Journal of Biological Control, 32(4): 246-251,2018, DOI: 10.18311/jbc/2018/21604

Research Article

Molecular characterization of an indigenous lepidopteran toxic *Bacillus thuringiensis* strain T532

K. V. NAYAN GANESH, A. L. REYAZ and N. BALAKRISHNAN*
Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore – 635110, Tamil Nadu, India
**Corresponding author E-mail*: bala8775@gmail.com

ABSTRACT: *Bacillus thuringiensis* Berliner (Bt) being an eco-friendly bioinsecticide is effectively used in pest management strategies and, therefore, isolation and identification of new strains effective against a broad range of target pests is important. In the present study, four indigenous *Bt* isolates (T489, T491, T495 and T532) were taken and investigated for their properties such as crystal morphology, *cry* gene(s) content and insecticidal activity. Bipyramidal, cuboidal and spherical crystals were observed. Among the four isolates only one isolate was found positive for lepidopteran specific *cry* genes. The isolate T532 which showed the presence of *cry1, cry2Aa* and *cry2Ab* genes was selected for protein profiling and bioassay. The isolate showed the presence of two proteins of molecular weights at ~135 and ~65 kDa sizes. Artificial diet based bioassay resulted in 100% mortality of the neonate larvae of *H. armigera* and *S. litura* for the isolate T532 which was comparable to that of the positive control (HD1). The full length *cry2A* gene was amplified and cloned into *E. coli* DH5α strain. The recombinant plasmids were sequenced and the sequence was deposited in GenBank nucleotide database. The accession number of the gene is MH475905. The gene was named by the *Bacillus thuringiensis* delta endotoxin nomenclature committee as *cry2Aa23*.

KEY WORDS: *Bacillus thuringiensis,* Cloning, *cry*2Aa, PCR

(Article chronicle: Received: 16-05-2018; Revised: 10-11-2018; Accepted: 15-12-2018)

INTRODUCTION

Bacillus thuringiensis Berliner (Bt) is a Gram positive, sporulating bacterium, which is a common inhabitant of soil and aquatic ecosystems (Bernhard *et al.,* 1997; Konecka *et al.,* 2012). The importance of Bt in the field of agriculture is in the form of a potential bioinsecticide which holds toxicity against a broad range of insects belonging to the orders Coleoptera, Lepidoptera, Diptera, Mallophaga, Hymenoptera and Homoptera (Schnepf *et al.,* 1998; Palma *et al*., 2014). This property of Bt is conferred by the molecules which they produce such as crystal (Cry) proteins and vegetative insecticidal proteins (Vip) which are formed during sporulation and vegetative phases respectively (Ali *et al*., 2010). The crystalline protein which is in a protoxin form is solubilized inside the midgut of the insect by the gut protease enzymes and the toxin is released (Milne and Kaplan, 1993). This toxin binds to the brush border membrane of the midgut epithelial tissue and creates pores which ultimately lead to the lysis of epithelial cells followed by the death of the larvae (Ali *et al*., 2010).

Various chemical pesticides such as organic phosphates and synthetic pyrethroids are applied in the field at the rate of 8×10^{24} and 3×10^{22} molecules/hectare respectively whereas the need of commercial Bt insecticides is only 10^{20} molecules/hectare (Feitelson *et al*., 1992). This shows the increased potential of Bt bioinsecticide over the commonly used chemical pesticides. As of 10th July 2018, a total of 319 Bt holotype toxins (Crickmore *et al.*, 2016; http://www.btnomenclature.info/) have been reported since 1981, in which the first ever report of cloning a *cry* gene was made by Schnepf and Whiteley. The characterization of Bt isolates and identification of potential toxins lead to identification of novel potential gene(s) and which in turn will be helpful for the development of transgenic crop plants with insect resistance. The reports of insects developing resistance against existing Bt toxins improved the scope of research on novel isolates with broad spectrum of insecticidal activity. Cloning and expression of the toxins in other microorganisms helped in identifying the individual effects of the toxins towards the target insects (Jain *et al.,* 2006; Yilmaz *et al.,* 2017).

The present study was conducted in order to characterize four Bt isolates taken from the collection being maintained in Bt lab, Dept. of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology (CPMB&B), Tamil Nadu Agricultural University (TNAU), Coimbatore.

