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REGULAR ARTICLE

Characterization of a native *Bacillus thuringiensis* isolates from Malaysia that produces exosporium-enclosed parasporal inclusion

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ABSTRACT

In this study, Bt-S84-13a, a native *B. thuringiensis* isolate from Penang Hill, Malaysia that has been previously reported to produce exosporium-enclosed parasporal inclusion, in addition to free parasporal inclusion, was characterized using a series of morphological, biochemical and molecular analysis. In terms of colony morphology, Bt-S84-13a showed 2.0-2.5 mm white colonies with a typical "fried-egg" appearance, glossy surface, slightly raised and entire margin. SDS-PAGE of crude protein extracted from sporulated cells of Bt-S84-13a showed four major bands at 46, 31, 28 and 26 kDa. However, the spore-crystal suspension of Bt-S84-13a was not pathogenic to the third instar larvae of *Aedes aegypti, Aedes albopictus* and *Culex quinquefasciatus*, although Bt-S84-13a was characterized with *cry4* gene through PCR analysis. No correlation was found between the protein profile and *cry* gene pattern of Bt-S84-13a. Plasmid profiling showed that Bt-S84-13a contains only megaplasmid. Despite the effort of characterizing this unique Malaysian isolate, the actual ecological role of this unique strain remains puzzle and unclear. Further experiments should be performed to unveil the hidden potential in this native Malaysian *B. thuringiensis* isolate.

Keywords: *Bacillus thuringiensis*; Bioassay; *cry* gene; *cyt* gene; Parasporal inclusion

INTRODUCTION

Bacillus thuringiensis, commonly known as Bt, is a Gram positive, rod-shaped, ubiquitous soil-dwelling and endospore forming bacterium (Hofte and Whiteley, 1989; Glare and O'Collaghan, 1998; Ibrahim et al., 2010). Colonies of *B. thuringiensis* are described as circular to irregular with entire or undulate edges, and have matt to granular surface textures (Logan and De Vos, 2011). *B. thuringiensis* belongs to the *Bacillus cereus* group but it is mainly characterized by its capability of producing crystalline protein inclusions that contain one or more insecticidal δ-endotoxins, during the sporulation phase of its life cycle (Glare and O'Collaghan, 1998; Helgason et al.,1998; Stotzky, 2004; Ibrahim et al., 2010).

The δ-endotoxins consist of Cry and/or Cyt proteins, which are expressed by their respective *cry* and/or *cyt* genes. The Cry protein is responsible towards the strain toxicity, and the family consists of diverse Cry protein classes, with each class bearing specific activity. For example, Cry1 protein (130-140 kDa) targets lepidopteran pests, Cry2 (65-71 kDa) protein for Lepidoptera and Diptera, Cry3 (66-77 kDa) protein is effective for coleopteran, and Cry4 protein (128-135 kDa) is active against dipteran (Hofte and Whiteley, 1989; van Frankenhuyzen, 2009; Mahadeva Swamy et al., 2013). Some Cry protein was also found with anticancer property (Mizuki et al., 2000; Jung et al., 2007) and nematocidal activity (Wei et al., 2003; Zhang et al., 2012). In contrast, the Cyt protein (25-30 kDa) is cytolytic against invertebrate and vertebrate cells including erythrocytes (Mizuki et al., 1999; Yasutake et al., 2008).

The application of polymerase chain reaction (PCR) in the detection of *B. thuringiensis* δ-endotoxin genes was first started by Carozzi et al. (1991). It is a rapid and reliable molecular tool used to identify the δ-endotoxin genes in *B. thuringiensis*. PCR is useful in the prediction of insecticidal activity in *B. thuringiensis*, however, interest in developing a potential strain into biopesticide would still require

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assessment through toxicity bioasays (Porcar and Juárez-Pérez, 2003; Vidal-Quist et al., 2009).

