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Distinct contributions of one Fe- and two Cu/Zn-cofactored superoxide dismutases to antioxidation, UV tolerance and virulence of *Beauveria bassiana*

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ABSTRACT

Beauveria bassiana, a filamentous entomopathogen, has five distinct superoxide dismutases (SODs), including cytosolic and mitochondrial MnSODs (Sod2/3) which have proved contributing primarily to intracelluar SOD activity and additively to antioxidation and virulence. Here we characterized cytosolic Cu/ZnSOD (Sod1), mitochondrial FeSOD (Sod4) and cell wall-anchored Cu/ZnSOD (Sod5). The latter two are unexplored despite existence in many filamentous fungi, and their subcellular localization was well confirmed with specifically stained cells expressing Sod4::eGFP or Sod5::eGFP fusion. Total SOD activity decreased by ~15% in Δ*sod1* but increased by 11–20% in three *sod4* knockdown mutants (Δ*sod4* was lethal) when co-cultivated with menadone and H_2O_2. Surprisingly, total catalase activity decreased much more in the *sod4* mutants (69–75%) than in Δ*sod1* (27–33%) under normal and oxidative conditions. However, Δ*sod5* showed little change in either SOD or catalase activity. Transcript levels of SOD partners and five catalases also changed more dramatically in the *sod4* mutants than in Δ*sod1* and Δ*sod5*. As a consequence of global effect, intracellular ROS levels induced by both oxidants were higher in Δ*sod1* than in the *sod4* mutants and Δ*sod5*. All the mutants were differentially more sensitive to the two oxidants and UV-A/UV-B irradiations and less virulent to *Galleria mellonella* larvae but not responsive to high osmolarity, cell wall stress and high temperature. Taken together with previously characterized Sod2 and Sod3, our results provide full insight into the SOD family, unveiling the interactions of each SOD with other partners and catalases in the antioxidant reaction associated with the fungal biocontrol potential.

1. Introduction

Superoxide dismutases (SODs) are a family of metalloproteins cofactored with the metal ions Mn^{2+}, Fe^{2+}, Li^{+}, Cu^{2+} or Zn^{2+} in prokaryotes and eukaryotes and can scavenge intracellular reactive oxygen species (ROS) that may impair biomolecules, such as DNA, proteins and lipids (Fridovich, 1995). These enzymes can prevent cells from ROS damage by detoxifying superoxide radical anions into molecular oxygen and hydrogen peroxide. Fungal SODs are usually cofactored with Cu^{2+}/Zn^{2+} or Mn^{2+}, localized in cytosol or mitochondria, and involved in cell differentiation and multi-stress responses *in vitro* and *in vivo* (Aguirre et al., 2005). Of those, mitochondrial MnSODs may prevent yeast cells from the damages of oxidative, osmotic and thermal stresses (Hwang et al., 2003). Single deletions of three SOD genes in *Aspergillus fumigatus* resulted in severe growth defects under thermal and oxidative stresses, contrasting to a slight growth delay of the same fungus at high temperature when a cytosolic MnSOD was inactivated (Lambou et al., 2010). Phenotypic changes caused by deleted SOD genes in some fungi include reduced growth in *Candida albicans* (Hwang et al., 2002), decreased conidiation capacity in *Oidiodendro maius* (Abba et al., 2009, delayed germination in *A. fumigatus* (Lambou et al., 2010), and attenuated virulence in *Cryptococcus neoformans* (Narasipura et al., 2005). However, a major Cu/ZnSOD is not essential for the pathogenicity of *Claviceps purpurea* (Moore et al., 2002). Nor is a MnSOD required for the virulence of *Colletotrichum graminicola* due to complementary effects from two other isoenzymes (Fang et al., 2002). These studies demonstrate functional diversity and differentiation of individual SODs in the fungal pathogens of animals and plants. However, full insights into the functions of all SODs in many filamentous fungi are lacking.