MATERIALS AND METHODS

Bacterial strains and plasmids

Four indigenous isolates of *Bacilllus thuringiensis* were chosen from the collection maintained in Bt lab, Department of Plant Biotechnology, CPMB&B, TNAU, Coimbatore. The isolates taken for characterization were T489, T491, T495 and T532. The isolates were revived from glycerol stocks and cultured in T_3 medium. The Bt isolates HD1 and 4G1, obtained from Bacillus Genetic Stock Center (Ohio State University, USA) were used as positive controls and the strain 4Q7 was used as negative control for the experiments. T/A cloning vector pTZ57R/T were used for cloning of the insert into *Escherechia coli* DH5α strain.

PCR analysis for lepidopteran toxic genes

According to the protocol given by Kalman *et al.,* (1993), total genomic DNA was isolated from the isolates and used for performing PCR screening. Universal primers (Ben-Dov *et al.,* 1997 and 1999) for various lepidopteran toxic genes such as *cry1, cry2Aa, cry2Ab* and *cry9* (Table 1) were used for identifying the presence of respective genes in the isolates. For 25µl of polymerase chain reaction, 0.1 µg of total genomic DNA and 50ng of each primer were mixed with 2.5µl of 10X buffer, 2.5µl of 2.5mM dNTPs, 0.5µl DNA polymerase of 3U/µl (GeNeiTM, Bengaluru, India). Bt reference strain HD1 was used as positive control for *cry1, cry2Aa* and *cry2Ab* genes and 4G1 was used as the positive control for *cry9*.

Harvesting spore-crystal mixture from the isolates

The bacterial mother culture was initiated in 5 ml of T_3 broth and incubated in a rotary shaker (Scigenics Orbitek, India) set at 180rpm at 30°C. From the overnight grown culture, 50 µl was inoculated in 25 ml T_3 broth and incubated at 30°C with shaking. After a period of 48 hours, the sporulation of the bacterial culture was monitored through phase contrast microscope. When it appeared that more than 90 per cent of the cells got lysed, the culture was transferred to 4°C for a minimum of half an hour. The culture was then centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet obtained was washed with 5 ml of ice-cold Tris-EDTA buffer [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM phenyl methyl sulphonyl fluoride (PMSF)]. After centrifugation, the pellet was washed with 5 ml of ice-cold 0.5 M sodium chloride which was followed by two more washes with 5 ml of Tris–EDTA buffer with 0.5 mM PMSF by centrifuging at the same speed and time. Finally, the spore-crystal pellet was suspended in 100 µl of sterile distilled water containing 1 mM PMSF and stored at -20°C.

Preliminary toxicity of the isolate T532 against *Helicoverpa armigera and Spodoptera litura*

Laboratory reared neonate larvae of *H. armigera* and *S. litura* were used for surface contaminated artificial diet based insect bioassay for the isolates. Artificial diet (Nagarkatti and prakash, 1974) was poured into cryovials and the spore-crystal mixture of the isolate was coated on the surface evenly. For each isolate, three replications were maintained and for each replication, 10 larvae were used in individual

Table 1: Oligonucleotide primers used for PCR screening of the *Bacillus thuringiensis* isolates

S. No.	Primer Sequences 5' to 3'	*cry genes*	Amplicon size (bp)	Reference
1.	FP: CATGATTCATGCGGCAGATAAAC RP: TTGTGACACTTCTGCTTCCCATT	*cry1*	277	Ben-dov *et al*. (1997)
2.	FP: GTTATTCTTAATGCAGATGAATGGG RP: GAGATTAGTCGCCCCTATGAG	*cry2Aa*	498	
3.	FP: GTTATTCTTAATGCAGATGAATGGG RP:TGGCGTTAACAATGGGGGGAGAAAT	*cry2Ab*	546	
4.	FP: CGGTGTTACTATTAGCGAGGGCGG RP: GTTTGAGCCGCTTCACAGCAATCC	*cry9*	354	Ben-dov *et al*. (1999)

vials. Artificial diet surface coated with spore crystal mixtures of HD1 and 4Q7 was used as positive and negative controls respectively. Besides, empty diet was also used a negative control. Observations were taken in every 24 hour intervals for one week.

