Current Research in Parasitology & Vector-Borne Diseases 7 (2025) 100245

Contents lists available at ScienceDirect

Current Research in Parasitology & Vector-Borne Diseases

journal homepage: www.sciencedirect.com/journal/current-research-in-parasitology-and-vector-borne-diseases

Larvicidal activity of *Bacillus thuringiensis* strains against *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes

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ARTICLE INFO

Keywords:
Biocontrol agents
Bacillus thuringiensis
cry gene
Delta endotoxins
Insecticidal activity
Mosquito control

ABSTRACT

Organophosphates, carbamates and synthetic pyrethroids are commonly used in Thailand to control mosquito vectors; however, long-term use of insecticides in vector control has led to the rapid development of insecticide resistance. In this study, we screened *Bacillus thuringiensis* strains as biological control agents for potential toxins against mosquito larvae as an additional control tool. Preliminary bioassays conducted on 434 strains demonstrated that 41 strains (11.64%) and 14 strains (3.97%) achieved 100% mortality against *Ae. aegypti* and *Cx. quinquefasciatus* larvae, respectively. Three strains (JC690, JC691, and JC699) showed toxicity to both mosquito species, compared with the reference Bti strain. Strain JC691 demonstrated the highest efficacy against *Ae. aegypti* and *Cx. quinquefasciatus*, with an LC_{50} value of 6.96×10^4 CFU/ml and 1.16×10^3 CFU/ml, respectively. A comparison of LC_{50} values revealed that JC691 exhibited higher efficacy against *Cx. quinquefasciatus* than that by Bti (Bti LC_{50}: 8.89×10^4 CFU/ml) but lower efficacy against *Ae. aegypti* (Bti LC_{50}: 1.99×10^3 CFU/ml). Scanning electron microscopy revealed that JC690, JC691, and JC699 are rod-shaped, have oval spores, and produce bi-pyramidal crystal proteins. Protein profile analysis using SDS-PAGE demonstrated distinct differences between these Thailand strains (JC690, JC691, and JC699) and the reference Bti strain. All three Thailand strains contained *cry*1I and *cry*2A genes, and only JC691 harbored the *cry*32 gene. Bayesian inference and maximum likelihood phylogenetic analyses of *cry*32 indicated that the partial sequences of *cry*32 in JC691 from Thailand were distinct from those of other *B. thuringiensis* strains from different countries. This study demonstrates the potential of JC690, JC691, and JC699 as biocontrol agents for *Ae. aegypti* and *Cx. quinquefasciatus*.

1. Introduction

Diseases transmitted by mosquitoes are a public health concern responsible for millions of deaths annually (Chilakam et al., 2023). The mosquitoes *Aedes aegypti* (L., 1762) and *Culex quinquefasciatus* Say, 1823 are widely distributed worldwide, particularly in tropical and subtropical regions (Juliano and Philip Lounibos, 2005; Kraemer et al., 2015). Although *Ae. aegypti* is the most important global vector of arboviruses, such as dengue, chikungunya, Zika, and yellow fever (Campos et al., 2020), *Cx. quinquefasciatus* transmits West Nile virus (Richards et al., 2012), St. Louis encephalitis virus (Diaz et al., 2013), Usutu virus (Clé et al., 2019), and the filarial nematode *Wuchereria bancrofti* (Cobbold, 1877) (Ant et al., 2020) in various tropical and temperate regions worldwide.

In Thailand, *Ae. aegypti* and *Cx. quinquefasciatus* are significant vectors for the transmission of mosquito-borne diseases, such as dengue, filariasis, and chikungunya (Jitpakdi et al., 1998; Triteeraprapab et al., 2000; Pumidonming et al., 2005). Pyrethroid-based space sprays have been used to control adult *Aedes* spp. and *Culex* spp. mosquitoes in outbreak areas (Plernsub et al., 2016). However, widespread pyrethroid resistance, driven by kdr and cytochrome P450 monooxygenase mechanisms, has been observed in *Cx. quinquefasciatus* (Yanola et al., 2015) and *Ae. aegypti* (Plernsub et al., 2016) populations. Temephos, an organophosphate insecticide, was traditionally recommended for

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https://doi.org/10.1016/j.crpvbd.2025.100245
Received 23 October 2024; Received in revised form 7 January 2025; Accepted 24 January 2025
Available online 27 January 2025

controlling mosquito larvae (WHO, 2012). Nevertheless, susceptibility assays performed on late third-instar to early fourth-instar *Ae. aegypti* larvae, following WHO guidelines, indicated varying degrees of resistance to this insecticide across Thailand (Saeung et al., 2020). Consequently, the excessive and long-term use of this insecticide has significantly contributed to the rapid emergence of insecticide resistance (Hemingway and Ranson, 2000; Saingamsook et al., 2017; Riveron et al., 2018; Sathantriphop et al., 2020; Nachaiwieng et al., 2021). Therefore, additional measures for mosquito vector control are required.

The use of entomopathogenic bacteria, such as *Bacillus thuringiensis* and *Bacillus sphaericus*, is a viable alternative approach for insect control (Regis et al., 2001). *Bacillus thuringiensis* is a ubiquitous, gram-positive, rod-shaped, sporulating bacterium distributed worldwide in a diverse range of ecosystems, including soil, water, dead insects, dust from silos, leaves from deciduous trees, diverse conifers, and insectivorous mammals (Höfte and Whiteley, 1989; Roh et al., 2007; Raymond et al., 2010). Several types of insecticidal proteins, known as delta endotoxins, are formed as crystals in bacterial cells during sporulation, including vegetative insecticidal protein toxins and secreted insecticidal proteins (Donovan et al., 2006). Delta endotoxins were categorized into two major types: Cry and Cyt. Cry consists of 79 families and 775 genes, whereas Cyt comprises 8 families and 44 genes (Crickmore et al., 2021; www.bpprc.org).

The larvicidal properties of *Bacillus thuringiensis israelensis* (Bti) was initially discovered, and it was found to contain six primary toxins: Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa, and Cyt2Ba (Guerchicoff et al., 1997). Subsequently, various *B. thuringiensis* strains, producing diverse Cry toxins, namely Cry2, Cry17, Cry19, Cry24, Cry25, Cry27, Cry29, Cry30, Cry32, Cry39, Cry40, Cry44Aa, and Cry54A, have been reported as effective against dipteran insects, including mosquitoes (Wu et al., 1994; Schnepf et al., 1998; Ibarra et al., 2003; Tan et al., 2009; van Frankenhuyzen, 2009).

