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RESEARCH ARTICLE

Oxidative stress in entomopathogenic fungi grown on insect-like hydrocarbons

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Abstract Entomopathogenic fungi mostly attack their insect hosts by penetration through the cuticle. The outermost insect surface is covered by a lipid-rich layer, usually composed of very long chain hydrocarbons. These fungi are apt to grow on straight chain hydrocarbons (alkanes) as the sole carbon source. Insect-like hydrocarbons are first hydroxylated by a microsomal P450 monooxygenase system, and then fully catabolized by peroxisomal β-oxidation reactions in *Beauveria bassiana*. In this review, we will discuss lipid metabolism adaptations in alkane-grown fungi, and how an oxidative stress scenario is established under these conditions. Fungi have to pay a high cost for hydrocarbon utilization; high levels of reactive oxygen species are produced and a concomitant antioxidant response is triggered in fungal cells to cope with this drawback.

Keywords *Beauveria bassiana* · Alkane degradation · Reactive oxygen species · Antioxidant enzymes

Introduction

Broad host range entomopathogenic fungi attack insect hosts via attachment to their surface. The production of degrading enzymes that help penetration through the insect cuticle was early reported in *Beauveria bassiana* s.l. and *Metarhizium anisopliae* s.l. (Ferron 1985; St. Leger et al. 1986a, b). Surface structure and the chemical composition of the cuticle are both believed to affect this process, which can be divided into three successive stages. (1) Adsorption at the interface between the propagules and the insect epicuticle: this first step may involve specific receptor–ligand and/or nonspecific hydrophobic and electrostatic mechanisms (Boucias and Pendland 1991). (2) Fungal germination and development on the insect cuticle: early events in spore germination require an exogenous carbon source. Both germination stimulators and inhibitors have been reported in the cuticle (Smith and Grula 1981; Saito and Aoki 1983; Lecuona et al. 1997). (3) Fungal penetration: germ tubes must pass through the different cuticular layers. This process depends both on the intrinsic properties of the germ tube and on the physiological state of the host and it is essential for infection occurrence. Successful pathogens overcome any defensive reaction and penetrate into the hemocoel. Finally, the fungus replicates as budding hyphal bodies invading the internal cavity; insect death takes place shortly after.

The ability of entomopathogenic fungi to degrade insect hydrocarbons and utilize them for energy production and for the biosynthesis of cellular components was first shown in *B. bassiana* s.l. and *M. anisopliae* s.l. (Napolitano and Juárez 1997). Furthermore, we have proposed a relationship between insect cuticular hydrocarbon composition and fungal virulence. Insects containing a blend of saturated straight and branched chains as major cuticular hydrocarbons are more susceptible to entomopathogenic fungi than those insects with predominance of unsaturated (alkenes and/or alkadienes) chains and/or the presence of some compounds showing an antibiotic role, such as quinone derivatives or short chain lipids (Pedrini et al. 2007). In addition,

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B. bassiana s.l. grown on an insect-like hydrocarbon containing media were more virulent than glucose-grown fungi producing higher insect mortality or lesser mean lethal time against different insect hosts (Crespo et al. 2002; Pedrini et al. 2009). These data indicate that alkane degradation by *B. bassiana* s.l. represents a key metabolic pathway related to the insect pathogenic nature of the fungus. Although it is a poorly studied field, it might be also important during its saprophytic stage as an alkane-assimilating microorganism in soil remediation processes.

Focusing on their pathogenic nature, it is likely that fungi are exposed to high levels of stress in all the stages described, in particular during penetration through the cuticle to reach the insect hemocoel. Aiming to contribute to a better understanding of the metabolic challenges faced by alkane-grown fungi, we will focus on the occurrence of oxidative stress and the antioxidant response during degradation of insect-like hydrocarbons.

