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

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20 Abstract

21 Based on genomic analysis, polyketide synthase (PKS) and nonribosomal peptide synthetase
22 (NRPS) pathways account for biosynthesis of the majority of the secondary metabolites produced by
23 the entomopathogenic fungus *Metarhizium robertsii*. To evaluate the contribution of these pathways to
24 *M. robertsii* fitness and/or virulence, mutants deleted for *mrppta*, the Sfp-type 4' phosphopantetheinyl
25 transferase gene required for their activation were generated. $\Delta mrppta$ strains were deficient in PKS
26 and NRPS activity resulting in colonies lacking the typical green pigment that failed to produce the
27 nonribosomal peptides destruxins, serinocylins, and the siderophores ferricrocin and metachelins, as
28 well as the hybrid polyketide-peptides NG-39x that are all produced by the wild type (WT) *M.*
29 *robertsii*. The $\Delta mrppta$ colonies were also auxotrophic for lysine. Two other mutant strains were
30 generated: $\Delta mraara$, in which the α -aminoadipate reductase gene critical for lysine biosynthesis was
31 disrupted, and $\Delta mrsida$, in which the L-ornithine N⁵-oxygenase gene that is critical for hydroxamate
32 siderophore biosynthesis was disrupted. The phenotypes of these mutants were compared to those of
33 $\Delta mrppta$ to separate effects of the loss of lysine or siderophore production from the overall effect of
34 losing all polyketide and non-ribosomal peptide production. Loss of lysine biosynthesis marginally
35 increased resistance to H₂O₂ while it had little effect on the sensitivity to the cell wall disruptor sodium
36 dodecyl sulfate (SDS) and no effect on sensitivity to iron deprivation. In contrast, combined loss of
37 metachelin and ferricrocin through the inactivation of *mrsida* resulted in mutants that were
38 hypersensitive to H₂O₂, iron deprivation, and SDS, and were either identical or marginally higher in
39 $\Delta mrppta$ strains. In contrast to $\Delta mrppta$, loss of *mrsida* did not completely abolish siderophore
40 activity, which suggests the production of one or more non-hydroxamate iron-chelating compounds.
41 Deletion of *mrppta*, *mrsida*, and *mraara* reduced conidium production and conidia of a GFP-tagged
42 $\Delta mrppta$ strain displayed a longer germination delay than WT on insect cuticles, a deficiency that was
43 rescued by lysine supplementation. Compared with WT, $\Delta mrppta$ strains displayed ~19-fold reduction
44 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.

Keywords

4' phosphopantetheinyl transferase, α -aminoadipate reductase, L-ornithine N⁵-oxygenase, siderophores, PKS, NRPS, entomopathogen.

Abbreviations

Aspergillus minimal media (AsMM), electrospray mass detection (ESIMS), High-Resolution ElectroSpray Ionisation Mass Spectrometry (HRESIMS), Reciprocal Best Blast (RBB), secondary metabolite (SM), bathophenanthroline disulfonic acid (BPS), sodium dodecyl sulfate (SDS), L-ornithine N⁵-oxygenase (ONO), α -aminoadipate reductase (AAR).

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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 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

92 activates the mitochondrial type II fatty acid synthase, exemplified by *Saccharomyces cerevisiae* Ppt2
 93 and *Aspergillus nidulans* pptB (Allen et al., 2011; Stuible et al., 1998); 2. the integrated Pptases
 94 responsible for self-pantetheinylation of the cytoplasmic fatty acid synthetase α -subunit, exemplified
 95 by *S. cerevisiae* Fas2, and *A. nidulans* fasA (Brown et al., 1996; Fichtlscherer et al., 2000); and 3. the
 96 Sfp-type Pptases dedicated to post-translational modification of NRPS, PKS, hybrid PKS/NRPS and
 97 NRPS-like enzymes, which include α -aminoadipate reductase (AAR) required for the biosynthesis of
 98 lysine, the NRPSs that produce hydroxamate siderophores (Horbach et al., 2009; Oberegger et al.,
 99 2003; Velázquez-Robledo et al., 2011; Wiemann et al., 2012; Zainudin et al., 2015), and pathways
 100 involved in secondary metabolism, like penicillin and conidial pigments in *A. nidulans* (Horbach et al.,
 101 2009; Keszenman-Pereyra et al., 2003; Leng and Zhong, 2012; Márquez-Fernández et al., 2007;
 102 Neville et al., 2005; Wiemann et al., 2012; Zainudin et al., 2015). Thus, mutation of the Sfp-type
 103 Pptase leads to both lysine auxotrophy and defects in iron metabolism which in turn can severely limit
 104 growth, viability, and virulence and overshadow the effects deriving from the loss of other NRPSs and
 105 PKSs.

106 Phenotypic changes specifically due to the deficiency in either lysine or siderophores can be
 107 investigated by inactivating their respective biosynthetic routes. Changes deriving from lysine
 108 auxotrophy, loss of siderophores and the rest of the PKSs and NRPSs can be distinguished by
 109 comparing Sfp-type Pptase minus strains to mutants blocked solely in lysine or siderophore
 110 biosynthesis. In fungi, strains auxotrophic for lysine are produced by deleting genes contributing to the
 111 aminoadipate pathway, including the α -aminoadipate reductase (AAR) *LYS2/aarA* (Chattoo et al.,
 112 1979; Xu et al., 2006). Fungal strains blocked in siderophore production can be generated by targeting
 113 genes orthologous to L-ornithine N^5 -oxygenase (ONO) *sidA/sidI*, which supplies the N^5 -hydroxy-L-
 114 ornithine units necessary for both ferrichrome-type and coprogen-type NRPSs to assemble their
 115 respective products (Eisendle et al., 2003; Hilty et al., 2011; Hissen et al., 2005; Mei et al., 1993).
 116 Thus, deletion of the *sidA/sidI* ortholog in *M. robertsii* is predicted to abolish biosynthesis of both

117 metachelin (a coprogen-type siderophore) and ferricrocin (a ferrichrome-type siderophore) (Donzelli et
118 al., 2015). As with lysine auxotrophy, loss of siderophores has been linked to a decline in virulence
119 (Oide et al., 2015; Oide et al., 2007; Oide et al., 2006; Schrettl et al., 2004). Association between Sfp-
120 type Pptase and pathogenicity has been studied mainly in plant pathogenic fungi. For instance, in both
121 the rice pathogen *Magnaporthe oryzae* and the maize anthracnose fungus *Colletotrichum graminicola*,
122 reduction of virulence following deletion of Sfp-Pptase was attributed both to lysine auxotrophy and
123 the inability of Sfp-Pptase mutants to form melanized appressoria, but there was no significant
124 contribution from siderophore loss (Horbach et al., 2009). In the rice pathogen *Fusarium fujikuroi*,
125 reduced virulence of Sfp-Pptase mutants was attributed to both lysine auxotrophy and loss of
126 siderophores with no implication of a role in virulence for PKSs and non-siderophore NRPS
127 metabolism (Wiemann et al., 2012). In several *Cochliobolus* species, lysine was recognized as an
128 important nutrient supporting growth on the host surface and, depending on the species, loss of either
129 siderophores or PKSs and NRPSs was more likely to be responsible for the reduced virulence of
130 mutants lacking the Sfp-type Pptase (Leng and Zhong, 2012; Zainudin et al., 2015). Although similar
131 studies have not been conducted in animal pathogenic fungi, the lesson from plant pathogenic fungi is
132 that the relative roles of lysine, siderophore, and SM biosynthetic pathways in virulence differ across
133 species.