The mosquitocidal activity of *B. thuringiensis* is an additive effect of each toxin and involves complex synergistic interactions among the toxins (Ibarra et al., 2003). Several studies have investigated highly effective native strains of *B. thuringiensis* that target mosquitoes and discovered novel *cry* genes capable of killing mosquito larvae (Monnerat et al., 2005; Patil et al., 2012; Elleuch et al., 2015; Lobo et al., 2018; Wu et al., 2021; da Costa Fernandes et al., 2022).

In this study, we aimed to identify and select Thailand strains of *B. thuringiensis* with high larvicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus*, comparable to Bti strain. To achieve this objective, we characterized the biochemical properties, morphological features, protein profiles, and *cry* gene content of the selected Thailand strains of *B. thuringiensis* to assess their suitability as biocontrol agents.

2. Materials and methods

2.1. Bacterial strains and preparation of spore-crystal suspensions

A total of 436 strains of *B. thuringiensis* were obtained from soil samples collected at various locations in Thailand (Attathom et al., 1995; Thaphan et al., 2008). *Bacillus thuringiensis israelensis* (Bti; Thailand Institute of Scientific and Technological Research [TISTR] 500), used as the reference strain, was obtained from TISTR, Bangkok, Thailand. All *B. thuringiensis* strains were maintained at −20 °C in nutrient broth (NB) (Merck, Darmstadt, Germany) supplemented with 30% glycerol for long-term storage.

An inoculation loop was used to move colonies of each isolate grown on nutrient agar (Merck, Darmstadt, Germany) to 5 ml of the NB medium, which was subsequently incubated at 37 °C for 72 h. Spores and crystals were concentrated and washed several times with ice-cold distilled water. The final bacterial pellet was resuspended in 4 ml 1 mM NaCl solution to create a solution containing a bacterial concentration of 10^9 colony-forming units (CFUs)/ml and then stored at −20 °C until further analysis.

2.2. Larvicidal bioassays

2.2.1. Experimental mosquito rearing

Eggs of *Ae aegypti* NIH strain and *Cx. quinquefasciatus* NIH strain were obtained on filter paper strips from the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. Mosquito larvae were allowed to hatch in dechlorinated tap water and fed with powdered fish food until they reached the fourth-instar stage, which is the appropriate stage for bioassays according to the World Health Organization guidelines (WHO, 2005). The larvae were maintained in controlled conditions (temperature of 25 ± 2 °C, relative humidity of 70–80%, and a 12 h/12 h light/dark cycle) (WHO, 2005) at the Research and Lifelong Learning Center for Urban and Environmental Entomology (RESCUE), Kasetsart University, Bangkok, Thailand. This protocol used the guidelines of the Thai Institutional Animal Care and Use Committee (IACUC) and the National Research Council of Thailand (NRCT) (license No. U1-03015-2559).

2.2.2. Screening and determination of the lethal concentration (LC_{50})

A two-step procedure was used to evaluate the larvicidal activity of the strains. First, screening was conducted to identify the most effective strains against *Ae. aegypti* and *Cx. quinquefasciatus*. Secondly, the toxicities of the selected strains were quantified by determining their LC_{50} values.

For the screening tests, *Ae. aegypti* and *Cx. quinquefasciatus* larvae were exposed to a spore-crystal suspension of *B. thuringiensis* at a concentration of 10^8 CFU/ml (El-Kersh et al., 2016). Five plastic cups containing 20 fourth-instar mosquito larvae in water at final volume of 100 ml were used. A plastic cup containing larvae without exposure to bacteria was used as a negative control. All experiments were conducted in triplicate under laboratory conditions (temperature of 25 ± 2 °C; relative humidity of 70–80%). Mortality rates were recorded at 24, 48, and 72 h after application of the bacterial suspension. Larvae were considered dead when no sign of movement was observed after prodding them with a sterile stick (Dulmage et al., 1990). Subsequently, *B. thuringiensis* strains that achieved 100% mortality in both *Ae. aegypti* and *Cx. quinquefasciatus* were selected to determine their average LC_{50} values.

To determine the LC_{50} values of the selected *B. thuringiensis* strains, serial dilutions of the spore-crystal suspensions at five concentrations (10^3, 10^4, 10^5, 10^6 and 10^7 CFU/ml) were prepared and used for each strain. Five plastic cups containing 20 fourth-instar mosquito larvae at a final volume of 100 ml were used. Cups containing larvae without the addition of *B. thuringiensis* strains (negative control) and with the addition of the Bti strain (positive control) were prepared. All experiments were conducted in triplicate under the aforementioned laboratory conditions. Mortality rates were recorded at 24, 48, and 72 h after application of the bacterial suspension. Larvicidal bioassays were performed using a completely randomized design with three replications.

2.3. Characterization of B. thuringiensis strains with high toxicity against Ae. aegypti and Cx. quinquefasciatus

2.3.1. Biochemical characterization

Bacillus thuringiensis strains were biochemically characterized using the API 50CHB system (BioMérieux, Mérieux Étoile, France), following the manufacturer's instructions.

2.3.2. Morphological characterization by scanning electron microscopy (SEM)

Spore-crystal suspensions of *B. thuringiensis* strains were placed on coverslips and air-dried on aluminum mounts. The samples were subsequently coated with a thin layer of platinum using a sputter coater (Quorum, Q150R ES). Finally, the dry samples were imaged using a Hitachi SU8020 scanning electron microscope (SU8020 FE-SEM, HITACHI, Ltd, Chiyoda City, Tokyo, Japan) with an acceleration

voltage of 5 kV.

2.3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of Cry and Cyt proteins

Bacillus thuringiensis strains were incubated in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl; pH 7.0) at 30 °C with shaking at 250 r/min for 72 h. Biomass containing spores and crystal proteins was harvested by centrifugation at 6000 rpm, 4 °C for 10 min and subjected to analysis of their protein composition using electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels (Laemmli, 1970). The gels were stained for 40 min with a 50% ethanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue R250 solution, followed by de-staining with a 6.75% glacial acetic acid and 9.45% ethanol solution. The molecular mass of the proteins was determined by comparison with a Precision Plus Protein™ Standard marker (BioRad, Hercules, CA, USA).