Biochemistry of alkane degradation by entomopathogenic fungi

Although the major bulk components of the insect cuticle are protein and chitin, the outermost epicuticular surface layer comprises a complex mixture of non-polar lipids, mainly composed by very long chain hydrocarbons. The catabolic pathway for hydrocarbon degradation by entomopathogenic fungi was first shown by Napolitano and Juárez (1997). Insect hydrocarbons were shown to be a much better fuel than synthetic analogs; fungal cultures grown on insect hydrocarbons produced a higher yield and were more compact than cultures grown on synthetic ones. However, fungi grown in either insect or synthetic hydrocarbons as the sole carbon source showed a significant delay in growth compared with fungi grown in rich media containing glucose and yeast extract (Napolitano and Juárez 1997). More recent data showed that *B. bassiana* s.l. is able to grow on a wide range of synthetic straight chain alkanes (up to *n*-C33), but failed to assimilate longer alkanes from 35 to 41 carbons (Pedrini et al. 2010). Using radio-labeled hydrocarbons, a hydrocarbon degradation pathway was proposed, in analogy with yeast systems. Radioactivity was incorporated into a variety of fungal lipids; and also evidence of fungal ability to fully degrade [1-^{14}C]*n*-hexadecane to [^{14}C]CO_2 was obtained (Napolitano and Juárez 1997; Crespo et al. 2000).

Growth on alkanes causes major changes in fungal metabolism. In this regard, an exhaustive study on lipid metabolism was conducted in the late 1990s by Juárez's group (Napolitano and Juárez 1997; Crespo et al. 2000; Juárez et al. 2000). The major lipid fractions of fungi grown in complete medium were sterols, whereas large amounts of triacylglycerols were evident in alkane-grown fungi. This difference was minimized after 14 days at 4 °C; the alkane-grown fungi showed a lipid profile similar to that of glucose-grown cultures. However, low-temperature storage did not alter the lipid profile of fungi cultured in complete medium without alkanes, suggesting that lipidic components of alkane-grown cells are present in a rather dynamic pool (Juárez et al. 2000). The unsaturated/saturated ratio of the fatty acid pattern was found to be markedly diminished and the relative composition was largely modified in alkane-grown *B. bassiana*. The higher levels of saturated fatty acids could cause a decrease in membrane fluidity, affecting permeability parameters and nutrient transport. Large amounts of odd-chain fatty acids (heptadecanoic and heptadecenoic acids) were reported (Juárez et al. 2000), in coincidence with previous reports in yeasts growing on alkanes (Bizzi et al. 1980). Also bacteria (e.g., *Pseudomonas oleovorans*) growing on hydrocarbons change their physiology, membrane morphology and properties (Chen et al. 1995), suggesting a common behavior for microbial assimilation of hydrocarbons. These results prove that alkane assimilation affect the fungal metabolism; thus, a scenario of oxidative stress caused by the accumulation of reactive oxygen species (ROS) might be expected in alkane-grown cells. Subsequently, the antioxidant defense system might be activated to avoid ROS harmful effects on biomolecules, such as damage to DNA, proteins, and membrane lipids.

Fungal genes and enzymes involved in hydrocarbon oxidation

The mechanism of hydrocarbon degradation starts with alkane activation by terminal, diterminal or subterminal oxidation to the corresponding primary or secondary alcohol. The primary alcohol is further oxidized by alcohol and aldehyde dehydrogenases. The resulting activated fatty acids might be incorporated in membranes, stored as triacylglycerides, or enter the β-oxidation pathway in peroxisomes (Juárez et al. 2004; Pedrini et al. 2007). Some of the genes potentially involved in this process and their putative functions are shown in Table 1.

The first oxidation round of the alkane substrate is catalyzed by a microsomal cytochrome P450 enzyme system. As part of a pre-genomic effort, we have characterized eight cytochrome P450 genes (CYP) encoding enzymes with putative specificity for alkanes (Pedrini et al. 2010); most of them have been partially characterized after heterologous expression in yeast (Zhang et al. 2012; Pedrini et al. 2013). Gene knockouts revealed a phenotype for only one (cyp52X1) of six genes examined to date. CYP52X1 oxidizes long chain fatty acids and participates in the

Table 1 Alkane degradation by entomopathogenic fungi: genes and enzymes involved in oxidation reactions and scavenging of the reactive oxygen species produced during this process

