The polyketide synthase PKS15 has a crucial role in cell wall formation in *Beauveria bassiana*

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Entomopathogenic fungi utilize specific secondary metabolites to defend against insect immunity, thereby enabling colonization of their specific hosts. We are particularly interested in the polyketide synthesis gene *pks15*, which is involved in metabolite production, and its role in fungal virulence. Targeted disruption of *pks15* followed by genetic complementation with a functional copy of the gene would allow for functional characterization of this secondary metabolite biosynthesis gene. Using a *Beauveria bassiana* Δ*pks15* mutant previously disrupted by a bialophos-resistance (bar) cassette, we report here an *in-cis* complementation at bar cassette using CRISPR/Cas9 gene editing. A bar-specific short guide RNA was used to target and cause a double-strand break in bar, and a donor DNA carrying a wild-type copy of *pks15* was co-transformed with the guide RNA. Isolate G6 of Δ*pks15* complemented with *pks15* was obtained and verified by PCR, Southern analyses and DNA sequencing. Compared to Δ*pks15* which showed a marked reduction in sporulation and insect virulence, the complementation in G6 restored with insect virulence, sporulation and conidial germination to wild-type levels. Atomic force and scanning electron microscopy revealed that G6 and wild-type conidial wall surfaces possessed the characteristic rodlet bundles and rough surface while Δ*pks15* walls lacked the bundles and were relatively smoother. Conidia of Δ*pks15* were larger and more elongated than that of G6 and the wild type, indicating changes in their cell wall organization. Our data indicate that PKS15 and its metabolite are likely not only important for fungal virulence and asexual reproduction, but also cell wall formation.

*Beauveria bassiana*, an entomopathogenic fungus, has a broad host spectrum and is considered to have high potential for insect biocontrol in agriculture. While *B. bassiana* can cause mycosis in several insect species¹², insect killing is fairly slow due to several limiting factors, particularly in the field. The fungus is also vulnerable to environmental stress factors such as UV radiation, high temperature and drought¹. A better understanding of the biological and physiological characteristics of this entomopathogen should allow us to improve its virulence and stress tolerance.

Secondary metabolites are abundant in entomopathogenic fungi and include polyketides, nonribosomal peptides, terpenes and alkaloids that play important roles in various aspects of the fungal life cycle. *B. bassiana* BCC2660, a widely used biocontrol fungus in Thailand, has 12 polyketide synthase (PKS) genes in its genome⁴. Two PKS genes, *pks15* and *pks14*, have crucial roles in virulence against insects, as previously demonstrated by targeted gene deletion⁴⁵. The *pks15* mutant exhibits loss in phagocytic survival ability, a phenotype likely associated with changes in the cell wall, the outermost layer of fungal conidia. Unfortunately, little is known regarding the relationship between polyketides and the fungal wall. In a few reports, melanin, the metabolite of a non-reducing PKS and other enzymes in melanin biosynthetic pathway, has been found in the cell walls of *Aspergillus fumigatus*, *Colletotrichum lagenarium*, *Neurospora crassa* and *Pestalotiopsis microspora*. The *P. microspora* PKS1 is also important for wall integrity and conidial germination in this endophytic fungus⁸. In

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addition, the green pigment citreoisocoumarin, synthesized by a non-reducing PKS, is present in the cell wall of mature *A. nidulans* conidia\(^{10,11}\). To the best of our knowledge, however, no report thus far has identified a role for a reducing PKS in modulating the fungal wall.

Previously, using a gene knock-out approach, we found that insect virulence of the *B. bassiana* \(\Delta pks15\) deletion mutant was considerably reduced\(^5\). Furthermore, our preliminary observation suggested that the spore wall surface of \(\Delta pks15\) was noticeably different from that of the wild type. In this study, we have set out to investigate whether \(\Delta pks15\) has impact on the fungal wall by performing genetic complementation of \(\Delta pks15\) by CRISPR/Cas9 to verify the role of PKS15 in cell wall formation.

So far, there have been a few reports on *B. bassiana* genome editing of using the CRISPR/Cas9 approach. This clustered regularly interspaced short palindromic repeat sequence (CRISPR)/Cas9 system has been developed to perform genome editing in various microorganisms\(^{43}\). This system consists of two components: the Cas9 endonuclease and a single chimeric guide RNA (sgRNA) containing 20-nucleotides matching the target DNA region followed by protospacer adjacent motif (PAM). Base pairing of the sgRNA and the target DNA results in recruitment of Cas9 to generate a specific double-strand break (DSB)\(^{13}\). DSBs can be repaired by either error-free homologous recombination called homology-directed repair (HDR) in the presence of a donor DNA or error-prone repair via non-homologous end-joining (NHEJ). The latter usually leads to a small insertion or deletion in the target sequence, resulting in gene mutation.

Here, we employed CRISPR/Cas9 with HDR to obtain a complemented isolate of *B. bassiana* \(\Delta pks15\) with a functional \(pks15\). The complemented isolate was verified by PCR and Southern analyses and examined various phenotypes, namely sporulation, germination. We report the impact of \(pks15\) in the cell wall formation in this study.