2.3.4. Detection of the cry gene

Polymerase chain reaction (PCR) was performed to identify the mosquito toxin-coding genes using various oligonucleotide pairs specific for the following genes/gene families: *cry*1, *cry*2, *cry*4, *cry*10, *cry*11, *cry*16, *cry*17, *cry*19, *cry*20, *cry*24, *cry*27, *cry*30, *cry*32, and *cry*39 (Bravo et al., 1998; Crickmore et al., 1998; Ibarra et al., 2003). Genomic DNA was extracted using the method of Harwood and Cutting (1990). Plasmid DNA was extracted using a rapid alkaline extraction procedure described by Birnboim and Doly (1979). Amplification was performed under the following conditions: 5 min of denaturation at 94 °C, followed by 30 cycles of amplification consisting of 1 min of denaturation at 94 °C, 45 s of annealing at a temperature dependent on the primer set used, and 2 min of extension at 72 °C. For the PCR products, 1% agarose electrophoresis was employed and the PCR product of each detected *cry* gene was purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany), and sent to Macrogen (Seoul, South Korea) for DNA sequencing.

2.3.5. Phylogenetic analysis of partial cry32 sequences

A phylogenetic tree of *B. thuringiensis* was constructed using Bayesian inference (BI) and maximum likelihood (ML). The best-fit evolution models of nucleotide substitution based on the Akaike information criterion (Akaike, 1974) for ML and the Bayesian information criterion (Schwarz, 1978) for BI were determined using the Kakusan4 program (Tanabe, 2007). The Treefinder program was used to perform ML analysis with 1000 bootstrap replicates to examine the branch confidence values. Tree topologies with bootstrap values of at least 70% were determined to be sufficiently resolved (Huelsenbeck and Hillis, 1993).

The BI analysis was performed using MrBayes software (version 3.2.6; Ronquist et al., 2012), with a Metropolis-coupled Markov chain Monte Carlo (MC-MCMC) sampling method. The four-chain MC-MCMC analyses were run twice in parallel for 100,000 generations with trees sampled every 1000 generations. The initial 25% of generations were discarded as "burn-in" samples. The remaining trees were used to investigate consensus tree topology and bipartition posterior probability (Huelsenbeck and Ronquist, 2001). A posterior probability value > 0.94 was considered notable support for consensus tree topology (Larget and Simon, 1999). To root the phylogenetic tree, we retrieved two *B. thuringiensis cry*2A sequences from GenBank (*B. thuringiensis cry*2Ad4: AM490199.1 and *B. thuringiensis* T405 *cry*2Aa: OM995804.1) used as outgroups. These sequences belonged to a more distantly related group and provided a more reliable representation of the *cry*32 phylogenetic tree.

2.4. Statistical analysis

LC_{50} values were estimated using Probit analysis (Finney, 1971) in SPSS v.13.0 on larval mortality data for *Ae. aegypti* and *Cx. quinquefasciatus* larvae exposed to various *B. thuringiensis* strain concentrations.

3. Results

3.1. Insecticidal activity

In the preliminary screening of 434 *B. thuringiensis* strains against mosquito larvae revealed that 352 strains (81.10%) exhibited larvicidal activity against both *Ae. aegypti* and *Cx. quinquefasciatus*, 67 strains (15.43%) against *Aedes aegypti* alone, and 13 strains (2.99%) against *Cx. quinquefasciatus* alone, when compared with the reference Bti strain. For *Ae. aegypti* larvae, 41 strains (11.64%) achieved 100% mortality. For *Cx. quinquefasciatus* larvae, only 14 strains (3.97%) achieved 100% mortality. Nine *B. thuringiensis* strains, exhibiting 100% mortality against both mosquito species, were selected for LC_{50} determination (Supplementary file S1: Table S1 and Table S2).

Among these nine *B. thuringiensis* strains, JC691 demonstrated the highest efficacy against *Ae. aegypti*, with an LC_{50} value of 6.96×10^4 CFU/ml. Strains JC690, JC691, and JC699 exhibited high efficacy against *Cx. quinquefasciatus*, with LC_{50} values of 7.30×10^3, 1.16×10^3, and 6.58×10^3 CFU/ml, respectively. The results suggested that strain JC691 was the most effective strain overall. However, a comparison of LC_{50} of JC691 with LC_{50} of Bti for *Ae. aegypti* (1.99×10^3 CFU/ml) and *Cx. quinquefasciatus* (8.89×10^4 CFU/ml) revealed that JC691 demonstrated higher efficacy against *Cx. quinquefasciatus* than Bti but not against *Ae. aegypti* (Table 1).

3.2. Morphological and biochemical characterization

Morphological analysis by scanning electron microscopy (SEM) showed that JC690, JC691, and JC699 strains are rod-shaped cells capable of producing oval spores and bi-pyramidal crystals (Fig. 1). Biochemical test results for the JC690, JC691, and JC699 strains and Bti strain are listed in Table 2. The biochemical profiles of the JC690, JC691, and JC699 strains exhibited slight differences. JC690 is capable

Table 1
Median lethal concentration (LC_{50}) of *Bacillus thuringiensis* strains pathogenic to *Culex quinquefasciatus* and *Aedes aegypti* at 72 h post-application.

