



DNM1, a Dynamin-Related Protein That Contributes to Endocytosis and Peroxisome Fission, Is Required for the Vegetative Growth, Sporulation, and Virulence of *Metarhizium robertsii*

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ABSTRACT Although dynamins and dynamin-related proteins (DRPs), a large GTPase superfamily, are involved in the budding of transport vesicles and division of organelles in eukaryotic cells, the function of these proteins in entomopathogenic fungi has not been reported to date. Here, DNM1, a DRP in *Metarhizium robertsii*, was characterized using gene disruption and complementation strategies. Mutant phenotype assays showed that the $\Delta Dnm1$ strain displayed increased defects in radial growth (~24%) and conidial production (~42%) compared to those of the wild type (WT), and reduced conidiation levels were accompanied by the repression of several key conidiation-related genes, including *fbaA*, *wetA*, and *fbdD*. Additionally, mutant bioassays revealed that disruption of *Dnm1* impaired the virulence (both topical inoculation and injection) of *M. robertsii* in the insect *Galleria mellonella*. Further analysis demonstrated that deleting *Dnm1* in fungi suppressed the transcriptional levels of several virulence genes in the insect hemocoel. Moreover, we found that DNM1 colocalized with peroxisomes and mitochondria. Importantly, disruption of *Dnm1* abolished normal fungal endocytosis, resulting in significantly decreased numbers of, as well as morphological changes in, peroxisomes. These findings indicate that deletion of *Dnm1* causes significant changes in the vegetative growth, sporulation, and virulence of *M. robertsii* due to changes in cell function and peroxisomes.

IMPORTANCE *Dnm1* was found to be involved in fungal development and virulence, mediated peroxisomal fission, and normal endocytosis. This finding provides new insights into the cellular processes and pathogenicity in entomopathogenic fungi.

KEYWORDS *Metarhizium robertsii*, dynamin-related proteins, peroxisomal, endocytosis, virulence

Entomopathogenic fungi play a crucial role in controlling insect populations, providing an attractive alternative to chemical insecticides (1–5). Extensive studies have revealed that fungal infection begins at the adhesion of conidia to the host cuticle, followed by germination and cuticle penetration for entry into the hemocoel where hyphal bodies proliferate via yeast-like budding until host death occurs from mummification (6–8). Unfortunately, the lethal actions of entomopathogenic fungi against target pests are relatively slow, restraining their wide applicability (2). Therefore, additional information on fungal infection mechanisms, such as virulence gene function, should be investigated to improve fungal virulence or accelerate lethal fungal action.

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Dynamins, which are cytosolic proteins, have typically been described as being composed of the following moieties: a GTPase domain, middle domain, PH domain, GTPase effector domain, and proline-rich domain (9). Furthermore, dynamin-related proteins (DRPs) lack one or more of these five domains or have additional domains. Dynamins and DRPs are members of a large superfamily of GTPases that play important roles in the budding of transport vesicles and the division of organelles in eukaryotic cells (10–12). The general mechanisms of action of dynamins and DRPs have been studied in some fungal species, particularly in yeast (*Saccharomyces cerevisiae*). Deletion of Vps1, a conserved fungal DRP, in *S. cerevisiae* causes mutant cells to exhibit major defects in vacuolar dynamics and cargo transport to the vacuole (13). In *Schizosaccharomyces pombe*, the primary roles of Msp1p, a conserved DRP in fungi, are to organize mitochondrial membranes, making them competent for fusion, and to maintain the integrity of mitochondrial DNA (14). A recent study reported that DNM1, a conserved DRP in fungi, mediates peroxisomal and mitochondrial fission, pexophagy, and mitophagy in the rice blast fungus *Magnaporthe oryzae* (15). Therefore, dynamins and DRPs are widely present in fungi and, as in plants and animals, are vital for many cellular functions that depend on membrane remodeling (9). Despite the clear importance of dynamins and DRPs in fundamental fungal cellular processes, no functional analysis of these proteins in entomopathogenic fungi has been reported to date.