### Results

**Directed mutagenesis of the bar cassette in the \(\Delta pks15\) mutant efficiently driven by NHEJ repair.** To study the function of PKS15 in *B. bassiana* BCC 2660, we were interested in generating a PKS15-complemented \(\Delta pks15\) mutant by CRISPR/Cas9. However, the CRISPR/Cas9 vector used in this study was previously developed for *Aspergillus aculeatus*\(^{33}\), so we first tested this vector for its ability to mediate targeted genome editing in the *B. bassiana* BCC 2660 \(\Delta pks15\) mutant. The sgRNA target site was designed based on the nucleotide sequence of the *bar* cassette previously inserted within \(pks15\) to disrupt gene expression\(^4\). A list of candidate bar protospacer sequences was generated using a web-based sgRNA analysis tool (https://bioinfo.imtech.res.in/manojk/gecrispr/index.php). The sequence with the lowest likelihood of off-target binding was selected.

Construction of the *bar*-targeting vector, pCas9sgBar, was performed by introducing the protospacer into the CRISPR/Cas9 backbone vector via USER cloning\(^{13}\). DNA sequencing was used to verify sequence integrity and the correct sequential order of pCas9sgBar elements. The elements of pCas9sgBar are shown in Supplemental Fig. S1. The vector pCas9sgBar was then transformed into the \(pks15\) mutant using PEG-mediated protoplast transformation. Transformsants were first screened for hygromycin resistance conferred by pCas9sgBar. Additionally, as the *bar* cassette enables growth on medium supplemented with glucosinate\(^4\), hygromycin-resistant transformants were subsequently screened for the inability to grow in the presence of glucosinate, which would indicate successful mutation of the *bar* cassette. Among 50 hygromycin-resistant transformants, two clones, namely A26 and C1, had lost glucosinate resistance (data not shown). Single spore isolation was performed to purify those mutants, and those isolates were again verified for hygromycin resistance and glucosinate sensitivity.

To determine the sequence of *bar* in A26 and C1, their *bar* fragments were amplified with a pair of *bar*-specific primers, cloned into the TA cloning vector pCR 2.1, and submitted for DNA sequencing. Sequencing results showed that clones A26 and C1 both had a one-base insertion in the *bar* cassette (Table 1). These genome alterations resulted in a frameshift mutation in *bar*, changing their amino acid sequences. The CRISPR/Cas9 system, therefore, can mediate targeted gene editing in *B. bassiana* BCC 2660.

| Insertion/deletion | Amino acid sequence |
|--------------------|---------------------|
| 1                  | ..THLLKSLEAQ        |
| 1                  | ..THLLKSLGGT        |

Table 1. Mutations in the *bar* locus of three mutants. The mutants A26 and C1 were directly mutagenized by CRISPR/Cas9. The one-base insertions in A26 and C1, highlighted in italics, caused frame shifts and changes in the amino acid sequence. The PAM site is underlined.

**In-cis genetic complementation of \(\Delta pks15\) using CRISPR/Cas9.** In addition to directed mutagenesis, the CRISPR/Cas9 system has the potential to mediate targeted genetic complementation via HDR. In \(\Delta pks15\), the *bar* cassette has been integrated in \(pks15\), thereby disrupting gene function\(^7\). To further verify the role of \(pks15\) in the fungus, a circular DNA donor pCR-PKS15-1k, which has a 1.0 kb *pks15* sequence serving as arms for homologous recombination, was co-transformed with pCas9sgBar into \(\Delta pks15\) for homologous replacement of *bar* (Fig. 1a).
In the genetic complementation of Δpks15, three transformants (G1, G2 and G6) grew on medium supplemented with hygromycin, but failed to grow on the glufosinate-supplemented one. This indicated the hygromycin resistance and glufosinate sensitivity of the aforementioned transformants (Fig. 2a). We performed molecular analysis of the pks15 locus in transformants G1, G2 and G6 by PCR amplification, in comparison to that of the wild type and Δpks15. The primers’ locations are mapped in Fig. 1a. G6 gave an amplification product of 3.2 kb similar to the wild-type amplicon, whereas G1 and G2 did not (Fig. 1b, left panel). The transformant G6, identified as a complemented isolate of Δpks15, was selected for further experiments and subjected to single spore isolation. It should be noted that, although we did not obtain a high number of transformants in our complementation experiment, one out of three (3%) had correctly repaired the pks15 locus. This recombination frequency is markedly higher than those of homologous recombination performed by conventional, non-CRISPR methods in various B. bassiana strains. For instance, 7–13% recombination was observed for strain Bb006214 and 7–25% for our BCC 2660 strain.4,5 After single spore isolation, all ten isolates were re-checked for hygromycin and glufosinate resistance as described above. Three single spore isolates, G6.1, G6.3 and G6.6, were randomly selected for PCR analysis. Those three isolates gave products of similar size as the wild type (Fig. 1a, right panel). Furthermore, we checked for the presence of bar cassette using the bar-specific primers. The pks15 mutant was the only one able to produce bar-derived PCR products, whereas isolates G6.1, 6.3, 6.6 and the wild type did not (Fig. 1c). Southern hybridization also showed that isolate G6.6 had a hybridized band similar to that of the wild type and noticeably shorter than that of Δpks15 (Fig. 1d). The PCR and Southern hybridization data confirmed that the bar cassette had been removed in the complemented isolate G6. Lastly, for the molecular analysis of the pks15 locus, DNA sequencing demonstrated the sequence integrity of pks15 in G6.6, being identical to that of the wild type (Supplemental Fig. S2). Together, these results indicated that replacement of the bar cassette with a wild-type copy of pks15 via CRISPR/Cas9-induced HDR was successful in this entomopathogenic fungus.