Gene	Putative function	Induced by[a]	References	Enzyme activity and/or characterization	References
CYP52X1	*n*-Alkane hydroxylation	*n*-C16, *n*-C20, *n*-C24, *n*-C28 and insect hydrocarbons	Pedrini et al. (2010)	Heterologous expression. Fatty acid hydroxylation	Zhang et al. (2012)
CYP655C1	*n*-Alkane hydroxylation	*n*-C16, *n*-C20, *n*-C24, *n*-C28 and insect hydrocarbons	Pedrini et al. (2010)	ND	
CYP5337A1	*n*-Alkane hydroxylation	*n*-C16, *n*-C20, *n*-C24, *n*-C28 and insect hydrocarbons	Pedrini et al. (2010)	Heterologous expression	Pedrini et al. (2013)
CYP52G11	*n*-Alkane hydroxylation	*n*-C16, *n*-C20, *n*-C24, *n*-C28 and insect hydrocarbons	Pedrini et al. (2010)	ND	
CYP539B5	*n*-Alkane hydroxylation	*n*-C16, *n*-C20, *n*-C24, *n*-C28 and insect hydrocarbons	Pedrini et al. (2010)	ND	
CYP617N1	*n*-Alkane hydroxylation	*n*-C16, *n*-C20, *n*-C24, *n*-C28 and insect HC	Pedrini et al. (2010)	Heterologous expression	Pedrini et al. (2013)
CYP53A26	*n*-Alkane hydroxylation	*n*-C16, *n*-C20, *n*-C24, *n*-C28 and insect hydrocarbons	Pedrini et al. (2010)	Heterologous expression. CO differential spectrum (P450 content)	Pedrini et al. (2013)
CYP584Q1	Unknown	–	Pedrini et al. (2010)	ND	Pedrini et al. (2013)
Acyl-CoA oxidase	β-Oxidation pathway	*n*-C16 and *n*-C28	Huarte-Bonnet and Pedrini, unpublished	Oxidation of acyl-CoAs from 16 to 24 carbons	Alconada and Juárez (2006)
catA (spore-specific)	H_2O_2 scavenging	*n*-C16 and *n*-C28	This work	Catalase activity induced in crude homogenate	Pedrini et al. (2006, 2007) and Ali et al. (2013)
catB (secreted)	H_2O_2 scavenging	*n*-C16	This work	Catalase activity induced in crude homogenate	Pedrini et al. (2006, 2007) and Ali et al. (2013)
catP (peroxisomal)	β-Oxidation pathway	*n*-C16 and *n*-C28	This work	Catalase activity induced in peroxisomes	Pedrini et al. (2006)
catC (cytoplasmic)	H_2O_2 scavenging	*n*-C16	This work	Catalase activity diminished in cytosol	Pedrini et al. (2006)
catD (secreted peroxidase/catalase)	H_2O_2 scavenging	–	This work	Catalase activity induced in crude homogenate	Pedrini et al. (2007) and Ali et al. (2013)
sod1 (Cu/Zn- SOD)	Superoxide radicals scavenging	*n*-C16 and *n*-C28	This work	Superoxide dismutase activity induced in crude homogenate	This work Ali et al. (2013)
sod2 (Mn-SOD)	Superoxide radicals scavenging	–	This work	Superoxide dismutase activity induced in crude homogenate	This work Ali et al. (2013)
sod3 (Mn-SOD)	Superoxide radicals scavenging	*n*-C28	This work	Superoxide dismutase activity induced in crude homogenate	This work Ali et al. (2013)
gpx	Hydroperoxides scavenging	*n*-C16	This work	GPx activity induced in crude homogenate	Ali et al. (2013)
gst	Detoxification	*n*-C16	This work	GST activity diminished in crude homogenate	This work

