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Pathogenicity and characterization of a novel *Bacillus cereus sensu lato* isolate toxic to the Mediterranean fruit fly *Ceratitis capitata* Wied.

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ABSTRACT

The lethal and sub-lethal effects of sporulated cultures of a novel *Bacillus cereus sensu lato* strain lacking detectable *cry* genes and identified through morphological and genetic analyses, have been studied on the Mediterranean fruit fly *Ceratitis capitata*. The lethal effects on young larvae were concentration dependent, with a median lethal concentration (LC_{50}) of 4.48×10^8 spores/g of diet. Sporulated cultures of this strain significantly extended development time and reduced immature survival, and the size of emerging fly adults. Besides spores, the toxicity has been associated to the insoluble extra-spore fraction characterized through a proteomic approach. The profile of the extra-spore protein fraction (ES) showed major protein bands within the 35–65 kDa range. The results of mass spectrometry analysis highlighted the presence of putative virulence factors, including members of protein families previously associated to the insecticidal action of other microbial entomopathogens. These proteins include metalloproteases, peptidases and other enzymes.

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1. Introduction

The Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) is a polyphagous species affecting more than 250 species of fruits and vegetables worldwide (White and Elson-Harris, 1994). In the Mediterranean basin, its host range includes several economically important crops, such as sour and sweet orange, grapefruit, loquat, apricot, peach, fig, pear, persimmon, prickly, pear and clementine (Liquido et al., 1991).

Due to its high reproductive potential, the management of this multivoltine pest is always necessary to protect fruits from the destructive action of larvae (Papadopoulos, 2008).

Most conventional management methods involve the application of various synthetic insecticidal formulations as foliage baiting or cover spraying. The negative impact of chemicals on non-target organisms (Damalas and Eleftherohorinos, 2011) has over time encouraged the research and development of safer methods of control including the use of low impact insecticides, mass trapping and the release of natural enemies. Efforts have been made to explore the opportunity to find and employ entomopathogenic microorganisms like fungi and bacteria against *C. capitata*. In this prospect, the potential of specific strains of fungal species *Beauveria bassiana* and *Metarhizium anisopliae* (Quesada-Moraga et al., 2006; Ortiz-Urquiza et al., 2010; Beris et al., 2013; Falchi et al., 2015), and of the entomopathogenic bacterium *Bacillus thuringiensis* (*Bt*) (Gingrich, 1987; Karamanlidou et al., 1991; Martinez et al., 1997) on diverse fly stages has been investigated. Similarly, the effects of *B. thuringiensis* isolates have been studied on other Tephritid species such as the olive fly *Bactrocera oleae* Gmelin (Alberola et al., 1999) and the Mexican fruit fly *Anastrepha ludens* (Loew) (Robacker et al., 1996). In this context, the insecticidal action of bacterial protein toxins produced by *B. thuringiensis* strains have been demonstrated, thus envisioning their possible employment in bait sprays against adults or as a resource to develop transgenic plants resistant to endophytic larval stages (Vidal-Quist et al., 2009 and Vidal-Quist et al., 2010). A main concern with different *B. thuringiensis* strains showing toxicity against flies is the production of unwanted secondary metabolites like the β-exotoxin, whose use in agriculture is prohibited (Liu et al., 2014). However, Vidal-Quist et al. (2010) have demonstrated the activity of Cyt1Aa protein from *B. thuringiensis* serovar *israelensis* against *C. capitata*.

The infectivity of other bacterial species to the Mediterranean fruit fly has been in a few cases reported (Sarakatsanou et al., 2011; Molina et al., 2010), even if the molecular implications and the role of specific bacterial metabolites in the interaction with the host have not been explained.

The *Bacillus cereus* phylogenetic cluster, which includes *B. thuringiensis*, is represented by close related bacterial species

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whose pathogenic properties are highly divergent. While their genetic relationships are still under debate, the insecticidal potential of new isolates in this group is of actual interest. Due to the production of typical parasporal crystals, *B. thuringiensis* can generally be phenotypically distinguished from other *B. cereus* group members (Vega and Kaya, 2012).

The present study investigates the effects of a recently isolated acrystalliferous *Bacillus cereus sensu lato* strain on immature *C. capitata* survival and development in relation to specific bacterial fractions characterized through a proteomic approach.

2. Materials and methods

2.1. Bacterial strain identification and preliminary characterization

The present study was conducted with a bacterial isolate selected for its toxicity against *C. capitata* larvae in a screening program including several *B. thuringiensis*-like isolates. Among more than 300 isolates assayed, this strain was the most effective against *C. capitata* larvae, and no toxicity was observed against adults. In addition, no effects were detected on other fly species like the olive fly *B. oleae* Gmelin (Diptera: Tephritidae) and the house fly *Musca domestica* L. (Diptera: Muscidae). Pathogenic effects were associated to whole spore suspensions and not to bacterial vegetative cells (Floris et al., 2007).

Spores of this strain were found in soil samples from Zimbabwe (Southern Africa) and were permanently stored in glycerol at -80 °C in the *B. thuringiensis*-like collection of the Dipartimento di Agraria (University of Sassari, Italy). For taxonomic identification, preliminary morphological examinations under light and transmission electron microscopy (TEM) were conducted. Subsequently, different genes were amplified and sequenced. These include the *16S rRNA* gene encoding for the Small subunit ribosomal RNA, the two house-keeping genes *gyrB* and *aroE* encoding for the subunit B protein of DNA gyrase (topoisomerase type I) and for the shikimate dehydrogenase, respectively. Specific flagellin (H antigen) genes (*hag*) were also used to complement phylogenetic analyses.