**Restoration of wild-type levels of sporulation and germination in the complemented isolate.** The sporulation assay has shown that Δpks15 produced significantly fewer conidia and blastospores than the wild type, as we previously reported. In this study, the complemented isolate G6 restored sporulation, with an increase in the percentage of conidia (37–88%) and blastospores (30–98%) compared to Δpks15, relative to wild-type levels (Table 2). Conidial germination was also restored from 34% in Δpks15 to 77% in the complemented isolate G6 (Table 2).

**Restoration of insect virulence and phagocytic survival ability of the complemented isolate.** Virulence against the beet armyworm was determined by inoculating low doses (300 conidia) of the wild type, Δpks15 or the complemented isolate G6 into larvae. Mortality data showed that the complemented isolate G6 resulted in similar insect mortalities to that of wild type over the observation period (Fig. 2b). On day 4 post-injection, mycosis and insect death were clearly observed in the worms inoculated by the wild type and the complemented isolate G6, resulting in 30% and 56% mortality, respectively (Fig. 2b). On the other hand, insect larvae injected with Δpks15 exhibited only 10% insect mortality at the same time point. At the end of experiment (day 7 post-injection), the mortality rate of worms inoculated with the wild type and complemented isolate G6 was at 95%, whereas Δpks15-associated mortality was at 60%. The mean lethal time (LT50) of insect larvae injected with the wild type, Δpks15 and complemented isolate G6 was 4.09, 4.71 and 3.95 days, respectively. Thus, the complemented isolate G6 was capable of killing larvae at a rate similar to that of the wild type. These data indicated that the impaired insect virulence of Δpks15 could be restored by complementation with a wild-type copy of pks15, thus further cementing a role for pks15 in insect virulence.

We previously investigated whether PKS15 is involved in the first line of insect host defense by assessing the ability of the wild type and Δpks15 in escaping phagocytosis using the soil-dwelling amoeba Acanthamoeba castellanii as a model of study. We found that phagocytic survival was impaired in Δpks15 compared to the wild type. Here, we studied G6 in a similar assay and conducted microscopy to monitor amoeboid mortality. Similar to the previous result, several Δpks15 were engulfed and lysed by the amoeba, whereas most wild-type and G6 blastospores escaped from phagocytosis (Fig. 2d). These blastospores then propagated extensively and switched to a vegetative phase, eventually leading to amoeboid death. Our quantitative data showed that co-culture of wild-type and G6 blastospores with phagocytotic amoeba resulted in 40–50% amoeba mortality, while only ~20% mortality was seen for Δpks15 at 48 and 72 h after mixing (Fig. 2c). This indicated that the ability of the complemented isolate G6 to counteract with phagocytosis was restored to wild-type levels.

**PKS15 is involved in cell wall formation.** Since the cell wall is the outermost part of the cell which actively interacts with the surrounding cells, we thus explored the cell wall surface characteristics in the wild type, pks15 mutant and G6. We performed a series of comparative analyses on cell wall characterization used scanning electron microscopy (SEM) to determine the cellular characteristics of conidia used in the insect bioasay and blastospores. The appearance of rodlet bundles on the cell wall surface was the most striking difference seen with the conidia of the wild type and G6 compared to Δpks15 (Fig. 3). Wild-type and G6 conidia had rough wall surfaces and smaller sizes, while mutant conidia had smoother surfaces and larger sizes. Size measurements derived from the scanning electron micrographs indicated that Δpks15 conidia were significantly larger in width, length and size (area) than the wild type and G6 (Fig. 3a,b). The average size of Δpks15 conidia was 1.65 × 2.09 μm (width and length) compared to 1.52 × 1.78 and 1.44 × 1.77 μm for the wild type and G6, respectively (Fig. 3b). The Δpks15 conidia appeared as an ellipse-like shape, compared to circular forms seen for the other two strains.

Atomic force microscopy (AFM) was also performed to visualize conidial wall surface. It revealed a dramatic difference between the wild type and the complemented isolate G6 and that of Δpks15, with wild-type and
G6 walls clearly possessing characteristic rodel bundles, whereas Δpks15 walls lacked such a feature (Fig. 4). However, the arrangement of bundles in the wild type appeared to be more uniform throughout the conidial surface than that of G6, which revealed a few bulges on the surface. The typical bundles disappeared or drastically reduced in size in the Δpks15 conidial surface, with rodellets barely visible. Quantitative analysis of the rodellets showed that the wild type and G6 had similar average numbers of rodellets in each bundle (6.7 ± 1.4 and 6.1 ± 1.3, respectively).