Peroxisomes are organelles surrounded by a single membrane and involved in diverse and important cellular processes in most eukaryotes (15, 16). Peroxisomal oxidation, biogenesis, and matrix protein import are necessary for fungal growth, sporulation, and virulence (17–19). In *M. oryzae*, MoDnm1 recruits MoFis1 (a mitochondrial fission protein) to peroxisomes and mitochondria through the adaptor protein MoMdv1, a WD40 repeat protein, and all of these proteins function as a complex that mediates peroxisomal fission; if one of the components of the complex is lacking, defects in fungal developmental processes and pathogenicity will occur (15). In the budding yeast *S. cerevisiae*, DNM1 functions as a complex with Fis1, Mdv1, and Caf4, a WD40 protein, for peroxisomal fission, and peroxisomal division depends on the compositional integrity of this complex; for example, deletion of *Dnm1* results in an abnormal morphology and a reduction of peroxisome numbers (20). These studies indicate that DRPs play important roles in both the division and fusion of peroxisomes.

Although dynamins and DRPs play important roles in fungal development, their roles are less understood in entomopathogenic fungi. In this study, DNM1 (MAA_00205), which is homologous to MoDnm1 in *M. oryzae*, was identified in *Metarhizium robertsii*, and its function was characterized by multiphenotypic analyses of the deletion mutant. These findings will allow a better understanding of the roles of DRPs in entomopathogenic fungal developmental processes and their pathogenic mechanisms.

RESULTS

Characteristics of DNM1 in *M. robertsii*. To investigate the function of DNM1 in the entomopathogenic fungus *M. robertsii*, a BLAST search was performed to identify dynamin proteins and DRPs in the *M. robertsii* genome database with *S. cerevisiae* DNM1 as a query (3). Two genes (*Dnm1* and *Vps1*) encoding DNM1 homolog proteins (MAA_00205 and MAA_09999) were found in the fungal genome (Fig. 1a). The *Dnm1* open reading frame consists of 2,597 nucleotides, contains three introns and four exons, and encodes a protein of 798 amino acids with a predicted molecular mass of 88.52 kDa and an isoelectric point of 7.05. The other open reading frame (*Vps1*) consists of 2,399 nucleotides, contains five introns and six exons, and encodes a protein of 699 amino acids with a predicted molecular mass of 77.2 kDa and an isoelectric point of 8.65. Conserved functional domain analysis revealed that both DNM1 and VPS1 lack two domains (PH and PRD) compared to those of classical dynamin and contain a GTPase domain, a middle domain, and a GTPase effector domain (GED), revealing that both are DRP proteins (Fig. 1b). Phylogenetic analyses showed that both DNM1 and VPS1 are more closely related to orthologues of entomopathogenic fungi (*Metarhizium*