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

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140 **2. Materials and methods**

141 **2.1. Strains, growth conditions and fungal transformation**

142 *M. robertsii* ARSEF 2575 and its mutants were maintained on ¼-strength Sabouraud dextrose
143 agar with yeast extract (SDAY/4). *Aspergillus* minimal medium (which included 1% glucose as the
144 main C source and 200 mM glutamine as the N source) or M100 medium (which included 1% glucose
145 as the C source and 30 mM KNO₃ as the N source) amended with lysine HCl (Sigma cat # L-5626)
146 were used in phenotypic tests (Moon et al., 2008; Pontecorvo et al., 1953). *Escherichia coli* XL1 Blue
147 was used for gene cloning and plasmid construction using routine cloning techniques (Sambrook et al.,
148 1989). *Agrobacterium*-mediated transformation was carried out as described previously (Moon et al.,
149 2008).

151 **2.2. Gene identification, targeted gene deletions and *mrppt1* complementation**

152 Candidate genes were identified by Reciprocal Best Blast (RBB) performed on translated gene
153 sequences from 52 fungal genomes followed by MCL clustering (Enright et al., 2002; Wall et al.,
154 2003). Orthologous groups corresponding to LYS5 from *S. cerevisiae* and *npgA/cfwA* from *A. nidulans*
155 (Pptase group); LYS2 from *S. cerevisiae* (AAR group); and *sidA* from *A. nidulans* (ONO group),
156 included single gene models belonging to *M. robertsii* which constituted our candidate genes (Eibel
157 and Philippsen, 1983; Hissen et al., 2005; Keszenman-Pereyra et al., 2003; Miller and Bhattacharjee,
158 1996). In all instances double crossover gene replacement constructs were assembled by inserting the
159 *bar* selection marker, which confers resistance to glufosinate ammonium, between matching regions
160 flanking the gene to be deleted. DNA fragments were produced by PCR with primers listed in
161 supplementary **Table S1** (Pall and Brunelli, 1993) and directionally assembled into the pBDU vector
162 employing a described USER cloning method (Donzelli et al., 2012; Geu-Flores et al., 2007).
163 Amplification templates were *M. robertsii* ARSEF 2575 genomic DNA and pUCAP *bar* NOSII
164 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 gray arrows) were used to confirm homologous recombination events. Candidates were purified by isolations of single conidium (Supplementary **Fig. S1A**, white arrows). For complementation of an *mrppta* mutant, a 3,483 bp fragment that included the endogenous *mrppta* promoter, coding region and polyadenylation regions was PCR-amplified using total DNA extracted from *M. robertsii* ARSEF 2575 as the template and the primers PPT-CF and PPT-CR (supplementary **Table S1**). This fragment was cloned in a pBDU vector as described previously (Donzelli et al., 2012). Candidate-complemented strains were selected on lysine-free M100 medium amended with carbenicillin 300 mg/L to kill *A. tumefaciens*, and then purified by single conidium isolation. Five colonies with WT green pigmentation and restored lysine prototrophy were selected.

2.3. Phenotypic tests

Effects of lysine, casein, Fe^{2+} chelator bathophenanthroline disulfonic acid (BPS), hemin, sodium dodecyl sulfate (SDS), H_2O_2 , and ketoconazole were assayed in microtiter plates as described elsewhere (Donzelli et al., 2015). Hemin was tested for free iron by washing ~15 mg in 250 μL of 10 mM citrate phosphate buffer pH 3.6 amended with 0.05% Silwet L-77 (hemin is water insoluble and hydrophobic) for 30 min. Clear supernatants were recovered by 5 min, 12,000 $\times$ g centrifugation and analyzed undiluted using a colorimetric method (Nilsson et al., 2002). Free iron was undetectable (the limit of detection of the method was ~0.1 μM Fe^{2+}). For experiments, 12.5 mM hemin stock solutions were prepared in DMSO. Stressors/nutrients were added at indicated concentrations to either M100 or AsMM amended with 2 mM lysine. Cultures were incubated at 24-26 °C for 6 to 15 d, depending on the growth rate allowed by the specific assay condition. Colony growth was assessed as absorbance at 900 nm (OD_{900}) using a plate reader (Bio-Tek Synergy HT) set in a 5x5 grid scanning mode. Colony

size was normalized to that of the same strain grown in the control condition, Cox Box-transformed and evaluated by ANOVA. Normalization was necessary to adjust for differences in strain growth observed in the absence of each treatment. Statistical analyses were conducted using JMP 11.0 (SAS Institute Inc.). Each treatment had four replicates.

Conidiation levels were compared on M100 amended with 1% casein in the presence of 0, 0.01, 0.1 and 1 mM lysine. Conidia from 14-day old colonies grown in 24-well microtiter plates were dislodged using a brush and 500 μ L 0.1% Silwet L-77. Each colony was scraped twice and the conidial suspensions combined. Conidia were washed 2x in 0.05 % Silwet L-77, centrifuged 3 min at 6,000 $\times$ g and resuspended in 1 mL 0.05 % Silwet L-77. A 75 μ L aliquot of each suspension was combined with 75 μ L molten 1% agar in 96-well microtiter plates. Once hardened, conidial suspensions in agar carrier retain a homogeneous and stable distribution of conidia. After 15 min incubation, OD₆₂₀ of the solidified suspensions was assessed using a microtiter plate reader. Spore concentration was estimated using reference curves built using hemocytometer-calibrated conidial suspensions from all the strains under analysis. Results were subjected to ANOVA and pairwise Student's t-test.

2.4. Germination on insect cuticles

Conidia from the sGFP-expressing strain NF2 and its derivative $\Delta mrppta$ #9 were resuspended in 0.01% Silwet L-77 at 1×10^5 /mL. A 100 μ L aliquot of the suspension was transferred to microfuge tubes containing eight wings from *Blattella germanica* and incubated on a rotary apparatus for ~1 h at RT to promote spore attachment. The suspension was allowed to collect at the bottom of the tube and then removed with a pipette. Inoculated wings were incubated at 26 °C for 40 h and observed by epifluorescent microscope at 200x magnification. The experiment was replicated four times.

2.5. *Bacillus cereus* inhibition

Bacillus cereus cultures were grown overnight in LB broth at 25 °C, 200 RPM, diluted 500-fold

215 with water, spread on SDAY/4 at 30 μ L/60 mm plate and dried for ~1 h. Fungal strains were pre-grown
216 for 48h at 26 °C on 5 mm filter paper disks placed on SDAY/4 medium. Disks were then transferred
217 onto SDAY/4 plates pre-inoculated with *B. cereus* suspension. Cultures were incubated at 26 °C for 48
218 h.