Genomic DNA of the bacterium was routinely isolated from overnight cultured cells employing the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) in compliance with the manufacturer's instructions. PCRs were performed using a Veriti™ Thermal Cycler (Applied Biosystems) in a total volume of 25 μl containing 1× reaction buffer (supplied with 2.0 mM Mg^{2+}); approximately 100 ng DNA; 0.3 μM of each primer; 0.3 mM of each dNTPs; and 0.5 U of KAPA HiFi HotStart DNA polymerase (KAPA-BIOSYSTEMS, Boston, USA). The following sets of primers were used: BcF 5′-GGATTAAGAGCTTGCTCTTAT-3′ (forward) and 16S2 5′-AAGCCCTATCTCTAGGGTTT-3′ (reverse) for *16S rRNA* (Chen and Tsen, 2002); gyrB-F1 5′-ATGGAACAAAAGCAAATGCA-3′ (forward) and gyrB-R1 5′-TTAAATATCAAGGTTTTTCA-3′ (reverse) for *gyr*; aroE-F1 5′-ATCGGAAATCCAATTGGACA-3′ (forward) and aroE-R1 (5′-CCTGTCCACATTTCAAAYGC-3′ (reverse) for the *aroE* gene (Soufiane and Côté, 2009); slt-F1, 5′-ATATGCAAGCACTTCTTTT ACT-3′ (forward) and fliC-R6, 5′-ATTHGCDGGATTATCMGAAGC-3′ (reverse) for the amplification of the flagellin sequences between *slt* and *fliC* genes (Xu and Coté, 2008). To amplify the *hag* allele internal sequences, the following primers pairs were used: BtHag-F1, 5′-AGTACATGCGCCAAAACCAAG-3′ (forward) and BtHag-R1, 5′-GTTTGCTTGAGAAAGCATGCT-3′ (reverse); BtHag-F2, 5′-GGGGTTCTTAATCATGAGAA-3′ (forward) and BtHag-R2, 5′-TAACTCAAATGGCTTATTGT-3′ (reverse) (Xu and Coté, 2006).

PCR conditions were set according to the above mentioned references and PCR products were routinely analyzed by agarose gel electrophoresis using SYBR® Safe DNA stain (Life Technologies Europe BV, Bleiswijk, The Netherlands) for DNA visualization using UV transillumination. The bands of interest were excised from gel, and the DNA was extracted using a QIAquick gel extraction kit (Qiagen). Purified amplicons were supplied to the Sanger sequencing facilities of BMR Genomics (Padova, Italy) and output sequences were analyzed with the NCBI Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

In addition to taxonomic studies, genomic DNA was enriched with the bacterial plasmid isolated with PureYield™ Plasmid Midiprep System (Promega®, Madison, USA) in compliance with manufacturer's instructions, and used as a template for the detection of possible *cry* genes, using the PCR primer system designed by Noguera and Ibarra (2010) on conserved regions of the *cry* family. These analyses were conducted using *B. thuringiensis kurstaky* HD1 as a positive control.

2.2. Bacterial preparations

Bacteria were routinely grown in conical flasks containing 1 l T3 medium (Travers et al., 1987) incubated at 30 °C with shaking at 180 rpm. An aliquot (10 ml) of an overnight LB pre-culture inoculated with 1 ml heat-activated spore suspension (70 °C for 30 min) was used as *inoculum* to facilitate culture synchronization. In these conditions, culture sporulation and sporangia lysis usually occurred within 72 h, as monitored by phase microscopy. Sporulated cultures were harvested by centrifugation at 10,000 × g for 10 min at 4 °C, washed three times in sterile water to eliminate main cell debris, and quantified using a Thoma chamber (E. Hartnack, Berlin, Germany). Culture supernatants and these spore suspensions at a concentration of 2×10^9 spores/ml were stored at −20 °C until use in bioassays. As confirmed by phase microscopy observations, no parasporal crystals were detected in these spore suspensions employed in bioassays.

At a later stage, an additional cleaning step was included in preparation procedures, in order to separate spores from other insoluble components that could be harvested by centrifugation. For this purpose, whole spore suspensions obtained as previously described, were submitted to five consecutive centrifugation cycles at 5,000 × g for 3 min at 4 °C. The resulting supernatant was ultracentrifuged for 1 h at 50,000 × g to collect the insoluble extra-spore (ES) fraction, while the remaining pellet was represented by pure spores.

2.3. Bioassays

Experiments were conducted with insects from a *C. capitata* colony established in the entomology laboratory of the Dipartimento di Agraria of the University of Sassari (Italy). Insect rearing methods and conditions have been described elsewhere (Falchi et al., 2015).

Dose–response bioassays were conducted to determine the lethal effects caused by different bacterial preparations, including whole spore suspensions, culture supernatants and, at a later stage, pure spores and the insoluble extra-spore fraction. For this purpose, the bioassay design involved four replications consisting of four groups of 24 s instar larvae to be maintained in 96-well polystyrene microplates (one larva per well) filled with an artificial diet made as described in Falchi et al. (2015). The diet was incorporated with the different bacterial preparations to be assayed or was left untreated (control). Insects were routinely incubated inside a growth chamber at 25 °C and 60% relative humidity, and were inspected daily. In line with similar experiments conducted with fly larvae, mortality data were assessed after 5 days (Floris et al., 2007; Ruiu et al., 2007).

In preliminary experiments, the spore suspension obtained harvesting and washing the whole sporulated culture was assayed at a

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