Lastly, we examined differences in cell wall carbohydrate characteristics by staining conidia and blastospores with concanavalin A, which binds to α-glucan and α-mannan, and calcofluor white, which binds to chitin. Concanavalin A-stained Δpks15 conidia, and blastospores to a lesser extent, exhibited brighter staining patterns compared to wild-type and G6 blastospores under identical staining and microscopic protocols (Figs. 5a, 6a). In contrast, the calcofluor-stained wild type, Δpks15 and G6 conidia (Fig. 5a) as well as blastospores (data not shown) displayed similar fluorescence intensity.

We also determined the size and cell characteristics of stained conidia and blastospores. From 2D images, overall conidial size (areas in 2D) of Δpks15 was larger than that of the wild type and complemented isolate G6 (Fig. 5b,c), similar to SEM analysis results. In the wild type and complemented isolate G6, more than 88% of conidia were smaller than 6.0 µm². In contrast, the ratio of Δpks15 conidia with areas less than to greater than 6.0 µm² was 55:45. A number of Δpks15 conidia were bigger than 10 µm³, which is unusual and strikingly larger than typical B. bassiana conidia. We then applied the shape factor (circularity) feature in the NIS-Elements D software version 5.10 (Nikon, USA) with the formula \[ \text{Shape Factor} = \frac{4 \pi \text{Area}}{\text{Perimeter}^2} \] to analyze conidia shapes. Nearly 40% of the Δpks15 conidial population had an elliptical shape rather than the circular form mostly seen with the wild type and G6 (Fig. 5d,e).

Comparisons of blastospore shapes and sizes did not reveal as large a difference among the three strains. The Δpks15 blastospores were noticeably smaller than the wild type and G6, however. When blastospores were classified as either smaller or larger than 15.0 µm², Δpks15 blastospores exhibited a 70:30 ratio compared to 76:24 and 80:20 for the wild type and G6, respectively (Fig. 6b). Nonetheless, the differences were not statistically different (p > 0.05). With respect to the shape of blastospores, we assessed their elongation values using the formula \[ \text{Elongation} = \frac{\text{MaxFeret}}{\text{MinFeret}} \]. The Δpks15 blastospores were more elongated than that of the wild type and G6, with nearly all (90%) Δpks15 blastospores being more elongated (elongation value of > 2.0) compared to 67–76% for wild type and G6 blastospores (Fig. 6b). In contrast, only 10% of Δpks15 blastospores were less elongated (elongation value of < 2.0), compared to 33% and 24% for the wild type and G6 (Fig. 6c), respectively.

Discussion

In our previous report, the gene pks15 was observed to be expressed in almost all culture conditions tested for B. bassiana\(^{15}\), including potato dextrose broth (PDB) (where conidia are produced) and Sabouraud dextrose broth supplemented with 1% yeast extract (SDY) (where in vitro blastospores are formed). We thus hypothesized that PKS15 could be important for fungal growth and development. Indeed, PKS15 is necessary for the formation of conidia and blastospores in this fungus\(^{4}\). SEM and AFM examination of the pks15 locus of transformants G1, G2 and G6 (from genetic complementation of Δpks15) was compared to those for the wild type (WT) and Δpks15 using PCR (b, c) and Southern (d) analyses. Primers used for PCR and their locations are shown in (a). (b) PCR amplification with primers PKS15-minus-895F and PKS15-2200R primer. (c) PCR amplifications with primers Bar100F and PKS15-1182R, and PKS15-StartF and Bar360R. (d) Southern blotting results for Δpks15, the complemented isolate G6.6 and the wild type. Genomic DNA was digested with EcoRI and hybridized with a pks15-specific probe, shown in (a).
a Growth of transformants on selective media

PDA  Hygromycin B  Glufosinate

Δpks15  WT  Δpks15  WT  Δpks15  WT

G1  G2  G6

b Virulence against beet armyworm

Cumulative insect mortality (%) vs Time post inoculation (days)

Time post inoculation (days)

Control  Wild type  Δpks15  Complemented G6

Phagocytic assay

Percentage amoeboid mortality vs Time (0 h, 24 h, 48 h, 72 h)