B. thuringiensis contains a set of self-replicating plasmids and they are transferred during conjugation (Thomas et al., 2000; Rolle at al., 2005). The set of plasmids carried by *B. thuringiensis* strains can vary in number and in size (4-150 MDa) (Fagundes et al., 2011). Plasmid patterns of *B. thuringiensis* can be divided into two different groups: small plasmids which are ≤30 MDa, and megaplasmids which are ≥30 MDa. In agarose gel electrophoresis, the small plasmids (usually present in high copy numbers) and the megaplasmids (usually present in low copy numbers) are separated by a chromosomal band (Reyes-Ramirez and Ibarra, 2008; Fagundes et al., 2011). The δ-endotoxin genes of *B. thuringiensis* are located on megaplasmids and/or chromosomes (Kronstad et al., 1983; Lereclus et al., 1993; Glare and O'Callaghan, 2000; Zujiao et al., 2008), whilst the smaller plasmids are referred as cryptic plasmids because no specific function has been found in them yet.

A screening program in Malaysia has led to the discovery of an unusual native *B. thuringiensis* strain, Bt-S84-13a. This strain has been previously reported to be able to produce exosporium-enclosed parasporal inclusion, in addition to the commonly seen spore-free inclusion (Chai et al., 2014). The present study is aimed to characterize this unique *B. thuringiensis* strain isolated from Penang Hill, Malaysia using colony morphology, SDS-PAGE, PCR, plasmid profile and mosquito bioassay.

MATERIALS AND METHODS

Bacterial strain

Bt-S84-13a was isolated from soil in Bukit Bendera, Penang, Malaysia. *Bacillus thuringiensis* var. *israelensis* (Bti) H-14 was obtained from the Institute for Medical Research, Kuala Lumpur, Malaysia.

Colony morphology

Bt-S84-13a was streaked onto Nutrient agar and the plate was incubated for 24 h at 28°C. The size, form, margin, elevation and color of the bacterial single colony were examined.

SDS-PAGE

Crude protein of autolyzed Bt-S84-13a grown in Nutrient broth was pelletized by centrifuged at 9,000 rpm in 4°C for 20 min (Universal 320, Hettich Zentrifugen). Concentration of the crude protein re-suspended in sterile distilled water was estimated using Bradford method (Bradford, 1976) and 30 µg of the crude protein was analyzed with 10% acrylamide gel (Laemmli, 1970).

PCR

Genomic DNA of Bt-S84-13a was extracted using i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology, Inc.). Primers were obtained from First Base Laboratories, Malaysia, and all other PCR buffers and reagents were acquired from Thermo Scientific Inc. The 20 µL of PCR mixture consisted of 1X *Taq* buffer with potassium chloride (KCl), 2.0 mM $MgCl_2$, 200 µM of dNTP mix, 0.2 µM of each forward and reverse primers, 1.5 U of *Taq* DNA polymerase, 100 ng of DNA and sterile deionized water. PCR was carried out in MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., USA) set for initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 1 min, annealing at the respective temperature for 1 min (Table 1), and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. Amplified products were analyzed in 1.0% agarose gel.

Plasmid profiling

Plasmids were extracted using a modified protocol of Reyes-Ramirez and Ibarra (2008). Bt-S84-13a was pre-cultured on Nutrient agar for 14-16 h at 28°C. A single colony of the bacteria was inoculated into a 250 mL conical flask containing 50 mL of Spizizen medium (Spizizen, 1958). Bacteria were incubated in room temperature (25±2°C) by shaking on an orbital shaker at 120 rpm until OD_{600} reached the range of 0.8-1.0.

Two milliliters of the late logarithmic phase bacteria culture was transferred into a microcentrifuge tube and the bacteria cells were pelletized at 20,200 g (Universal 320, Hettich Zentrifugen) for 1 min in 4°C. Supernatant was completely removed while pellet was washed with 1 mL of cold TES buffer (30 mM Tris base, 5 mM EDTA, 50 mM NaCl; pH 8.0). The suspension was centrifuged under the same conditions. Pellet which contained the bacteria cells was resuspended in 100 µL of TES buffer containing 20% sucrose. Later, 3 µL of 100 mg/mL lysozyme (iNtRON Biotechnology, Inc.) and 5 µL of 10 mg/mL RNase (iNtRON Biotechnology, Inc.) were added into the suspension. The suspension was mixed by vortexing and was incubated in a 37°C water bath for 2-3 h.