Filamentous entomopathogens, such as *Beauveria bassiana*, are arthropod biocontrol agents that have been developed into a large number of mycoinsecticides and mycoacaricides, in which

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unicellular conidia are active ingredients (de Faria and Wraight, 2007). The biological control potential of such fungi depends not only on virulence to target pests but on tolerance to high temperature and solar UV irradiations, which affect the field persistency and efficacy of a fungal formulation after application (Wang and Feng, 2014). In fact, many outdoor stresses, such as UV, heat, drought and applied agrochemicals, are all possible inducers of ROS, such as superoxide radical anions, in the fungal cells. Thus, it is important to understand the roles of different SODs in protecting the fungal cells from damages under the stresses.

There are five SOD genes (*sod1-5*) in the annotated genome of *B. bassiana* (Xiao et al., 2012). Of those, Sod2 (cytosolic MnSOD) and Sod3 (mitochondrial MnSOD) were proven to dominate intracellular SOD activity and hence contribute additively to antioxidant capability, UV tolerance and virulence in *B. bassiana* (Xie et al., 2010a, 2012). Sod1, a cytosolic Cu/ZnSOD, which can be activated only by the insertion of copper chaperon into active sites in some fungi (Casareno et al., 1998; Schmidt et al., 2000; Furukawa et al., 2004), became highly active when the point mutations P143S and P145L occurred, making the chaperon insertion unnecessary (Xie et al., 2010b). However, the connection of Sod1 to cellular stress responses and virulence has been unexplored. Two other SODs, i.e., Sod4 and Sod5, are unknown at all. Our preliminary sequence analysis demonstrates that Sod5 could be another Cu/ZnSOD with an N-terminal signal peptide, which indicates a likelihood of its localization on cell wall. Surprisingly, Sod4 was predicted as a FeSOD with a C-terminal domain typical for FeSODs, which are common in prokaryotes, protozoan, and chloroplasts of plants and algae but rare in filamentous fungi (Wintjens et al., 2004; Dufernez et al., 2006; Kang et al., 2008). This study sought to characterize the functions of Sod1, Sod4 and Sod5 in *B. bassiana* by transcriptional, enzymatic and multi-phenotypic analyses of single-gene deletion or knockdown mutants. We found that the three SODs were positive ROS scavengers and hence contributed significantly to some or all of examined phenotypes, including conidiation, antioxidant response, UV-A/UV-B tolerance and virulence. Taken together with the previously characterized Sod2 and Sod3, our findings provide full insight into the significance of the whole SOD family for the life of *B. bassiana* as well as the subcellular localization of Sod5, a cell wall-anchored Cu/ZnSOD, and of Sod4, a mitochondrial FeSOD characterized for the first time in fungi.

2. Materials and methods

2.1. Microbial strains and culture conditions

The wild-type strain *B. bassiana* ARSEF 2860 (WT hereafter) was routinely cultured at 25 °C and 12: 12 h (light/dark cycle) on Sabouraud dextrose agar (4% glucose, 1% peptone and 1.5% agar) plus 1% yeast extract (SDAY) as a rich medium or Czapek agar (CZA: 3% sucrose, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$ and 0.001% $FeSO_4$) as a minimal medium. *Escherichia coli* DH5α and *E. coli* BL21 (DE3) from Invitrogen (Shanghai, China) were cultured in Luria–Bertani medium at 37 °C for vector propagation. *Agrobacterium tumefacien* AGL-1 used in the mediation of fungal transformation (Fang et al., 2004) was incubated in the broth of 0.5% sucrose, 1% peptone, 0.1% yeast extract and 0.05% $MgSO_4$ at 28 °C.