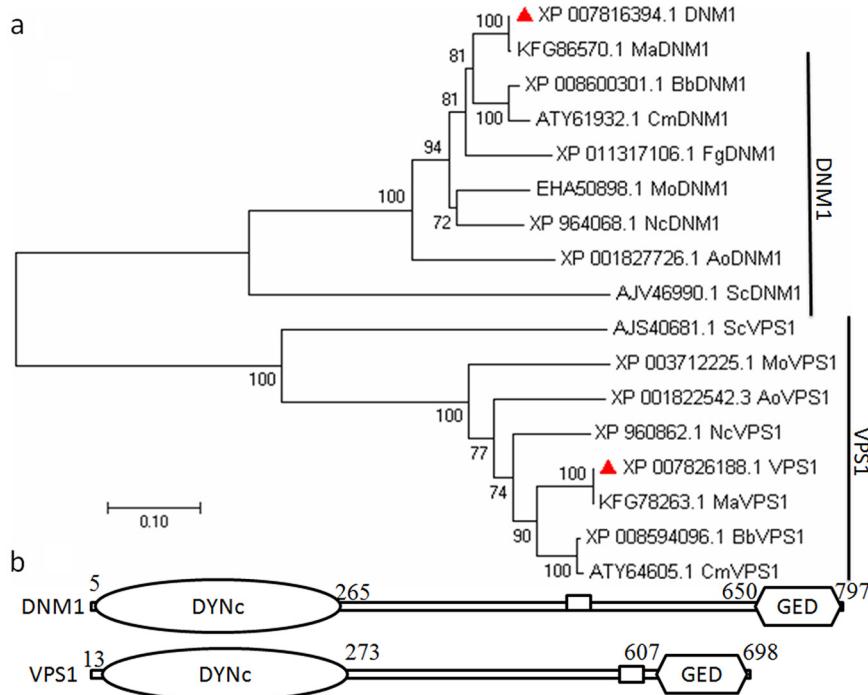


FIG 1 Bioinformatics analysis of fungal dynamin-related proteins. (a) Phylogenetic analysis of dynamin-related proteins from several organisms. Triangle, *Metarhizium robertsii*; Ma, *Metarhizium anisopliae*; Bb, *Beauveria bassiana*; Cm, *Cordyceps militaris*; Fg, *Fusarium graminearum*; Mo, *Magnaporthe oryzae*; Nc, *Neurospora crassa*; Ao, *Aspergillus oryzae*; Sc, *Saccharomyces cerevisiae*. (b) Structure domain analysis of the DNM protein. DYNC, large GTPase domain; GED, GTPase effector domain.

anisopliae, *Beauveria bassiana*, and *Cordyceps militaris*) than to nonentomopathogenic fungi (Fig. 1).

A $\Delta Vps1$ (MAA_09999) mutant could not be obtained despite screening thousands of transformants, which also have been reported in *M. oryzae* (15); therefore, only functions of DNM1 in *M. robertsii* were further explored in this study.

Construction and validation of DNM1 mutants. To investigate the role of DNM1 in the entomopathogenic fungus *M. robertsii*, *Dnm1* was deleted based on the identified sequences. The $\Delta Dnm1$ strain was constructed by replacing the coding region of *Dnm1* with the bar gene cassette (see Fig. S1 in the supplemental material). A complementation strain was obtained by transforming Com-pBen-DNM1 into the $\Delta Dnm1$ strain. To investigate the cellular localization of DNM1 and peroxisomal fission, the *Dnm1* gene with a green fluorescent protein (GFP) or mCherry tag was inserted into the binary pDHT-SK-ben vector and transformed into a $\Delta Dnm1$ strain (see Fig. S2 in the supplemental material). All of the mutant strains were confirmed by PCR and reverse transcription-PCR (RT-PCR) (see Fig. S1 and S2).

DNM1 is required for fungal development. The effect of disrupting *Dnm1* on vegetative growth was examined to elucidate the function of DNM1. The colony diameters of the $\Delta Dnm1$ strain were significantly reduced, with decreases of 23%, 24.4%, and 28% compared to those of both the wild-type (WT) and the complemented strains cultured on potato dextrose agar (PDA), Sabouraud dextrose agar plus yeast extract (SDAY), and 1/4 SDAY medium, respectively (Fig. 2a and b). Consequently, the number of conidia produced by the colonies significantly differed among these strains (Fig. 2c). Conidiation of the $\Delta Dnm1$ strain was decreased by 41.99% compared to those of the WT and complemented strain (CP) (Fig. 2c). Furthermore, the expression of key conidiation-associated genes was assessed in different strains to further explore the functional patterns of DNM1 in fungal conidiation. The real-time quantitative PCR (qPCR) results demonstrated that *fbaA*, *AbaA*, *WetA*, and *FlbD* were downregulated in the

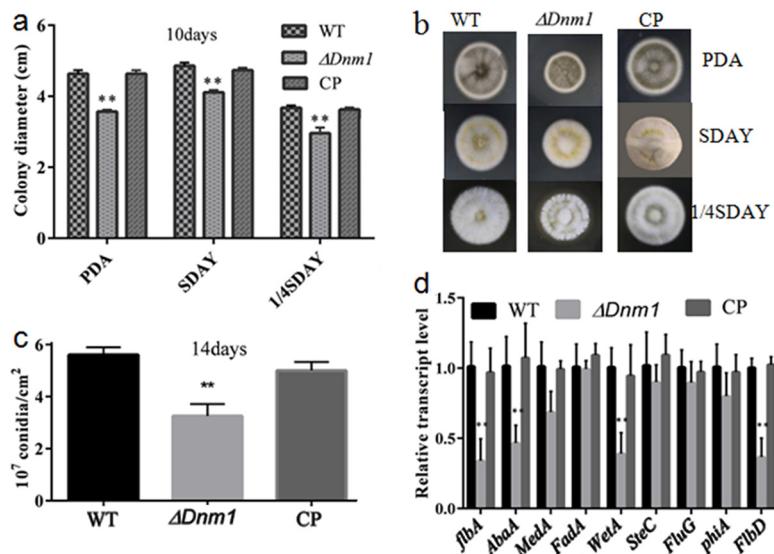


FIG 2 DNM1 is required for conidial production and vegetative growth. (a) Fungal colony diameters of different strains grown on PDA, SDAY, and 1/4 SDAY plates for 10 days. (b) Colony characteristics of different strains grown on PDA, SDAY, and 1/4 SDAY plates for 14 days. (c) Conidial production of different strains cultured on PDA for 14 days. (d) qPCR of sporulation-related genes. **, $P < 0.05$.

