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Insect-toxic secreted proteins and virulence of the entomopathogenic fungus *Beauveria bassiana*

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ABSTRACT

Fungal virulence has been mostly associated with cuticle-degrading enzymes that can be regulated depending on nutrient conditions. However, few studies have related fungal virulence to insect-toxic secreted proteins. Here, we describe how the presence of secreted toxic proteins may be linked to conidial virulence, which can be affected by nutrient factors. In this study we evaluated: (1) the virulence of the conidia of four *Beauveria bassiana* strains (EABb 01/103-Su, EABb 01/12-Su, EABb 01/88-Su and EABb 01/110-Su) grown on three different media (malt extract agar (MA), Rice (Rice), Sabouraud dextrose agar (SDA) and harvested from the cadavers of fungal-infected *Galleria mellonella* larvae (CAD) and (2) the toxicity of the crude soluble protein extracts (CSPEs) obtained from Adamek's liquid medium inoculated with these conidia. Conidial suspensions were obtained from the four media, assessed on *G. mellonella* larvae and used to produce CSPEs that were injected into healthy *G. mellonella* larvae. The larvae were also injected with conidia obtained from MA and CAD cultures to expose them to *in vivo*-secreted proteins. For all isolates, the CAD conidia were by far the most virulent, followed by conidia grown on SDA, Rice and MA. The injected CSPEs showed the same toxicity trends as the conidial suspensions. In addition, the outcomes of injection of the *in vivo*-secreted proteins showed that the toxic proteins secreted *in vitro* by the EABb 01/110-Su strain are not produced *in vivo*. However, the other strains produced toxic proteins both *in vivo* and *in vitro*, suggesting that these toxic proteins may be virulence factors involved in invertebrate pathogenesis.

1. Introduction

The entomopathogenic mitosporic ascomycete *Beauveria bassiana* (Bals.) Vuill. is an important natural pathogen of insects that has been developed as a microbial insecticide for use against many major arthropod pests in agricultural, urban, forest, livestock and aquatic environments (Charnley and Collins, 2007; Faria and Wraight, 2007). Mycoinsecticides based on *B. bassiana* are currently the most common (33.9%), followed by those derived from *Metarhizium anisopliae* (33.9%), *Isaria fumosorosea* (5.8%) and *Beauveria brongniartii* (4.1%) (Faria and Wraight, 2007). However, increasing the market share of these fungi, particularly *B. bassiana*, will depend upon accelerating their killing speed, probably the major obstacle limiting their exploitation as mycoinsecticides (St Leger and Wang, 2009). Research and development efforts in the last decade have led to some optimization of the preparation and application of fungal inoculum (Wraight et al., 2001); thus, one suggested approach to improve the virulence of mycoinsecticides is genetic modification (St Leger and Wang, 2009). This approach requires precise information on the mechanisms involved in pathogenesis and host death, key components that are amenable to improvement via biotechnology (Hegedus and Khachatourians, 1995; St Leger and Screen, 2001).

Liquid culture screening has been shown to be an attractive approach for identifying putative environmental signals because it requires little knowledge of biosynthetic loci and because it is more adaptable to the simultaneous detection of multiple metabolites. It has been reported in the last few decades that factors identified *in vitro* using liquid culture screening do indeed act as important virulence factors of phytopathogenic fungi and of microbial control agents such as entomopathogenic bacteria and entomopathogenic fungi (Bowen and Ensign, 1998; Fang et al., 2005; Siddiqui and Shaukat, 2004; St Leger et al., 1997; St Leger and Screen, 2001; St Leger and Wang, 2009). Based on this approach, most studies on the virulence factors of entomopathogenic fungi have been directed at elucidating the most relevant cuticle-degrading enzymes (Griesch and Vilcinskas, 1998; Khachatourians, 1996; St Leger et al., 1996) because their overexpression in engineered strains results in more fungi that are more deadly towards insects (Fang et al., 2005; St Leger et al., 1996). However, little attention has been focused on post-penetration events, particularly the release of soluble toxic fungal proteins during colonization by the fungus.

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Regardless of the spectrum of diverse toxic compounds – including small secondary metabolites and cyclic peptides that are identified in *B. bassiana* cell-free culture supernatants (beauvericin, bassianolide, oosporein, oxalic acid, etc.) (Vey et al., 2001; Zimmermann, 2007), very little is known about the *in vitro* or *in vivo* secretion of toxic proteins by *B. bassiana* during the infection process. The only insect-toxic proteins secreted by *B. bassiana* that have so far been purified to homogeneity are bassiacridin (Quesada-Moraga and Vey, 2004) and a chitosanase-like protein (Fuguet et al., 2004), which were highly toxic when injected into orthopteran and lepidopteran hosts, respectively.

The aim of this work was to determine whether a correlation exists between the *in vitro* and *in vivo* production of insect-toxic proteins and the virulence of four *B. bassiana* strains against *Galleria mellonella* (L.) (Lepidoptera; Pyralidae), a model insect host for the study of pathogenesis (Mylonakis, 2008).

2. Materials and methods

2.1. Fungal strains

The four strains of *B. bassiana* were isolated from diverse places in the southern Iberian Peninsula (Table 1). These strains were selected because their protein extracts (Quesada-Moraga et al., 2006) induce high mortality within a few hours and caused melanization of the integument in preliminary assays (Quesada-Moraga, unpublished data). Slant monoconidial cultures of each strain were grown on malt extract agar (MA) at 25 °C in the dark and then stored at 4 °C.