0 h  24 h  48 h  72 h

A  A + Sc  A + Wild type  A + Δpks15  A + complemented G6

Δpks15  WT  Δpks15  WT  Δpks15  WT

G1  G2  G6

A + G6  A + Sc
(a) Growth of the B. bassiana wild type (WT), Δ pks15 and transformants G1, G2 and G6 (from genetic complementation of Δ pks15) on PDA with/without hygromycin B or on a minimal medium containing glucose. The three isolates G1, G2 and G6 were resistant to hygromycin B but became sensitive to glucose. (b) Virulence against beet armyworm larvae determined by cumulative insect mortalities (%) caused by the B. bassiana wild type, Δ pks15 and complemented isolate G6 using a low-dose inoculum (1 × 10^5 conidia ml^-1). Saline was used as the control. (c) Phagocytic assay using the soil amoeba A. castellanii. Mortality rates (%) of the amoebae (A) after incubation with blastospores of the B. bassiana (WT), Δ pks15 and the complemented isolate G6 and S. cerevisiae cells (control). Data shown are mean ± S.E.M. Asterisks indicate statistical significance between the wild type or the complemented isolate G6 and Δ pks15 (Student's t test: *p < 0.05; **p < 0.01). (d) Light micrographs of amoebae (A) after incubation with blastospores of the B. bassiana wild type, Δ pks15 and G6 and S. cerevisiae cells for 24 h. The wild type and G6 grew to generate hyphae at this early time point. Bars, 10 μm.

| Strains                  | Relative sporulation (%) | Conidial germination (%) |
|--------------------------|--------------------------|--------------------------|
|                          | Conidia | Blastospores | Conidia |
| Wild type                | 100*     | 100*         | 74* ± 6.0 |
| Δ pks15                  | 30.5 ± 1.0 | 37.5 ± 1.3  | 34 ± 3.1  |
| Complemented isolate G6 | 98.7* ± 4.8 | 88.9* ± 2.2 | 77* ± 4.0 |

Table 2. Comparative sporulation and germination of the B. bassiana wild type, Δ pks15 and the complemented isolate G6. Relative sporulation (%) was determined by the number from spores in each strain relative to that of the wild type. Conidia and blastospore yields were determined on 5-day-old PDA and in 2-day-old SDY broth, respectively. Germination analysis was performed by incubation of conidia in 5% (v/v) PDB for 20 h. Data shown are mean ± S.E.M. Asterisks indicate statistical significance relative to that of Δ pks15 (Student's t-test, p < 0.05).

Generally, fungal walls are composed of chitin, β-1,3-glucan, β-1,6-glucan, glycoprotein, mannoprotein, and galactomannoprotein, which could influence binding to host receptors and immune invasion. For instance, Candida albicans mannoproteins, Paecilomyces farinosus galactomannan and Nomuraea rileyi galactose induce opsonization of fungal cells by insect hosts. Changes in the wall surface may therefore affect host–pathogen interactions, as seen when the loss of chitin and β-1,3 glucan increases the ability of fungal cells to escape insect immune responses. In mycobacteria, the type I polyketide synthase gene pks13 is important for biosynthesis of mycolic acid, a major wall component of such pathogenic bacteria, and its deletion results in a change in the cell envelope structure. Mycolic acid-containing glycolipids are also important for virulence in mice. These observations further support the concept that pks15 plays a role in cell wall formation and subsequently affects virulence in insect hosts.

There are a few cases where fungal polyketides have been associated with insect virulence, sporulation or cell wall formation. For insect virulence, the red pigment oosporein has been reported to be important for B. bassiana virulence against insects, with the authors noting the effects of oosporein on host immunity modulation and limiting bacterial growth after host death. The Δ pksA mutant of the saprophytic fungus Aspergillus parasiticus, which is deficient in biosynthesis of dothistromin (a polyketide structurally related to aflatoxin), was also reported to have reduced sporulation. Notably, sporulation rates were only a third that of the A. parasiticus wild type, similar to the sporulation impairment seen for B. bassiana Δ pks15. In two ascomycetes, Sordaria macrospora and Neurospora crassa, mutants lacking the putative dehydrogenase gene fbm1, which is in a polyketide biosynthesis cluster, were impaired in sexual development, particularly in perithecia formation. In our current study, the role of PKS15 in cell wall integrity was clearly demonstrated by targeted gene disruption and genetic complementation. We therefore hypothesize that PKS15 and its metabolite might have a mechanistic role in cell signaling during developmental processes, including sporulation, germination and spore wall formation, some of which clearly affect insect virulence. Alternatively, the PKS15 metabolite could directly be a spore wall component. Nonetheless, these two possibilities remain hypothetical and require further experimental proof. While involvement of non-reducing PKSs in cell wall formation have been reported, our study is the first to report a role for a reducing PKS in cell wall formation. Cell wall alteration seen for Δ pks15 could account for the impaired phagocytic survival and consequently affect virulence against insects.