$\Delta Dnm1$ strain compared to those in the WT and complemented strains (Fig. 2d). These results indicate that *Dnm1* is involved in vegetative growth and conidiation. Furthermore, the effects of osmotic (NaCl), oxidative (H_2O_2), and cell wall (Congo red) stressors on the growth and conidial tolerance of the $\Delta Dnm1$ strain to UV irradiation and heat shock were assessed (see Fig. S3 in the supplemental material).

DNM1 contributes to fungal virulence. Insect bioassays (topical and injection) were employed to assess the consequences of the loss of *Dnm1* on pest virulence. In topical infection bioassays, $\Delta Dnm1$ strain-infected larvae had higher survival rates than those infected with the wild-type and control strains (Fig. 3a and b). The insect groups infected with the $\Delta Dnm1$ strain displayed mean lethal times to death (LT_{50}) of 4.54 ± 0.1 days, with a significant ($P < 0.05$) attenuation of virulence, while the wild-type and the CP groups displayed LT_{50} s of 4.07 ± 0.14 and 4.11 ± 0.09 days, respectively. In the injection bioassays, $\Delta Dnm1$ strain-infected larvae exhibited higher survival rates than those infected with the wild-type and control strains (Fig. 3c and d). The insect groups infected with the $\Delta Dnm1$ strain exhibited mean lethal times to death (LT_{50}) of 4.31 ± 0.18 days, with a significant ($P < 0.05$) attenuation of virulence, while the wild-type and CP groups displayed LT_{50} s of 3.6 ± 0.12 and 3.65 ± 0.11 days, respectively.

Moreover, the rates of appressorium formation on hydrophobic surfaces were determined and showed no significant differences between the $\Delta Dnm1$ and control strains, suggesting that DNM1 is unnecessary for fungal appressorium formation, which is required for cuticular penetration during the course of infection (Fig. 3e and f).

To investigate the functional patterns of DNM1 with respect to *M. robertsii* virulence, the expression of several fungal virulence genes in the insect hemocoel was detected by qPCR. The expressions of *snf1* (encoding a sucrose-nonfermenting protein kinase), *Msn2* (a transcription factor), and *DtxS1* (a nonribosomal peptide synthetase) were significantly downregulated in the $\Delta Dnm1$ strain versus those in the WT and the CP strains (Fig. 3g). *snf1* is involved in carbon source utilization, the function of *Msn2* is attributed to multistress responses, and *DtxS1* suppresses both cellular and humoral immune responses, which occur in the insect hemocoel, and revealed that virulence was attenuated, which may again be associated with virulence gene downregulation due to suppressed growth (21–23).