ND non determined

[a] Genes showing at least twofold induction, compared with a reference culture grown on glucose-containing media, are detailed. CYP gene expression was evaluated in fungi grown on each of the following hydrocarbons: *n*-C16, *n*-C20, *n*-C24, *n*-C28, and insect cuticle hydrocarbons. All other genes were evaluated in fungi grown either on *n*-C16 or *n*-C28

degradation of specific epicuticular lipid of the model host *Galleria mellonella* (Zhang et al. 2012). However, no phenotype with respect to growth or germination on *G. mellonella* lipids or virulence against the same host has been noted for other mutants obtained (Pedrini et al. 2013). Single gene knockout may not display any noticeable phenotypes, perhaps due to the potential redundancy and/or overlapping substrates specificities of these enzymes. The recent release of *B. bassiana* s.l. complete genome (Xiao et al. 2012) has revealed the existence of 77 CYP genes, reinforcing the hypothesis that overlapping specificity of several CYP genes is a key factor on targeting the surface lipids of a wide variety of insect hosts. Besides producing a fatty alcohol as the primary oxidation product, a single P450 form in yeast was also shown to catalyze the production of a cascade of monooxidation products, followed by diterminal oxidation and finally yielding α-ω acids (Scheller et al. 1998). Further studies in entomopathogenic fungi are needed to identify all the genes involved in each stage, and their role to achieve the efficient hydrocarbon degradation observed.

The fatty alcohol—or eventually the fatty acid—will traverse the peroxisomal membrane, and after successive transformations by the concerted action of alcohol dehydrogenase, aldehyde dehydrogenase and acyl-CoA synthetases, the appropriate fatty acyl-CoA will be available for complete β-oxidation. Little is known about the potential role of these enzymes involved in the oxidation of the fungal P450 products. In *M. anisopliae* s.l., two aldehyde dehydrogenase genes were shown to be up-regulated when the fungus is grown in the presence of insect cuticles (Freimoser et al. 2005). This finding is probably related to the availability of intermediate metabolites of the cuticle hydrocarbon oxidation pathway.

Fatty acids synthesized by these enzyme systems are completely catabolized through peroxisomal β-oxidation; proliferation of small-size peroxisomes was evident in alkane-grown *B. bassiana* (Crespo et al. 2000). The first enzyme involved in very long chain fatty acids peroxisomal β-oxidation is an acyl-CoA oxidase, which donates electrons to molecular oxygen. This oxidase reaction accompanies the emission of toxic by-product reactive oxygen molecules including superoxide anion and hydrogen peroxide; thus, superoxide dismutase and catalase activities are essential to detoxify them in peroxisomes. A search into *B. bassiana* genome revealed that only one acyl-CoA oxidase seem to be present, in contrast with the alkane-assimilating yeast *Yarrowia lipolytica* that possesses five acyl-CoA oxidase genes (Wang et al. 1999). A significant increment in acyl-CoA oxidase activity was observed in peroxisome fractions of *B. bassiana* grown either on *n*-C16, *n*-C24 or *n*-C28 as compared with the same fraction of glucose-grown fungi. Moreover, when different acyl-CoA substrates with chain length from 16 to 24 carbons were used as substrates, acyl-CoA activity was increased progressively with carbon number, from palmitoyl to lignoceroyl-CoA (Alconada and Juárez 2006), suggesting that only one enzyme is capable of β-oxidizing acyl-CoAs of different chain length with different specificities.

