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Disruptions of the genes involved in lysine biosynthesis, iron acquisition, and secondary metabolisms affect virulence and fitness in *Metarhizium robertsii*

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A B S T R A C T

Based on genomic analysis, polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) pathways account for biosynthesis of the majority of the secondary metabolites produced by the entomopathogenic fungus *Metarhizium robertsii*. To evaluate the contribution of these pathways to *M. robertsii* fitness and/or virulence, mutants deleted for *mrpptA*, the Sfp-type 4′ phosphopantetheinyl transferase gene required for their activation were generated. Δ*mrpptA* strains were deficient in PKS and NRPS activity resulting in colonies that lacked the typical green pigment and failed to produce the nonribosomal peptides (destruxins, serinocylins, and the siderophores ferricrocin and metachelins) as well as the hybrid polyketide-peptides (NG-39x) that are all produced by the wild type (WT) *M. robertsii*. The Δ*mrpptA* colonies were also auxotrophic for lysine. Two other mutant strains were generated: Δ*mraarA*, in which the α-aminoadipate reductase gene critical for lysine biosynthesis was disrupted, and Δ*mrsidA*, in which the L-ornithine N^5-oxygenase gene that is critical for hydroxamate siderophore biosynthesis was disrupted. The phenotypes of these mutants were compared to those of Δ*mrpptA* to separate effects of the loss of lysine or siderophore production from the overall effect of losing all polyketide and non-ribosomal peptide production. Loss of lysine biosynthesis marginally increased resistance to H_2O_2 while it had little effect on the sensitivity to the cell wall disruptor sodium dodecyl sulfate (SDS) and no effect on sensitivity to iron deprivation. In contrast, combined loss of metachelin and ferricrocin through the inactivation of *mrsidA* resulted in mutants that were as hypersensitive or slightly more sensitive to H_2O_2, iron deprivation, and SDS, and were either identical or marginally higher in Δ*mrpptA* strains. In contrast to Δ*mrpptA*, loss of *mrsidA* did not completely abolish siderophore activity, which suggests the production of one or more non-hydroxamate iron-chelating compounds. Deletion of *mrpptA*, *mrsidA*, and *mraarA* reduced conidium production and conidia of a GFP-tagged Δ*mrpptA* strain displayed a longer germination delay than WT on insect cuticles, a deficiency that was rescued by lysine supplementation. Compared with WT, Δ*mrpptA* strains displayed ~19-fold reduction in virulence against *Drosophila suzukii*. In contrast, lysine auxotrophy and loss of siderophores accounted for ~2 and ~6-fold decreases in virulence, respectively. Deletion of *mrpptA* had no significant effect on growth inhibition of *Bacillus cereus*. Our results suggest that PKS and NRPS metabolism plays a significant role in *M. robertsii* virulence, depresses conidium production, and contributes marginally to resistance to oxidative stress and iron homeostasis, but has no significant antibacterial effect.

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1. Introduction

Metarhizium robertsii, a cosmopolitan entomopathogenic Ascomycete with a broad host range (Bischoff et al., 2009; St. Leger et al., 2011), has been studied intensively as a biocontrol agent for insect pests. This organism encodes a plethora of secondary metabolite (SM) pathways whose biological roles and products

Abbreviations: AsMM, *Aspergillus* minimal media; ESIMS, electrospray mass detection; HRESIMS, high-resolution electrospray ionisation mass spectrometry; RBB, Reciprocal Best Blast; SM, secondary metabolite; BPS, bathophenanthroline disulfonic acid; SDS, sodium dodecyl sulfate; ONO, L-ornithine N^5-oxygenase; AAR, α-aminoadipate reductase.

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are largely unknown (Donzelli and Krasnoff, 2016). Those with known genetic bases have shown minimal effects on fitness and virulence (Donzelli and Krasnoff, 2016). For instance, the cyclic peptides destruxins have been touted as likely virulence factors because of their *in vitro* toxicity towards a wide range of insect species (Pedras et al., 2002). However, several lines of evidence indicate that their contribution to pathogenicity is either small or masked by the production of other unknown virulence factors (Donzelli et al., 2012; Wang et al., 2012). The latter scenario implies that loss of SMs might have a large effect on virulence only when multiple pathways are inactivated simultaneously. One way to achieve sweeping SM inactivation is through deletion of the 4′ phosphopantetheinyl transferase (Pptase) - encoding gene responsible for activation of all nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs), the core enzymes that constitute the majority of SM biosynthetic pathways (Lambalot et al., 1996). Fungal genomes usually carry three Pptase classes: 1. the holo-acyl carrier protein synthases (AcpS-type Pptase), which activates the mitochondrial type II fatty acid synthase, exemplified by *Saccharomyces cerevisiae* Ppt2 and *Aspergillus nidulans* pptB (Allen et al., 2011; Stuible et al., 1998); 2. the integrated Pptases responsible for self-pantetheinylation of the cytoplasmic fatty acid synthetase α-subunit, exemplified by *S. cerevisiae* Fas2, and *A. nidulans* fasA (Brown et al., 1996; Fichtlscherer et al., 2000); and 3. the Sfp-type Pptases dedicated to post-translational modification of NRPS, PKS, hybrid PKS/NRPS, and NRPS-like enzymes, which include α-aminoadipate reductase (AAR), which is required for the biosynthesis of lysine, the NRPSs that produce hydroxamate siderophores (Horbach et al., 2009; Oberegger et al., 2003; Velázquez-Robledo et al., 2011; Wiemann et al., 2012; Zainudin et al., 2015), and pathways involved in secondary metabolism, like penicillin and conidial pigments in *A. nidulans* (Horbach et al., 2009; Keszenman-Pereyra et al., 2003; Leng and Zhong, 2012; Márquez-Fernández et al., 2007; Neville et al., 2005; Wiemann et al., 2012; Zainudin et al., 2015). Thus, mutation of the Sfp-type Pptase leads to both lysine auxotrophy and defects in iron metabolism which in turn can severely limit growth, viability, and virulence and overshadow the effects deriving from the loss of other NRPSs and PKSs.

