Fungal Biology 123 (2019) 274–282

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

An efficient gene disruption system for the nematophagous fungus *Purpureocillium lavendulum*

Lu Liu [a, 1], Yan-Ru Cao [b, 1], Chen-Chen Zhang [a], Hai-Feng Fan [a], Zhi-Yi Guo [a], He-Yu Yang [a], Mi Chen [a], Jing-Jing Han [a], Jianping Xu [a, c], Ke-Qin Zhang [a, **], Lian-Ming Liang [a, *]

[a] State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan and The Key Laboratory for Southwest Microbial Diversity of the Ministry of Education, Yunnan University, Kunming, 650091, China
[b] Key Laboratory of Special Biological Resource Development and Utilization of Universities in Yunnan Province, Department of Life Science and Technology, Kunming University, Kunming, 650091, China
[c] Department of Biology, McMaster University, Hamilton, Ontario, L8S 4K1, Canada

ARTICLE INFO

Article history:
Received 31 January 2018
Received in revised form
20 September 2018
Accepted 23 October 2018
Available online 5 February 2019

Corresponding Editor: Gustavo Henrique Goldman

Keywords:
Biocontrol
Gene knockout
Ku80
Nematophagous fungi
Serine protease

ABSTRACT

The fungus *Purpureocillium lavendulum* (formally *Paecilomyces lilacinus*) is a natural enemy of insects and plant-parasitic nematodes, and has been used as an important bio-control agent against agricultural pests all over the world. In order to understand the genetic mechanisms governing its biocontrol efficiency and other biological processes, an effective gene disruption system is needed. Here we report the development of an efficient system which integrates selective markers that differ from *Purpureocillium lilacinum*, a one-step construction method for gene knockout plasmids, and a ku80 knockout strain for efficient homologous recombination. With this system, we effectively disrupted the transcription factors in the central regulation pathway of sporulation and a serine protease which were contributed to nematode infection, demonstrating this system as an efficient gene disrupting system for further characterization of genes involved in the development and pathogenesis of this fungus.

1. Introduction

Plant parasitic nematodes cause enormous crop and economic losses amounting to $157 billion annually worldwide (Abad et al., 2008). The nematophagous fungi prey on free-living as well as plant-parasitic nematodes and are potential bio-control agents. Among these fungi, a species previously named *Paecilomyces lilacinus* has shown to be capable of parasitizing clusters of nematode eggs in a gelatinous structure of root-knot nematodes and the cysts of *Globodera* spp. and *Heterodera* spp. (Esser and Gholl, 1993). *P. lilacinus* has been considered to have the greatest potential for application as a biocontrol agent in sub-tropical and tropical agricultural soils (Morgan-Jones et al., 1983). Indeed, it is the most widely used commercial bionematicide in the world. However, *P. lilacinus* is an opportunistic human pathogen and can infect the eye, lung, skin and heart of immunocompromised humans. Its pathogenicity on humans has restricted its application in biocontrol of nematodes (Esser and Gholl, 1993). By comparing the sequences at the 18S rRNA gene, internal transcribed spacer (ITS) and partial translation elongation factor 1-a (TEF) of clinical isolates of *P. lilacinus* with those isolated from soil, insects and nematodes, Luangsaard et al. proposed a new genus *Purpureocillium*, which contains all the isolates of *P. lilacinus* (Luangsaard et al., 2011). This new genus contains two species, *Purpureocillium lilacinum* and *Purpureocillium lavendulum* (Perdomo et al., 2013). The major difference between the two species is that *P. lavendulum* cannot grow at above 35 °C and thus is of limited clinical relevance and probably a safer biocontrol agent for plant-parasitic nematodes.

In contrast to the abundant studies of *P. lilacinum*, there has been no molecular study of *P. lavendulum*. When we tried to knock out genes in this fungus, we were unable to use the selective markers commonly used in *P. lilacinum*, including the *bar* gene (for

* Corresponding author. Fax: +86 871 65034838.
** Corresponding author. Fax: +86 871 65034838.
E-mail addresses: kqzhang1@ynu.edu.cn (K.-Q. Zhang), lianglm@ynu.edu.cn (L.-M. Liang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.funbio.2018.10.009
1878-6146/

glufosinate resistance) and CBX gene coding for carboxin resistance (Yang et al., 2015). Another restriction for studying this fungus is the low homologous recombination frequency, which is a common issue in molecular manipulation of filamentous fungi (Ninomiya et al., 2004; Carvalho et al., 2010).