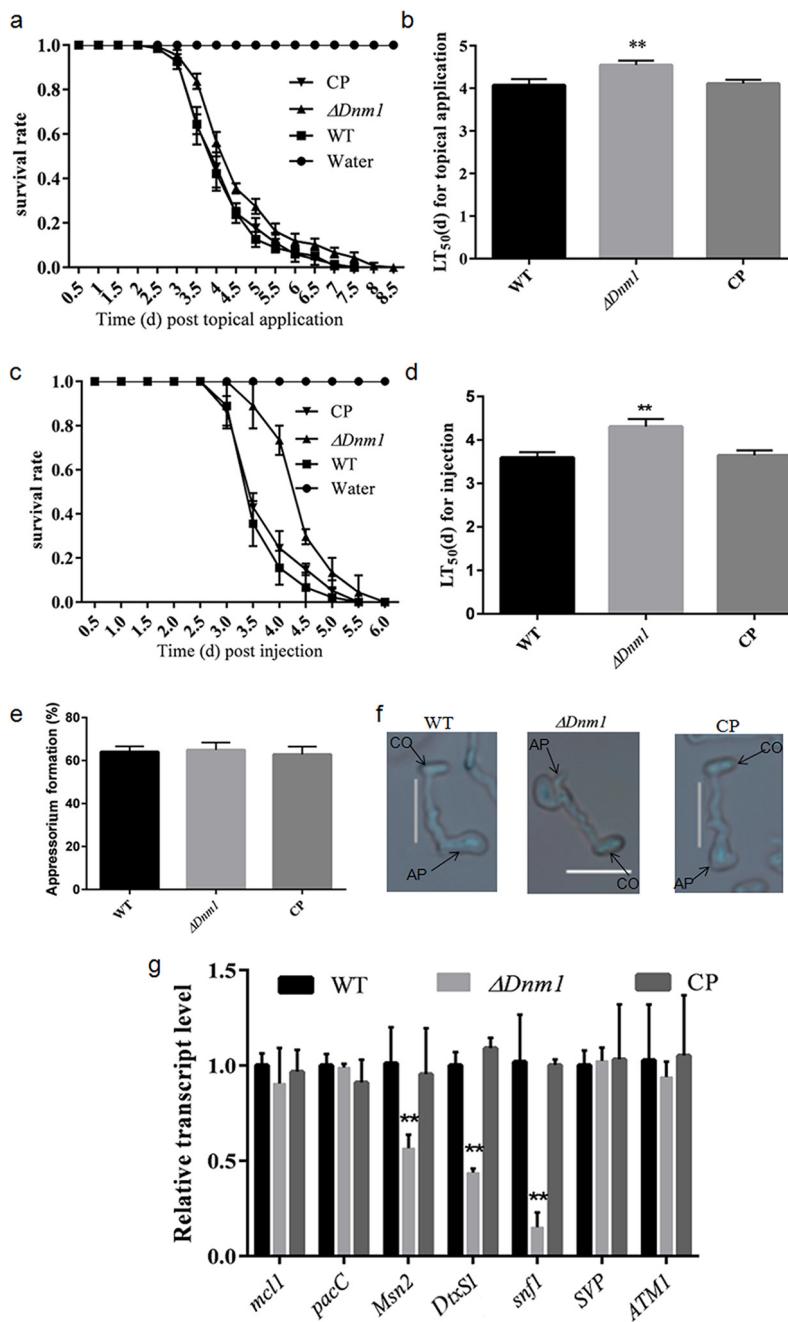


FIG 3 Effect of DNM1 deletion on fungal virulence and virulence-related genes. (a) Survival of *G. mellonella* after topical application of conidial suspensions from different strains. Control insects were treated with sterile water. (b) LT₅₀ (days) of different strains after topical inoculation. (c) Survival of *G. mellonella* after injection of conidial suspension from different strains. Control insects were injected with 10 μ l sterile water. (d) LT₅₀ (days) of different strains after injection. (e) Percentage of appressorium formation incubated on a plastic hydrophobic surface. (f) Microscopic images of appressorium (AP) formation induced on plastic hydrophobic surface. CO, conidia. Scale bar = 5 μ m. (g) Expression levels of virulence-associated genes. **, P < 0.05.

DNM1 is localized to peroxisomes and mitochondria. To further characterize the functions of *Dnm1*, we examined the subcellular localization of DNM1 fused with a GFP tag at the N terminus. mCherry-PTS1 (PTS1 expresses peroxisomal targeting signal 1) was introduced into the same strain. The red and green fluorescence results showed that punctate green fluorescence and red fluorescence were detected and overlapped, suggesting that DNM1 is localized to peroxisomes (Fig. 4A). Furthermore, mutant

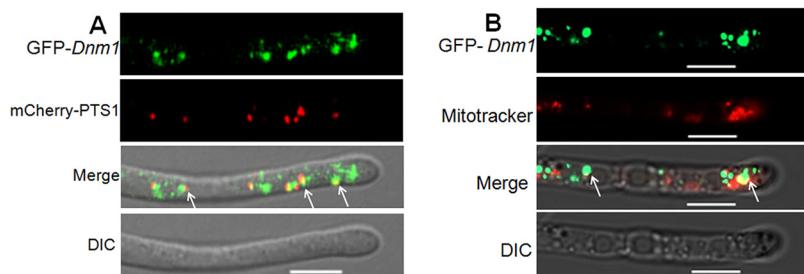


FIG 4 DNM1 is localized to peroxisomes and mitochondria. (A) Confocal fluorescence microscopy showing that DNM1 colocalizes with the peroxisome marker mCherry-PTS1 in hyphae. Scale bar = 5 μ m. (B) Confocal fluorescence microscopy showing DNM1 colocalized with the mitochondrial marker Mito-Tracker in hyphae. Scale bar = 5 μ m.

hyphae with GFP-DNM1 were incubated with a far-red-fluorescent dye (MitoTracker; absorption/emission, ~644/665 nm), followed by observation of GFP fluorescence as distinct spots that partially colocalized with the mitochondrial structures (Fig. 4B).