Protein profiling of the potential isolate

The isolate showing potential toxicity against the neonate larvae of *H. armigera* and *S. litura* were selected and performed SDS-PAGE analysis to identify the protein profile of them. The experiment was carried out following the protocol provided by Laemmli (1970). Stacking gel of 4 per cent and separating gel of 9 per cent were used for SDS-PAGE. HD1 was used as positive control and 4Q7 as negative control. The molecular weight of proteins was determined using higher range protein molecular weight marker (myosin rabbit muscle 205 kDa, phosphorylase b 97.4 kDa, bovine serum albumin 65 kDa, ovalbumin 43 kDa and carbonic anhydrase 29 kDa) obtained from GeNeiTM, Bangalore, India.

Cloning of *cry2A* gene

The full length *cry2A* gene was amplified from Bt isolate T532 using the forward primer 2AFS (ATGGTACC ATGAATAATGTATTGAATAGTGGAA) and reverse primer 2ARS (GTTCTAGACTCAAACCTTAATAAAGTGGTG). The PCR reaction was carried out in a total volume of 25 µl in a Thermal cycler (Eppendorf Mastercycler personal, Germany). The PCR product was purified using a PCR clean up kit (Thermo Scientific, USA) and was ligated into pTZ57R/T vector and cloned into *E. coli* DH5α strain and the plasmid was isolated and sequenced at Eurofins Genomics India Pvt. Ltd. The sequence was deposited in GenBank and accession number was obtained. The sequence was submitted to *B. thuringiensis* delta endotoxin nomenclature committee.

RESULTS AND DISCUSSION

Bacillus thuringiensis is a ubiquitous bacterium found in various habitats such as soil, phylloplane, dead insects, dust, spider web etc. (Mizuki *et al.,* 1999; Asokan and Puttaswamy, 2007; Ramalakshmi and Udayasuriyan, 2010; Unalmis *et al.,* 2015 and Reyaz *et al.,* 2017). In the present study, the isolates preserved in glycerol stocks were revived and cultured in T_3 medium. The cultures were monitored for the presence of more than 90 per cent lysis and the crystal morphology was studied at this stage using phase contrast microscope. Among the isolates studied, the isolate T532 showed the presence of three types of crystal proteins such as bipyramidal, cuboidal and spherical (Fig. 1). Only bipyramidal crystals were observed in T491 and T495 whereas cuboidal crystals were observed in T489. Jain *et al*. (2017) and Reyaz *et al.* (2017) reported the presence of various kinds of crystals such as spherical, bipyramidal, cuboidal and irregular shapes. Earlier the predominance of bipyramidal crystals was also reported by Bernhard *et al*. (1997), Martin and Travers (1989) and Ramalakshmi and Udayasuriyan (2010).

Crickmore *et al.* (1998) reported that lepidopteran specific toxins are produced by *cry* genes. The isolates were subjected to PCR screening for the presence of lepidopteran specific *cry* gene(s) using primers as described by Ben-Dov *et al.,* 1997 and 1999. While screening by PCR with gene specific primers *viz., cry1, cry2Aa* and *cry2Ab*, the Bt isolate T532 only showed the amplicon sizes of ~277 bp, ~500 bp and ~550 bp respectively (Fig. 2). No amplification was obtained for *cry9* gene. Patel and Ingle (2012) performed PCR screening for the selected isolates and found the presence of *cry1* and *cry2* genes in two and one isolates respectively. The abundance of *cry* genes in the Bt strains from Bangladesh was screened by Shishir *et al.* (2014) and reported *cry1* gene as the most abundant with (30.8%) followed by *cry2* (25.5%), *cry3* (22.2%) and *cry9* (7.2%) genes.

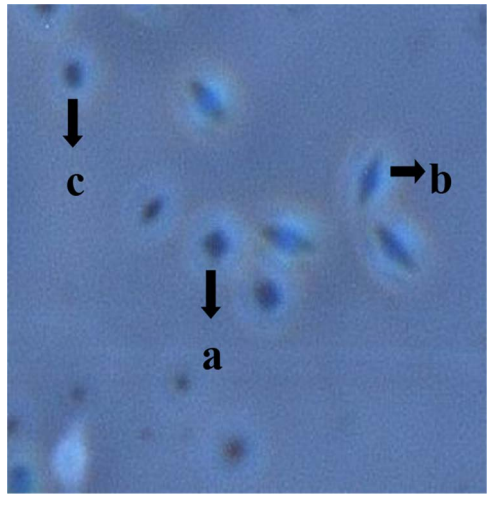

Fig. 1. Different types of crystals present in the *Bt* isolate T532 a. Cuboidal, b. Bipyramidal and c. Spherical.

