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Protein Patterns and Larvicide Activity of Crystalline Inclusions of *Bacillus thuringiensis* ssp. *kumamotoensis DSM 6070*

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ABSTRACT

Morphological and electrophoretic analysis of the crystalline inclusions (parasporal crystals) of sporulated cultures of *B. thuringiensis ssp. kumamotoensis* DSM 6070 (Bt 6070) was conducted via phase contrast and scanning electron microscopy and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The activity of the spore-crystal suspensions against house fly (*Musca domestica*) larvae was also assessed. Bipyramidal and smaller, irregular shaped crystals were observed in the sporulated cultures. 130, 75 and 25 kDa bands were detected in the protein pattern. The presence of 25 kda proteins in Bt6070 has not been reported earlier. The spore-crystal suspension showed significant larvicide activity against housefly larvae. Larvicide activity of *B. thuringiensis* ssp. *kumamotoensis* against any dipteran species has not been detected yet. Further studies are needed on identifying the dipteran- active fraction.

Keywords: *Bacillus thuringiensis,* Parasporal crystals, Polyacrylamide gel-electrophoresis, Scanning electron microscopy.

INTRODUCTION

Bacillus thuringiensis is a Gram-positive spore-forming soil bacterium with entomopathogenic properties. In the past decades, several *B. thuringiensis* strains have been used in biological pest control (de Maagd, 2014; Cannon, 1995). *B. thuringiensis* strains produce insecticidal proteins during the sporulation phase of their life cycle as parasporal crystalline inclusions of different shape, size and composing proteins, also called delta-endotoxins. The crystals are predominantly comprised of one or more Crystalline (Cry) proteins, or consist of associated Cry and Cytolytic (Cyt) proteins, (Palma *et al.,* 2014). The toxins are specific to their target insect order (Höfte and Whiteley, 1989; Crickmore *et al.,* 1998; van Frankenhuyzen, 2009). They are classified according to nucleotide sequence homologies (Crickmore *et al.,* 1998).

Phase contrast and electron microscopy are accepted methods for the detection and morphological description of parasporal crystals (Ibarra *et al.,* 2003; Hernandez-Rodriguez and Ferré, 2005; Kati *et al.,* 2007). Generally, morphology of crystals is determined by the type of the composing protoxin proteins (Höfte and Whiteley, 1989; Federici *et al.,* 2010) that makes it possible to predict the group of target insect species. For the separation of protein components of the crystalline inclusions, Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is a widely used technique (Rupar *et al.,* 1991; Ibarra *et al.,* 2003; Kati *et al.,* 2007).

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Isolation and characterization of novel *B. thuringiensis* strains with insecticidal activity is the subject of extensive research worldwide. Strains with known protein patterns of their insecticidal protoxins and target insect spectra are routinely used to aid the finding of presumably insecticidal proteins of new *B. thuringiensis* isolates, and to reveal their probable susceptible insect species.

Bacillus thuringiensis ssp. *kumamotoensis* strains have not been studied in much detail regarding the morphology and composition of crystals. First described by Ohba *et al.* (1981), the strain was isolated from silkworm (*Bombyx mori* larvae) litter and was found to produce typically bypiramidal shaped parasporal inclusions. The spore-parasporal inclusion complex of the strain was found to be toxic for first and fourth instar silkworm, but not toxic to mosquito (*Aedes aegypti*) larvae. The strain is also known for its *Cry3* protoxin producing property, which is toxic against coleopteran species (Palma *et al.,* 2014; van Frankenhuyzen, 2009; Donovan *et al.,* 1992). Takács *et al.,* (2010) have found that not only the rate of proliferation and sporulation, but the type and amount of the produced protoxins vary with culturing conditions, mainly pH and temperature. In our study, the crystal morphology, the patterns of crystalline proteins and the larvicidal activity of sporulated *B. thuringiensis* ssp. *kumamotoensis* cultures against housefly larvae were examined in more detail.