DNM1 is necessary for peroxisomal fission. Peroxisomes duplicate through fission during each cell division, which is a fundamentally important process in eukaryotes. To examine the potential functions of DNM1 in peroxisomal fission, peroxisomes in conidia and hyphae of different transformants were analyzed. The morphological features of peroxisomes in conidia demonstrated obvious changes between the WT and Δ Dnm1 strains (Fig. 5a). In the wild-type strain, 83.7% of peroxisomes from conidia were globular, and only 13.8% and 2.5% of them were in plaque and tubular forms, respectively. The percentage of globular peroxisomes was significantly reduced, and the percentages of tubular and plaque peroxisomes were significantly decreased in Δ Dnm1 strain conidia, accounting for 29.46 and 38.22%, respectively (Fig. 5a and b). In hyphae, peroxisomes were globular and evenly distributed in WT specimens, while most of them were dramatically enlarged and irregular with significantly reduced

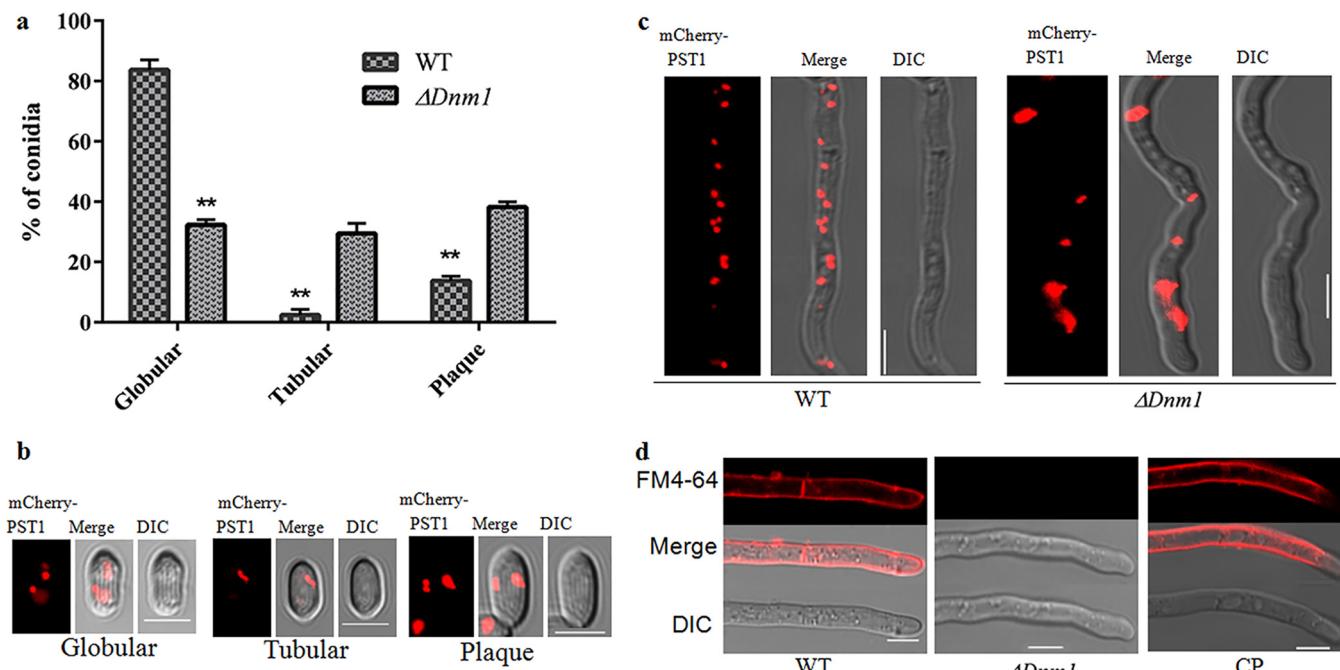


FIG 5 DNM1 is important for peroxisomal fission and normal endocytosis. (a and b) Observation and statistical analysis of peroxisomes in conidiospores of different indicated strains. **, $P < 0.05$. (c) Observation of peroxisomes in different fungal hyphae. (d) Different strains were stained with FM4-64 after culture for 24 h on GB medium. Scale bar = 5 μ m.

numbers in $\Delta Dnm1$ strain specimens (Fig. 5c). The altered peroxisomal morphology and numbers indicate that DNM1 is a key regulator of peroxisomal proliferation.