CRISPR/Cas9 has recently become one of the most popular molecular tools for genome editing due to its extraordinary capability for modifying genomes of various organisms. However, application of CRISPR/Cas9 in fungal molecular genetic research by performing an in-cis genetic complementation of Δ pks15 in B. bassiana using this technique. Interestingly, we successfully used a CRISPR/Cas9 vector previously developed for A. aculeatus without any modification, despite the phylogenetic distance between A. aculeatus and B. bassiana and the rare occurrence of homologous recombination in filamentous fungi. This success may also be attributed to consideration of circumstances affecting CRISPR/Cas9-driven HDR, as DNA donor characteristics such as DNA form (circular versus linear) and arm sizes affect the repair success rate. For instance, as linear DNA donors are prone to degradation and are considered less efficient than circular DNA donors, we used a circular DNA donor as the template for HDR. In contrast, attempts with linear donor DNA of similar length...
Figure 3. (a) Scanning electron micrographs (SEMs) of conidia from the wild type (WT), Δpks15 and the complemented isolate G6. It is noted that wild-type and G6 conidia have characteristic rodlet bundles on the wall surface but the pks15 mutant lacks these bundles. Also, this Δpks15 conidium, as a representative of most of the mutant conidia, is larger and more elongated than that of the wild type and G6. Bars, 500 nm. (b) Measurement of width, length and area of conidia from the three strains, as analyzed from the electron micrographs taken. Data shown are mean ± S.E.M. Asterisks indicate statistical significance between the wild type or the complemented isolate G6 and Δpks15 (Student's t test: *p < 0.05). (c) SEMs of blastospores from WT, Δpks15 and G6. Bars, 1 µm.
were unsuccessful in driving HDR in this fungus (unpublished data). We also used longer flanking regions to increase the efficiency of homologous recombination, as arms 1 kb or longer on either side of a target gene are generally used to facilitate successful recombination in filamentous fungi\textsuperscript{34}. Our study demonstrated that a 600-bp arm was also sufficient to mediate HDR in \textit{B. bassiana}. This finding is in agreement with a previous report that successfully used 250-bp arms for homologous recombination via CRISPR/Cas9\textsuperscript{32}. Our study therefore supports the use of CRISPR/Cas9 as a powerful tool for site-specific mutagenesis and genetic complementation in this fungal entomopathogen.

Methods

Strains, culture conditions and genomic DNA preparation. \textit{Beauveria bassiana} strain BCC 2660 was obtained from Thailand's BIOTEC Culture Collection, and the \textit{pks15} mutant was previously generated to contain a disruption in \textit{pks15}, rendering the PKS gene nonfunctional\textsuperscript{4}. The two strains were grown on half-strength potato dextrose agar (PDA; Difco, USA) at 25 °C for 5–7 days for the production of conidia. To produce blastospores, fungal conidia were inoculated in Sabouraud dextrose broth (Difco) supplemented with 1% yeast extract (SDY), and shaken at 150 rpm, 25 °C for 2 days. \textit{Escherichia coli} strain DH5α was employed for plasmid propagation. For genomic DNA preparation, all fungal strains were grown in SDY as described above. All the cells were collected by centrifugation at 7,500 × g and fungal genomic DNA was extracted as previously described\textsuperscript{7}.

\textit{Acanthamoeba castellanii}, obtained from the Faculty of Tropical Medicine, Mahidol University, Thailand, was grown in peptone-yeast extract-glucose (PYG) broth as previously described\textsuperscript{4}.

General molecular methods. Standard molecular techniques were performed\textsuperscript{35} for plasmid purification, restriction enzyme analysis and DNA ligation. For PCR amplifications, we used the following thermal cycling program: 5 min at 95 °C; 35 cycles of 15 s at 95 °C, 25 s at 55 °C, and 1–5 min at 72 °C depending on the length of expected PCR products (1 min for a 1-kb product, 2 min for a 2-kb product and 5 min for products 5 kb or longer); and 5 min at 72 °C.

Ligated fragments were transformed into \textit{Escherichia coli} DH5α, and bacterial clones were screened by PCR amplification with the primers CSN389 and CSN390 to verify the presence of the sgRNA insert. Clones with the

Figure 4. Atomic force micrographs of conidial surfaces from the wild type (WT), Δ\textit{pks15} and the complemented isolate G6. Amplitude images are shown. Rodlet bundles were found on the surface of wild-type and G6 conidia, but not detected for Δ\textit{pks15}. Bars, 500 and 100 nm for upper and lower panels, respectively.
**Figure 5.** Size and shape of fluorescently-stained conidia in the wild type (WT), Δpks15 and the complemented isolate G6. (a) Calcofluor- and FITC-tagged concanavalin A staining (upper and lower panels). Bars, 5 μm. (b) Distribution of sizes in the conidial populations of the wild type, Δpks15 and G6 from a single representative experiment. (c) Frequency of conidial sizes for each of the three strains. Size data were from three independent experiments. (d) Distribution of shapes in the conidial populations of wild type, Δpks15 and G6 from a single representative experiment. Shape factor (circularity) was determined using the NIS-Elements D software. A shape factor of 1.0 indicates a circle, whereas a shape factor less than 1.0 indicates an ellipse. (e) Frequency of conidial shapes for each of the three strains. Data shown are mean ± s.e.m. Asterisks indicate statistical significance between the wild type or the complemented isolate G6 and Δpks15 (Student’s t-test: *p*<0.05; **<0.01).
Figure 6. Size and shape of fluorescently-stained blastospores in the wild type (WT), Δpks15 and the complemented isolate G6. (a) FITC-tagged concanavalin A staining. Bars, 5 μm. (b) Frequency of blastospore sizes for each of the three strains. (c) Frequency of blastospore elongation for each of the three strains. Data shown are mean ± S.E.M. Asterisks indicate statistical significance between the wild type or the complemented isolate G6 and Δpks15 (Student’s t-test: *p < 0.05; ns not significant).
Eco-mented isolate G6.6 were digested to completion with a 1.4-kb-long Bio-Sciences, USA) according to the manufacturer's instructions. To prepare a DNA probe for Southern analysis, phoresis in a 1% agarose gel, and transferred and cross-linked to a nylon membrane (Hybond N+; GE Healthcare) was hybridized with the biotinylated-