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220 **2.6. Mycelium and conidium hydrophobicity assay**

221 Colony wetting propensity was assessed using 5-d old non-conidiating mycelial mats formed on
222 SDAY/4 plates at 26 °C and wetted with 30 μ L droplets containing variable proportions of water and
223 ethanol, which afforded solutions with known surface tension and wetting properties (Chau et al., 2010;
224 Lundgren et al., 2002; Vazquez et al., 1995). The minimum concentration of ethanol needed to wet
225 fungal mats at 23 °C was documented and the stability of the droplets was observed for 60 min. Each
226 treatment had 3 replicates. Conidium hydrophobicity was tested using a modification of the assay
227 described previously (Kim et al., 2010). Briefly, conidia were scraped from a 3 x 3 mm area of the
228 colony with a metal spatula and transferred to a 1.5 mL microfuge tube containing 500 μ L of water. In
229 water conidia tend to adhere to polypropylene walls and float on the water surface. A stock solution of
230 0.1% Silwet L-77 was added to each suspension in 5 μ L steps and tubes gently inverted each time.
231 Hydrophobicity levels were estimated by measuring the minimum volume of 0.1% Silwet L-77 needed
232 to disrupt conidial floating. We avoided transferring conidia as agar plugs as suggested in the original
233 protocol because in preliminary tests, we noticed that they can carry surfactants originating from the
234 medium or secreted by the fungus which may alter the assay outcome. The assay was replicated three
235 times.

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237 **2.7. Casein utilization test**

238 Ability to exploit proteinaceous substrates as the source of lysine by lysine-auxotrophic strains
239 was tested on M100 medium in which KNO₃ was replaced by 1% casein as the only N source. To

240 assess concentration required to restore growth, the experiment was conducted in the presence of 0,
241 0.01, 0.1 or 1 mM lysine. Hammerstein grade casein was prepared as a 10% stock solution in 0.5x
242 maleic acid buffer (maleic acid 2.9 g/L, NaCl 2.2 g/L, pH 7.5) and autoclaved for 15 min at 121 °C.
243 Casein stock was then added to sterile M100 medium. Plates were inoculated by pricking the medium
244 surface with a 10 µL pipette tip previously dipped in a 1×10⁷/mL conidial suspension in 0.01% Silwet
245 L-77. Plates were incubated for 7 d at 24 °C with a 15h:9h (light: dark) photoperiod. The assay was
246 replicated four times.

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248 **2.8. Iron chelation activity**

249 We used the universal Chrome Azurol S (CAS) assay to compare siderophore activity among
250 WT, *Δmrppta*, *Δmrsida* and *Cmrppta* strains (Schwyn and Neilands, 1987). Briefly, fungi were grown
251 for 7 d at 24 °C in AsMM containing 80 nM iron. Culture fluids were centrifuged for 5 min at
252 12,000×g to remove mycelia. One hundred µL of clarified culture fluids were combined with an equal
253 volume of CAS reagent within wells of a 96-well microtiter plate, incubated at RT for 20 min, then
254 absorbance at 620 nm was measured using a microtiter plate reader. Due to the extremely fast reaction
255 time, samples of WT, *Δmrsida*, and *Cmrppta* were diluted 10-fold. CAS clearing activity was
256 normalized to that of the WT using the following formula:

$$\frac{1 - OD_{620x}}{1 - OD_{620wt}}$$

257 where OD_{620x} and OD_{620wt} are the absorbance at 620 nm recorded for the strain x and the WT,
258 respectively.

259

260 **2.9. Insect assays**

261 Virulence assays were conducted using *Drosophila suzukii* as the host. Flies were reared at
262 22°C and 15 h:9 h (light:dark) cycle on a diet containing 50 g/L inactive yeast, 60 g/L yellow

263 cornmeal, 40 g/L sucrose, 7 g/L fly agar, 26.5 mL/L of a 30% methylparaben solution in EtOH, 12 mL
264 acid mix (836 mL propionic acid + 80 mL phosphoric acid in 1 L water). Batches of ~50 adult females
265 were dipped in a 2×10^7 conidia/mL suspension in 0.01% Silwet L-77 for 5 sec. Insects were held for 24
266 h in Petri dishes lined with moist filter paper and survivors (usually >90%) transferred to assay
267 chambers constructed from 50 mL conical centrifuge tubes. One day prior to inoculation insects were
268 switched to a methylparaben-free diet which was then replaced every 24-48 h during the assay.
269 Mortality was recorded daily for 10 d. Differences in virulence among strains were quantified as
270 difference in relative mortality risks (or risk ratios) by Cox's regression. Statistical analyses, including
271 tests of hazard proportionality were conducted using the library “survival” in the software program R
272 (Team, 2011; Therneau, 2015).

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274 **2.10. Chemical analyses**

275 **Destruxins.** 100 mL batches of HB medium (Donzelli et al., 2010) inoculated with 10^6 conidia
276 of WT, $\Delta mrpptA$, and $CmrpptA$ strains were fermented in duplicate with shaking at 150 RPM for 8 d at
277 25°C. Culture broths were harvested by filtration (Whatman #1) at 10 d post-inoculation. Fermentation
278 fluids were extracted 3× with 50 mL dichloromethane. The combined organic layers were dried *in*
279 *vacuo*, reconstituted in MeOH and analyzed by HPLC-MS using a Waters Acquity UPLC system with
280 a 2.1 x 50 mm Acquity UPLC® BEH C18 column eluted at 0.5 mL/min with a mobile phase
281 consisting of 0.1% formic acid in MeCN : 0.1% formic acid in HPLC-grade H₂O (40:60). High-
282 resolution mass detection (HRESIMS) was accomplished using a Waters XEVO G2 QTOF
283 spectrometer scanning the mass range from m/z 150-1300 in 0.5 sec in positive ion mode with sampling
284 cone and capillary voltages set at 40 V and 3.5 kV, respectively.

285 **NG-39x.** Fungal strains were grown and extracted as for destruxins (*vide supra*), but were
286 harvested at 4 d post-inoculation. Extracts reconstituted in MeOH were analyzed by HPLC using a
287 Waters 600E pump and 717 autosampler, with a 4.6 x 250 mm Fluophase RP column (Thermo

288 Scientific part # 82505-254630) eluted at 1.0 mL/min with the same mobile phase used for destruxins.
289 UV detection at 363 nm was accomplished with a Waters 2487 detector.

290 **Metachelins.** Strains were grown as for destruxins (*vide supra*) but in AsMM with harvesting at
291 12 d post-inoculation. Fermentation filtrates were extracted 3x with EtOAc (50 mL) and the aqueous
292 layers chromatographed in 100 mL batches on Amberlite XAD-16 in 1.4 cm ID x 9.0 cm
293 polypropylene columns eluted with 5 bed volumes of H₂O and then 10 bed volumes of MeOH. The
294 MeOH eluate containing the metachelin fraction was dried *in vacuo* and reconstituted in MeOH (50%):
295 1.0 % formic acid (50%) and analyzed by HPLC-MS using a Waters 2695 HPLC system with a 2.1 x
296 150 mm Fluophase RP column (Thermo Scientific part # 82505-152130) eluted at 0.25 mL/min with a
297 mobile phase consisting of 0.1% formic acid in MeCN: 0.1% formic acid in HPLC-grade H₂O (36:64).
298 ESIMS was accomplished with a Waters ZQ single quadrupole spectrometer scanning the mass range
299 from *m/z* 50-1300 in 1.0 sec in positive ion mode using sampling cone and capillary voltages of 40 V
300 and 3.0 kV, respectively.