Antioxidant defense system in alkane-grown fungi

Superoxide dismutases

B. bassiana s.l. has three genes encoding for superoxide dismutases (Xie et al. 2010, 2012). The *sod1* gene is translated as a Cu/Zn-SOD, which is active in cytoplasm and requires a posttranslational activation by a specific metallochaperon. This protein inserts the Cu^{2+} cofactor into active sites and catalyzes the oxidation of a conserved disulfide bond that is essential for the SOD activity. Cytosolic SOD activity was found to be influenced by carbon, nitrogen, and metal ion sources in the fungus *Cordyceps militaris* (Wang et al. 2006). Ali et al. (2013) reported an increment in SOD activity measured in crude homogenate of the entomopathogenic fungus *Isaria fumosorosea* grown either in chitin, chitosane, or hydrocarbons (*n*-C16, *n*-C24, and *n*-C28) compared with the controls. In *B. bassiana* s.l., SOD activity was significantly higher in fungi grown in *n*-C28 as the sole carbon source than in the controls grown in a glucose-containing medium (Fig. 1a). Regarding the gene expression pattern, Fig. 2 show that *B. bassiana sod1* was highly induced in *n*-C28 (22.0 ± 7.4 fold induction) and in *n*-C16 (6.7 ± 0.01 fold induction). On the other hand, manganese-containing superoxide dismutases (Mn-SODs) were described in mitochondrial matrix, peroxisomes, and cytosol of several fungi (Fridovich 1995; Fang et al. 2002; Karlsson et al. 2005). In *B. bassiana* s.l., cytosolic *Mn-sod2* and mitochondrial *Mn-sod3* have been characterized; by knockout and RNAi mutants approaches both genes showed significant phenotypic alterations, co-contributing to the biocontrol potential of *B. bassiana* s.l. (Xie et al. 2012). Unfortunately, alkane assimilation in these mutants has not yet been examined. Although little or no induction was observed for *sod3* (<2.5-fold induction) and *sod2* (<1 fold induction) in alkane-grown *B. bassiana* (Fig. 2), both genes were more expressed than *sod1* in *B. bassiana* s.l. exposed to the pyrethroid insecticide deltamethrin (Forlani et al. 2014); suggesting functionally complementary effects among them to cope with the superoxide ions generated by different sources. Although no information about enzyme activity in subcellular fractions is available, the gene expression results suggest that *sod1* rather than other *sod* genes are mostly responsible for the high activity detected in crude homogenate of alkane-grown fungi (Fig. 1a).