Following that, 200 µL of TES buffer containing 8% of SDS was supplemented to the suspension and incubated in a 68°C water bath for 20 min. Then, 100 µL of 3 M sodium acetate 3-hydrate ($CH_3COONa•3H_2O$) (pH 4.8) was added, gently mixed by inverting the microcentrifuge tubes a few times, and the suspension was allowed to stand at -20°C for 30 min. The suspension was centrifuged at 20,200 g (Universal 320, Hettich Zentrifugen) for 10 min at 4°C and the translucent supernatant was collected into a new microcentrifuge tube. Two volumes of cold absolute ethanol were added to the supernatant and they were

Table 1: Primers selected for PCR

Primer	Direct sequence (d) and reverse sequence (r)	Gene recognized	Product size (bp)	References	Annealing temperature (°C)
gral-cry1	(d) 5'-CTGGATTTACAGGTGGGGATAT-3' (r) 5'-TGAGTCGCTTCGCATATTTGACT-3'	*cry1*	543-594	Bravo et al., 1998	53.0
Un2	(d) 5'-GTTATTCTTAATGCAGATGAATGGG-3' (r) 5'-CGGATAAAATAATCTGGGAAATAGT-3'	*cry2*	689-701	Ben-Dov et al., 1997	54.0
CJIII20 CJIII21	(d) 5'-TTAACCGTTTTCGCAGAGA-3' (r) 5'-TCCGCACTTCTATGTGTCCAAG-3'	*cry3*	652-733	Ceron et al., 1995; Öztürk et al., 2008	55.5
Un4	(d) 5'-GCATATGATGTAGCGAAACAAGCC-3' (r) 5'-GCGTGACATACCCATTTCCAGGTCC-3'	*cry4*	439	Ben-Dov et al 1997; Öztürk et al., 2008	58.6
Un9	(d) 5'-CGGTGTTACTATTAGCGAGGGCGG-3' (r) 5'-GTTTGAGCCGCTTCACAGCAATCC-3'	*cry9*	351-354	Ben-Dov et al., 1999	56.0
gral-cry11	(d) 5'-TTAGAAGATACGCCAGATCAAGC-3' (r) 5'-CATTTGTACTTGAAGTTGTAATCCC-3'	*cry11*	305	Bravo et al., 1998	50.5
gral-nem	(d) 5'-TTACGTAAATTGGTCAATCAAGCAAA-3' (r) 5'-AAGACCAAATTCAATACCAGGGTT-3'	*cry5, cry12, cry14, cry21*	474-489	Bravo et al., 1998; Vidal-Quist et al., 2009	48.0
cry6(+) cry6(-)	(d) 5'-TGGCGTAGAGGCTGTTCAAGTA-3' (r) 5'-TGTCGAGTTCATCATTAGCAGTGT-3'	*cry6*	302	Ejiofor and Johnson, 2002; Salehi Jouzani et al., 2008	52.0
ps2	(d) 5'-GTTATTCAAGAATACCTTACG-3' (r) 5'-GACATAGCTGATATTCAAGAT-3'	*ps2*	1104	Ashwini, 2006; Yadav, 2007	41.0
ps1	(d) 5'-ATCAAGAATTTTCCGATAATC-3' (r) 5'-CCAAAAGTGCCAGAATG-3'	*ps1*	1000	Yasutake et al., 2006; Uemori et al., 2007; Poornima et al., 2012	43.0
cyt1gral	(d) 5'-CCTCAATCAACAGCAAGGGTTATT-3' (r) 5'-TGCAAACAGGACATTGTATGTGTAATT-3'	*cyt1*	477-480	Ibarra et al., 2003; Mahalakshmi et al., 2012	56.0
cyt2gral	(d) 5'-ATTACAAATTGCAAATGGTATTCC-3' (r) 5'-TTTCAACATCCACAGTAATTTCAAATGC-3'	*cyt2*	355-356	Ibarra et al., 2003; Mahalakshmi et al., 2012	54.0

mixed by gentle inversion for a few times. The mixture was incubated overnight at -20°C.

The plasmid-enriched DNA was pelletized by centrifuged at 20,200 g (Universal 320, Hettich Zentrifugen) for 20 min at 4°C. Supernatant was removed and ethanol residue was dried by exposing the microcentrifuge tube containing pellet of plasmid DNA under a laminar hood air flow for 15 min. Pellet was then resuspended in 25 µL of sterile TE buffer (pH 8.0). Plasmid pattern of Bt-S84-13a was analyzed on 0.5% (w/v) agarose gel. Bti was used as reference strain.