Isolate	*Culex quinquefasciatus*			*Aedes aegypti*		
	LC_{50} (CFU/ml) (fiducial limit)	χ^2	*P*	LC_{50} (CFU/ml) (fiducial limit)	χ^2	*P*
JC20	5.90×10^5 (4.40×10^5–8.03×10^5)	14.16	0.36	3.82×10^7 (1.85×10^7–1.07×10^8)	9.15	0.76
JC411	9.60×10^5 (7.36×10^5–1.27×10^6)	13.74	0.39	5.78×10^6 (3.74×10^6–9.79×10^7)	13.21	0.43
JC412	1.62×10^6 (1.23×10^6–$2.17 \times 10^{6)}$	11.79	0.55	1.44×10^7 (9.17×10^6–2.60×10^7)	6.68	0.98
JC690	7.30×10^3 (4.95×10^3–1.64×10^4)	15.53	0.28	5.06×10^6 (3.65×10^6–7.44×10^6)	7.72	0.86
JC691	1.16×10^3 (5.42×10^2–2.11×10^3)	22.01	0.06	6.96×10^4 (4.47×10^4–1.07×10^5)	3.38	0.99
JC693	3.03×10^5 (2.36×10^5–3.91×10^5)	20.21	0.09	1.85×10^6 (1.27×10^6–2.84×10^6)	7.68	0.86
JC699	6.58×10^3 (4.30×10^3–9.59×10^3)	19.25	0.12	1.74×10^6 (1.27×10^6–2.48×10^6)	13.02	0.45
JCPT17	6.03×10^5 (4.64×10^5–7.90×10^5)	10.09	0.69	3.50×10^6 (2.37×10^6–5.79×10^6)	12.42	0.49
JCPT22	2.68×10^5 (2.10×10^5–3.41×10^5)	17.09	0.19	1.24×10^6 (8.94×10^5–1.78×10^6)	10.99	0.61
Bti	8.89×10^4 (5.80×10^3–1.38×10^5)	10.05	0.69	1.99×10^3 (1.04×10^3–3.36×10^5)	15.53	0.28

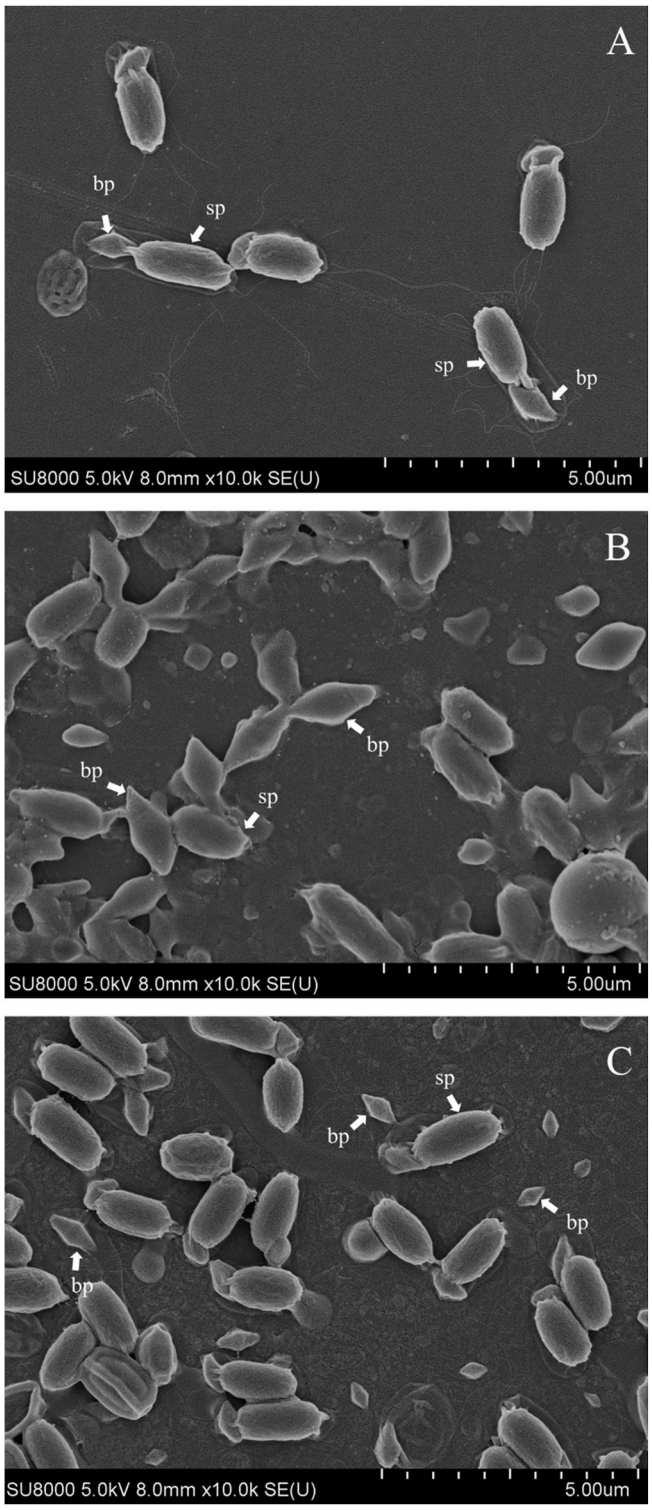

Fig. 1. Scanning electron microscopy of spores and crystal proteins of *Bacillus thuringiensis* strains JC690 (**A**), JC691(**B**), and JC699 (**C**). *Abbreviations*: bp, bipyramidal crystal; sp, spore.

of fermenting D-mannose sugar, JC691 can ferment alpha-methyl-D-mannoside and alpha-methyl-D-glucoside, and JC699 can ferment gluconate sugars. Based on the API kit database and biochemical characteristics, the strains JC690, JC691, and JC699 were identified as *B. thuringiensis* with shared identities of 99.2, 99.0, and 99.1%, respectively.

Table 2
Biochemical profiles of *Bacillus thuringiensis* strains JC690, JC691 and JC699 compared with the reference strain *B. thuringiensis israelensis*.