MATERIALS AND METHODS

Bacterial Strains

B. thuringiensis ssp. kumamotoensis DSMZ 6070 (other collection name NRRL HD-867) (briefly Bt 6070) was obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Production of Sporulated Cultures

For the production of crystalline inclusions, the strain was cultured in DSMG medium [0.4% (wt/vol) Nutrient Broth (Difco) 0.5% (wt/vol) glucose, 25 mM K_2HPO_4, 25 mM KH_2PO_4 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.01 mM $MnCl_2$, 0.01 mM $FeSO_4$] at 28ºC, shaken at 300 rpm until sporulation (48-72 hours) (Rupar *et al.,* 1991). Sporulation was traced and confirmed by phase contrast microscopy.

Processing of Samples for Scanning Electron Microscopy and SDS-PAGE

The sporulated culture were harvested by centrifugation (10,000 rpm, 20 minutes, 6 ℃), washed twice in TETX buffer (10 mM Tris-HCl, pH 7,5; 1 mM EDTA, 0.005% [vol/vol] Triton X-100) and suspended with TETX buffer to a concentration of 100 mg per ml (wet weight, primarily spores plus crystals), and stored at 4°C (Rupar *et al.,* 1991).

Phase Contrast Microscopy (PCM)

Native suspensions of 24-72h cultures grown in DSMG medium were observed by phase contrast microscopy (Carl Zeiss, Jenalumar, 500× magnification; Canon G10 digital camera, 2× optical zoom).

Scanning Electron Microscopy (SEM)

Processed samples (primarily spore-crystal suspensions) were filtered onto polycarbonate filter with a pore size of 0.2 µm (Millipore) and frozen in liquid nitrogen, freeze-dried until 2×10^2 mbar, at -60℃ for 6-8 hours (Edwards), mounted, coated with gold-palladium (Polaron SC7630) and viewed by HITACHI S-2600N scanning electron microscope at an accelerating voltage of 15 kV.

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SDS-PAGE

The method of Rupar *et al.* (1991) was followed. The processed samples were mixed with an equal volume of the sample buffer [0.13 M Tris-HCl (pH 8.5), 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 0.05% (wt/vol) bromphenol blue, 10% (vol/vol) glycerol], incubated at 100°C for 7 min, vortexed for 10 seconds, centrifuged at 10,000 rpm for 5 minutes, and 10 µl aliquots of the supernatants were loaded onto 10.0% [wt/vol] polyacrylamide gel containing 0.1% SDS. Protein components were separated by a Mini Protean Tetra Cell electrophoresis system (Bio-Rad Laboratories, Inc., USA) following the manufacturer's prescriptions and stained with Coomassie blue dye. Gels were dried by Gel Air Drying System (Bio-Rad Laboratories, Inc., USA). Protein patterns were digitally recorded by Kodak Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, USA).

Larvicide Tests against Housefly Larvae

Twenty ml of the sporulated cultures were centrifuged at 10,000 rpm for 20 minutes (Haereus Megafuge, Thermo Scientific Inc, USA) and the spore-crystal sediment was resuspended in 1.5 ml TETX buffer. 0.5 ml of the concentrated culture were mixed in 3-3 ml of an agar based culture medium (Heins *et al.,* 2000) in triplicate and plated onto the bottom of 30 ml vials. For control, only 3ml of the agar based medium was used in triplicate. Ten eggs of the common housefly (*Musca domestica*) laid within an hour were placed in the vials, on the surface of the agar medium. The vials were incubated at 27°C. Hatching and mortality of the fly larvae were monitored through a 14-day experimental period. The mortality rates were compared by one-way ANOVA and Fisher's Least Significant Difference (LSD) test. The level of significance was set at P= 0.05.

RESULTS AND DISCUSSION

Phase Contrast Microscopic (PCM) Observations

PCM micrograph of the sporulated culture of Bt 6070 is shown in Figure 1. Free spores and crystals appeared predominantly after 72 hours of incubation.