301 **Ferricrocin and serinocyclin.** Conidia were produced either on SDAY1/4 or M100 plus 1%
302 casein and 0.01 mM lysine. Plates were incubated at 24°C for 10 d and a 15 h:9 h (light:dark)
303 photoperiod. Harvested conidia were extracted sequentially in hexane, MeOH, and MeOH (50%):1.0%
304 formic acid in HPLC-grade H₂O (50%) as described previously (Donzelli et al., 2015; Krasnoff et al.,
305 2007). The latter fractions, which contained both serinocyclins and ferricrocin, were reconstituted in
306 MeOH : 1.0% formic acid in HPLC-grade H₂O (50:50) diluted again by 10-fold in MeOH. Samples (5
307 µL) were analyzed directly by flow injections into the XEVO G-2 mass spectrometer using the Waters
308 Acquity autosampler and pump (*vide supra*) with 0.1% formic acid in MeCN flowing at 0.5 mL/min.
309 HRESIMS detection was accomplished by scanning the mass range from *m/z* 150-1300 in 0.5 sec in
310 positive ion mode with sample cone and capillary voltages set at 70 V and 2.8 kV, respectively.

311

312 3. Results

313 3.1. Identification of *mrppta*, *mrsida* and *mraara*

314 Through RBB we identified unique candidates as Sfp-type Pptase, ONO and AAR genes in *M.*
315 *robertsii* ARSEF 2575. These corresponded to the GenBank accessions EXV02093, EXV05491, and
316 EXV03721, and were annotated as *mrppta*, *mrsida*, and *mraara* respectively. All genes were present
317 as single copies. *mrppta* included an 873 bp open reading frame (ORF) interrupted by a 64 bp intron.
318 In many Hypocreales, including *Metarhizium*, the *mrppta* gene is located next to a diene lactone
319 hydrolase although the placement of this gene pair within the respective genomes varies
320 (Supplementary **Fig. S2A**). *Agrobacterium*-mediated gene knockout targeting the *mrppta* gene in both
321 *M. robertsii* ARSEF 2575 and the ARSEF 2575-derivative NF2 constitutively expressing sGFP yielded
322 three independent mutants for each strain (Supplementary **Fig. S1B**). All six strains displayed identical
323 phenotypes including albino mycelium and light pink conidia. Further experiments were conducted
324 with either $\Delta mrppta$ #7 or $\Delta mrppta$ NF2 #9. *mraara* and *mrsida* consisted of 3,522 bp and 1,800 bp
325 ORFs interrupted by six and two introns, respectively. Reintroduction of *mrppta* in $\Delta mrppta$ #7
326 produced several isolates which were able to grow on minimal medium (MM) without lysine
327 supplementation. These strains were also easily recognizable from $\Delta mrppta$ #7 because of the
328 restoration of the WT-like conidium color and the ability to produce pigmented colonies. Five
329 complemented mutants were selected and one (*Cmrppta* #1) was used in this study. The location of
330 *mraara* is conserved in most *Metarhizium* genomes (Gao et al., 2011; Hu et al., 2014), with the
331 exception of both *M. acridum* and *M. album* (Supplementary **Fig. S2B**). The location of *mrsida* in the
332 *M. robertsii* genome (GenBank accession PRJNA262044) was described earlier (Donzelli et al., 2015).
333 *Agrobacterium*-mediated gene transformation targeting either *mraara* or *mrsida* produced three and
334 four independent knockouts respectively. In both instances, two isolates were used for this study.

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336 3.2. *M. robertsii* $\Delta mrpptA$ and $\Delta mraarA$ mutants are auxotrophic for lysine

337 As expected, both *mrpptA* and *mraarA* mutants were not able to grow on M100 MM without
338 lysine supplementation (**Fig. 1**) while for all other strains lysine had no significant effect (**Fig. 1A** and
339 B). At lysine concentrations above 1 mM, *mraarA* mutants grew faster than WT and displayed thicker
340 aerial mycelium (**Fig. 1B**). Both $\Delta mrpptA$ and $\Delta mraarA$ strains failed to grow when 1% casein was
341 included as the only N source in M100 medium (**Fig. 1C** and D). Ten μ M lysine was sufficient to
342 restore growth of both $\Delta mrpptA$ and $\Delta mraarA$ mutants on casein-supplemented MM (**Fig. 1C** and D).
343 Based on visual assessment of the colony pigmentation, the impact of the mutations on conidiation
344 depended on the growth medium. $\Delta mraarA$ did not conidiate well on AsMM plus 1 mM lysine and
345 higher production of conidia was observed on M100 plus 1 mM lysine; for $\Delta mrsidA$ the effect of
346 medium on conidia production was the opposite of that seen for $\Delta mraarA$. $\Delta mrpptA$ failed to conidiate
347 repeatedly on both AsMM plus lysine and M100 plus 1 mM lysine (data not shown). Surprisingly,
348 SDAY/4 supported good $\Delta mrpptA$ conidiation while both $\Delta mrsidA$ and $\Delta mraarA$ conidiated poorly
349 (data not shown). Consistent production of conidia for all the strains was observed only on M100 + 1%
350 casein + lysine; thus we used this medium to compare the effect of the mutations on conidium
351 production. In M100 plus 1% casein amended with 0.01 mM lysine, $\Delta mrpptA$, $\Delta mrsidA$ and $\Delta mrpptA$
352 produced significantly fewer conidia than the WT. Levels of lysine had a suppressive effect on
353 conidiation for all deletion strains: in the presence of 1 mM lysine conidium production recorded for
354 $\Delta mrpptA$, $\Delta mraarA$ and $\Delta mrsidA$ dropped significantly (17.5%, 52.3% and 68.6%, respectively)
355 compared to the medium containing 0.01 mM lysine (**Fig. 2A**). WT and $\Delta mrpptA$ conidiation was not
356 significantly affected by high lysine concentration (**Fig. 2A**). Lysine also increased the production of
357 aerial mycelium (**Fig. 2B**).

358

359 **3.3. *M. robertsii* $\Delta mrppta$ mutants are defective in the biosynthesis of all known polyketides**
360 **and nonribosomal peptides**

361 The most obvious phenotype of $\Delta mrppta$ mutants was the altered pigmentation. Young colonies
362 were white and conidiating colonies displayed a salmon color that darkened in time (**Fig. 1** and **Fig. 2**).
363 The nonribosomally produced destruxins, serinocyclins, ferricrocin, metachelins and the hybrid
364 polyketide-peptide NG-39x were readily detected in extracts from fermentation fluids or primary
365 conidia from WT. None of these types of compounds were detected in extracts from $mrppta$ mutants
366 and biosynthetic capacity was restored in the complemented strains (**Fig. 3**). Disruption of $mrsida$
367 abolished production of metachelins and ferricrocin but not serinocyclin A (Supplementary **Fig. S3**),
368 which does not contain N^5 -hydroxy-L-ornithine, the product of the ONO encoded by $mrsida$. A mass
369 peak at m/z 780.25 that was present in WT extracts of spores was conspicuously absent in $\Delta mrsida$
370 extracts (Supplementary **Fig. S3**). This peak could not be ascribed to any known compound, suggesting
371 the possibility that *M. robertsii* produces an as yet uncharacterized product incorporating an N^5 -
372 hydroxy-L-ornithine-containing unit.

