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Protocol for the fast isolation and identification of insecticidal *Bacillus thuringiensis* strains from soil

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Abstract *Bacillus thuringiensis* (Bt) is an ubiquitous, Gram-positive and sporulating bacterium that synthesizes insecticidal proteins with specificity against a wide range of insects during sporulation (Cry and Cyt) and vegetative growth (Vip and Sip). These proteins have portrayed Bt as an environment-friendly alternative to chemical insecticides. However, the intensive use of this resource has promoted the evolution of insect resistance to some of the most used Bt strains to date. Continuous efforts are needed to obtain novel strains exhibiting new specificities in order to overcome insect resistance and maintaining the insecticidal potential of this bacterium. In this work, an experimental procedure for the fast isolation and identification of insecticidal Bt strains is described.

Keywords *Bacillus thuringiensis* (Bt); Bt toxins; biological control

Introduction

Bacillus thuringiensis (Bt) is a Gram-positive and sporulated bacterium that synthesizes a number of invertebrate active toxins with specific activity against a wide range of insects (de Maagd et al., 2003; Schnepf et al., 1998; van Frankenhuyzen, 2009). The δ-endotoxins, including Crystal (Cry) and cytolytic (Cyt) protein families are the best characterized group of insecticidal proteins and are synthesized during the stationary growth phase as crystalline parasporal inclusions (Schnepf et al., 1998; van Frankenhuyzen, 2009). In addition, Bt also produces other insecticidal proteins during the vegetative growth, the vegetative insecticidal proteins (Vips) and the secreted insecticidal protein (Sip), with toxicity against coleopteran (binary Vip1/Vip2 and Sip toxins) and lepidopteran (Vip3) pests (Donovan et al., 2006; Estruch et al., 1996; Warren et al., 1998). Strains producing these insecticidal proteins are commonly used in insecticidal formulations and their encoding genes, in the construction of transgenic plants (Sanchis, 2011), making the Bt-based products, the most marketed microbial pesticides worldwide (Roh et al., 2007; Schnepf et al., 1998). The broad diversity of insecticidal genes encoded by this bacterium strongly suggest that they resulted from high selective evolutionary pressures (de Maagd et al., 2003; Wu et al., 2007a; Wu et al., 2007b), that led to the enlargement of their target ranges and made Bt a broad source of insecticidal proteins (Palma et al., 2014). On the other hand, insects targeted by Bt-based insecticides, also suffered from selective pressures and began to show symptoms of resistance to some of the most frequently used insecticidal strains and toxins (Ferré and Van Rie, 2002; Tabashnik et al., 2009). These emerging insect resistance events are threatening the efficiency of the most used Bt-based biopesticides and demand the searching of novel insecticidal strains exhibiting novel specificities and host ranges. This protocol specifies the experimental procedure for the fast isolation and identification of insecticidal Bt strains from soil. In this work, an experimental procedure for the fast isolation and identification of insecticidal Bt strains is described.

1 Sampling

In this protocol, soil samples were obtained with a tubular soil sampler and consisted of ~20 g of cultivated or non-cultivated soil after removing the 2-3 cm of the top layer, to prevent low abundance levels of viable Bt spores due the effect of UV radiation from sun (Iriarte et al., 1998). After collection procedure, samples can be stored at 4 ℃ in

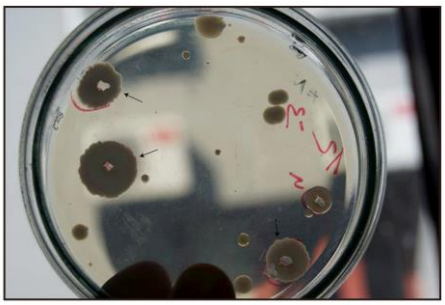

Figure 1 Isolated Bt-like colonies from soil samples (indicated by black arrows)

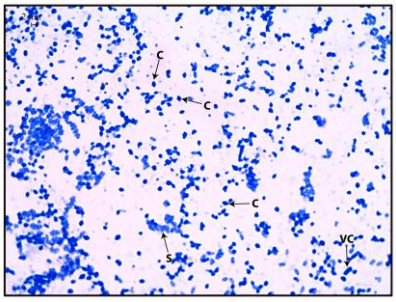

Figure 2 Sporulated Bt culture of *Bacillus thuringiensis* subsp. *kurstaki* HD1 strain showing rhomboidal parasporal crystals (C), spores (S) and vegetative cells (VC). In order to highlight parasporal crystals, the culture has been heat fixed and stained with Coomassie Blue (Ammons et al., 2002). Microphotograph (1000×) was obtained with a Leica DM750 microscope and its incorporated ICC50 digital camera (Leica Microsystems)