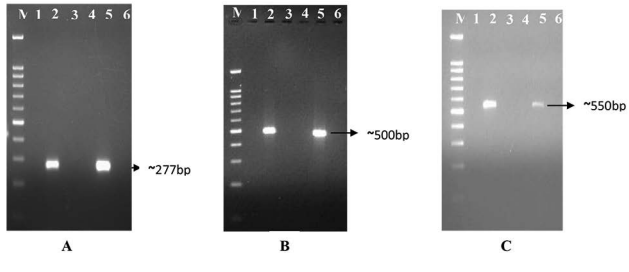

Fig. 2. A) PCR screening of *cry*1 gene, B) PCR screening of *cry*2Aa gene and C) PCR screening of *cry*2Ab gene. Lane M: 100bp Ladder, Lane 1: Negative control, Lane 2: Positive control (HD1), Lane 3: T489, Lane 4: T491, Lane 5: T532 and Lane 6: T495.

Preliminary bioassay with spore-crystal mixture of T532 Bt isolate showed 100 per cent mortality of the neonate larvae of both *Helicoverpa armigera and Spodoptera litura* using surface contaminated artificial diet based bioassay. This was comparable to the toxicity with the positive control Bt HD1 (Fig. 3). No mortality was observed in negative controls (i.e.,) diet coated with 4Q7 spore crystal mixture and empty diet. Reyaz *et al.* (2017) also followed surface contamination method for bioassay against *S. litura* larvae and found one among the 68 isolates with 100 per cent mortality. Unalmis *et al.* (2015) reported a reduced toxicity of the local Bt isolates against two stored grain pests, *Ephestia kuehniella and Plodia interpunctella* when compared to the reference strain.

SDS-PAGE analysis of spore-crystal mixture of T532 Bt isolate showed the presence of two distinct proteins of ~135 and ~65 kDa similar to that of the reference strain HD1 (Fig. 4). The reference strain 4Q7 was used as negative control which showed no proteins. The presence of a protein of ~130 to 135 kDa molecular weight indicates the presence of either *cry1* or *cry9* gene(s) and a protein of molecular weight ~60 to 70 are considered to be the result of *cry2* gene (Jain *et al.,* 2006; Zheng *et al.,* 2010 and Reyaz and Arulselvi, 2016). Ramalakshmi and Udayasuriyan (2010) reported earlier that out of the 70 Bt isolates analyzed, 17 isolates (24.2%) representing *cry2A* gene exhibited two major polypeptide bands with molecular weights in the range of ~135 and ~65 kDa.

The full ORF of the gene *cry2A* was amplified using gene specific primers and an amplified product of ~1.9 kb was obtained (Fig. 5). The purified PCR product was ligated into a T/A cloning vector pTZ57R/T and was transformed into *E. coli* DH5α cells. The recombinant cells were identified by blue-white screening and confirmed by colony PCR. The recombinant plasmid was isolated from transformed colony and sequenced at Eurofins Genomics India Pvt. Ltd. using an automated DNA sequencer (ABI 3730xl Genetic). The sequence was aligned with an existing *cry2Aa* gene sequence (M31738.1) which showed a similarity of 100 per cent among the sequences. The sequence was deposited in GenBank nucleotide database and the accession number is MH475905. The gene sequence and its deduced amino acid sequence were submitted to *Bacillus thuringiensis* delta endotoxin nomenclature committee and it was named as *cry2Aa23.* Manikandan *et al.* (2015) reported the cloning of *cry2A* gene from an indigenous *Bt* isolate using T/A cloning vector and the sequencing results revealed 99 per cent homology of the sequence with that of *cry2Aa1.* Likewise cloning of *cry1Ab* (Darsi *et al.,* 2010), *cry2Ab* (Jain *et al.*, 2006 and Pan *et al*., 2014), *cry2Ac* (Sasaki *et al.,* 1997) and *cry9* (Ben-Dov *et al*., 1999; Wasano *et al.*, 2005) genes were cloned and characterized.