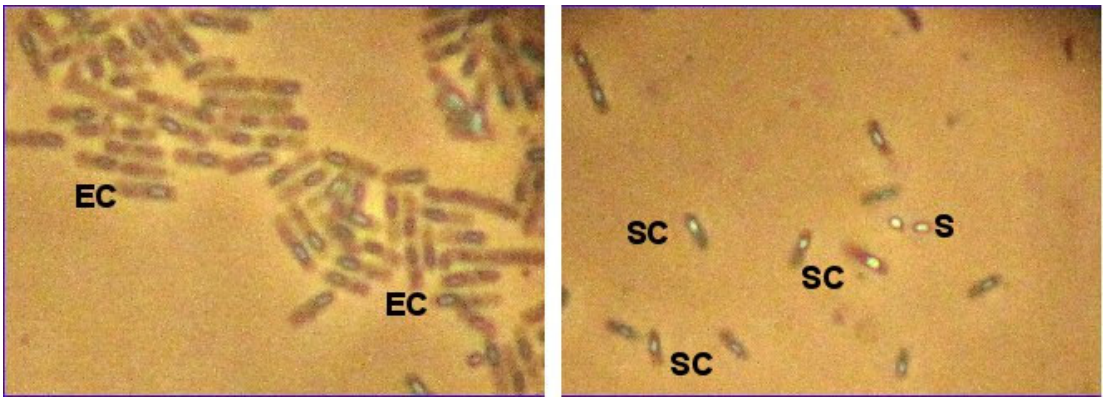

Figure 1. Sporulated cultures of *Bacillus thuringiensis ssp. kumamotoensis* after 48-72 hours of incubation. EC: Endospores and parasporal Crystals; SC: Spore-Crystal association, S: Spores.

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Morphology of Crystals

Scanning electron microscopic observations confirmed the presence of distinctively shaped crystals in the sporulated cultures. In the spore-crystal suspension bipyramidal, spherical and multiple, flat crystal shapes can be distinguished (Figure 2.). Ohba *et al.* (1981) have identified only bipyramidal-shaped inclusions in the cultures of the *B. thuringiensis* isolate that was later classified as *B. thuringiensis* ssp. *kumamotoensis*. Payne and Fu (1994) reported on bipyramidal crystals in the sporulated cultures of the B. thuringiensis HD-867 strain, comprising of *Cry7* and *Cry8* proteins. Difference in sporulating temperatures can lead to the absence of certain protein fractions (Takács, 2010). Though the process of crystallization of parasporal bodies is not fully known, it is likely that protoxins of a higher molecular weight (130-140 kDa) can spontaneously form crystals, while proteins of a lower molecular weight (Cry2 71 kDa, Cyt1a 27 kDa) need the presence of accessory proteins (Schnepf *et al.,* 1998). Considering that *Cry1* proteins form bipyramidal, *Cry2* proteins form cubic, *Cry3* proteins form flat or irregular shaped crystals (Schnepf *et al.,* 1998; Federici, 1999), some presumptions on the composition of parasporal inclusions can be made on the basis of crystal morphology. Irregular shaped crystals, that are smaller in diameter than larger bipyramidal crystals are usually observed in the sporulated cultures of e.g. *B. thuringiensis* ssp. *israelensis* (Ibarra *et al.,* 2003; Takebe *et al.,* 2007). Protoxin proteins of the *B. thuringiensis* ssp. *israelensis* are known to form aggregates during crystallization, creating irregular crystal shapes (Federici *et al.,* 2010). Our assumption is that the smaller, irregular (or spherical) shaped crystals might correspond to protoxins that were not yet described in *B. thuringiensis* ssp. *kumamotoensis* which was tested by molecular weight comparisons.

Evaluation of Electrophoretograms

Electrophoretograms of the processed spore-crystal suspensions of the strains are displayed in Figure 3. It confirmed the presence of 130 kD, 75-73 kDa doublet and 25 kDa bands. *Cry7* and *Cry8* proteins of 130 kDa were produced by *B. thuringiensis* ssp. *kumamotoensis* HD-867 have been reported by Payne and Fu (1994). The *Cry3Bb* crystal proteins produced by Bt 6070 are known to have a molecular weight of approximately 74 kDa (Takács, 2010, Chen *et al.,* 2005, Donovan *et al.,* 1992), however, 25 kDa proteins were not

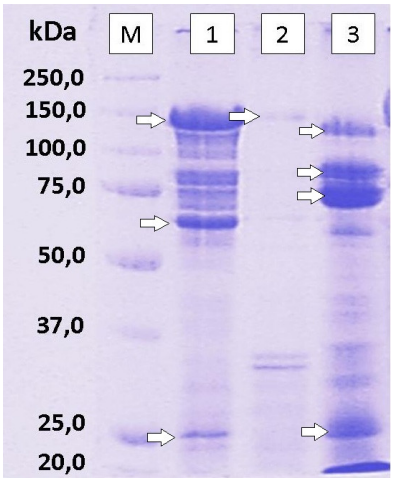

Figure 3. SDS-PAGE electrophoretogram of the spore-crystal suspensions. (M) Precision Plus Protein Standard (Bio-Rad Laboratories, Inc., USA), (3) Bt ssp. kumamotoensis DSM 6070.