2.2. Cloning and analysis of sod1, sod4, sod5 sequences in B. bassiana

The coding sequences of *sod1*, *sod4* and *sod5* (tag loci: BBA_02311, BBA_04317 and BBA_01984 respectively) were amplified from the WT genome under the NCBI accession NZ_ADAH00000000 (Xiao et al., 2012) with degenerate primers (Table S1), which were designed by referring to the conserved regions of their sequences aligned with those of other fungal homologues in NCBI database. The amplified fragments were verified by sequencing at Invitrogen. The proteins deduced from the verified sequences were compared with the SOD counterparts of other fungi in NCBI protein database via online BLAST analysis (http://www.ncbi.nlm.nih.gov/Blast.cgi/), followed by phylogenetic comparison with MEGA5 software (Tamura et al., 2011). An iron-binding site in a conserved domain of the deduced Sod4 and both the mitochondrium-targeting probability and 5′ transit leading signal of the same protein were predicted using the software MitoprotII 1.0a4 (Claros and Vincens, 1996). A signal peptide of the deduced Sod5 and its cleavage sites were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/).

2.3. Subcellular localization of Sod4 and Sod5

The open reading frames (ORF) of *sod4* and *sod5* were amplified from WT cDNA with paired primers in Table S1, and each was fused to the ORF of the enhanced green fluorescence protein gene *eGFP* (GenBank ID: U55763) from pEGFP-C1 (BD Biosciences Clontech, CA) via splicing-by-overlap extension (Lu et al., 2006). Each fusion gene was then digested with *Bam*HI and inserted into pAN52-bar that vectors the P*gpdA* and T*trpC* of *Aspergillus nidulans* and the phosphinothricin resistant *bar* marker (Ying and Feng, 2006), forming pAN52-*x*-bar (*x* = *sod4::eGFP* or *sod5::eGFP*). This new plasmid was linearized with *Nde*I and integrated into WT for expression via blastospore transformation (Ying and Feng, 2006). A positive transformant expressing *sod4::eGFP* (T1) or *sod5::eGFP* (T2) at high level was used in subcellular localization as follows.

T1 and T2 were grown for 2 days in 20 ml of SDB (agar-free SDAY) containing 5×10^4 conidia/ml by shaking (150 rpm) at 25 °C. The resultant T1 hyphae were stained with 500 nM Mito-Tracker Red CMXRos (Invitrogen), a mitochondrium-specific dye, and examined for the presence of red and green fluorescence under a Confocal microscope. The same images of bright, red (stain) and green (expressed eGFP) in different channels were merged using an image browser. Similarly, the T2 hyphae and blastospores from the SDB culture were stained with calcofluor white (Sigma), a stain specific to cell wall. Their images of bright, blue (stain) and green fluorescence were collected under the Confocal microscope.

2.4. Generation of sod mutants

The 3′ and 5′ ends of *sod1* (1519 and 1874 bp) or *sod5* (1720 and 1822 bp) were cloned from WT via PCR with paired primers (Table S1) and inserted into p0380-bar (Xie et al., 2012), yielding p0380-*x*up-bar-*x*dn (*x* = *sod1* or *sod5*) for *sod* deletion. The full-length DNA sequences of *sod1* (3643 bp) and *sod5* (4052 bp) with flanking regions were amplified from WT and ligated into p0380-sur-gateway (Xie et al., 2012) to replace the gateway fragment, forming p0380-sur-*x* for target gene complementation. Each *sod* was deleted by transforming the *bar*-inclusive cassette into WT for homogeneous replacement and complemented by ectopic integration of the *sur* cassette into the Δ*sod* mutant via *Agrobacterium*-mediated transformation (Fang et al., 2004). Putative mutant colonies grown on a selective medium were screened in terms of the *bar* resistance to phosphinothricin (200 μg/ml) or the *sur* resistance to chlorimuron ethyl (10 μg/ml) and identified via PCR, quantitative real-time PCR (qRT-PCR) and Southern blot analyses with paired primers and amplified probes (Table S1).

Since the *sod4* deletion with the same method failed in numerous attempts perhaps due to its indispensability for the fungus, *sod4* was silenced via hairpin RNA interference (RNAi) (Xie et al., 2012). To construct RNAi plasmids, two partial coding fragments (466 and 414 bp) of *sod4*, designated as S1 and S2, were cloned from WT cDNA with sense/antisense primers (Table S1). The RNAi

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