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374 **3.4. *M. robertsii* $\Delta mrppta$ and $\Delta mrsida$ mutants are hypersensitive to both iron depletion and**
375 **oxidative stress**

376 Since deletion of either $mrppta$ or $mrsida$ blocked the production of both the intracellular
377 siderophore ferricrocin and the extracellular siderophore, metachelin A (**Fig. 3B**, Supplementary **Fig.**
378 **S3**) (Donzelli et al., 2015), both $\Delta mrppta$ and $\Delta mrsida$ were expected to have a similar phenotype with
379 regard to iron acquisition and sensitivity to iron depletion. To test this hypothesis, we compared the
380 growth of these two strains and controls (WT, $Cmrppta$, $\Delta mraarA$) in media with different levels of
381 iron availability: 10 μM iron (iron replete environment); 1 μM iron (borderline iron replete/iron deplete
382 environment); and 10 μM hemin (complexed iron) with and without the Fe^{2+} chelator BPS. WT,
383 $Cmrppta$ and $\Delta mraarA$, displayed similar responses to iron availability, including a relatively small

384 effect of BPS chelation on growth (**Fig. 4A**).

385 *Δmrppta* and *Δmrsida* strains, on the other hand, were strongly affected by low iron availability and
386 iron source and their sensitivity to iron supply was identical up to day 6 (**Fig. 4A**). However, after day
387 6, *Δmrsida* was able to overcome BPS inhibition at both 10 μM iron and 10 μM hemin while *Δmrppta*
388 was able to do so only in the presence of 10 μM hemin (**Fig. 4B and C**), which indicates a distinctive
389 response of *Δmrppta* to reduced iron supply compared to *Δmrsida*. To test whether this unexpected
390 difference might be due to the presence of extracellular siderophores beyond the product of *mrsidD*
391 (Donzelli et al., 2015) we compared the iron chelating activity of WT, *Δmrppta*, *Δmrsida*, and
392 *Cmrppta* strains using a CAS assay. *Δmrppta* had negligible CAS clearing, while *Δmrsida* displayed
393 49% of the iron chelation activity measured in the WT (Supplementary **Fig S4**). We conclude that
394 *Δmrsida* can produce one or more iron-scavenging metabolite(s) whose biosynthesis is abolished in
395 *Δmrppta*.

396 Both *Δmrppta* and *Δmrsida* mycelia were highly sensitive to H₂O₂ compared to WT, *Cmrppta*
397 and *Δmraara*. In M100 medium amended with 2 mM lysine and 200 μM iron, 4 mM H₂O₂ was able to
398 completely inhibit growth of both strains while *Cmrppta* and *Δmraara* were significantly more tolerant
399 to H₂O₂ compared to WT (**Fig. 5**). In contrast, 2 mM H₂O₂ had a strong stimulatory effect on WT,
400 *Cmrppta* and *Δmraara* strains while *Δmrsida* and *Δmrppta* displayed a significant and distinct
401 difference in inhibition (66% and 86% growth reduction, respectively, **Fig. 5**). *Δmrppta* and *Δmrsida*
402 strains were also significantly more sensitive to 0.1 % SDS (p<0.01) compared to the other three strains
403 analyzed (**Fig. 5**) which points to a role in cell wall integrity of *mrppta* and *mrsida*. Deletion of
404 *mrppta*, *mrsida*, or *mraara* did not affect sensitivity to the membrane stressor ketoconazole (data not
405 shown) indicating that plasma membranes are unaffected by these mutations.

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407 **3.5. Disruption of *mrppta* reduces germination speed on insect cuticles**

408 We compared germination of WT and *Δmrppta* strains expressing sGFP on cockroach cuticles

to determine whether this substrate was able to sustain growth in the absence of both lysine and siderophore biosynthesis. Spores were incubated for 40h at 25 °C with or without exogenous lysine. As observed in axenic cultures, WT growth was unaffected by lysine (Supplementary **Fig S5**). Deletion of *mrppta* strongly depressed germination and addition of 2 mM lysine to the spore suspension largely restored germination pace (Supplementary **Fig S5**).

3.6. Inactivation of *mrppta* slightly reduces mycelium hydrophobicity and has no significant effect on conidium hydrophobicity

A mycelium hydrophobicity test based on the water drop penetration time performed poorly as all tested strains failed to absorb water even after >3h of incubation. When treated with ethanol/water mixtures, the ethanol concentration needed to wet the colony was 42.5% (v/v) for the $\Delta mrppta$ strains vs 50.0% (v/v) in both WT and complemented strains (**Fig. 6**). Using the charts published by Vazquez *et al.* (Vazquez *et al.*, 1995), we calculated the surface tensions of the alcohol solutions to be 29.7 and 28.1 mN/m at 23.5°C, respectively, which in turn indicates a ~6% increase in wettability due to the loss of *mrppta*. Hydrophobicity of $\Delta mrppta$, *Cmrppta*, and WT conidia were not significantly different (ANOVA, $\alpha=0.05$) as the volume of 0.1% Silwet L-77 required to inhibit conidial floating was between 70 and 75 μ L (0.013% Silwet L-77 final concentration) for those strains.

3.7. Inactivation of *mrppta* does not affect *B. cereus* inhibition

We have previously observed that *M. robertsii* ARSEF 2575 has antibiotic activity against *B. cereus*, which can be readily visualized as a zone of inhibition around growing fungal colonies (unpublished data). We tested whether loss of both PKS and NRPS pathways had any effect on *B. cereus* inhibition by co-cultivating the bacterium and $\Delta mrppta$. Surprisingly, $\Delta mrppta$ strains exhibited zones of inhibition very similar to those around the WT and other control strains (**Supplementary Fig. S6**) suggesting that NRPS- and PKS-dependent pathways do not contribute to this antibacterial activity.

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3.8. Deletion of *mrppta*, *mraarA* and *mrsidA* resulted in significant loss of virulence

The effect of *mrppta* deletion on virulence was assessed using adult female *D. suzukii* as the insect host. $\Delta mrppta$ strains were compared with WT, $\Delta mrppta$, $\Delta mraarA$, $\Delta mrsidA$, the ferricrocin synthetase (*mrsidC*) and metachelin synthetase (*mrsidD*) deletion mutants (Supplementary **Fig. S7**) (Donzelli et al., 2015). Deletion of the Sfp-type Pptase *mrppta* had the largest effect on virulence. $\Delta mrppta$ – infected *D. suzukii* had an 18.7-fold reduction in mortality risk compared to those inoculated with *M. robertsii* WT (mortality risk = 1), and complementation of $\Delta mrppta$ completely reversed the loss of virulence (**Table 1**). Deletion of either *mraarA* or *mrsidA* had a lesser impact on *D. suzukii* survival as these strains displayed 1.8- and 5.7-fold reduction in *D. suzukii* mortality risk, respectively, compared to the WT (**Table 1**). Loss of either ferricrocin or metachelin resulted in ~2-fold decline in virulence (**Table 1**).