Test	JC690	JC691	JC699	*israelensis*
Glycerol	+	+	+	+
Erythritol	–	–	–	–
D-Arabinose	–	–	–	–
L-Arabinose	–	–	–	–
Ribose	+	+	+	+
D-xylose	–	–	–	–
L-xylose	–	–	–	–
Adonitol	–	–	–	–
Beta-Methylxyloside	–	–	–	–
Galactose	–	–	–	–
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	–	–	+
L-Sorbose	–	–	–	–
Rhamnose	–	–	–	–
Dulcitol	–	–	–	–
Inositol	–	–	–	–
Mannitol	–	–	–	–
Sorbitol	–	–	–	–
Alpha-Methyl-D-mannoside	–	+	–	–
Alpha-Methyl –D-glucoside	–	+	–	–
N-Acethylglucosamine	+	+	+	+
Amygdalin	+	+	+	–
Arbutin	+	+	+	+
Esculin	+	+	+	+
Salicin	+	+	+	+
Cellobiose	+	+	+	+
Maltose	+	+	+	+
Lactose	–	–	–	–
Melibiose	–	–	–	–
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Inulin	–	–	–	–
Melezitose	–	–	–	–
D-Raffinose	–	–	–	–
Starch	+	+	+	+
Glycogen	+	+	+	+
Xylitol	–	–	–	–
Beta-Gentiobiose	–	–	–	–
D-Turanose	–	–	–	–
D-Lyxose	–	–	–	–
D-Tagatose	–	–	–	–
D-Fucose	–	–	–	–
L-Fucose	–	–	–	–
D-Arabitol	–	–	–	–
L-Arabitol	–	–	–	–
Gluconate	–	–	+	+
2-Ketogluconate	–	–	–	–
5-Ketogluconate	–	–	–	–

Key: +, positive reaction; –, negative reaction.

3.3. The profile of SDS-PAGE proteins in Bacillus thuringiensis strains

Protein profile analyses using SDS-PAGE revealed that the *B. thuringiensis* JC690, JC691, and JC699 isolates differed from those of Bti (Fig. 2). JC690 and JC699 exhibited proteins with molecular masses within the range of 20–80 kDa, whereas JC691 contained proteins with molecular masses within the range of 25–150 kDa. Proteins with molecular weights of approximately 65 and 75 kDa were detected in all strains. A well-defined protein band with a molecular mass of 100–150 kDa was observed exclusively for isolate JC691.

3.4. Determination of cry gene content

PCR analysis of the highly effective strains (JC690, JC691, and JC699), using general and specific primers for *cry* genes, revealed that all three strains tested positive for *cry*1I and *cry*2A in chromosomal DNA, with only JC691 testing positive for *cry*32. Plasmid DNA analysis showed that *cry*1I was present in all three strains, whereas *cry*32 was

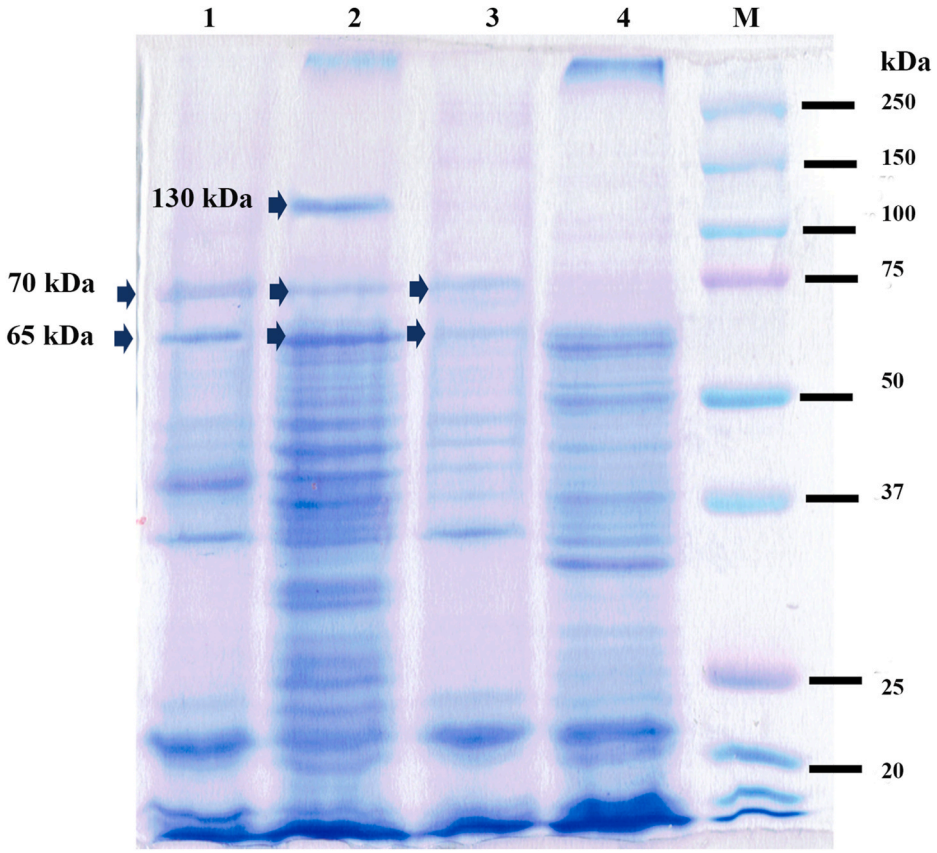

Fig. 2. SDS-PAGE proteins profile in *Bacillus thuringiensis* strains. Lane 1, *B. thuringiensis* strain JC690; Lane 2, *B. thuringiensis* strain JC691; Lane 3, *B. thuringiensis* strain JC699; Lane 4, *B. thuringiensis israelensis*; Lane M, molecular mass markers, where numbers on the right indicate the molecular masses of standard marker proteins. Arrows indicate the expected molecular weights (approximately 65, 70 and 130 kDa).

present only in JC691. All PCR products were cloned, sequenced, and subjected to a Basic Local Alignment Search Tool (BLAST) search using the National Center for Biotechnology Information (NCBI) database.

Sequence comparisons with other *cry* genes in the database showed that the sequences of *cry*1I and *cry*2A from all strains were highly homologous to sequences of *cry*1I (GenBank: U07642.1) and *cry*2A (GenBank: DQ341378.1), with shared identities of 98% and 93%, respectively. The *cry*32 sequences shared 98% identity with that of *cry*32 (GenBank: GU324274.1). Based on our results, portions of the *cry*32 sequence were deposited in GenBank under accession no. OQ316632, according to its previously reported toxicity of *cry*32 to mosquito larvae (van Frankenhuyzen, 2009). Therefore, the genetic structure of *cry*32 was further examined and compared with available data from other sources.