Phenotypic changes specifically due to the deficiency of either lysine or siderophores can be investigated by inactivating their respective biosynthetic routes. Changes deriving from lysine auxotrophy, loss of siderophores and the rest of the PKSs and NRPSs can be distinguished by comparing Sfp-type Pptase minus strains to mutants blocked solely in lysine or siderophore biosynthesis. In fungi, strains auxotrophic for lysine are produced by deleting genes contributing to the aminoadipate pathway, including the α-aminoadipate reductase (AAR) LYS2/*aarA* (Chattoo et al., 1979; Xu et al., 2006). Fungal strains blocked in siderophore production can be generated by targeting genes orthologous to L-ornithine N^5-oxygenase (ONO) *sidA*/*sid1*, which supplies the N^5-hydroxy-L-ornithine units necessary for both ferrichrome-type and coprogen-type NRPSs to assemble their respective products (Eisendle et al., 2003; Hilty et al., 2011; Hissen et al., 2005; Mei et al., 1993). Thus, deletion of the *sidA*/*sid1* ortholog in *M. robertsii* is predicted to abolish biosynthesis of both metachelin (a coprogen-type siderophore) and ferricrocin (a ferrichrome-type siderophore) (Donzelli et al., 2015). As with lysine auxotrophy, loss of siderophores has been linked to a decline in virulence (Oide et al., 2015, 2007, 2006; Schrettl et al., 2004). Association between Sfp-type Pptase and pathogenicity has been studied mainly in plant pathogenic fungi. For instance, in both the rice pathogen *Magnaporthe oryzae* and the maize anthracnose fungus *Colletotrichum graminicola*, reduction of virulence following deletion of Sfp-Pptase was attributed both to lysine auxotrophy and the inability of Sfp-Pptase mutants to form melanized appressoria, but there was no significant contribution from siderophore loss (Horbach et al., 2009). In the rice pathogen *Fusarium fujikuroi*, reduced virulence of Sfp-Pptase mutants was attributed to both lysine auxotrophy and loss of siderophores with no implication of a role in virulence for PKSs and non-siderophore NRPS metabolism (Wiemann et al., 2012). In several *Cochliobolus* species, lysine was recognized as an important nutrient supporting growth on the host surface and, depending on the species, loss of either siderophores or PKSs and NRPSs was more likely to be responsible for the reduced virulence of mutants lacking the Sfp-type Pptase (Leng and Zhong, 2012; Zainudin et al., 2015). Although similar studies have not been conducted in animal pathogenic fungi, the lesson from plant pathogenic fungi is that the relative roles of lysine, siderophore, and SM biosynthetic pathways in virulence differ across species.

Herein we analyze the phenotypes of *M. robertsii mrpptA* mutants and compare them to strains incapable of lysine biosynthesis (Δ*mraarA*) and to strains deficient in siderophore production (Δ*mrsidA*) to shed light on the role of secondary metabolism in the biology of this insect pathogen.

2. Materials and methods

2.1. Strains, growth conditions and fungal transformation

M. robertsii ARSEF 2575 and its mutants were maintained on ¼-strength Sabouraud dextrose agar with yeast extract (SDAY/4). *Aspergillus* minimal medium (which included 1% glucose as the main C source and 200 mM glutamine as the N source) or M100 medium (which included 1% glucose as the C source and 30 mM KNO_3 as the N source) amended with lysine HCl (Sigma cat # L-5626) were used in phenotypic tests (Moon et al., 2008; Pontecorvo et al., 1953). *Escherichia coli* XL1 Blue was used for gene cloning and plasmid construction using routine cloning techniques (Sambrook et al., 1989). *Agrobacterium*-mediated transformation was carried out as described previously (Moon et al., 2008).

2.2. Gene identification, targeted gene deletions and mrppt1 complementation

Candidate genes were identified by Reciprocal Best Blast (RBB) performed on translated gene sequences from 52 fungal genomes followed by MCL clustering (Enright et al., 2002; Wall et al., 2003). Orthologous groups corresponding to LYS5 from *S. cerevisiae* and *npgA*/*cfwA* from *A. nidulans* (Pptase group); LYS2 from *S. cerevisiae* (AAR group); and *sidA* from *A. nidulans* (ONO group), included single gene models belonging to *M. robertsii* which constituted our candidate genes (Eibel and Philippsen, 1983; Hissen et al., 2005; Keszenman-Pereyra et al., 2003; Miller and Bhattacharjee, 1996). In all instances double crossover gene replacement constructs were assembled by inserting the *bar* selection marker, which confers resistance to glufosinate ammonium, between matching regions flanking the gene to be deleted. DNA fragments were produced by PCR with primers listed in Supplementary Table S1 (Pall and Brunelli, 1993) and directionally assembled into the pBDU vector employing a described USER cloning method (Donzelli et al., 2012; Geu-Flores et al., 2007). Amplification templates were *M. robertsii* ARSEF 2575 genomic DNA and pUCAP *bar* NOSII plasmid DNA (Donzelli et al., 2012). The resulting vectors were used to transform either *M. robertsii* ARSEF 2575 or a nourseothricin-resistant derivative expressing sGFP under the control of the *A. nidulans gpd* promoter (Oide et al., 2006; Punt et al., 1987). Identification of deletion mutants was carried out by PCR. Primers annealing outside each targeted region and primers annealing to the *bar* cassette (Supplementary Fig. S1A, black and

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