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The *Adh1* gene of the fungus *Metarhizium anisopliae* is expressed during insect colonization and required for full virulence

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ABSTRACT

Zymography of alcohol dehydrogenase (ADH) activity in the entomopathogenic fungus *Metarhizium anisopliae* grown under various conditions revealed that micro-aerobic growth was associated with increased ADH activity. The major ADH protein, AdhIp, was purified to homogeneity by affinity chromatography and has an estimated molecular weight of 41 kDa and an isoelectric point (p*I*) of 6.4. Peptide mass fingerprint analysis allowed the identification and cloning of the gene that encodes this protein, *Adh1*, as annotated in the *M. anisopliae* genome database. AdhIp is related to the medium-chain dehydrogenase/reductase (MDR)/zinc-dependent alcohol dehydrogenase-like family and contains conserved ADH sequence motifs, such as the zinc-containing ADH signature, the FAD/NAD binding domain and amino acid residues that are conserved in most microbial ADHs. Semi-quantitative RT-PCR analysis revealed that *Adh1* gene expression occurs at low levels during early *Plutella xylostella* infection and that the *Adh1* gene was primarily expressed at larval death and as mycelia emerge from the insect cuticle before conidiation. Antisense-RNA experiments indicated that NAD^+-dependent ADH activity was diminished by 20–75% in the transformants, and the transformants that had lower ADH activity showed allyl alcohol resistance, which indicates that reduction in ADH activity also occurs *in vivo*. Bioassays performed using antisense *adh1* transformants, which have lower ADH activity, showed that LC_{50} values were two to five times higher than the wild-type, indicating that AdhIp is required for full capability of the fungus to penetrate and/or colonize the insect.

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Introduction

Metarhizium anisopliae is an entomopathogenic fungus that has been used for biological control of several insect pests, including adult African malaria vectors (Scholte et al., 2003; Roberts and St Leger, 2004; Arruda et al., 2005; Blanford et al., 2005; Bukhari et al., 2011). Recently, another member of the *Metarhizium* genus, *M. robertsii*, was reported to be rhizosphere-competent and able to stimulate plant root development (Sasan and Bidochka, 2012). This tight interaction is evidenced by the ability of *M. robertsii* to translocate insect-derived nitrogen to plants (Behie et al., 2012).

Like other entomopathogenic fungi, *M. anisopliae* invades its host directly through the insect exoskeleton or cuticle (Clarkson and Charnley, 1996). Under aerobic conditions and proper humidity, conidia germinate on the host surface and differentiate to form a specialized adhesion structure called an "appressorium." An infection hypha emerges from this structure, penetrates the host cuticle through a combination of mechanical pressure and enzymatic degradation, and eventually enters the insect's hemocoel (Clarkson and Charnley, 1996).

Currently, little is known about the events that take place after the fungus enters the insect. After the initial penetration steps, the fungus must confront changes in micro-ambient conditions and nutrient availability. *M. anisopliae* conidia germination was found to be accompanied by a significant increase in O_2 consumption under aerobic conditions (Braga et al., 1999). Regarding nutrient availability, disaccharide trehalose is the main sugar in the hemolymph of insects (Becker et al., 1996), and *M. anisopliae* has been shown to secrete high levels of the enzyme trehalase into the hemolymph (Xia et al., 2002a,b). Purified acid trehalase can efficiently hydrolyze hemolymph trehalose into glucose *in vitro* (Zhao et al., 2006); thus, trehalose could serve as an important carbon source for pathogenic fungi that invade the insect hemolymph. If *M. anisopliae* cells growing under aerobic conditions on the insect surface have a primary oxidative metabolism, it is reasonable to speculate that these cells must adjust their metabolism to grow efficiently under the new environmental conditions during the invasion process. Given the micro-ambient conditions present in insect hemolymph, a fermentative metabolism would likely facilitate the invasion process. *M. anisopliae* grown *in vitro* on insect hemolymph exhibited increased expression of genes involved in carbohydrate metabolism (Wang and St Leger, 2005). The common final products of microbial fermentative metabolism are ethanol and CO_2. Alcohol is produced by the reduction of acetaldehyde, with $NADH^+$ acting as the reducing agent *via* alcohol dehydrogenase (ADH). Under anaerobic conditions, regeneration of NAD^+ by ADH is an essential process for energy production and growth (Breunig et al., 2000; Zeeman et al., 2000). In addition to its role in fermentative metabolism, ADH is involved in other metabolic and physiological processes in a variety of organisms, including the oxidation of alcohols as carbon and energy sources (Pateman et al., 1983; Saliola and Falcone, 1995), protection against anaerobic stress (Kelly et al., 1990), and maintenance of the intracellular redox balance (Bakker et al., 2000).