4. Discussion

M. robertsii is known primarily as an insect pathogen but it can also subsist in the soil as a saprobe and in close associations with plants (Behie et al., 2012; Bidochka et al., 1998; Keyser et al., 2014; Liao et al., 2013; St Leger et al., 1997). This species possesses a complex secondary metabolism that may have an important role in its adaptation to these very different lifestyles (Donzelli and Krasnoff, 2016). In this study we examined the contribution of PKS and NRPS metabolisms to some aspects of *M. robertsii* biology using mutants deleted for the Sfp class 4' phosphopantetheinyl transferase *mrppta* and compared them to deletion mutants impaired either in lysine biosynthesis ($\Delta mraarA$) or the biosynthesis of hydroxamate siderophores ($\Delta mrsidA$). As expected, deletion of *mrppta* abolished production of NRPSs and PKSs-derived compounds including a conidial pigment recently linked to the activity of MrPks1, siderophores, and other characterized natural products (Chen et al., 2015; Donzelli et al., 2010; Donzelli et al., 2015; Donzelli et al., 2012; Moon et al., 2008). Like

459 *mraarA* mutants, Δ *mrpptA* strains were lysine auxotrophic but in contrast to Δ *mraarA* strains, lysine
460 supplementation alone did not fully restore WT colony size, which may indicate a beneficial role of
461 NRPS and PKS metabolism in *M. robertsii* growth. Inability of Δ *mrpptA* strains to germinate
462 efficiently on cockroach cuticles suggested that conidia of lysine auxotrophs might be unable to exploit
463 complex substrates as lysine sources, including cuticular proteins. To test this hypothesis, we compared
464 the growth of lysine auxotrophic strains Δ *mraarA* and Δ *mrpptA* to non-auxotrophic controls on MM in
465 which casein (which contains 7.5% lysine) was used as the only N and lysine source (Vickery and
466 White, 1933). Neither *mrpptA* nor *mraarA* mutants were able to obtain lysine from casein; on both
467 cockroach cuticles and casein-amended MM, addition of lysine partially compensated for the
468 germination defect of Δ *mrpptA*. The inability of Δ *mrpptA* and Δ *mraarA* strains to use casein as a lysine
469 source is reminiscent of *Aspergillus fumigatus* homocitrate synthase (*hcsA*) null mutants which were
470 also lysine auxotrophic and failed to germinate on either albumin or casein (Schöbel et al., 2010).
471 Earlier studies of *A. fumigatus* showed that upregulation of the α -aminoadipate pathway occurs within
472 30 min of conidium inoculation in yeast extract/peptone/dextrose (YPD) medium, and out of 17 amino
473 acid transporters only Afu3g01560 was upregulated during germination (Lamarre et al., 2008).
474 Interestingly, Afu3g01560 is an ortholog of the *S. cerevisiae* lysine permease LYP1 (Sychrova and
475 Chevallier, 1993). Thus, during germination two taxonomically unrelated fungi appear to rely on *de*
476 *novo* biosynthesis of lysine or free amino acids and not on either reserves or proteins present in the
477 medium (Kasuga et al., 2005; Lamarre et al., 2008). Failure of *M. robertsii* to germinate on casein also
478 indicated that peptidases leached from conidia are ineffective in releasing amino acids from this
479 substrate or cuticular proteins and that their function may not be nutritional (Qazi and Khachatourians,
480 2007; Santi et al., 2010).

481 Loss of *mrpptA* strongly increased sensitivity to both iron deprivation and H₂O₂. Both
482 phenotypes can be attributed predominantly to loss of siderophore biosynthetic capability since *mrsidA*
483 and *mrpptA* mutants behaved almost identically and loss of lysine biosynthesis did not diminish

484 protection against those stresses. However, our results hint at the presence of distinctive and
 485 multifaceted iron uptake strategies in *M. robertsii*. *M. robertsii* is able to efficiently use hemin as an
 486 iron source and to do so, it uses both hydroxamate siderophores and a reductive iron assimilation
 487 system (RIA) since siderophore-minus strains $\Delta mrsidA$ and $\Delta mrppta$ grew more slowly than the WT
 488 on hemin and their growth was further reduced by BPS (**Fig. 4**). The residual growth of both $\Delta mrppta$
 489 and $\Delta mrsidA$ on hemin in the presence of BPS suggests the existence of a specialized pathway(s)
 490 dedicated to the exploitation of heme iron. In contrast, in *Candida albicans* heme utilization is
 491 independent of both RIA and siderophore-mediated iron uptake systems (Santos et al., 2003) and relies
 492 instead on the activity of heme receptors RBT5 and RBT51 located at the cell surface (Weissman and
 493 Kornitzer, 2004) and the heme oxygenase CaHMX1, which frees iron intracellularly by degrading the
 494 porphyrin ring. The human pathogens *Cryptococcus neoformans* and *Histoplasma capsulatus* also
 495 possess specialized receptors dedicated to the exploitation of heme as an iron source (Cadieux et al.,
 496 2013; Foster, 2002; Hu et al., 2013). The human pathogen *A. fumigatus*, on the other hand, does not
 497 possess specialized heme binding proteins or a CaHMX1 ortholog and cannot use hemin as an iron
 498 source (Schrettl et al., 2004). The *M. robertsii* genome, encodes at least five heme oxygenase-domain
 499 proteins, however preliminary studies in our lab have shown that none of their corresponding genes,
 500 including the ortholog of the *C. albicans* CaHMX1, is upregulated under iron starvation (unpublished
 501 data). Sensitivity to BPS exhibited by hydroxamate siderophore minus strains ($\Delta mrsidA$ and $\Delta mrppta$)
 502 compared to the insensitivity displayed by hydroxamate siderophore plus strains (WT, $\Delta mraarA$ and
 503 $\Delta mrppta$) suggests that both siderophores and a yet-to-be characterized RIA contribute to non-heme
 504 iron uptake. It is noteworthy that *M. robertsii* possesses orthologs of the Fe^{2+} transport pathway which
 505 include multicopper oxidase FET3, the ferrous permease FTR1, and 15 proteins belonging to the ferric
 506 reductase family (Stearman et al., 1996). Despite being equally impaired in the biosynthesis of the
 507 hydroxamate siderophores ferricrocin and metachelin, $\Delta mrppta$ and $\Delta mrsidA$ differed significantly in
 508 their sensitivity to BPS-mediated iron sequestration, and H_2O_2 . This led us to the hypothesis that,

509 contrary to our expectations, $\Delta mrsidA$ and $\Delta mrppta$ may differ in their siderophore activity.
510 Surprisingly, when tested using a CAS assay $\Delta mrsidA$ still retained about half of the iron chelating
511 activity of the WT, whereas $\Delta mrppta$ had none at all. This result suggests that the siderophore system
512 in *M. robertsii* may be more complex than appreciated earlier (Donzelli et al., 2015; Krasnoff et al.,
513 2014) and may comprise *mrsidA* -independent compounds as well as the known *mrsidA* -dependent
514 hydroxamate siderophores. Other fungi are known to produce multiple siderophores as in the case of
515 *Gibberella zeae* which produces two extracellular iron scavenging peptides (triacetyl fusarinine C and
516 malonichrome) and one intracellular siderophore (ferricrocin) (Oide et al., 2015). These hydroxamate
517 siderophores are encoded by distinct synthetases that can be identified using phylogenetic and domain
518 structure analyses (Bushley et al., 2008; Bushley and Turgeon, 2010). When these analyses were
519 applied to classify all *M. robertsii* NRPSs, no additional hydroxamate siderophore synthetases were
520 identified beyond those linked to ferricrocin (*mrsidC*) and metachelin (*mrsidD*) biosynthesis (Donzelli
521 and Krasnoff, 2016), which further supports the possibility that the residual siderophore activity
522 observed in $\Delta mrsidA$ derives from an unknown non-hydroxamate natural product. Additionally,
523 increased resistance of $\Delta mrsidA$ to oxidative stress compared with $\Delta mrppta$ might be conferred by yet
524 another unknown natural product not involved in iron homeostasis. It should be noted that orthologs of
525 the *M. robertsii* NRPS-like EXU99674 and hybrid PKS-NRPS EXV04383 have been implicated in
526 oxidative stress protection in other fungi (Awakawa et al., 2013; Gallagher et al., 2012; Ohm et al.,
527 2012; Reeves et al., 2006; Wang et al., 2015).

