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Targeted disruption of the polyketide synthase gene *pks15* affects virulence against insects and phagocytic survival in the fungus *Beauveria bassiana*

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ABSTRACT

The reducing clade III polyketide synthase genes, including *pks15*, are highly conserved among entomopathogenic fungi. To examine the function of *pks15*, we used targeted disruption to investigate the impact of *Beauveria bassiana pks15* on insect pathogenesis. Southern analysis verified that the Δ*pks15* mutant was disrupted by a single integration of the transformation cassette at the *pks15* locus. The Δ*pks15* mutant had a slight reduction in radial growth, and it produced fewer spores. Our insect bioassays indicated the Δ*pks15* mutant to be significantly reduced in virulence against beet armyworms compared to wild type (WT), which could be partially accounted for by its markedly decreased ability to survive phagocytosis. Total haemocyte count decreased sharply by 50-fold from days 1–3 post-inoculation in insects infected with WT, compared to a 5-fold decrease in the Δ*pks15* mutant. The mutant also produced fewer hemolymph hyphal bodies than WT by 3-fold. In co-culture studies with amoebae that have phagocytic ability similar to that of insect haemocytes, at 48 h the mortality rate of amoebae engulfing Δ*pks15* decreased by 72 %, and Δ*pks15* CFU decreased by 83 % compared to co-culture with WT. Thus, the Δ*pks15* mutant had a reduced ability to cope with phagocytosis and highly reduced virulence in an insect host. These data elucidate a mechanism of insect pathogenesis associated with polyketide biosynthesis.

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Introduction

Microorganisms synthesize a large and diverse array of secondary metabolites. These secondary metabolites supply defensive or offensive small molecules for the producing microbe to fit their ecological niche. Microorganisms producing these compounds experience remarkable advantages over nonproducing ones by gaining access to and utilizing nutrient sources that are inaccessible to other groups of microorganisms. In fungi, there have been several fungal genomes reported, many of which are rich in secondary metabolite biosynthetic gene clusters (Xiao *et al.* 2012; Inglis *et al.* 2013). Nevertheless, few of these clusters have been characterized regarding their roles in their interaction with other organisms, with the surrounding environment, or in normal growth and development. Fungal secondary metabolites include polyketides, which are synthesized by multifunctional enzymes called polyketide synthases (PKSs) using acetyl-CoA as the starter unit and malonyl-CoA as extenders (Khosla 2009). Three major domains of PKSs include β-ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). Modification of a newly formed polyketide is then mediated by accessory domains including but not limited to β-ketoreductase (KR), dehydratase (DH), and trans-acting enoyl (ER) domains (Schwarzer & Marahiel 2001; Staunton & Weissman 2001). PKSs are classified into three types: types I, II, and III (Hertweck 2009; Weissman 2009). Fungal PKSs are type I and can be further divided into two major classes: nonreducing (NR; subclades I-III) and reducing (R; I-VIII) (Kroken *et al.* 2003; Punya *et al.* 2015).

Entomopathogenic fungi are a remarkable and beneficial group of microbes used for the biocontrol of insects. In the absence of insect hosts, they can be saprobic in the soil. However, upon contact with the insect cuticle, these fungi switch to their pathogenic phase and cause mycosis (Roberts & Hajek 1992; Bidochka *et al.* 2000). *Beauveria bassiana* is one of the most well-known and studied entomopathogenic fungi used for insect control. The fungus has a broad host range and is widely used as a biocontrol agent for agricultural pests, such as white flies, aphids, mealybugs, thrips (Shah & Goettel 1999), corn earworms and beet armyworms (Wraight *et al.* 2010). *Beauveria bassiana* infects insect hosts upon contact, distinguishing its group from entomopathogenic bacteria and viruses that require ingestion by the host. After fungal conidia adhere to the host cuticle, they can germinate and grow given favourable environmental conditions such as pH, temperature, and host surface compounds (St Leger *et al.* 1986). The growing hyphae then penetrate into the haemocoel, form hyphal bodies that can evade the host immune system, and develop into the vegetative stage (Amnuaykanjanasin *et al.* 2013). Finally, the fungus emerges and hyphae cover the host surface. During invasion into the haemocoel, insect hosts defend themselves using innate immune responses, including humoural and cellular systems (Lavine & Strand 2002). The cellular immune response is associated with production and mobilization of haemocytes (Russo *et al.* 2001), the phagocytosis ability of which is crucial for removal of infectious microbes (Borges *et al.* 2008). Two entomopathogenic fungi, *Metarhizium anisopliae* and *B. bassiana*, have been examined for susceptibility to phagocytosis by the soil amoeba *Acanthamoeba castellanii* (Bidochka *et al.* 2010), which has properties analogous to insect haemocytes. The results showed that these phagocytosed insect pathogens were able to survive and grow within the amoebae, leading to amoeboid death.

In host–pathogen interactions, some secondary metabolites produced by fungal pathogens play a major role in successful infection and colonization of a host. In another large family of fungal secondary metabolites, nonribosomal peptides, beauvericin, and bassianolide were shown by targeted gene disruption studies to be crucial insect virulence factors for *Galleria mellonella*, *Spodoptera exigua*, and *Helicoverpa zea* (Xu *et al.* 2008, 2009). In the polyketide family, some PKS genes have been studied using a similar genetic knockout approach. A functional study of tenellin revealed that this polyketide is not important for infection of *G. mellonella* (Eley *et al.* 2007). A more recent study on the oosporein biosynthetic cluster demonstrated that this red polyketide pigment contributes to fungal virulence on an insect host, perhaps by modulating the host's immunity (Feng *et al.* 2015).

Previously, we reported that fungal PKSs in reducing clade III are extremely conserved among insect-pathogenic fungi, including *B. bassiana*, *Cordyceps pseudomilitaris*, *Isaria javanica*, *Metarhizium flavoviride*, and *Paecilomyces tenuipes* (Amnuaykanjanasin *et al.* 2009; Punya *et al.* 2015). Moreover, PKS genes in this group have been found as single-copy genes in these fungal genomes, in contrast to other reducing clades, of which more than one gene can be found (Amnuaykanjanasin *et al.* 2009). In this study, the function of the reducing clade III PKS gene *pks15* in the fungus *B. bassiana* BCC2660 was investigated by targeted disruption. Differences in colony morphology, radial growth, sporulation, and gene expression levels between the wild type (WT) and mutant were evaluated. To investigate the influence of the *pks15*-derived polyketide(s) in virulence against insects, the disrupted mutant was used in bioassays against beet armyworms (*S. exigua*) and silkworm (*Bombyx mori*). Moreover, phagocytic survival against *A. castellanii* was determined to study the potential effect of gene disruption on insect immune response escape. Here, we showed that deletion of *pks15* in this fungal entomopathogen reduces its ability to survive phagocytosis and reduces virulence.

Materials and methods

Fungal, bacterial, and amoeboid strains and culture conditions

Beauveria bassiana BCC2660 was obtained from the BIOTEC Culture Collection, Thailand, and grown on half-strength PDA (Difco, USA) at 25 °C for 5–7 d to obtain conidia. For production of blastospores, the fungal conidia were shaken in Sabouraud dextrose broth (Difco) supplemented with 1 % yeast extract (SDY) at 150 rpm and 25 °C for 2 d. The fungus was grown in PDB for 5–7 d for genomic DNA extraction and in SDY for 3–7 d for total RNA isolation.

Agrobacterium tumefaciens EHA105 was obtained from the Enzyme Technology Laboratory (BIOTEC, Thailand) and