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described in Bt 6070. Assuming that the 25 kDa fraction potentially infers entomocidal proteins, the known types of protoxins similar in molecular weight are the cytolitic toxins (Insell and Fitz James, 1985; Federici, 2010, Ben-dov, 2014). Larvicide tests against housefly larvae were conducted to test the assumption.

Larval Toxicity Tests

Twenty-four out of the 30 housefly eggs hatched and 100% of the housefly larvae have deceased in the vials added with the sporulated cultures of Bt 6070. Hatching rate was 100% and the average mortality rate in the control vials was 10% ($P< 0.0001$). The larvicide activity of *B. thuringiensis* ssp. *kumamotoensis* against dipteran species was not yet described. Ohba *et al.* (1981) found that the spore-parasporal inclusion complex of the culture did not increase mortality in mosquito (*Aedes aegypti*) larvae. Considering that temperature and pH influence the synthesis and crystallization of insecticidal proteins (Takács *et al.,* 2010), we suggest that in the study of Ohba *et al.,* (1981) the spore-crystal complex did not contain the 25 kDa fraction, which might carry the toxic activity against dipteran species. The mosquito-specific mode of action of cytolitic toxins and synergy between crystalline and cytolitic protoxins are well recognized (Ben-dov, 2014). The activity of *B. thuringensis* ssp. *israelensis* delta-endotoxins against houseflies have also been confirmed *in vitr*o (Singh, 1986) and *in vivo* (Merdan, 2012; Mwamburi *et al.,* 2011), however, the larvicide activity of *B. thuringiensis* ssp. *kumamotoensis* against any dipteran species has not been detected yet. This is a limitation of our study that further examinations to identify the specific fraction responsible for the observed larvicide activity and to confirm the presence of known *Cry*- or *Cyt*-genes could not be carried out.

CONCLUSIONS

The toxicity of parasporal crystals of *B. thuringiensis* ssp. *kumamotoensis* DSM 6070 against dipteran species was confirmed by larvicide studies against housefly larvae. It suggests that the larvicide spectrum of an enthomopathogenic strain of the *Bacillus* genus cannot be fully predicted on the basis of the source of isolation. Considering that *B. thuringiensis* ssp. *kumamotoensis* has already been known for its enthomopathogenic activity against coleopteran and certain lepidopteran species, the finding of the present study suggests that the larvicide spectrum of the strain is even broader and can be utilized in research and development of biopesticides. Further studies are needed to identify the dipteran-active fraction.

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الگوهای پروتئین و فعالیت لاور کشی اجزا کریستالی *Bacillus thuringiensis* ssp. *kumamotoensis DSM 6070*

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چکیده

بررسی مورفولوژیک و الکتروفورتیک اجزاء کریستالی (کریستال پاراسپورال) محیط اسپورزای *B. thuringiensis ssp. kumamotoensis* DSM 6070 (Bt 6070) از طریق میکروسکوپ فازکنتراست و میکروسکوپ الکترونی روبشی و الکتروفورز ژل سدیم دودسیل سولفات- پلی اکریل آمید انجام شد. همچنین فعالیت سوسپانسیون های اسپور-کریستال روی لارو مگس خانگی *(Musca domestica)* نیز بررسی شد. اشکال کریستالی هرمی (بی پیرامیدال) و کوچکتر و غیر معمول در محیط اسپورزا مشاهده شد. باندهای 130 kDa, 75 kDa و 25 kDa در الگوهای پروتئینی شناسایی شد. سوسپانسیون اسپور-کریستال، لاروکشی بالایی روی مگس خانگی نشان داد. فعالیت لاروکشی *B. thuringiensis ssp. kumamotoensis* DSM 6070 (Bt 6070) روی گونه های دوبالان هنوز بررسی نشده و نیاز به مطالعات بعدی وجود دارد.

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