYNRQVERAGDYSDHCV
KWYSTGLNNLRGTNAESWVRYNQFRRDMTLMVLDLVALFPSYDTQMYPIKTTAQLT
REVYTDAIGTVHPHPSFTSTTWYNNNAPSFSAIEAAVVRNPHLLDFLEQVTIYSLLSRWS
NTQYMNMWGGHKLEFRTIGGTLNISTQGSTNTSINPVTLPFTSRDVYRTESLAGLNLFL
TQPVNGVPRVDFHWKFVTHPIASDNFYYPGYAGIGTQLQDSENELPPEATGQPNYESYS
HRLSHIGLISASHVKALVYSWTHRSADRTNTIEPNSITQIPLVKAFNLSSGAAVVRGPGF
TGGDILRRTNTGTFGDIRVNINPPFAQRYRVRIRYASTTDLQFHTSINGKAINQGNFSAT
MNRGEDLDYKTFRTVGFTTPFSFLDVQSTFTIGAWNFSSGNEVYIDRIEFVPVEVTYEAE
YDFEKAQEKVTALFTSTNPRGLKTDVKDYHIDQVSNLVESLSDEFYLDEKRELFEIVKY
AKQLHIERNM

>WP_001067062.1 MULTISPECIES: pesticidal protein Cry2Ab [Bacillus cereus group]

MNNVLNSGRTTICDAYNVVAHDPFSFEHKSLDTIQKEWMEWKRTDHSLYVAPVVGTV
SSFLLKKVGSLIGKRILSELWGIIFPSGSTNLMQDILRETEQFLNQRLNTDTLARVNAELI
GLQANIREFNQQVDNFLNPTQNPVPLSITSSVNTMQQLFLNRLPQFQIQGYQLLLLPLFA
QAANMHLSFIRDVILNADEWGISAATLRTYRDYLRNYTRDYSNYCINTYQTAFRGLNT
RLHDMLEFRTYMFLNVFEYVSIWSLFKYQSLMVSSGANLYASGSGPQQTQSFTAQNW
PFLYSLFQVNSNYILSGISGTRLSITFPNIGGLPGSTTTHSLNSARVNYSGGVSSGLIGATN
LNHNFNCSTVLPPLSTPFVRSWLDSGTDREGVATSTNWQTESFQTTLSLRCGAFSARGN
SNYFPDYFIRNISGVPLVIRNEDLTRPLHYNQIRNIESPSGTPGGARAYLVSVHNRKNNIY
AANENGTMIHLAPEDYTGFTISPIHATQVNNQTRTFISEKFGNQGDSLRFEQSNTTARYT
LRGNGNSYNLYLRVSSIGNSTIRVTINGRVYTVSNVNTTTNNDGVNDNGARFSDINIGNI
VASDNTNVTLDINVTLNSGTPFDLMNIMFVPTNLPPLY

>WP_001039073.1 MULTISPECIES: vegetative insecticidal protein Vip3A family protein [Bacillus cereus group]

MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLN
DISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVY
LPKITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKY
VNEKFEELTFATETSSKVKKDGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDV
MVGNNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAKAFLTLTTCRKLL
GLADIDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGH
ALIGFEISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNI
VFPNEYVITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDG
VYMPLGVISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFI
SNIVENGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKT
EYVIQYTVKGKPSIHLKDENTGYIHYEDTNNNLEDYQTINKRFTTGTDLKGVYLILKSQ
NGDEAWGDNFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQL
DSFSTYRVYFSVSGDANVRIRNSREVLFEKRYMSGAKDVSEMFTTKFEKDNFYIELSQG
NNLYGGPIVHFYDVSIK

Interestingly, the three-candidate insecticidal *cry8Ea1* genes detected in the plasmids of *Bt* SY27.1 had diversity in their protein sequences. The identity between SY271_000932 and SY271_000262 is 86%, while that of SY271_000932 and SY271_000263 is 39%. Molecular phylogenetic analysis, using MEGA11 (Tamura et al., 2021), showed diversity in *cry8Ea1* genes produced by different *B. thuringiensis* strains and *Bt* SY27.1 (Figure 4.2)