Based on the PCR screening, preliminary insect bioassay

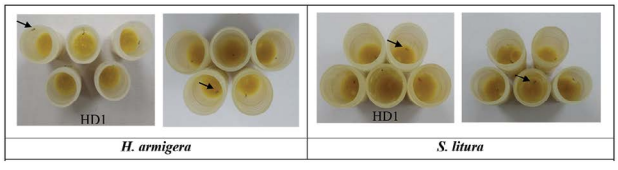

Fig. 3. Artificial diet based bioassay of the Bt isolate T532.

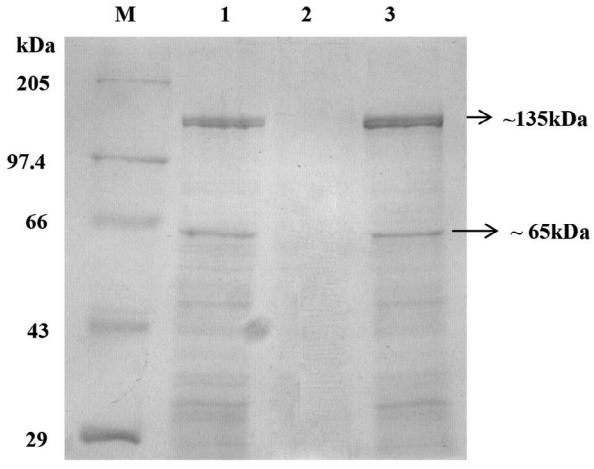

Fig. 4. SDS-PAGE protein profiling of spore-crystal mixture. Lane M: High MW Protein marker, Lane 1: HD1, Lane 2: 4Q7 and Lane 3: T532.

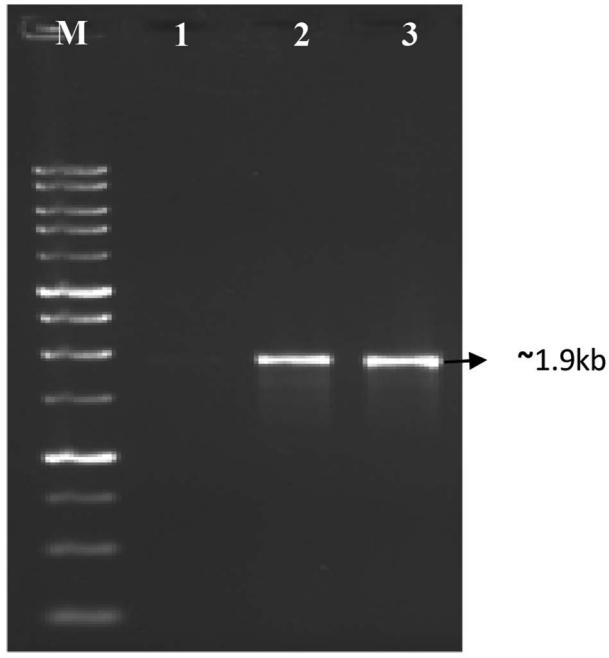

Fig. 5. Amplification of full length cry2A gene. Lane M: 1kb Ladder, Lane 1: Negative control, Lane 2: Positive control (HD1) and Lane 3: T532.

and protein profile, it is evident that T532 is a potential indigenous Bt isolate against lepidopteran insect pests (*H. armigera* and *S. litura*). Further work need to be done to evaluate its full potential.

ACKNOWLEDGEMENT

The authors are thankful to Tamil Nadu Agricultural University for providing all the facilities to carry out the study.

REFERENCES

Ali S, Zafar Y, Ali GM, Nazir F. 2010. *Bacillus thuringiensis* and its application in agriculture. *Afr J Biotechnol.* **9**(14): 2022-2031.

Asokan R, Puttaswamy. 2007. Isolation and characterization of *Bacillus thuringiensis* Berliner from soil, leaf, seed dust and insect cadaver. *J Biol Control*. **21**(1): 83-90.