membrane hybridization and signal detection was performed using North2South™ Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The nylon membrane (Amersham Hybond-N + GE Healthcare, Life Sciences) was hybridized with the biotinylated-

probe at 55 °C overnight. After a high stringency wash with 2x saline sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 55 °C, the membrane was incubated with a streptavidin–horseradish peroxidase conjugate solu-
tion. The hybridized membrane was analysed by a CCD high-resolution chemiluminescence detection system (ChemiDoc® XRS + System, Bio-Rad, USA).

**Insect bioassay and determination of sporulation and germination.** To assess insect virulence, conidia of the *B. bassiana* wild type, Δpks15 mutant, and a complemented isolate were separately harvested in saline (0.85% NaCl), and the density was adjusted to 1 × 10^6 cells ml^-1 using a haemocytometer. Third- to fourth-instar beet armyworm (*Spodoptera exigua*) larvae were injected with a 3-µl conidial suspension of one of the three fungal strains using a specialized 33-gauge needle-syringe set (Hamilton, USA). Injected larvae were transferred individually into a 24-well plate and fed with an artificial  medium. Saline-injected worms were used as controls. Ten insect larvae were treated for each fungal strain and the saline control. The experiment was repeated three times. Larval mortality was determined on days 1–7 after fungal inoculation. Mean lethal time (LT₅₀) was determined using Probit analysis (SPSS package version 11.5).

To assess sporulation, production of conidia and blastospores were determined on 5-day-old half-strength PDA and in 2-day-old SDY cultures, respectively. PDA and SDY cultures were prepared as described above using 100 µl and 1 ml of conidial suspension at 1 × 10^7 conidia in sterile water, respectively. To determine conidial germination, conidia were harvested from PDA as described above and resuspended in 5% (v/v) PDB in sterile water. Germination percentage was determined after incubation for 20 h. There were three replicates for each strain, and the experiment was repeated twice.

**Phagocytic survival assay.** Blastospores were assayed for phagocytic survival in *A. castellanii* as previously described, with the yeast *S. cerevisiae* as a control. Briefly, the *B. bassiana* wild type and mutants were grown in SDY broth. *S. cerevisiae* and *A. castellanii* were grown in YPD broth and PYG broth respectively. After incubation for two days, all cells were collected by centrifugation, filtered (for removal of mycelia in fungal cul-
ture) and adjusted to 1 × 10^6 cells ml^-1. Each fungal strain was co-cultured with *A. castellanii* in 96-well plates at a 1:1 ratio for 72 h in order to determine post-challenge amoeboid survival rates and fungal CFUs. Determination of amoeboid survival rates was performed as previously described. Trypan blue dye was used to determine the viability of amoebae. The experiment was repeated twice.

**Ultrastructural characterization of cell wall surface.** For scanning electron microscopy (SEM), PDA-
grown conidia were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences (EMS), USA) and 2% para-
formaldehyde (EMS) in phosphate buffer (0.1 M KH2PO4 and Na2HPO4, pH 7.2), followed by fixation in 1% OsO4 (EMS), as previously described. The cells were then dehydrated with an ethanol gradient from 30, 50, 70, 80, 90, 95, to 100% ethanol, then critical-point dried using a CO2 drier model HCP-2 (Hitachi, Japan) and sputter-coated with gold-platinum with the Q150R Au/Pt coater (Quorum Technologies, UK). Photographs were taken with SEM model FE-SEM SU-5000 (Hitachi).

For atomic force microscopy (AFM), conidia were prepared by growing fungal strains on PDA for 7 days before conidia were dislodged with 10 ml of sterile water. Conidial suspensions were filtered through eight layers of cheesecloth and the flow-through centrifuged at 13,700 × g for 2 min, rinsed with PBS buffer twice, and finally resuspended in 1 ml of PBS buffer. Sixty microliters of the conidial suspension were dropped on a membrane filter with 1.2 µm pore size (MF-Millipore, Germany) and air-dried overnight. Each conidial sample was mounted on a glass slide and subjected to atomic force microscopy (AFM).