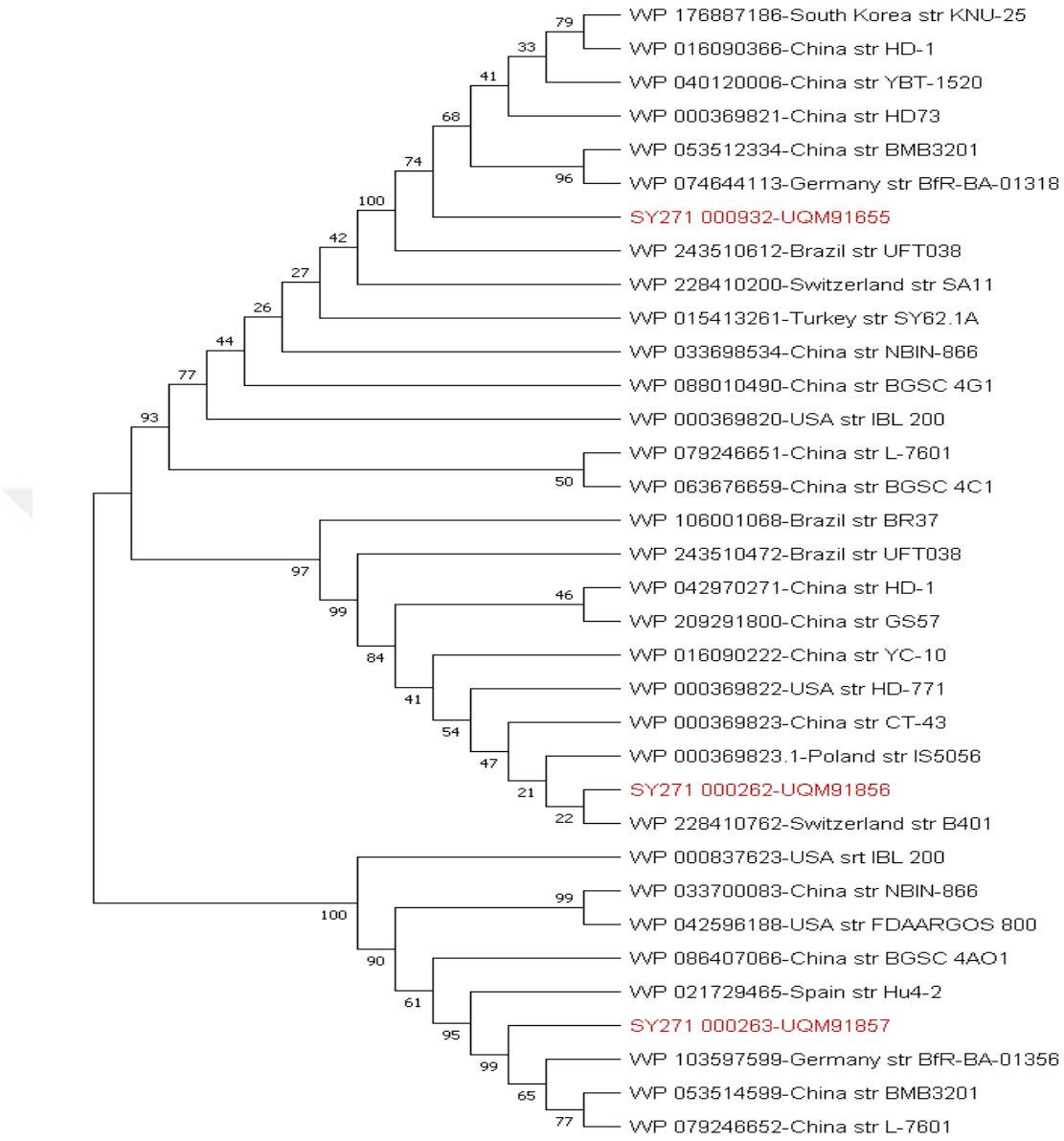


Figure 4.2. Neighbour-Joining Phylogenetic tree showed diversity in cry8Ea1 genes produced by different *B. thuringiensis* strains, including Bt SY27.1. The percentage of replicate trees (1000 replicates) is presented next to the branches, and the evolutionary distance was computed using the Jones-Taylor-Thornton (JTT) matrix-based method.

Besides insecticidal toxins, other toxins with specific functions are produced by the *Bacillus cereus* group. In plasmids carried by SY56.3, a cereulide biosynthesis alpha/beta hydrolase CesH peptide was identified. This is a small, heat and acid-tolerant emetic toxin synthesized during vegetative growth of *Bacillus cereus* group (Ehling-Schulz, Fricker, et al., 2006). This toxin is associated with large scale foodborne outbreaks, with extreme

cases of cereulide intoxication causing multi-organ failure and fatalities being reported (Kranzler et al., 2021). Another important toxin designated as HBL/NHE enterotoxin family protein was also identified in SY27.1. Hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE) constitute a family of tripartite toxins implicated in *Bacillus cereus* food poisoning (Böhm et al., 2016; Worthy et al., 2021). Other toxins included; Type II toxin-antitoxin system death-on-curing family toxin, which encodes a toxic protein and an antidote protein that inhibit the activities of the same toxin through direct interaction (Jurėnas & Van Melderen, 2020); sporulation delaying protein family toxin, which is an antimicrobial peptide involved *Bacillus* cannibalism (González-Pastor et al., 2003; Morales et al., 2013); and zeta toxin family protein, capable of poisoning insect and other Gram-negative bacteria through global inhibition of peptidoglycan synthesis (Chelliah et al., 2019). Protein sequences and accession numbers of toxin peptides with emetic and enterotoxin activities are shown below:

>WP_000291475.1 MULTISPECIES: cereulide biosynthesis alpha/beta hydrolase CesH [Bacillus]

MYYKEFGVEHERMMVYIHGGGVSGWMWDKQVDFFSQNFHCLVLDLPEHGYSRKDE
QIFSIDYAAEQIIKLIEEKREGKSVVAIGFSLGSQVLLSMLSKKKDLIDFAMINSALVKPIP
FANILNKIMTMALPLVKNKTFSKIQAKTLYIGEEYFNLYYQESLQVNKDAFLRVMNEN
SSFKIPKTFREYPHNILVTVGEKEKKIMKDSMKEIMANNPNCQGVIIPKIGHGVPLANPK
LFNKMLEEWLDSNLIIDEVKLASNS

>WP_000790998.1 MULTISPECIES: HBL/NHE enterotoxin family protein [Bacillus cereus group]

MKNKIMTGFLITSIVTGATIPINTLATPIVQAETKQENIDISSALRKIGAHSKLTQTFIDGA
LSSPNVQLEEVPSLNTTQFLIKQDMKEWSSELYPKLILLNSKSKGFVTKFNSYYPTLKGF
VDNKEDKEGFTDRLEVLQDMTITNQESVQRQINELTDLKLQVDKKLKNLDTDVAKTQ
SVLNSEGTGKIDKLKNEMLDTKKSIQNDLQQIALLPGALNEQGLKVFQEIYSLSKDIIEP
AAQTAVVAYNKGKEINNAIVDAENKAEQEAKEKGKSAIEIEAAKKEAREAIEKSKKGEI
AAAAVTKTKEYDLMKVIDPEKIKKTYNTFAEINKLTAEQRAYLNDLEKQNQKLYDLTT
KLTVADLQKSMILFMQNDLHTFANQVDGEIELMKRYKEDLDLINNSITKLSTEVDTNN
TQSQKDTLRRLKSVTTQLEEQVYKF

Several peptides involved in *Bacillus cereus sensu lato*'s virulence were identified in plasmids carried by the three strains. One of these virulence factors characterized as

hemolysin XhlA family protein was found in SY27.1 and SY62.1A. This peptide is similar to *Bacillus subtilis* protein XhlA and it acts to lyse the granulocytes and plasmatocytes immune cells of insects upon ingestion. It is necessary for full virulence towards the larvae of the insect pest *Manduca sexta* (Cowles & Goodrich-Blair, 2005). Also, an endospore germination permease which facilitates the formation of stress-resistant and spore under nutrient-deficient conditions (Southworth et al., 2001), was detected. Other peptides conferring virulence factors were also identified (Table 3.8). The accession numbers and protein sequences of the hemolysin peptides are shown below:

>WP_016090502.1 MULTISPECIES: hemolysin XhlA family protein [Bacillus cereus group]

MDSLQEVKGDVQEIRQDIKEIRLEIRSLEMRTTGNEKDIININKQLDKISANTTWILRLIIG
GLIGAALTFFLKDQL

>WP_016090409.1 MULTISPECIES: hemolysin XhlA family protein [Bacillus cereus group]

MSEQKNDDFKELLVGLTRVETKLDTLGNVKDVAIEAQQSAKSAHLRIDRLDKLVFWIG
TTVVGAIITGGIMALFKFAGK

ATP-binding cassette (ABC) transporters may play a crucial role in the transport of toxicity or antibiotics (Rismondo & Schulz, 2021). One of these peptides is the ABC transporter ATP-binding protein, which belongs to a family of macrolide-specific ABC-type efflux carriers (MacAB) and confers resistance to macrolides (Gaudet & Wiley, 2001). This peptide may have been responsible for the erythromycin resistance by strain SY27.1. Another peptide characterized as ABC transporter ATP-binding protein/permease comprises both ATPase and permease components of an ABC-type multidrug transport system involved in resistance to multiple antibiotics (Decottignies et al., 1998; Kuwano et al., 1999). The ABC-2 transporter permease belongs to the ABC-2 membrane transport family, which functions to catalyze drug and carbohydrate export (Reizer et al., 1992). The rest of the proteins are listed in Table 3.9. Accession numbers and protein sequences of these peptides are displayed below:

>WP_016090619.1 MULTISPECIES: ABC transporter ATP-binding protein [Bacillus cereus group]

MEVVRLENVVKTYGEGETQVQALKGINLSIRRGEFVTIVGASGSGKSTLLHILGGLDRP
SSGNVFIGDENIYNYKDNELSIFRRRKVGFIFQFFNLIPVLNVQENIALPALLDEEQVDDH
YLDEIIRTLGLNERRDHLPSELSGGQQQRVSIGRSLINKPDIILADEPTGNLDTKNTKEVL
NLLKVTAKKYNQTVILITHDPAIASNSDRIITITDGMIISDKQLVQSQEKYGGEEY

>WP_016090599.1 MULTISPECIES: ABC transporter ATP-binding protein/permease [Bacillus cereus group]

MNKKINKQDEIQISYKELLFLYPYLKNTKTLLALGLIGMVIASLIIAPIPYIIGYIIDKVILL
NKSYDQLLKTTLILLLIYCINYLISIGYEYLFTRVQQNVVNEIRLSMISNIIDAPLSYINQK
EKGYILSRIAESGNVSALFSPNLLRVFSGIFDFFFALFIMFNLSVKLTGIIVIIIPIYFIISKHS
SKMISKSTTNVYESSAVLNAEMYETLNGIEDIKLLNGKNIQTNKIKSKLNNVIKSALKQS
LHFIFFMQNLIVTNNFVTVIVLLVSGILILQNQLTIGVYTSFSIYMAKLLATTQALGSLDIT
LKPVCISIKRIKEFFNLDVENTLGTNIMKEPINSIEFKDVCFKYNGNSDLIFDHLNVQIEK
GDKLLIDGINGSGKSTFIKLLTGLYQPESGKILINNIDYNLIHKESVRDRIGIVSQNIFLFK
GTILDNILYGQTNKTKEDVVKLMKRYHLTSHLNRFENGLDTIIVQDGSSVSGGQAQFIA
FLRAILTKRDIIILDEAASNLHIDTRNLMYEILQNYNLCNILIMISHQQDGLYFLNKTLEL
THGNKRIALNGSML

>WP_000906771.1 MULTISPECIES: ABC-2 transporter permease [Bacillus]

MLINLVMKDFLLVKKYFLILLVFAAIAPIYLSSQLQLNDGGLVSFLLTVILMEYILFGTVS
KFEDQYKGAALLCATPYTRSAFVKAKYLFLFVIFICAAIIRIIISVVAPSGIESLSASALGIT
FLTLSILFGVLLPVQFKFGYDKTKIISLLTAFLIPFVAPTLIRGLQSSDIDFKSIFPFSPTVLT
WMPCFISIIIGFISMIISLKVYAKKDL

Several other genes coding for proteins involved in host colonization and immune evasion were also detected. Bacterial pathogens deploy diverse immune evasion strategies in the host to succeed in host invasion and colonization (Williamson, 2022). Bacteria employ these tactics by releasing proteins that inhibit or degrade host immune system and sometimes by mimicking the host's molecules (Bizzell, 2018). Proteins found in these strains that play a direct and indirect role in immune evasion and host colonization are shown in Table 3.9.

4.2. Conclusion and Future Works

In conclusion, *Bacillus cereus sensu lato* strains were highly susceptible to ciprofloxacin, levofloxacin, chloramphenicol, and meropenem, while mostly resistant to

sulfamethoxazole/trimethoprim, penicillin, and ampicillin. Some strains were able to hydrolyze imipenem. Further phenotypic analyses are recommended to examine the imipenem hydrolyzing potential of these strains. Also, with the help of Next Generation Sequencing and bioinformatics tools, this study revealed the contributions of megaplasmids carried by *B*t SY27.1, *Bt* SY62.1A, and *Bt* SY56.3 to the virulence, toxigenicity, and multiple antibiotic resistance these strains. This shows the significance of these technologies and how they can be utilized to provide modern solutions in the health and agriculture sectors. Although the sample size of isolates was small, this study represents preliminary data regarding the antibiotic susceptibility patterns of the *Bacillus cereus* group in Adana, Turkey. Further studies, including whole genome sequencing and proteomics analyses, are recommended to achieve a better understanding of the *Bt* SY27.1, *Bt* SY62.1A, and *Bt* SY56.3 pathogenicity. Furthermore, future studies should try to increase the sample size and isolate strains from diverse areas such as hospitals, food, water, currencies, etc. This will give a better representation of antibiotic resistance patterns of the *Bacillus cereus* group in such areas.

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