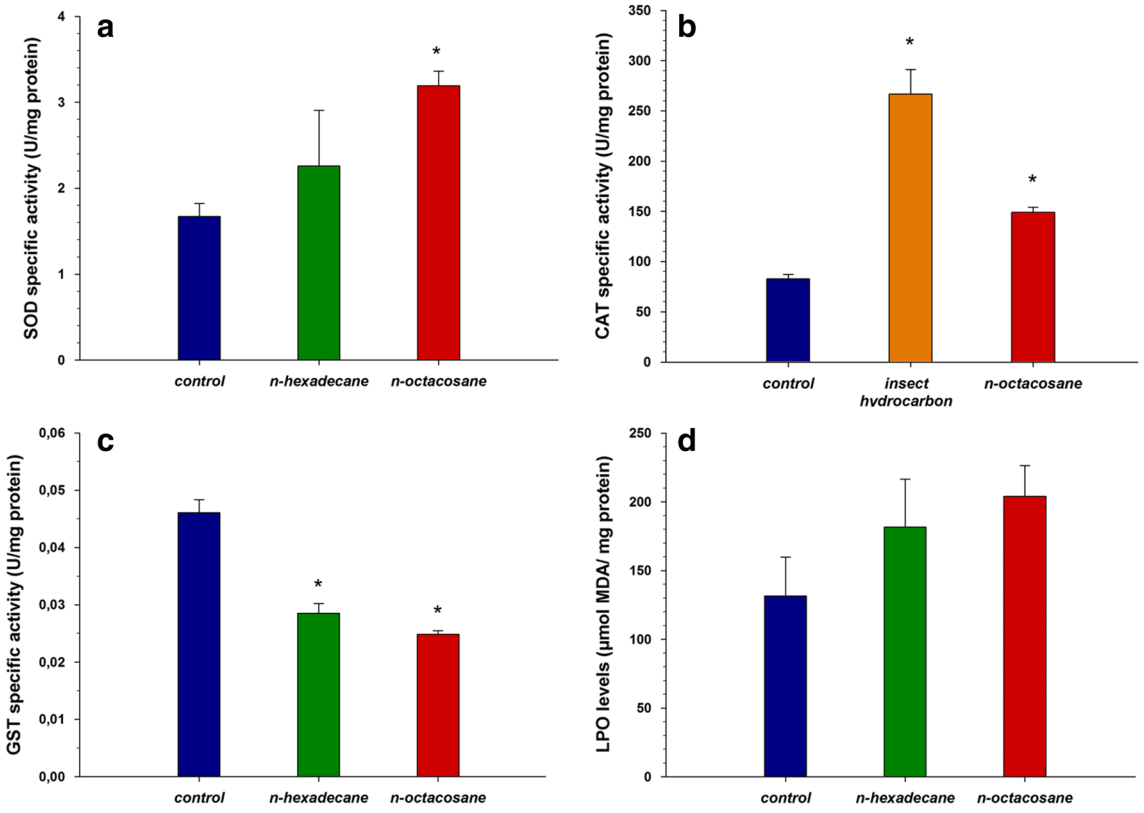

Fig. 1 The antioxidant system of *B. bassiana*. Fungal cultures were homogenized by cell disruption (Forlani et al. 2014) after growth in minimal media containing either different synthetic hydrocarbons or *Triatoma infestans* cuticle hydrocarbons as the sole carbon source. Control cultures were grown in complete media containing glucose and yeast extract. **a** Superoxide dismutase (SOD). **b** Catalase (CAT). **c** Glutathione-*S*-transferase (GST). **d** Lipid peroxidation (LPO). All the parameters were evaluated by spectrophotometry as described [Marklund and Marklund 1974 (SOD), Beers and Sizer 1952 (CAT), Habig et al. 1974 (GST), Buege and Aust 1978 (LPO)]. Values are mean ± SEM ($n = 3$). The *asterisk* indicates significant differences ($P < 0.05$) between glucose- and alkane-grown fungi by the Student's *t* test. CAT activity is reproduced from Pedrini et al. (2007)

Thus, the cytosolic activity corresponding to the *sod1* gene expression appear to be important scavenging intracellular ROS and might act as the first-line enzymes in the cellular defense system against superoxide damage triggered by growth in alkanes.

Catalases

The typical peroxisomal marker enzyme is catalase, a hemoprotein that decomposes excess H_2O_2 produced by β-oxidation. Peroxisomal catalase induction was observed in *n*-C28-grown *B. bassiana*, a high increment in the catalase activity (14-fold) was measured in these fungi compared with control cultures grown in complete medium (Pedrini et al. 2006). There are several potential sources of H_2O_2 other than β-oxidation; thus, catalase is also a marker enzyme of oxidative stress. Catalase activity was also detected in the cytosolic fraction of *B. bassiana* s.l., although this isoform was not induced in the same culture condition (Pedrini et al. 2006). Figure 1b shows the increment in catalase activity in a crude homogenate of *B. bassiana* grown in media containing either *n*-C28 or insect cuticular hydrocarbons, compared with the controls, in coincidence with the results reported in alkane-grown *I. fumosorosea* (Ali et al. 2013). The catalase family of *B. bassiana* s.l. consists of five genes namely, *catA* (spore-specific), *catB* (secreted), *catC* (cytoplasmic), *catD* (secreted peroxidase/catalase), and *catP* (peroxisomal) (Wang et al. 2013). The latter was induced in alkane-grown *B. bassiana*, the expression level was 4.5 ± 2.2 (*n*-C28) and 2.5 ± 0.1 (*n*-C16) higher than glucose-grown fungi (Fig. 2). *catC* was similarly induced after growth in *n*-C16, but no induction was observed after growth in *n*-C28, in harmony with the lower cytosolic enzyme activity previously reported for this growth condition (Pedrini et al. 2006). Other *cat* genes induced under these conditions were both spore-specific and secreted catalases. In *n*-C28-grown fungi, *catA* transcript expression was 5.8 ± 1.6 folds higher than controls, whereas *catB* expression was 4.5 ± 0.8-fold-induced in medium containing *n*-C16 as the sole carbon source.