Bioassay

Autolyzed Bt-S84-13a cells cultured in Nutrient broth were pelletized at 9,000 rpm (Universal 320, Hettich Zentrifugen) for 20 min at 4°C. The pellet was resuspended in sterile distilled water and protein content was estimated using Bradford method (Bradford, 1976). The spore-crystal suspension was diluted to 100 mg/L working stocks. Late third instar larvae of *Ae. aegypti* (strain VCRU), *Ae. albopictus* (strain VCRU) and *Cx. quinquefasciatus* (strain VCRU) were provided by Vector Control Research Unit (VCRU), School of Biological Sciences, Universiti Sains Malaysia. Bioassays were designed based on the methods described by World Health Organization (WHO) (1981) and Skovmand and Becker (2000). Bioassays were carried out at 27±1°C in 250 mL plastic cups. Batches of 25 late third instar larvae were firstly placed in 50 mL of distilled water. Then using separate plastic cups, 50 mL of concentrated crude protein suspensions were prepared from 100 mg/L spore-crystal working stocks which will eventually diluted to the testing concentrations of 0.05, 0.10, 0.50, 1.00, 5.00, and 10.0 mg/L at a final volume of 100 mL after the introduction of 50 mL distilled water containing 25 mosquito larvae. Meanwhile, batches of 25 larvae in 100 mL of distilled water were served as negative control. The study comprised six replicates of 25 larvae for every concentration tested. Larval mortality was recorded at 24 and 48 h post-dose.

RESULTS

Bt-S84-13a showed 2.0-2.5 mm white colonies with "fried-egg" appearance, glossy surface, slightly raised and entire margin (Fig. 1). The morphology was different from *B. thuringiensis* var. *israelensis* (Bti) H-14, which displayed 5-6 mm, slightly raised and entire colonies with smooth-looking center and granular side (Fig. 2).

SDS-PAGE

SDS-PAGE of crude protein extract of Bt-S84-13a showed four distinct bands at 46, 31, 28 and 26 kDa (Fig. 3).

PCR

Bt-S84-13a showed positive amplification for *cry4* gene with expected product of 439 bp (Fig. 4). No results were obtained from 14 others δ-endotoxin genes (*cry1*, *cry2*, *cry3*,

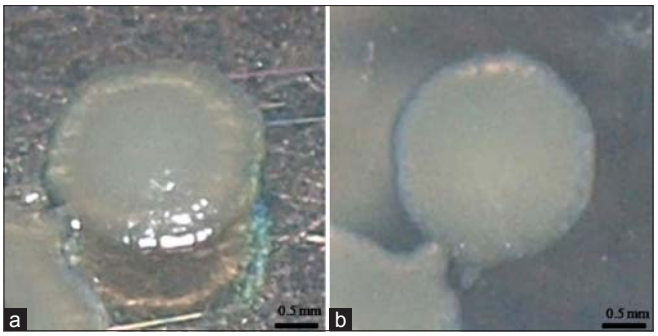

Fig 1. Colony morphology of Bt-S84-13a on NA. The colonies are 2-2.5 mm in size with glossy surface, slightly raised and entire margin. (a) Fried-egg appearance of the colony viewed from top of the Petri plate; (b) Colony morphology viewed from reverse surface of the Petri plate. Bar = 0.5 mm.

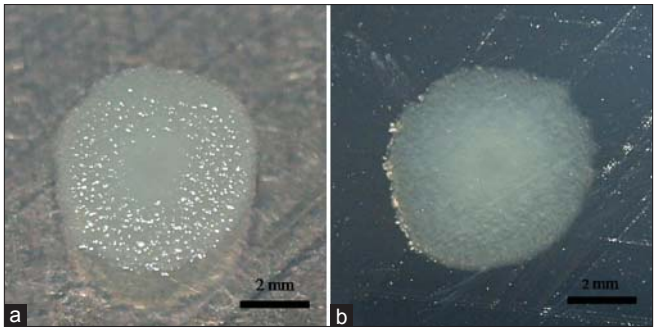

Fig 2. Colony morphology of Bti on NA. The colonies are 5-6 mm in size, slightly raised, entire with smooth-looking center and encircled by granular side. (a) Fried-egg appearance of the colony viewed from top of the Petri plate; (b) Colony morphology viewed from reverse surface of the Petri plate. Bar = 2 mm.

cry5, *cry6*, *cry9*, *cry11*, *cry12*, *cry14*, *cry21*, *cry31*, *cry46*, *cyt1* and *cyt2*) examined.