ADHs (E.C. 1.1.1.1) are oxidoreductases that catalyze the reversible oxidation of alcohols to aldehydes or ketones with the concomitant reduction of NAD^+ or $NADP^+$. ADH activity has been extensively studied in various organisms, including fungi. However, the presence or physiological role of ADH activity during the interaction between entomopathogenic fungus and their insect hosts currently remains uncharacterized. We investigated ADH activity in *M. anisopliae* grown under various conditions, and the enzyme responsible for the majority of ADH activity during aerobic and micro-aerobic growth of this fungus was purified and characterized. To understand the role of ADH activity, an internal fragment of the *Adh1* gene from *M. anisopliae* was used to reduce *Adh1* gene expression by antisense expression. The pathogenic activity against *Plutella xylostella* larvae was monitored in these antisense-expressing transformants, and a significant increase in the dose required to kill 50% of the hosts (LC_{50}) was observed.

Materials and methods

Organism and culture conditions

M. anisopliae strain Ma10 was obtained from the Centro Nacional de Referencia (Colima, Mexico). The fungus was propagated in minimal medium, consisting of 0.2% NH_4NO_3, 0.3% KH_2PO_4, and 2%, 50X salt stock (25 g $MgSO_4$, 0.09 g $ZnSO_4$, 0.05 g $FeSO_4$, 0.015 g $MnSO_4$, and 0.02 g $CuSO_4$), as previously described (Morales Hernandez et al., 2010). To obtain aerobically grown mycelia, conidia (1×10^6 mL^{-1}) were inoculated in liquid minimal medium containing either 2% glucose or 2% glycerol as the carbon source, complex Sabouraud dextrose medium or Sabouraud trehalose medium. The cultures were incubated on an orbital shaker at 28 °C with shaking at 160 rpm. To produce mycelia under micro-aerobic conditions, conidia were grown as previously described (Salcedo-Hernandez and Ruiz-Herrera, 1993). Briefly, 250-mL Erlenmeyer flasks containing 250 mL Sabouraud dextrose medium or Sabouraud trehalose medium and a magnetic stir bar were inoculated with 1×10^6 conidia mL^{-1}. Flasks were sealed with rubber stoppers, and needles were inserted into the stoppers to permit the escape of excess CO_2; under these conditions, a low O_2 tension was imposed after 2–3 h of incubation (Salcedo-Hernandez and Ruiz-Herrera, 1993).

Obtaining cell-free extracts

Mycelia were produced as described above, and cell-free extracts were obtained as previously described (Torres-Guzman et al., 1994) with some modifications. Briefly, mycelia were washed and suspended in 500 μL of 0.05 M Tris–HCl (pH 8.5) containing complete protease inhibitor cocktail (Roche). The cell suspension was mixed with glass beads and disrupted using a Braun MSK cell homogenizer (B. Braun, Biotech) 18 times for 30 s each using a CO_2 stream. The cell homogenate was centrifuged at $126{,}963 \times g$ for 1 h at 4 °C in an Optima XL-100 ultracentrifuge (Beckman-Coulter). Both the mixed membrane fraction (pellet) and soluble fraction (supernatant) were stored at −70 °C for subsequent protein and enzymatic analysis.

Enzyme assays and protein determination

ADH activity was determined as previously described (Bergmeyer et al., 1983) and monitored using a Beckman DU 650 spectrophotometer (Beckman-Coulter). Enzymatic assays were performed in 1 mL reaction mixtures containing 50 mM Tris–HCl (pH 8.5), 2 mM NAD^+, cell-free extract (100–200 mg protein) or 10 μg of purified protein, and 0.8 M ethanol. The reaction was initiated by the addition of ethanol and NAD^+, and reduction was monitored by the increase in absorbance at 340 nm over a 3-min period. One unit of enzyme activity was defined as the amount required to reduce 1 μmol of NAD^+ per minute at 25 °C. Specific ADH activity was expressed as units per milligram of protein. The values shown in the results are the means from three experiments carried out in duplicate; the variation coefficient (*i.e.*, the standard deviation expressed as a percentage of the mean) was less than 20%. Zymogram detection of ADH activity was performed following electrophoresis on non-denaturing 12% polyacrylamide gels, as previously described (Torres-Guzman et al., 1994). The protein concentration was measured as previously described (Lowry et al., 1951) using bovine serum albumin as a standard.