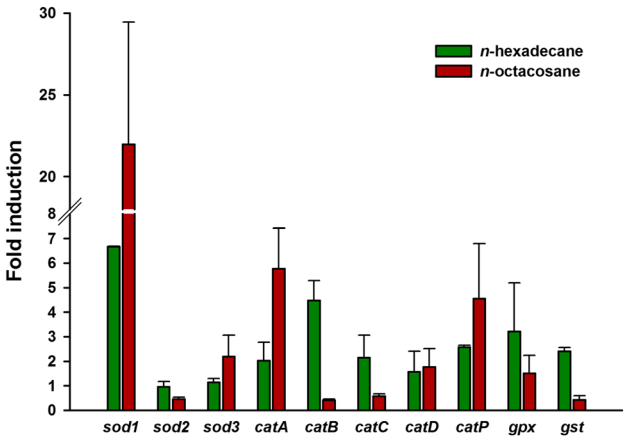

Fig. 2 *B. bassiana* antioxidant gene expression analysis as determined by qRTPCR during growth on either *n*-hexadecane or *n*-octacosane as the sole carbon source. Data are normalized to the expression values obtained during growth on glucose-containing media. Actin was used as housekeeping gene. The primers and run conditions for amplification of the different genes encoding for superoxide dismutase (*sod*), catalase (*cat*), glutathione peroxidase (*gpx*) and glutathione transferase (*gst*) were previously described (Forlani et al. 2014)

Finally, secreted catalase/peroxidase displayed <1.8-fold induction in both alkane-containing media (Fig. 2). Some functions of these genes and their protein products have been studied by single targeted gene disruption mutant strains (Wang et al. 2013). Both *catP* and *catB* genes seem to be the most important contributors to the total catalase activity, with losses of 56 and 89 % for Δ*catP* and Δ*catB*, respectively. Both *catA* and *catD* appear to be important in other stressful situations, such as heat shock (45 °C) and resistance to UV-B radiation (Wang et al. 2013). Deletion of *catP*, the enzyme most likely to be involved in hydrocarbon assimilation pathways, resulted in increased sensitivity to oxidative stress both on the conidial germination level and during vegetative growth. Δ*catP* mutants were essentially unaffected in thermal sensitivity and UV-B tolerance, but displayed ~50 % decrease in virulence indicating that it is an important enzyme involved in the pathogenic process.

An increase in catalase activity was reported during conidiation, germination and early exponential growth of the filamentous fungus *Neurospora crassa* (Michán et al. 2002). In this regard, the high expression levels of both *catA* and *catB* genes detected in alkane-grown *B. bassiana*, and the correlation between higher catalase activity and faster germination reported in alkane-grown *B. bassiana* (Pedrini et al. 2007) and *I. fumosorosea* (Ali et al. 2013) compared to the controls, suggest an important function for catalases in H_2O_2 consumption by fungi, and hence facilitating conidia germination on these carbon sources. In *B. bassiana* s.l., a transcriptome analysis of fungi grown on insect cuticle showed that peroxidases and catalases are up-regulated in cuticle-germinated conidia compared with cuticle-grown mycelia (Mantilla et al. 2012). Transgenic strains of *M. anisopliae* s.l. overexpressing *cat1* gene duplicated the catalase activity of the wild type, and this gene seems to be responsible for the faster germination observed in the mutant compared with the wild type (Morales Hernandez et al. 2010). Coincidently with the situation for superoxide dismutase mutants, and although data on alkane assimilation in catalase mutants is not available, current information suggests that peroxisomal catalases might be crucial factors for adaptation to oxidative stress generated during fungal growth on insect hydrocarbons.

Glutathione system enzymes

Glutathione peroxidases (GPx) catalyze the reduction of peroxides by reduced glutathione. These enzymes are part of the glutathione system, which is known to be one of the main thiol antioxidant systems in the cells in addition to the thioredoxin system. GPx are known to reduce peroxides in the presence of two molecules of reduced glutathione (GSH), forming one molecule of glutathione disulfide (GSSG) as a by-product. Glutathione peroxidase gene expression has been studied in *Saccharomyces cerevisiae* and induction of these genes was observed in response to oxidative stress or glucose repression (Inoue et al. 1999). The two glutathione peroxidases of the human pathogen fungus *Cryptococcus neoformans* are differentially expressed in response to stress; *Gpx1* is induced in the presence of organic peroxides, while *Gpx2* is induced in response to hydrogen peroxide (Missall et al. 2005). The only one *gpx* gene in *B. bassiana* s.l. was also induced in alkane-grown fungi, with values of 3.2 ± 1.9 (*n*-C16) and 1.5 ± 0.7 (*n*-C28) fold-induction (Fig. 2). However, GPx activity in *I. fumosorosea* was reported to be higher in *n*-C28-grown fungi compared with cells grown in *n*-C16 (Ali et al. 2013). H_2O_2 can be detoxified by two different enzymes, CAT and GPx. Cooperativity between both activities has been reported (Michiels et al. 1994); CAT is often vulnerable at high levels of H_2O_2, but *gpx* expression is up-regulated to maintain the catalase activity (Baud et al. 2004). Thus, working together they help overcome the cytotoxic effect of the hydroxyl radicals and H_2O_2 generated by this stressful growth condition.