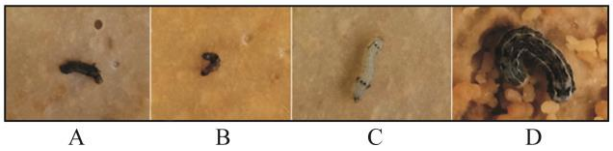

Figure 3. Preliminary bioassays results obtained with *S. cosmioides larvae*. (A) Dead *S. cosmioides* larvae treated with HD1 Bt strain; active against Lepidoptera (B) Dead *S. cosmioides* larvae treated with HD133 Bt strain, also active against Lepidoptera (C) *S. cosmioides* larvae exhibiting impaired growth when treated with IPS82 Bt strain, a strain active against Diptera (mosquitoes and black flies). (C) Negative control (sterile water)

50 ml (sterile) centrifuge tubes or zip-lock bags until isolation.

2 Isolation and identification

Vegetative cells from sporulated bacteria (Figure 1) were isolated by homogenization of 3 g of each soil sample in 10 ml of sterile distilled water, which was later mixed by intense vortexing for 1 min. and incubated at 70 ℃ for 15 min., after that, samples were vortexed and heated again as previously described. Each sample was then subjected to five ten-fold dilutions and 50 µl (from 10^{-3} to 10^{-5} dilutions) were plated using a Drigalsk spatula onto Petri dishes containing nutrient agar (0.5% Peptone, 0.3% beef extract, 0.5% NaCl and 1.5% agar). Plates were incubated at 28 ℃ for at least 72 h. Bacterial colonies exhibiting Bt-like phenotype (matte white colour, flat, dry and uneven borders) (Figure 2) were sub-cultured for single-colony isolation with a 5 µl inoculating loop and incubated as before. Each sporulated culture was then heat fixed and stained (0,133 % Coomassie Blue stain in 50 % acetic acid) in a glass microscope slide (Ammons et al., 2002). The Bt-like cells identification was performed by microscopic examination using a Leyca DM 750 bright field microscope (Leyca Microsystems). Those colonies showing Coomassie Blue stained parasporal crystals were stored at -20 ℃ in 50 % glycerol

3 Determining the insecticidal activity of novel Bt isolates against *Spodoptera cosmioides*.

Preliminary bioassays (Figure 3) were performed by surface contamination of artificial diet for lepidopteran (1.3 g benzoic acid, 128,4 g corn grits, 32.1 g wheat germ, 34.3 g beer yeast, 4.5 g ascorbic acid, 1.1 g methylparaben (Nipagin), 0.5 mL formaldehyde, 12 g of Agar and 779.5 mL of distilled water), using first instar *Spodoptera cosmioides* (Walker) larvae (Figure 3). Sporulated cultures of Bt isolates (mix of spores and crystals) were obtained from nutrient agar plates by scrapping the surface of agar with an inoculating loop. This material, corresponding to the entire Petri plate surface, was then diluted in 1 ml of distilled water and homogeneizated by vortexing vigorously for 1 min. at room temperature (RT). After mixing, 100 µl were loaded onto the surface of the solid lepidopteran artificial diet, spread with a Drigalski spatula and allowed to dry at RT. Bioassays were conducted with neonate larvae that were placed onto the surface of contaminated artificial diet. Sterile water was used as a negative control and the commercial Bt strains HD1 (Bt subsp. *kurstaki*), HD133 (Bt subsp. *aizawai*) and IPS82 (Bt subsp. *israelensis*) were used as positive controls. Ten larvae were used for each plate and each bioassay was

repeated twice. Bioassays were conducted at 28℃ ± 2℃ and a 16: 8-h (light/dark) photoperiod. Absolute mortality and functional mortality rates (dead larvae plus larvae remaining in the first instar) were scored after 7 days.

4 Notes

By developing the experimental procedure described in this protocol, the Bt-like isolates can be preliminary identified as *Bacillus thuringiensis* in 5 days of work, from the isolation to the preliminary determination of its toxic activity. However, further studies such as PCR amplification and sequencing of 16S rDNA, PCR amplification (and sequencing) of insecticidal genes commonly harboured by this bacterium (e.g. *cry*, *cyt* and *vip*), analysis of their SDS-PAGE protein profiles and bioassays involving additional insect species should be also performed in order to complement the preliminary characterization of the isolated strains.

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