Ben-Dov E, Wang Q, Zaritsky A, Manasherob R, Barak Z, Schneider B, Khamaraev A, Baizhanov M, Glupov V, Margalith Y. 1999. Multiplex PCR screening to detect cry9 genes in *Bacillus thuringiensis* strains. *Appl Environ Microbiol*. **65**(8): 3714-3716. PMid:10427071 PMCid:PMC91556

Ben-Dov E, Zaritsky A, Dahan E, Barak ZE, Sinai R, Manasherob R, Khamraev A, Troitskaya E, Dubitsky A, Berezina N. (1997). Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis*. *Appl Environ Microbiol*. **63**(12): 4883-4890. PMid:9406409 PMCid:PMC168816

Bernhard K, Jarrett P, Meadows M, Butt J, Ellis DJ, Roberts GM, Pauli S, Rodgers P, Burges HD. 1997. Natural isolates of *Bacillus thuringiensis* worldwide distribution, characterization and activity against insect pests. *J Invertebr Pathol.* **70:** 59-68 https://doi.org/10.1006/jipa.1997.4669

Crickmore N, Baum J, Bravo A, Lereclus D, Narva K, Sampson K, Schnepf E, Sun M, Zeigler DR. 2016. *Bacillus thuringiensis* toxin nomenclature. Available from: http://www.btnomenclature.info

Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH 1998. Revision of nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev*. **62**: 807-813. PMid:9729610 PMCid:PMC98935

Darsi S, Prakash GD, Udayasuriyan V. 2010. Cloning and characterization of truncated *cry*1Ab gene from a new indigenous isolate of *Bacillus thuringiensis*. *Biotechnol Lett.* **32**(9): 1311-1315. https://doi.org/10.1007/s10529-010-0301-1 PMid:20480206

Feitelson JS, Payne J, Kim L. 1992. *Bacillus thuringiensis*: insects and beyond. *Nature Biotechnol*. **10**: 271-275. https://doi.org/10.1038/nbt0392-271

Jain D, Sunda SD, Sanadhya S, Nath DJ, Khandelwal SK. 2017. Molecular characterization and PCR-based screening of *cry* genes from *Bacillus thuringiensis* strains. *3 Biotech* **7**(1): 4. https://doi.org/10.1007/s13205-016-0583-7 PMid:28391468 PMCid:PMC5385171

Jain D, Udayasuriyan V, Arulselvi PI, Dev SS, Sangeetha P. 2006. Cloning, characterization, and expression of a new *cry*2Ab gene from *Bacillus thuringiensis* strain 14-1. *Appl Biochem Biotechnol*. **128**(3): 185-194. https://doi.org/10.1385/ABAB:128:3:185

Kalman S, Kiehne KL, Libs JL, Yamamoto T. 1993. Cloning of a novel *cry*IC-type gene from a strain of *Bacillus thuringiensis* subsp. galleriae. *Appl Environ Microbiol*. **59**(4): 1131-1137. PMid:8476286 PMCid:PMC202250

Konecka E, Baranek J, Hrycak A, Kaznowski A. 2012. Insecticidal activity of *Bacillus thuringiensis* strains isolated from soil and water. *Sci World J.* Article ID: 710501. doi:10.1100/2012/710501. https://doi.org/10.1100/2012/710501

Kumar S, Singh A. 2015. Biopesticides: Present status and the future prospects. *J Fertil Pestic.* **6**(2): 1-2. https://doi.org/10.4172/2471-2728.1000e129

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259): 680-685. https://doi.org/10.1038/227680a0 PMid:5432063

Manikandan R, Ramalakshmi A, Balasubramani V, Udayasuriyan V. 2015. Characterization and cloning of the *cry*2A gene from indigenous isolates of *Bacillus thuringiensis. Mol. Bio*. **49**(4): 520-526. https://doi.org/10.1134/S0026893315040111

Martin PA, Travers RS. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl Environ Microbiol*. **55**(10): 2437-2442. PMid:16348022 PMCid:PMC203101

Milne R, Kaplan H. 1993. Purification and characterization of a trypsin-like digestive enzyme from spruce budworm *(Choristoneura fumiferana)* responsible for the activation of δ- endotoxin from *Bacillus thuringiensis. Insect Biochem Molec Biol*. **23**: 663-673. https://doi.org/10.1016/0965-1748(93)90040-Y

Mizuki E, Ichimatsu T, Hwang SH, Park Y, Saitoh H, Higuchi K, Ohba M. 1999. Ubiquity of *Bacillus thuringiensis* on phylloplanes of arboreous and herbaceous plants in Japan. *J Appl Microbiol*. **86**(6): 979-984. https://doi.org/10.1046/j.1365-2672.1999.00778.x

Nagarkatti S, Prakash S. 1974. Rearing of *Heliothis armigera* (Hub.) on artificial diet. *Tech Bull Commonwealth Inst Biol Control* **17**: 169-173.