528 Deletion of *mrppta* increased sensitivity to SDS, which has both membrane- and cell wall-
529 disrupting activities (Santos and Snyder, 2000; Shimizu et al., 1994), relative to WT, *Cmrppta*, and
530 $\Delta mraarA$ strains. The response of $\Delta mrppta$ to SDS was not significantly different from that induced by
531 the deletion of *mrsidA*, hence it is likely that the $\Delta mrppta$ phenotype arose exclusively from loss of
532 siderophores. Since both $\Delta mrppta$ and $\Delta mrsidA$ strains were as sensitive as the WT to the membrane-
533 damaging ketoconazole, increased SDS toxicity against these strains is likely to derive from weakened

534 cell walls.

535 Several fungal PKS and NRPS metabolites exhibit antibacterial activity (Fleming, 1929; Smith
536 et al., 1990). In preliminary tests, we observed that *M. robertsii* strongly inhibits growth of the gram⁺
537 bacterium *B. cereus*, and we hypothesized that a natural product might be responsible for this activity.
538 For instance, *M. robertsii* harbors a PKS pathway resembling that leading to the production of
539 viridicatumtoxin in *Penicillium aethiopicum*, a tetracycline-like antibiotic active against gram⁺ bacteria
540 (Chooi et al., 2010; Gibson et al., 2014; Nicolaou et al., 2014). However, inactivation of *mrppta* did
541 not reduce *M. robertsii* antibacterial activity indicating that this effect is not mediated by a non-
542 ribosomal peptide or polyketide or *mrppta* does not activate all PKSs and NRPSs present in the *M.*
543 *robertsii* genome. This is highly unlikely because no additional Sfp-type 4'-phosphopantetheinyl
544 transferase is found in the *M. robertsii* genome and all known PKSs and NRPSs pathways were
545 affected by the loss of *mrppta*. This unexpected finding suggests that either terpenes or other small
546 molecules of unknown biogenetic origin are responsible for the antibacterial activity.

547 Deletion of the *mrppta* gene resulted in nearly avirulent *M. robertsii* strains that never produced
548 mycosed cadavers. In comparison, lysine auxotrophy or loss of siderophores attenuated virulence but
549 nearly all dead insects inoculated with the $\Delta mrsidA$ or $\Delta mraarA$ produced conidiating cadavers (data
550 not shown). The extreme severity of the virulence reduction observed in $\Delta mrppta$ relative to the
551 moderate and small reduction observed in $\Delta mrsidA$ and $\Delta mraarA$ strains, respectively, strongly
552 supports a contribution to pathogenicity stemming from the combined activity of NRPS and PKS
553 pathways. In contrast to other fungal systems where lysine auxotrophy strongly depresses virulence
554 (Leng and Zhong, 2012; Liebmann et al., 2004; Wiemann et al., 2012), in *M. robertsii* inactivation of
555 the α -aminoadipate pathway resulted in a small reduction in *D. suzukii* mortality, despite the strong
556 effect of lysine auxotrophy on germination when free lysine is unavailable. This suggests that on
557 cuticles of living insects, lysine might be not as limiting as indicated by *ex-vivo* assays (Supplementary
558 **Fig. S5**). Moreover, the hemocoele is not expected to be a growth-limiting environment for lysine

auxotrophs as lysine concentration in hemolymph exceeds 1 mM (Bennett and Shotwell, 1973; Locke, 1980; Piyankarage et al., 2008). Loss of siderophores, on the other hand, was associated with a much larger decline in virulence than lysine auxotrophy. Elimination of both intracellular and extracellular siderophores had a dramatic effect on virulence in some fungal species (Hissen et al., 2005; Oide et al., 2015) but not in others in which RIA is the primary iron acquisition route (Hwang et al., 2008; Mei et al., 1993; Ramanan and Wang, 2000; Saikia et al., 2014). In *M. robertsii*, deletion of *mrsidA* or *mrsidD* resulted in partially reduced virulence. This indicates that this pathogen relies on a combination of iron acquisition systems, including hydroxamate and unknown non-hydroxamate siderophores, to gain iron from its hosts. The reduced virulence of $\Delta mrsidA$ could be ascribed to loss of both metachelin ($\Delta mrsidD$) and ferricrocin ($\Delta mrsidC$), which contributed to virulence to about the same extent. Thus, the significance of metachelin for *M. robertsii* virulence against *D. suzukii* stands in contrast to our previous finding that $\Delta mrsidD$ mutants were as virulent as WT against *Spodoptera exigua* larvae (Donzelli et al., 2015), suggesting that the contribution of iron sequestration to the innate host immune response to *M. robertsii* infection is host-dependent.

573

574 **Conclusions**

In this study, we demonstrated the involvement of the Sfp type Pptase *mrppta* in the activation of known PKS and NRPS pathways in *M. robertsii*. The mutation resulted in lysine auxotrophy, significant reduction in resistance to iron deprivation, hypersensitivity to oxidative stress, and a dramatic drop in virulence. Lysine auxotrophy was *mraarA*-dependent and strongly reduced, but did not abolish germination on insect cuticles. Other phenotypes, including decreased virulence, increased sensitivity to both oxidative stress and iron deficiency, were likely due to the combined effect of known siderophores and unknown nonribosomal peptides or polyketides, of which some may act as additional iron chelators. Further work will be needed to unravel the various roles played by the more than 50 PKSs and NRPSs predicted from the *M. robertsii* genome (Donzelli and Krasnoff, 2016).

584

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587 proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S.
588 Department of Agriculture and does not imply its approval to the exclusion of other products or vendor
589 that may also be suitable.

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Fig. 1. Effect of lysine and casein supplements on growth on M100 minimal medium. (A) Growth measured after 7 days at 24 °C in the presence of variable amounts of lysine, (B) colonies after 12 days of incubation, (C) growth in the presence of 1% casein and variable amounts of lysine measured after 7 days at 24 °C and (D) colonies at 10 days. Error bars represent the standard error (n = 4). *ΔmrpptA* and *ΔmraarA* are lysine auxotrophs and are unable to initiate utilization of casein unless “primed” with lysine.

Fig. 2. Conidiation of *M. robertsii* WT and mutants grown on M100 + 1% casein + 200 μM iron and variable amounts of lysine. The *ΔmrpptA*-complemented strain *CmrpptA* was also included as a control. (A) Conidiation levels, (B) Colonies 15 days after incubation at 24 °C. Error bars represent the standard error (n = 3). Strains not connected by the same letter are statistically different within the same lysine supplementation level as determined by one-way ANOVA and Student's t-test ($\alpha=0.05$). Mutants lacking *mrpptA* and *mrsidA* were consistently depressed in conidiation while conidiation of *mraarA* mutants depended on the amount of supplemented lysine. WT and *ΔmrpptA* complemented strain *CmrpptA* were unaffected by lysine levels.