In several filamentous fungi and yeasts, the targeted gene-disruption frequencies were improved when the non-homologous end joining (NHEJ) pathway was disabled (Takahashi et al., 2006; He et al., 2013; Koh et al., 2014). The specific operation to disrupt the NHEJ pathways was to knock out the *ku80* or *ku70* genes of the Ku protein complexes (Wang et al., 2006), so that the probability of homologous recombination will increase. For example, knocking out *ku80* in *Magnaporthe grisea* increased the frequency of disruption of the *MgADE4* gene from 5 % to 80 % (Villalba et al., 2008). Similarly knocking out *ku80* in *Neurospora* increased the disruption frequencies of genes *mtr* and *ad-3A* from 10 %-30 % to 100 % (Ninomiya et al., 2004); and knocking out *ku80* in *Kluyveromyces lactis* increased the disruption frequency of gene *KlADE2* from 0 % to 88 % to greater than 97 % (Kooistra et al., 2004).

In this study, we knocked out the *ku80* gene of *P. lavendulum* and the mutant showed a higher homologous recombination frequency than the wild type strain. Constructing an effective genetic manipulation system will facilitate molecular studies in *P. lavendulum* and a more comprehensive understanding of the fungus-nematode interaction at the molecular level.

2. Materials and Methods

2.1. Fungal and bacterial strains

P. lavendulum YMF1.00683 was isolated from a soil sample in Yunnan, China. *Escherichia coli* DH5α was used for plasmid construction and propagation. *Agrobacterium tumefaciens* AGL-1 was used for fungal transformation (Duarte et al., 2007).

2.2. Plasmids

Two plasmids were constructed for gene disruption experiments in this study. To construct pPK2-neo-GFP, we replaced the *ccdB* cassette in pPK2-OSCAR-GFP(Xu et al., 2014) (digested with *Xba*I) with the *neo* cassette (cut off with *Xba*I from the pSK-neo (Pei et al., 1994) plasmid, a kind gift from Prof. Shaojie Li, Institute of Microbiology, Chinese Academy of Sciences).

To construct the second plasmid, a 3094bp benomyl resistance gene *bml* was amplified by PCR from the plasmid pBT6 (Orbach et al., 1986; Duarte et al., 2007) (All the primers used in this study were described in Table 1). This fragment was then inserted into the plasmid pPK_2-OSCAR-GFP (Xu et al., 2014) (digested with *Xba*I) by In-Fusion technology, to obtain the plasmid pPK_2-bml-GFP.

2.3. Identification and characterization of PlKu80 in P. lavendulum

We performed a whole-genome sequencing of *P. lavendulum* strain YMF1.00683 using HiSeq4000 PE150 and PacBio RSII. The raw sequences were assembled by SMRT portal and genes were predicted by Genewise (version 2.4.1) and annotated by BLAST against databases like GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Cluster of Orthologous Groups of proteins), NR (Non-Redundant Protein Database), etc. (unpublished). Based on the annotated Ku80 protein sequence of this strain as well as other published Ku80 sequences from the Ascomycetes, a phylogenetic tree was constructed by software MEGA7.0 using the Basidiomycetes *Cryptococcus neoformans* sequence as an outgroup (Kumar et al., 2016).