AFM imaging was performed with the NanoWizard 3 AFM system (JPK Instruments, Bruker, USA) mounted on an isolation platform to minimize surrounding noise. Amplitude and phase images were collected in the AC (non-contact) mode under ambient conditions. An amplitude image records the amplitude changes of the oscillating cantilever during the scan, thus reflecting the vertical distance (Z-direction) of the sample. A phase image records the phase difference between the cantilever input signal cycle and the responsive signal cycle, reflecting the mechanical properties of the sample such as stiffness, and adhesion. The AFM cantilever for this study was a non-contact mode silicon AFM cantilever (product no. ACTA, AppNano, USA) with a nominal spring constant of 37 N m⁻¹ and a tip radius of curvature of approximately 6 nm. The images were recorded at a scanning speed of 0.5 Hz at different resolutions, including 256 × 256 pixels, or 512 × 512 pixels.

**Cell wall staining.** *Beauveria bassiana* conidia and blastospores were collected from 7-day-old PDA and 2-day-old SDY cultures, respectively, and rinsed once with PBS. Spores were stained with concanavalin A con-
jugated with the fluorophore FITC (Sigma-Aldrich) and calcofluor white (Sigma-Aldrich). For conA staining, spores were fixed with 3.7% formaldehyde (in 100 µl) for 20 min and rinsed with PBS once. Then, the spores were incubated in 20 µl of 1 mg ml⁻¹ conA in PBS (pH 7.4) for 60 min and rinsed once with PBS. For calcofluor staining, spores were incubated in a 1:1 mixture of 5 mg ml⁻¹ calcofluor white in PBS (pH 7.4) and 1 M KOH in a total volume of 200 µl. The cell staining mixture was incubated at room temperature for 2 min. Stained spores were collected by centrifugation at 6,729 × g for 1 min and rinsed once with PBS.

### Confocal and fluorescence microscopy and cell measurements of stained fungal spores.

The concanavalin A- and calcofluor-stained fungal spores were wet-mounted onto glass slides and covered with No.1 (0.13-0.16 um thickness) cover glass (BRAND, Germany). Fluorescence images were acquired by the Nikon Eclipse Ti-2E using the Nikon Plan Apo VC 100 × oil objective lens (numerical aperture (N.A.) = 1.40). Images were captured at 2,880 × 2,048 pixels in TIFF format.

For confocal laser scanning microscopy, we used the Olympus FV1000 confocal laser scanning system configured with the Olympus IX81 inverted microscope with the Olympus UIS2 UPLSAPO 100 × oil objective lens (N.A. = 1.4). All images were recorded at a resolution of 1,600 × 1,600 pixels using a 100 × oil immersion objective lens with a 3 × digital zoom. Parameters for confocal staining images were as follows: laser power, 20%; HV, 400–500; gain, 1 and pinhole, 150–155 (auto). Parameters for confocalanil A staining images were as follows: laser power, 30%; HV, 600–650; gain, 1–3 and pinhole, 150–155 (auto). For each image, the offset voltage was adjusted until the background appeared black or nearly so. Kalman filtering (n = 6) was generally used to improve the signal-to-noise ratio of images. All images were captured as TIFF files.

Spore sizes and shapes were compared using two dimensional image analysis of conA- or calcofluor-stained spores taken by the Eclipse Ti-2E and the NIS-Elements software (Nikon). For determination of spore sizes, the ‘5 Point Ellipse’ feature for area measurement of NIS-Elements was manually used to mark the boundary of a conidium and the ‘Auto Detect’ feature was used to mark the boundary of a blastospore (Supplemental Fig. S3). Consequently, ‘size’ was automatically determined as ‘area’ by the software. Budding blastospores were excluded from the analysis.

In NIS-Elements, shape factor, calculated using the formula shape factor = 4πA/P² where A = area and P = perimeter, was used to determine whether a spore is circular or elliptical in shape. The shape factor value of a circle equals 1.00, whereas values less than 1.00 indicates an ellipse.

### Statistical analyses.

All experiments in this study were repeated two or three times. There were three replicates for each fungal strain in a given experiment. Data were analyzed for statistical significance using ANOVA in SPSS package version 11.5 and the student’s t-test. LT₅₀ was determined using Probit analysis in the SPSS package.

Received: 3 June 2019; Accepted: 30 June 2020
Published online: 28 July 2020

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Acknowledgements
We greatly thank Drs. Nodvig and Mortensen for kindly providing the CRISPR/Cas9 vectors pFC331-334 used in this study. Dr. Samaporn Teeravechyan for critically reading and editing the manuscript and Dr. Kalabaheti, Department of Microbiology and Immunology, Faculty of Topical Medicine, Mahidol University, Thailand, for the gift of Acanthamoeba castellanii. We are grateful for use of the AFM system at the National Nanotechnology Center (NANOTEC), Thailand. This research was supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC) postdoctoral fellowship to SU and grants from the Research Development Innovation Management for National Strategic and Network Division (RNS) and BIOTEC, NSTDA, Thailand.

Author contributions
A.A., S.U. and M.T. designed the research. A.A. and S.U. wrote the manuscript. S.U. and W.T. constructed recombinant DNAs for CRISPR/Cas9 experiments. S.U. performed fungal transformation and molecular and phenotypic analyses of B. bassiana strains. R.W. and C.S. carried out insect bioassay. U.S., S.U., N.W., N.A.S.N. and A.A. conducted cell wall characterizations.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-69417-w.

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