Fig. 3. Loss of selected metabolites in $\Delta mrppta$ compared with *M. robertsii* WT and complemented mutant *Cmrppta*. (A) LC/MS extracted mass chromatograms for the $[M+H]^+$ of destruxin A at m/z 578.3554. Extracts from $\Delta mrppta$ were dissolved in 1 mL MeOH whereas WT and *Cmrppta* were further diluted 100-fold. Estimates for DTX A in WT and *Cmrppta* mutants shown were ~100 ng on column or ~1 mg/100 mL broth. Destruxin production was tested in HB broth. (B) LC/MS extracted ion chromatograms selecting the pseudomolecular ion of metachelin A at m/z 1095.5. WT and *Cmrppta* extracts were diluted 10-fold relative to the $\Delta mrppta$. Estimates for metachelin A in the WT and *Cmrppta* samples shown were ~500 ng on column or 6.2 mg/100 mL broth. Small peaks at ~2 min are generated by non-specific ions in the solvent. Metachelin production was tested in low-iron *Aspergillus* Minimal medium. (C) HPLC analyses showing NG-391 (t_R = 15.3 min) and NG-393 (t_R = 13.4 min) in extracts of WT and *Cmrppta*. Injection volumes were 10 μ L for WT and *Cmrppta* and 20 μ L for $\Delta mrppta$ mutants, thus the second trace represents a 2-fold increase in concentration relative to the original 100 mL broth sample. Estimates for NG-391 were ~5 and 4 μ g on column or 0.5 and 0.4 mg/100 mL of broth for WT and *Cmrppta*, respectively. (D) Accurate mass spectra from flow injections showing the mass range including pseudomolecular ions $[M+K]^+$ of serinocyclin A, and the ferrated form of ferricrocin (calculated exact masses = 711.2716 and 809.2046, respectively). Estimates for serinocyclin A for the spectra shown (based on ion counts for the relevant mass peaks) were 246 ng for the injection or 984 μ g/100 mL broth for WT and 219 ng for the injection, 876 μ g/100 mL broth for *Cmrppta*. Estimates for ferricrocin were 12 ng for the injection or 48 μ g/100 mL broth for WT and 8 ng for the injection or 34 μ g/100 mL broth for *Cmrppta*. The peak at ca. m/z 780.25 is from an unknown compound. For this experiment conidia were produced on SDAY/4.

Fig. 4. Effect of iron supply on the growth in *Aspergillus* Minimal Medium of *M. robertsii* WT and mutants lacking *mrppta*, *mraarA*, *mrsida*. $\Delta mrppta$ -complemented strain (*Cmrppta*) was also included as a control. (A) Colony growth at day 6 normalized to that of the same strain grown in 10 μ M iron and

634 2 mM lysine (relative growth), (B) Relative growth of $\Delta mrpptA$ and $\Delta mrsidA$ colonies at 11 days, (C)
635 Colonies at 11 days. Error bars represent the standard error ($n = 4$). Relative growth levels were
636 compared within each treatment using ANOVA and Student's t-test ($\alpha=0.05$) after Box-Cox
637 transformation. Strains not connected by same letter are significantly different. Iron depletion had little
638 or no effect on growth of WT, $\Delta mrsidA$, and $\Delta mraarA$ but negatively affected growth of $\Delta mrpptA$ and
639 $\Delta mrsidA$ which at day 6 were statistically indistinguishable. In time, $\Delta mrsidA$ strains were able to
640 overcome bathophenanthroline disulfonic acid (BPS) inhibition at 1 mM $FeCl_3$ while $\Delta mrpptA$ strains
641 were not.

642

643 **Fig. 5.** Effect of hydrogen peroxide (H_2O_2) or sodium dodecyl sulfate (SDS) included in M100 minimal
644 medium on the growth of *M. robertsii* WT, the *mrpptA*-complemented strain *CmrpptA* and mutants
645 lacking *mrpptA*, *mraarA*, or *mrsidA*. Growth was estimated as OD_{900} normalized to that of the same
646 strain grown in medium containing no stressors. Strains were compared within each treatment using
647 ANOVA and Student's t-test ($\alpha=0.05$) after Box-Cox transformation. Strains not connected by same
648 letter are significantly different. Deletion of *mraarA* and *mrsidA* had opposite effects on resistance to
649 oxidative stress. Deletion of *mrpptA* had the largest negative effect on H_2O_2 sensitivity. Sensitivity to
650 SDS was reduced to the same extent in *mrsidA* and *mrpptA*.

651

652 **Fig. 6.** Deletion of *mrpptA* gene has a small but significant effect on colony hydrophobicity when
653 tested with water/ethanol mixtures. Droplets (30 μ L) were placed on mycelial mats and the minimum
654 concentration of ethanol needed for hyphal wetting was recorded. The test was conducted on SDAY/4
655 plates and replicated three times.

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Table 1

Pairwise comparison of mortality risks and survival times calculated for *M. robertsii* WT,

$\Delta mrpptA$, $\Delta mraarA$, $\Delta mrsidD$, $\Delta mrsidC$ strains. Comparisons are expressed as relative mortality risks (RMR). Nonsignificant differences are marked in bold.

A	B	RMR (B/A)	95 % CI	Wald (z)	Pr(> z)	1/RMR (A/B)
WT	$\Delta mraarA$	0.55	0.41 - 0.74	-3.953	<0.0001	1.80
WT	$\Delta mrpptA$	1.25	0.95 - 1.66	1.575	0.115	0.79
WT	$\Delta mrsidC$	0.45	0.32 - 0.62	-4.737	<0.0001	2.23
WT	$\Delta mrsidD$	0.51	0.36 - 0.71	-3.957	<0.0001	1.96
WT	$\Delta mrpptA$	0.05	0.03 - 0.09	-10.237	<0.0001	18.68
WT	$\Delta mrsidA$	0.17	0.13 - 0.24	-11.344	<0.0001	5.74
$\Delta mrpptA$	$\Delta mraarA$	10.35	5.79 - 18.49	7.893	<0.0001	0.10
$\Delta mrpptA$	$\Delta mrpptA$	23.40	13.06 - 41.94	10.593	<0.0001	0.04
$\Delta mrpptA$	$\Delta mrsidC$	8.35	4.59 - 15.18	6.960	<0.0001	0.12
$\Delta mrpptA$	$\Delta mrsidD$	9.50	5.22 - 17.3	7.359	<0.0001	0.10
$\Delta mrpptA$	$\Delta mrsidA$	3.25	1.83 - 5.78	4.028	<0.0001	0.30
$\Delta mrsidA$	$\Delta mrsidC$	2.57	1.77 - 3.71	5.004	<0.0001	0.39
$\Delta mrsidA$	$\Delta mrsidD$	2.92	2.01 - 4.24	5.645	<0.0001	0.34
$\Delta mrsidA$	$\Delta mraarA$	3.18	2.27 - 4.46	6.6940	<0.0001	0.31
$\Delta mrsidA$	$\Delta mrpptA$	7.19	5.11 - 10.13	11.3070	<0.0001	0.14
$\Delta mraarA$	$\Delta mrsidC$	0.81	0.56 - 1.17	-1.135	0.256	1.24
$\Delta mraarA$	$\Delta mrsidD$	0.92	0.63 - 1.33	-0.45	0.653	1.09
$\Delta mraarA$	$\Delta mrpptA$	2.26	1.62 - 3.15	4.8120	<0.0001	0.44
$\Delta mrsidC$	$\Delta mrpptA$	2.80	1.94 - 4.05	5.482	<0.0001	0.36
$\Delta mrsidC$	$\Delta mrsidD$	1.14	0.76 - 1.7	0.629	0.529	0.88
$\Delta mrsidD$	$\Delta mrpptA$	2.46	1.7 - 3.57	4.779	<0.0001	0.41

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6