2.2. Insect host

The bioassays were performed with 5th-instar *G. mellonella* larvae from a healthy colony established at our laboratory. *G. mellonella* was selected as an experimental host because it provides some unique advantages for the study of fungal pathogenesis (Mylonakis, 2008). Moreover, this lepidopteran has been previously used by several authors to study the pathogenesis of entomopathogenic fungi (Fuguet and Vey, 2004; Quesada-Moraga et al., 2006; Quesada-Moraga and Vey, 2003; Schuhmann et al., 2003). Larvae were reared in glass jars at 28 °C and 40% relative humidity, and they were fed with a mixture of 30.8 g of corn flour, 30.8 g of wheat germ, 30.8 g of wheat bran, 10.8 g of brewer's yeast, 27 ml of glycerol and 48.6 ml of honey per 200 g of artificial diet.

2.3. Fungal preparations

Based on previous studies (Quesada-Moraga and Vey, 2003), we used two mycological media to obtain conidia from the four fungal strains with different degrees of virulence: malt agar (MA) and sabouraud dextrose agar (SDA). We also used two other substrates to harvest conidia: cooked sterilized Rice (Rice) and cadavers of fungal-infected *G. mellonella* (CAD). The four fungal strains were sequentially subcultured three times on each substrate: MA, SDA, Rice and CAD. Mycological media (MA and SDA) were purchased from Panreac (Barcelona, Spain). To obtain conidia from *G. mellonella* cadavers, 8 μl of a spore suspension adjusted to 10^5 conidia/ml was injected into 5th-instar larvae and dead insects were collected and placed in humid chambers (100% RH) at 25 °C to stimulate sporulation. This procedure was repeated to pass the fungus three times through the host. Sterile cooked Rice plates were prepared by placing 100 g of Rice grains with 300 ml of dH_2O in 200-mm glass Petri dishes before autoclaving the plates at 120 °C for 20 min. The conidia harvested from every third subculture were used either to assess the virulence of the fungi or the *in vitro* and *in vivo* production of toxins. Conidial suspensions were prepared by scraping the conidia into sterile 0.1% Tween 80 aqueous solution. All the conidial suspensions were filtered through cheese cloth to remove mycelia and their concentration was determined by direct counting using a hemocytometer. Before the preparation of suspensions, the viability of conidia was checked using germinating tests in liquid Czapek–Dox medium supplemented with 1% (w/v) yeast extract. Germination rates were greater than 95% in all the experiments. The conidia obtained after three passages through MA, SDA, Rice and CAD were respectively designated $conidia_{MA}$, $conidia_{SDA}$, $conidia_{Rice}$ and $conidia_{CAD}$.

Table 1
Identity of *Beauveria bassiana* isolates from the culture collection at C.R.A.F. Department of the University of Cordoba assayed against *Galleria mellonella* larvae.

Isolate	Habitat	Place and year of isolation
EABb 01/12-Su	Soil (non-cultivated)	Seville (Spain), 2001
EABb 01/88-Su	Soil (sunflower)	Vila Velha (Portugal), 2001
EABb 01/103-Su	Soil (forest)	Seville (Spain), 2001
EABb 01/110-Su	Soil (oak)	Seville (Spain), 2001

2.4. Production of insecticidal fungal proteins in Adamek's liquid medium

Four conidial suspensions were obtained from each conidial source and strain, as described previously. Each conidial suspension was then used to obtain a different crude soluble protein extract (CSPE), such that there were four CSPEs per strain. The CSPEs obtained from liquid cultures inoculated with $conidia_{MA}$, $conidia_{SDA}$, $conidia_{Rice}$ and $conidia_{CAD}$ were designated as $CSPE_{MA}$, $CSPE_{SDA}$, $CSPE_{Rice}$ and $CSPE_{CAD}$. The different CSPEs were obtained as follows. To prepare a primary culture, 1 ml of conidial suspension (adjusted to 10^7 spores/ml) was inoculated into 100-ml Erlenmeyer flasks containing 25 ml of Adamek's liquid medium and cultured at 25 °C on a rotatory shaker (OVAN Multimix, Badalona, Spain) at 110 r.p.m. for 4 days. For large-scale growth of the fungus, 2 ml of the primary culture were transferred into 1-L Erlenmeyer flasks containing 250 ml of the same medium as above and cultured in the same way for 7 days before removing the mycelial material by filtration through Whatman No. 3chr filter paper (Whatman, Kent, UK). The CSPE was obtained by precipitating the cell-free culture with ammonium sulfate (90% saturation, Panreac, Barcelona, Spain) and centrifuging at 10,000*g* for 30 min. The resultant pellets were dissolved in dH_2O and desalted by dialyzing against 40 volumes of dH_2O for 24 h at 4 °C using dialysis tubing with a 6- to 8-KDa cutoff membrane (Spectrum Europe, Breda, The Netherlands). The desalted fractions were concentrated at 4 °C by embedding the membrane in polyethylene glycol 20,000 (Merck-Schuchardt, Hohenbrunn, Germany). Afterwards, the CSPEs were centrifuged at 10,000*g* for 10 min and filtered with a syringe filter (Sartorius, Goettingen, Germany). Finally, a protease inhibitor cocktail (Sigma, St. Louis, MO) was added to the CSPEs (20 μl/ml of CSPE) to preserve the proteins of the extracts. The concentration of soluble protein in all CPSEs was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (Merck-Schuchardt, Hohenbrunn, Germany) as the standard.

2.5. Virulence assays

The virulence of the different conidial suspensions was assessed for every strain on 5th-instar *G. mellonella* larvae. The larvae were directly immersed in groups (maximum four larvae per group) into 2 ml of the different conidial suspensions for 10 s. The conidial suspensions were applied at four different concentrations: 1.0×10^5, 1.0×10^6, 1.0×10^7 and 1.0×10^8 conidia/ml and sterile 0.1%