Glutathione-*S*-transferases (GSTs) are enzymes involved in cellular detoxification of both xenobiotic and endobiotic compounds. They are mostly localized in cytosol and are major phase II detoxification enzymes. These enzymes catalyze the conjugation of GSH with various electrophilic substances, playing an important role in preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (Ketterer et al. 1983). There is not much information about fungal GSTs, the genes characterized in

Phanerochaete chrysosporium, *Mucor circinelloides* and *Y. lipolytica* fit in the Theta class of the well-studied mammalian GSTs (Sheehan et al. 2001). In alkane-grown *B. bassiana*, GST activity was significantly lower than that of controls (Fig. 1c), in a similar fashion as the activity decay found in the same strain exposed to high doses of deltamethrin (Forlani et al. 2014). The expression of the unique *B. bassiana gst* gene was not significantly altered either in insect-like hydrocarbons (Fig. 2) or exposed to this xenobiotic (Forlani et al. 2014); further studies are needed to understand its role in this fungi.

Lipid peroxidation

Reactive oxygen species accumulation often correlates with high levels of lipid peroxidation (LPO), which in turn can produce serious damage in biological membranes. ROS readily attack the polyunsaturated fatty acids of the membranes, initiating a self-propagating chain reaction. To prevent this toxic situation, several enzymatic and non-enzymatic mechanisms are trigged to remove ROS from cytosol (Mylonas and Kouretas 1999). A decrease in LPO levels was reported in hydrocarbon-grown *I. fumosorosea* (Ali et al. 2013). In similar growth conditions, *B. bassiana* LPO was slightly induced, although not statistically significant (Fig. 1d). These evidences might suggest that LPO occurrence is not relevant in alkane-grown fungi, because enhanced SOD and CAT activities might be sufficient to eliminate ROS successfully. In this regard, *B. bassiana* exposed to high doses of deltamethrin significantly increased their LPO levels with a concomitant diminution in SOD and CAT activities, since the antioxidant system was surpassed by the insecticide exposure (Forlani et al. 2014). Lipoperoxidation is also an important source of organic hydroperoxides; the similar LPO levels detected in both glucose- and alkane-grown fungi could explain the null or little induction of GST measured by both enzyme activity and gene expression.

Conclusion

A hyperoxidant state is defined as an unstable and transient situation trigged by different stress conditions, such as microbial differentiation processes, dimorphic growth and anaerobic conditions (Hansberg and Aguirre 1990). It was described during cell differentiation in *N. crassa*, in correlation with high levels of catalase activity (Michán et al. 2003). During a hyperoxidant state, the amount and ratio of NAD(P)H/NAD(P) and glutathione/glutathione disulfide changes dramatically (Toledo et al. 1991, 1995) and this misbalance might affect many metabolic fluxes. A hyperoxidant state might also be generated by other stress situations that can surpass the antioxidant response of the cell. To overcome the stressful scenario of growth on insect-like hydrocarbons as the sole carbon source, entomopathogenic fungi must address important challenges. The induction of antioxidant enzymes in hydrocarbon-grown fungi seems to be a key factor. Gene silencing approaches including targeted knockout by homologous DNA recombination and/or gene knockdown carried out by antisense RNAs techniques are needed to elucidate the physiological role of these genes and enzymes, as well as to confirm whether a hyperoxidant state is established during alkane assimilation processes by entomopathogenic fungi.

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