3.5. Phylogenetic analysis of the partial nucleotide sequence of the cry32 gene

The ML and BI analyses based on the partial *cry32* gene of the nuclear genome, showed consistency in tree topologies; thus, only the ML tree is shown (Fig. 3). ML bootstrap values of 100% and a Bayesian posterior probability of 1.0 clearly revealed that *B. thuringiensis* formed a monophyletic clade. According to the phylogenetic tree, four distinct groups were identified. Notably, the sequence of *B. thuringiensis* voucher JC691 (GenBank: OQ316632) was clearly separated from that of voucher JC51 (GenBank: KX685157.1).

4. Discussion

Of the 434 *Bacillus thuringiensis* strains, JC690, JC691, and JC699 obviously exhibited lower lethal concentration (LC_{50}) values of 1.16–7.30 $\times$ 10^3 CFU/ml against *Cx. quinquefasciatus* compared with those of Bti (LC_{50}: 8.89 $\times$ 10^4 CFU/ml), demonstrating their potential for controlling this mosquito species. Notably, JC691 was the most toxic to *Ae. aegypti* (LC_{50}: 6.96 $\times$ 10^4 CFU/ml); however, its efficacy was not as potent as Bti (LC_{50}: 1.99 $\times$ 10^3 CFU/ml). Similarly, Chowanadisai et al. (1995) reported that the strains S-KB1802, S-KB1001, and S-KB2701 were toxic to the larvae of aedine mosquitoes, with LC_{50} values of 1.28 $\times$ 10^2, 3.59 $\times$ 10^2, and 9.80 $\times$ 10^2 spores/ml, respectively.

Rajchanuwong et al. (2019) reported that *B. thuringiensis chanpaisis* (JC51) showed promise as a mosquito larvicide, being effective against *Ae. aegypti* and *Cx. quinquefasciatus* with LC_{50} values of 1.48 $\times$ 10^4 and 1.0 $\times$ 10^4 CFU/ml, respectively. However, in screening the spore-crystal suspension of 434 Thailand strains of *B. thuringiensis*, including the reference strain Bti, against culicine mosquito larvae, we observed lower mortality rates for *Ae. aegypti* and *Cx. quinquefasciatus* by JC51 at 61.67% and 25%, respectively, compared with their reported 100% mortality for both species. This difference might be influenced by the mosquito strains used because we tested *Ae. aegypti* and *Cx. quinquefasciatus* NIH strains, whereas the existing study used the Bora Bora strain (Koou et al., 2014). Additionally, JC51 was first described in 1995, while JC690, JC691, and JC699 are new, not as yet studied strains.

In this study, three Thailand strains JC690, JC691, and JC699 of *B. thuringiensis* demonstrated lower LC_{50} than the strains reported to date. For example, the BU55 strain had an LC_{50} value of 2.20 $\times$ 10^7 CFU/ml against *Cx. quinquefasciatus* (Roy et al., 2021), and the SB1 strain had LC_{50} values of 5.82 $\times$ 10^8 CFU/ml and 6.32 $\times$ 10^8 CFU/ml against *Ae. aegypti* and *Cx. quinquefasciatus* larvae, respectively (Chatterjee et al., 2018).

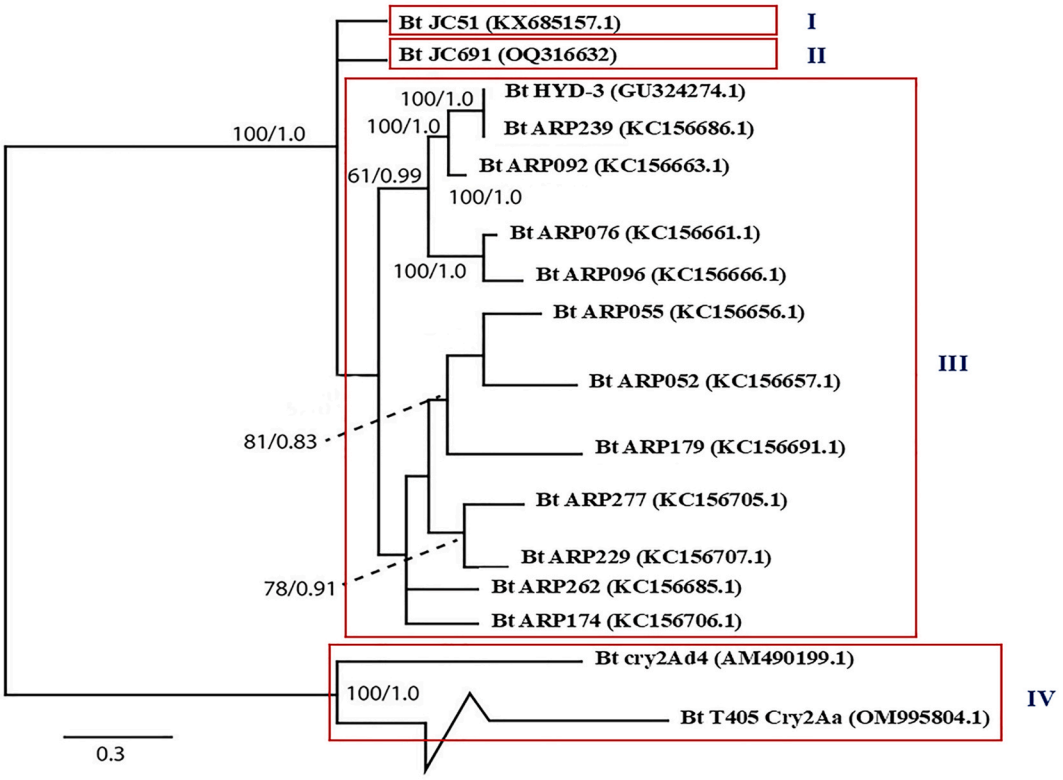

Fig. 3. Phylogenetic tree of *Bacillus thuringiensis* in Thailand based on maximum likelihood analysis of the *cry*32 gene. Outgroups: *B. thuringiensis cry*2Ad4: AM490199.1 and *B. thuringiensis* T405 *cry*2Aa: OM995804.1) Maximum likelihood (ML bootstrap values) and BI nodal support (posterior probability values) are shown as ML/BI.

The spore-crystal mixture is an established technique for evaluating the effectiveness of *B. thuringiensis*, especially when analyzing extensive sample sets. Several preparations of spore-crystal mixtures of *B. thuringiensis* strains exhibiting significant larvicidal properties have been isolated from different regions worldwide (Soares-da-Silva et al., 2017; da Costa Fernandes et al., 2022; Nair et al., 2020; Salamun et al., 2021).