Deletion of DNM1 abolishes endocytosis. To investigate whether the loss of *Dnm1* results in defects in endocytosis and intracellular transport in *M. robertsii*, FM4-64 was used to stain hyphae from different strains to evaluate vacuolar membrane internalization. The results demonstrated that FM4-64 was internalized within 1 min after exposure in the wild-type and CP strains, while no FM4-64 was internalized over approximately 30 min in the $\Delta Dnm1$ mutant strain, revealing that endocytosis was severely affected in fungi in the absence of *Dnm1* (Fig. 5d). These data suggest that fungal endocytosis is abolished in response to the deletion of *Dnm1* in *M. robertsii*.

DISCUSSION

Membrane budding and fission are essential to eukaryotic life and were originally seen as resulting from the action of mechanoenzymes, such as the DRP family. Fungal DRPs mediate peroxisomal and mitochondrial fission, pexophagy, and mitophagy, resulting in a reduction in pathogenicity in the rice blast fungus *M. oryzae* (15). However, no functional analysis of these proteins in entomopathogenic fungi has been reported to date. In this study, we characterized the functions of *Dnm1* in *M. robertsii*. Disruption of *Dnm1* revealed serious defects, not only in fission and division of lipid membranes but also in virulence to the pest insect. It also revealed that virulence was attenuated and may again be associated with virulence gene downregulation due to suppressed growth.

In fungi, DNM1 has been identified in *M. oryzae* and yeast (15, 24). Our phylogenetic analysis showed that DNM1 is present in entomopathogenic, phytopathogenic, and saprophytic fungi and that DNM1 from distinct fungi contained the same three conserved domains (GTPase, MD, and GED), revealing that *Dnm1* is widely present and has highly conserved functions across different fungi.

Dnm1 colocalizes with peroxisomes and mitochondria, and deletion of *Dnm1* impaired peroxisomal fission and normal endocytosis in *M. robertsii*, consistent with a previous report in *M. oryzae* (15). However, disruption of *MoDnm1* in *M. oryzae* showed only that the number of peroxisomes was significantly decreased, while deletion of *Dnm1* in this study showed both a reduction in the number of peroxisomes and an irregular morphology of peroxisomes (15). In addition, the *M. oryzae* *Dnm1* mutant showed an FM4-64 internalization delay of approximately 7 min in the $\Delta MoDnm1$ mutant strain, in contrast to ≤ 1 min for the wild type, while the dye was not detected for over 30 min in the $\Delta Dnm1$ strain, suggesting that disruption of *Dnm1* abolished endocytosis (15). These data demonstrate that DNM1 may participate in cellular biological processes with different function mechanisms in diverse fungal species.

As an entomopathogenic fungus applied in pest biocontrol, a major limitation to the efficacy of pest control and consistency of *Metarhizium* is cell sensitivity to UV, heat, and stressful chemicals (25). Contrary to our expectations, the $\Delta Dnm1$ strain showed a similar growth capability in the presence of stressful chemicals and conidial tolerance to UV irradiation (germination time of $\Delta Dnm1$ strain conidia was in advance) and heat shock as that of the control strains. These data indicate that DNM1 may not be responsible for the fungal stress response.

In conclusion, DNM1 plays important roles in fungal development and virulence of entomogenous fungus, and DNM1 colocalizes with peroxisomes and mitochondria and is also responsible for peroxisomal fission and normal endocytosis. Thus, deletion of *Dnm1* exhibits significant differences in the vegetative growth, sporulation, and virulence of *M. robertsii* due to changes in cell function and peroxisomes. Our findings provide insight into the function of DNM1 in entomopathogenic fungi. Further analyses of this protein are required to fully understand its mechanisms and role in fungal developmental and insect pathogenesis.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type *M. robertsii* strain ARSEF 23 (ATCC MYA-3075) was grown on potato dextrose agar (PDA) (20% potato, 2% dextrose, and 2% agar [wt/vol]) in the dark at 28°C

for 12 days. Conidia were harvested in a 0.05% Tween 80 aqueous solution, and the conidial suspension was filtered through a sterile nonwoven fabric to remove mycelial debris. The spore concentration was determined using a hemocytometer and standardized by dilution.