Plasmid profiling

Bt-S84-13a was found with only megaplasmid while exhibited no small plasmids (Fig. 5).

Bioassay

Spore-crystal suspension of Bt-S84-13a did not exhibit any activity against the third instar larvae of *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* at the highest testing concentration of 10.0 mg/L after 48 h post-dose. In contrast, the control strain *B. thuringiensis* var. *israelensis* H-14 showed strong activity by killing all the larvae of three species at 24 h post-dose with the lowest concentration of spore-crystal suspension used (0.05 mg/L).

DISCUSSION

There are various descriptions of *B. thuringiensis* colony morphology available in the literature. Logan and De Vos (2011) defined *B. thuringiensis* colonies as circular to irregular with entire or undulate edges, and have matt

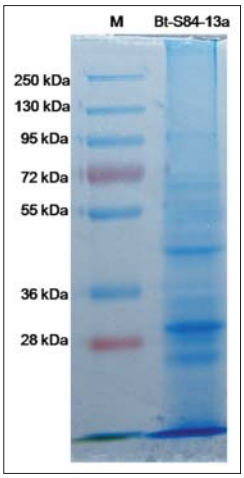

Fig 3. SDS-PAGE of Bt-S84-13a. The native isolate showed four major bands at 46, 31, 28 and 26 kDa. M = Molecular weight marker (Fermentas PageRuler Plus Prestained Protein Ladder).

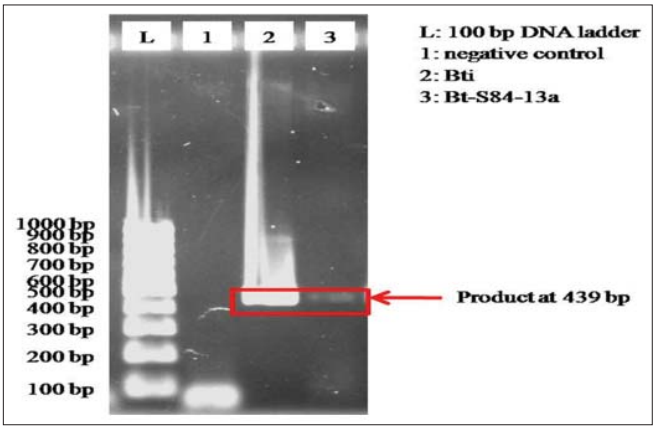

Fig 4. Agarose gel electrophoresis (1.0%) of PCR products using *cry4* universal primers. Native isolate Bt-S84-13a showed expected product at 439 bp. L = GeneRuler™ 100 bp DNA ladder (Fermentas).

to granular surface textures. De Respinis et al. (2006) mentioned that the colony morphology of *B. thuringiensis* and *B. cereus* is indistinguishable and both species show the typical morphology of circular or irregular colonies in white or grey; granular, milky or mat aspect and minimum 5 mm of diameter. In addition to smooth colonies with irregular shape, Lima et al. (2002) also characterized a *B. thuringiensis* isolate that produced dark yellowish colonies. Apart from the characteristics stated, many also reported that *B. thuringiensis* strains formed "fried-egg" appearance on agar plate (Travers et al., 1987; El-kersh et al., 2012; Mahalakshmi et al., 2012). The descriptions for colony morphology are very subjective. Bt-S84-13a showed different colony morphology when compared with Bti, however, it possessed the common morphological appearance as described in the literature, and most importantly, it also displayed the "fried-egg" morphology, which is in agreement with the earlier reports.

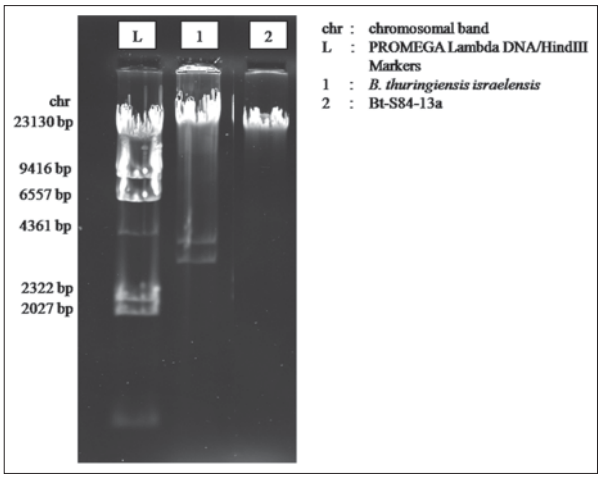

Fig 5. Agarose gel electrophoresis (0.5%) of plasmid DNA of Bt-S84-13a.