Generally, *B. thuringiensis* strains exhibit insecticidal activity primarily against Lepidoptera (Armengol et al., 2007; Gobatto et al., 2010; Silva et al., 2012) and Coleoptera (Chilcott and Wigley, 1993; Kaelin et al., 1999; Quesada-Moraga et al., 2004) and rarely show mosquitocidal activity. In this study, 4.88% and 3.02% of the tested strains caused mortality of *Ae. aegypti* and *Cx. quinquefasciatus* larvae, respectively. A small proportion of the strain actively targeted mosquito larvae and most remained inactive. These findings are consistent with those of several studies conducted in different geographical regions, which have revealed a low occurrence of active mosquitocidal strains (Bernhard et al., 1997; Park et al., 2008; El-Kersh et al., 2014). The low frequency of isolates with the potential for mosquito control may be related to the smaller number of mosquitocidal toxins (Soares-da-Silva et al., 2017) compared with approximately 95 active toxins catalogued for the control of lepidopterans and coleopterans (van Frankenhuyzen, 2009, 2013).

The isolates JC690, JC691, and JC699 were further characterized in terms of their morphological, biochemical, and molecular features, as well as their protein profiles. Scanning electron microscopy revealed that crystal proteins and spores could be differentiated by their shapes: crystal proteins exhibited a bi-pyramidal shape, and spores were spherical. This result is in line with those of existing studies reporting that most *B. thuringiensis* strains including those from Thailand exhibit a bi-pyramidal crystal morphology (Meadows et al., 1992; Bernhard et al., 1997; Thaphan et al., 2008; Lee et al., 2012). Despite having identical crystal protein shapes, the three strains exhibited variations in the protein crystal size, with JC691 producing larger crystals compared to JC690 and JC699.

The protein profiles of *B. thuringiensis* strains were analyzed using SDS-PAGE. The molecular weights of the proteins in the strains JC690 and JC699 ranged from 20 to 80 kDa, and those of JC691 ranged from 25 to 150 kDa. Most of these proteins were highly expressed, as illustrated by the SDS-PAGE results.

Protein bands with molecular weights of approximately 65 kDa (Cry1I toxin) and 75 kDa (Cry2A toxin) were observed for all strains. Strain JC691 additionally revealed the presence of a band at approximately 130 kDa, corresponding to the Cry32 toxin. A cluster of bands between 25 and 37 kDa was detected, suggesting the presence of the Cyt1 and Cyt2 toxins (Chow et al., 1989; Juárez-Pérez et al., 2002).

PCR data showed that all toxic strains carried the *cry*1I and *cry*2A genes, with bands of approximately 80 and 70 kDa, respectively, corresponding to the size of the protoxin forms of Cry1I and Cry2A toxins (Ruiz de Escudero et al., 2006; Ramalakshmi et al., 2022). The Cry2A protein has been reported to exhibit a wide range of insecticidal toxicities against *Ae. aegypti* and *Cx. quinquefasciatus* (van Frankenhuyzen, 2009; Liang et al., 2011). In the present study, none of the tested strains produced PCR products of expected sizes for *cry*4, *cry*10, *cry*11, *cry*16, *cry*17, *cry*19, *cry*20, *cry*24, *cry*27, *cry*30, or *cry*39 after using the appropriate primers for gene detection. In contrast, *cry*4 and *cry*11 genes were detected in approximately 8% of the Mexican strain collection (Bravo et al., 1998) and *cry*4 genes were the second most abundant in 215 *B. thuringiensis* strains collected from Israel, Kazakhstan, and Uzbekistan (Ben-Dov et al., 1997).

However, several studies have reported the absence of specific *cry* genes in certain *B. thuringiensis* populations. For instance, Sivaji and Girija (2017) found 20 *B. thuringiensis* strains isolated from the Western Ghats of Kerala, India, that did not contain *cry*16, *cry*17, and *cry*19 genes. Similarly, Konecka et al. (2014) did not detect *cry*17, *cry*18, *cry*19, *cry*20, *cry*22, *cry*24, *cry*26, *cry*27, *cry*28, *cry*29, *cry*30, *cry*32,

*cry*39, and *cry*40 genes in 18 *B. thuringiensis* strains obtained from the intestinal tract of *Cydia pomonella* larvae. Furthermore, Ibarra et al. (2003) found only *cry*10, *cry*17, *cry*27, and *cry*30 genes in *B. thuringiensis* strains from Latin America, with no detection of *cry*24, *cry*29, *cry*32, and *cry*40 genes.

The absence of certain *cry* genes in *B. thuringiensis* populations can be attributed to the genetic diversity within this species. *Bacillus thuringiensis* comprises numerous strains, each possessing a unique combination of genes, leading to notable variations in the presence or absence of specific *cry* genes (Tetreau et al., 2021). Furthermore, several *cry* genes are located on plasmids, which are extrachromosomal DNA molecules (Lereclus et al., 1993). These plasmids can be readily acquired or lost during bacterial growth and reproduction, resulting in variations in gene content across different *B. thuringiensis* strains (Sarrafzadeh et al., 2007; Fagundes et al., 2011).

The PCR products of *cry*1I and *cry*2A, which are commonly found in Asian *B. thuringiensis* strains (Chak et al., 1994; Ben-Dov et al., 1997; Kim, 2000; Zhang et al., 2000), were detected in all tested strains in this study. High frequencies of *cry*1 and *cry*2 have been described in *B. thuringiensis* samples throughout Thailand (81.3% and 80.6%, respectively) (Thammasittirong and Attathom, 2008).

The toxicity of JC690 and JC699 to *Ae. aegypti* and *Cx. quinquefasciatus* is attributed to the presence of *cry*1I and *cry*2A genes, which is consistent with findings reported by many researchers (van Frankenhuyzen, 2009; Liang et al., 2011; Ricoldi et al., 2018). Cry1I proteins are toxic to various lepidopteran larvae (Tailor et al., 1992; Gleave et al., 1993; Song et al., 2003; Berretta et al., 2020), coleopteran (Tailor et al., 1992; Martins et al., 2008) and dipteran insects (Crickmore et al., 1998). These toxins target the midgut, binding to the peritrophic membrane and disrupting epithelial cells (Feng et al., 2015). However, the mechanism of Cry1I action on mosquito larvae remains unstudied.