Palma L, Mu-oz D, Berry C, Murillo J, Caballero P. 2014. *Bacillus thuringiensis* toxins: an overview of their biocidal activity. *Toxins* **6**(12): 3296-3325. https://doi.org/10.3390/toxins6123296 PMid:25514092 PMCid:PMC4280536

Pan Z, Xu L, Zhu Y, Shi H, Chen Z, Chen M, Chen Q, Liu B. 2014. Characterization of a new *cry*2Ab gene of Bacillus thuringiensis with high insecticidal activity against Plutella xylostella L. *World J Microbiol Biotechnol*. **30**(10): 2655-2662. https://doi.org/10.1007/s11274-014-1689-x PMid:24943249

Patel KD, Ingle SS. 2012. Molecular characterization of novel serovars of *Bacillus thuringiensis* isolates from India. *Indian J Microbiol*. **52**(3): 332-336. https://doi.org/10.1007/s12088-011-0240-0 PMid:23997321 PMCid:PMC3460129

Ramalakshmi A, Udayasuriyan V. 2010. Diversity of *Bacillus thuringiensis* isolated from Western Ghats of Tamil Nadu state, India. *Curr Microbiol*. **61**(1): 13-18. https://doi.org/10.1007/s00284-009-9569-6 PMid:20033169

Reyaz AL, Arulselvi PI. 2016. Cloning, characteriztion and expression of a novel haplotype cry2A-type gene from *Bacillus thuringiensis* strain SWK1, native to Himalayan valley Kashmir. *J Invertebr Pathol*. **136**: 1-6. https://doi.org/10.1016/j.jip.2016.02.005 PMid:26906447

Reyaz AL, Gunapriya L, Arulselvi PI. 2017. Molecular characterization of indigenous *Bacillus thuringiensis* strains isolated from Kashmir valley. *3 Biotech* **7**(2): 143. https://doi.org/10.1007/s13205-017-0756-z PMid:28597156 PMCid:PMC5465046

Sasaki J, Asano S, Hashimoto N, Lay BW, Hastowo S, Bando H, Iizuka T. 1997. Characterization of a cry2A gene cloned from an isolate of *Bacillus thuringiensis* serovar *sotto*. *Curr Microbiol*. **35(**1): 1-8. https://doi.org/10.1007/s002849900201 PMid:9175551

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler D, Dean D. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev*. **62**(3): 775-806. PMid:9729609 PMCid:PMC98934

Schnepf HE, Whiteley H. 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli. Proc Natl Acad Sci.* **78**(5): 2893-2897. https://doi.org/10.1073/pnas.78.5.2893

Shishir A, Roy A, Islam N, Rahman A, Khan S, Hoq MM. 2014. Abundance and diversity of *Bacillus thuringiensis* in Bangladesh and their *cry* genes profile. *Front Environ Sci.* **2**: 1-10. https://doi.org/10.3389/fenvs.2014.00020

Unalmis S, Ayvaz A, Yilmaz S, Azizoglu U. 2015. Molecular screening and bioactivity of native *Bacillus thuringiensis* isolates. *J Agric Sci Technol.* **17**(5): 1197-1207.

Wasano N, Saitoh H, Maeda M, Ohgushi A, Mizuki E, Ohba M. 2005. Cloning and characterization of a novel gene *cry*9Ec1 encoding lepidopteran-specific parasporal inclusion protein from a *Bacillus thuringiensis* serovar *galleriae* strain. *Can J Microbiol*. **51**(11): 988-995. https://doi.org/10.1139/w05-084 PMid:16333339

Yilmaz S, Azizoglu U, Ayvaz A, Temizgul R, Atciyurt ZB, Karaborklu S. 2017. Cloning and expression of *cry*2Aa from native *Bacillus thuringiensis* strain SY49-1 and its insecticidal activity against *Culex pipiens* (Diptera: Culicidae). *Microb Pathogenesis* **105**: 81-85. https://doi.org/10.1016/j.micpath.2017.02.016 PMid:28215855

Zheng A, Zhu J, Tan F, Guan P, Yu X, Wang S, Deng Q, Li S, Liu H, Li P. 2010. Characterisation and expression of a novel haplotype *cry*2A-type gene from *Bacillus thuringiensis* strain JF19-2. *Ann Microbiol*. **60**: 129–134. https://doi.org/10.1007/s13213-009-0011-x