The protein profile of Bt-S84-13a determined through SDS-PAGE did not show any correlation with the δ-endotoxin gene detected, which was *cry4*. The absence of correlation between protein profile and *cry* gene pattern was also reported by Armengol et al. (2007). These variations could be caused by proteins that are coded by new gene types or that the primers did not amplify the genes. It is also possible that the detected genes may code for some non-active or low expressed proteins (Armengol et al., 2007).

Despite being characterized with the dipteran *cry4* gene, yet Bt-S84-13a did not show its predicted insecticidal activity. The lack of correspondence between δ-endotoxins genotype of Bt-S84-13a and its biological activity may be due to various factors, for example, the production of inactive crystal proteins whereby the gene that synthesize the crystal proteins could be under the control of a weak promoter (Masson et al., 1998; Ferrandis et al., 1999; Armengol et al., 2007). Some *cry* genes may be also expressed in low levels which may lead to the low expression of the respective Cry proteins. Porcar and Juárez-Pérez (2003) mentioned that the detection of a *cry* gene by PCR has no direct proof of its level of expression, but variation in the expression level of individual *cry* genes could weaken the correlation between *cry* gene content and toxicity. Therefore, it is very common that strains differ greatly in their insecticidal efficacy although they carried the same δ-endotoxin genes, owing to differences in the level of *cry* genes expression.

PCR analysis is important for the identification of the δ-endotoxin genes in a strain, however, it could neither indicate which genes are eventually translated nor the relative percent composition of the δ-endotoxin (Masson et al., 1998). The results of this study may suggest that the isolate might possess other pathogenic potentials which are yet to be explored.

The characteristics of Bt-S84-13a appeared similar to *B. thuringiensis* subspecies *finitimus*. Both were capable of producing two parasporal inclusions; one within the exosporium and one exterior to the exosporium. In terms of plasmid expressions, Debro et al. (1986) detected only two megaplasmids of 98 and 77 MDa in *B. thuringiensis* subp. *finitimus*. Plasmid-cured strains produced only the free inclusion which proved that the free-inclusion protein of subspecies *finitimus* is encoded in the chromosome.

Previous studies have discovered that most of the δ-endotoxin genes of *B. thuringiensis* are positioned on the megaplasmids (Gonzalez et al., 1982; Ward and Ellar, 1983) and/or occasionally, in the chromosome (Kronstad et al., 1983; Lereclus et al., 1993). Plasmid patterns of *B. thuringiensis* can be divided into two groups; the small plasmids and the megaplasmids. According to Reyes-Ramirez and Ibarra (2008) and Fagundes et al. (2011), these two groups were separated in the agarose gel electrophoresis by a chromosomal band. The small plasmids were less than 30 MDa and located below the chromosomal band whereas the megaplasmids were larger than 30 MDa and located above the chromosomal band. In the characterization of *B. thuringiensis* plasmid profiles, strains are usually firstly distinguished by observing the migration patterns of the small plasmids. Megaplasmids are always the second option for characterization due to their limited migration in the agarose gel during electrophoresis, thus causing difficulties in discriminating the comigrating bands (Reyes-Ramirez and Ibarra, 2008; Fagundes et al., 2011). In addition, megaplasmids tend to degrade during storage that leads to information loss on the plasmid patterns (Reyes-Ramirez and Ibarra, 2008).

CONCLUSIONS

A series of morphological, biochemical and molecular analysis had been performed on Bt-S84-13a, which was known to produce exosporium-enclosed parasporal inclusion. However, the actual ecological role of this unique strain is still not properly understood. Extensive study for Bt-S84-13a is needed to fully elucidate its ecological role and its probable use in the agriculture or medical industry.

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Authors' contributions

Pui Fun Chai carried out the project and drafted the manuscript. Xavier Rathinam and Amir Hamzah Ghazali participated in data analysis. Sreeramanan Subramaniam

was responsible for the final review of the manuscript. All authors read and approved the final manuscript.

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