Cry2A toxin is a toxic protein to mosquito larvae, including *Aedes aegypti* (Liang et al., 2011; Ricoldi et al., 2018) and *Culex pipiens* (Zghal et al., 2006). The mechanism of Cry2 action on mosquito larvae involves specific and saturable binding of the toxin to brush border membrane vesicles of midgut epithelial cells. This binding precedes membrane insertion, as reported by Hernández-Rodríguez et al. (2008).

Generally, Cry toxin insertion causes the formation of lytic pores in the microvilli of apical membranes (Aronson and Shai, 2001; Bravo et al., 2005). These characteristics result in cell lysis, damage to the midgut epithelium, and the release of cellular content, creating a favorable environment for spore germination. Severe septicemia ultimately leads to insect death (de Maagd et al., 2001; Bravo et al., 2005).

The mosquitocidal activity of isolate JC691 in this study may be due to the presence of protein bands between 25 and 29 kDa which is comparable to Cyt toxins (25–28 kDa). Cyt toxins have been reported to be toxic in the cell membrane of the larval gut (Soberón et al., 2013; Mendoza-Almanza et al., 2020). Nevertheless, the potential role of 25–29 kDa proteins in mosquito larvae mortality remains unclear and requires further investigation.

The presence of *cry*32 in JC691 and its absence in JC690 and JC699 suggests its potential as the most effective strain against *Ae. aegypti* and *Cx. quinquefasciatus* larvae. The protein profile of JC691 had molecular masses ranging from 20 to 75 kDa and 140 kDa; nevertheless, only 140 kDa was identical with the Cry32 protein. The Cry32Aa protein is expected to have a molecular mass of 139.2 kDa (Balasubramanian et al., 2002) and is considered toxic to dipteran insects, especially mosquito larvae (van Frankenhuyzen, 2009). This finding is consistent with another study reporting that *B. thuringiensis chanpaisis* contained only *cry*32 and exhibited mortality against *Ae. aegypti* and *Cx. quinquefasciatus* (Rajchanuwong et al., 2019). Furthermore, the Cry32 protein can act in synergy with Cry toxins to substantially increase their potential as control agents for mosquito populations (Crickmore et al., 1998; Bravo et al., 2006; Jouzani et al., 2008; Ben-Dov, 2014). However, the mode of action of the Cry32 protein remains to be investigated.

The Bacterial Pesticidal Protein Resource Center database currently lists 29 Cry32 proteins (Crickmore et al., 2021). However, only three genes (Cry32Aa1, Cry32Ha1, and Cry32Hb2) have been shown to be toxic to insects (Balasubramanian et al., 2002; Sampson et al., 2013). Therefore, the discovery of Cry32 proteins with toxicity to mosquito strains in this study prompted their selection for further analysis. This analysis included the construction of a phylogenetic tree using information from the NCBI database.

Based on the phylogenetic tree, there were four distinct groups; the largest group was group III, which included non-Thailand strains (strains from China and the USA). The *cry*32 gene from the Thailand strains JC51 and JC691 formed unique groups, separate from non-Thailand strains and distinct from each other. Accordingly, a full-gene sequence analysis of the *cry*32 gene, a less frequently studied gene for controlling mosquito larvae, is necessary.

Despite its contributions, this study has limitations. The main limitation of this study was that using spore-crystal suspensions (spores/ml) for LC_{50} determination limits direct comparison with studies using purified toxin concentrations (mg/ml). Another limitation was the use of laboratory-reared mosquito strains, which might not accurately reflect insecticide resistance levels in field populations. Consequently, further research should prioritize determining LC_{50} values by using purified *B. thuringiensis* toxins (mg/ml) against field-collected mosquito populations with known insecticide resistance levels.

5. Conclusions

Among the 434 native strains of *B. thuringiensis*, three (JC690, JC691, and JC699) harbored *cry*1I and *cry*2A, and were most effective against *Ae. aegypti* and *Cx. quinquefasciatus* larvae. Notably, *cry*32 was also detected in the highly effective strain (JC691), which may exhibit synergistic interactions with other *cry* genes, thus enhancing its larvicidal activity for mosquitoes. These three strains, demonstrating strong mosquito larvicidal activity, are excellent candidates for developing biocontrol products as an alternative to using chemical insecticides for controlling *Ae. aegypti* and *Cx. quinquefasciatus* populations in Thailand. The present results provide insights that represent an important step towards enhancing the phylogenetic understanding of the *cry* gene and its potential application, and the potential application of specific strains of *B. thuringiensis* as biocontrol agents.

CRediT authorship contribution statement

Prakai Rajchanuwong: Conceptualization, Methodology, Data curation, Investigation, Supervision, Funding acquisition, Visualization, Project administration, Resources, Writing – original draft, Writing – review & editing. **Sawaporn Peaboon:** Methodology, Investigation. **Ratchadawan Ngoen-Klan:** Software, Data curation, Validation, Formal analysis, Writing – review & editing. **Atsalek Rattanawannee:** Data curation, Writing – review & editing. **Atirat Noosidum:** Data curation, Formal analysis, Writing – review & editing. **Boonhiang Promdonkoy:** Data curation, Formal analysis, Writing – review & editing. **Jariya Chanpaisaeng:** Investigation, Formal analysis, Writing – review & editing. **Theeraphap Chareonviriyaphap:** Project administration, Resources, Writing – review & editing.

Ethical approval

The study protocol used the guidelines of the Thai Institutional Animal Care and Use Committee (IACUC) and National Research Council of Thailand (NRCT) (license No. U1-03015-2559).

Funding

This research was supported the Kasetsart University Research and Development Institute, (KURDI), Bangkok, Thailand (Grant # FF (KU) 52.67).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research received support from the Kasetsart University Research and Development Institute (KURDI) (Grant # FF (KU) 52.67). We thank Assistant Professor Akekawat Vitheepradit for providing the SPSS program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2025.100245.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary file.

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