Bioinformatics analysis of DNM1 homologues in different fungi. DNM1 in different fungi (*M. robertsii*, *M. anisopliae*, *B. bassiana*, *C. militaris*, *Fusarium graminearum*, *M. oryzae*, *Neurospora crassa*, *Aspergillus oryzae*, *S. cerevisiae*) was searched in different fungal genome databases. Protein domains were predicted by online BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and phylogenetic analysis was performed by MEGA7 (<https://www.megasoftware.net/>) using the neighbor-joining method.

Generation of DNM1 deletion and complementation mutants. *Dnm1* in *M. robertsii* was deleted as previously described (26). Briefly, the 5' and 3' flanking regions of the *Dnm1* gene were inserted into the binary pDHt-SK-bar vector for gene deletion, and the entire *Dnm1* gene and the upstream and downstream sequences were inserted into the binary vector pDHt-SK-ben for complementation vector construction. Vectors were transformed into *Escherichia coli* cells and verified by DNA sequencing. Positive plasmids were subsequently transformed into fungi through *Agrobacterium*. The primers used in this study are listed in Table S1 in the supplemental material.

Phenotype assays and insect bioassays. Phenotype assays were performed as previously described (26). In brief, 1-μl aliquots of 10^5 conidia ml⁻¹ suspensions were centrally spotted on PDA, SDAY, and 1/4 SDAY (amended with 1/4 nutrients of SDAY) plates. The radial growth (diameter) rates were measured daily, and the sporulation capacity was quantified from PDA cultures incubated for 20 days at 28°C. All experiments were performed using three replicates for all phenotypic assays. Fifth-instar *Galleria mellonella* larvae were used to assay the virulence of the fungal strains. Conidia were applied by immersing larvae in an aqueous 10^7 conidia ml⁻¹ suspension. Each snorkeling consisted of three replicates (60 larvae per replicate). Experiments were performed three times. After inoculation, mortality was recorded every 12 h for 10 days.

Transcriptional profiling of phenotype-related genes. Real-time quantitative PCR was performed as previously described (2, 27). Total RNA was extracted using TRIzol reagent (Invitrogen) from strains cultured on PDA plates for 2.5 days for sporulation-associated gene detection and from *G. mellonella* larvae infected for 48 h for virulence-associated gene detection. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a control, and values were obtained by normalization to the control gene. The $2^{-\Delta\Delta CT}$ method was used for calculating the gene relative expression levels. Three independent experiments were performed for each examined gene. The primers used in this study are listed in Table S2 in the supplemental material.

Subcellular localization and observation of peroxisomal morphology. To investigate the cellular localization of DNM1 and peroxisomal fission, two fragments (*Dnm1* gene with upstream sequence, GFP tag, and the *trpC* terminator; *gpdA* promoter with an mCherry tag, a type I peroxisomal targeting signal, and the *trpC* terminator) were inserted into the binary pDHt-SK-ben vector and transformed into the deletion mutant. Green and mCherry fluorescence were observed as previously reported (15). Briefly, WT and mutant strains were cultured on GB (0.5% peptone and 2% sucrose [wt/vol]) liquid medium for 24 h for collection of vegetative hyphae and were cultured on PDA medium for 10 days for collection of conidia. Confocal fluorescence microscopy (Zeiss LSM 880, Oberkochen, Germany) was used for fluorescence observation.

Endocytosis assays. For endocytic assays, hyphae of different strains from 24-h cultures in GB liquid medium were stained with *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylamino-phenyl-hexatrienyl) pyridinium dibromide (FM4-64) (Molecular Probes, Inc., Eugene, OR, USA) followed by observation by confocal fluorescence microscopy (Zeiss LSM 880, Oberkochen, Germany).

Statistical analysis. Three biological replicates were performed for each experiment. All data were analyzed using SPSS v23.0 software (IBM SPSS, IL, USA). A *P* value of <0.05 was considered statistically significant.

Data availability. Additional data regarding the identified forms of DNM1 in different fungi and sets of phenotype-related genes of *M. robertsii* are available in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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The authors have no conflicts of interest to declare.

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