The *ku80* gene disruption plasmid was constructed by the In-Fusion technology. Two pairs of primers (PLku80-5f/PLku80-5r and PLku80-3f/PLku80-3r) were designed with *Bss*HII and *Apa*I restriction sites, respectively. Through the In-Fusion® HD Cloning Kit, the above two PCR fragments were ligated to pPK2-neo-GFP. Confirmation of the plasmid construct is described in Fig. 1. This *PlKu80* disruption plasmid was firstly transformed into *A. tumefaciens* AGL-1 and confirmed by colony PCR (using primers PLku80-5f/PLku80-5). The transformed *A. tumefaciens* AGL-1 strain was then co-cultured with *P. lavendulum* on a microporous filtering film on the IM media at 22 °C for 48 h and transfered onto M-100 media with 500 μg/ml cephamycin and 250 μg/ml G418. The resulting transformants were firstly screened by PCR with primers Ku80-F/Ku80-R. A second round of PCR with primers GFP-5F/GFP-3R was done to ensure single integration.

To complement *ΔPlKu80*, a genomic DNA fragment of *PlKu80* including the promoter region, ORF and termination region was cloned by PCR using primers Sbfl5F-PlKu80/XbaI3R-PlKu80. This construct was then inserted into pPK2-bml-GFP (cut by *Sbf*I/*Xba*I) to form pPK2-bml-GFP-Plku80, which was transformed into *ΔPlKu80* by *A. tumefaciens* - mediated transformation. The transformants selected from M100 media with 10 μg/ml benomyl were screened by PCR using primers ku80-5/ku80-5 (the two primers were designed based on the pPK2-bml-GFP sequence which were at the upper and lower stream of the *PLKu80* gene, respectively) to obtain *PlKu80* complemented strain (*ΔPlKu80::PlKu80*). The knockout and complement strain of *PlKu80* were all confirmed by Southern blotting analysis.

2.4. Comparison of the growth and conidiation rates of among strains

The conidia of the *ku80* knockout (*ΔPlKu80*), the ku80 complemented (*ΔPlKu80::PlKu80*), and the wild-type(*Pl*) strains were obtained. For each strain, 300 μl (1×10^7/ml) conidia were spread evenly on the PDA medium, incubated at 28 °C for 3 d. They were then used to compare colony morphology and conidiation rates.

To determine the morphology of colonies, 8 mm agar plugs containing mycelia were taken from the above plates with each inoculated onto a new PDA plate. The colony morphology and growth rate of these three strains were observed and measured every 24 h. Three repetitions were done for each strain.

In order to calculate conidiation rates, three blocks of agar containing mycelia of 8 mm size were taken for each strain and placed in a 1.5 ml tube. Conidia were released from the mycelia using 1‰ Tween 80 and the concentration determined with a hemocytometer.

2.5. Nematicidal bioassay

To observe the infection of *Caenorhabditis elegans* more clearly, we used a strain transformed with pPK2-Sur-GFP. About 50 L4 nematodes were transferred onto NGM plates containing 500 μl (4×10^8/ml) conidia and *E. coli* OP50 and observed using a Zeiss Axioskop2 plus epifluorescent microscope (Zeiss, Jena, Germany). To compare the nematicidal activity of *ΔPlKu80*, *ΔPlKu80::PlKu80* and the wild-type strain of *P. lavendulum,* the bioassays were conducted with the conidia of the above strains. In the control group, *C. elegans* was added to the NGM plate only with *E. coli* OP50. The number of live nematodes was counted every 24 h on each plate under a Olympus BX51 light microscope (Olympus, Tokyo, Japan).

2.6. Recombination rate comparisons

We used the OSCAR technology originally constructed by Paz et al. (2011) and improved by Prof. Wei-Guo Fang to construct the

Table 1
Primers used in this study.

Primers	Sequence (5′-3′)	Descriptions
pBT6-bml-5F	CACGAGGACTTCTAGAGG CCAGCAGTAGACACTTGG	For amplification of the *bml* gene
pBT6-bml-3R	GCCCTGCAGCTCTAGAGA AGCGTGCCGAGATGGAGG	
PLku80-5f	TTGTAAAACGACGGCCAGTGAGCGCGCTGGCCCTTCATTCACTCTTGTT	For amplification of the *PlKu80* gene 5′flank
PLku80-5r	CCCTATAGTGAGTCGTATTACGCGCGCTTCTGTGGATGGATGGGTTGTA	
PLku80-3f	TTCCTATTCTCTAGAGCTGCAGGGCCC AGAGGCTGCTACAGGTTCATCA	For amplification of the *PlKu80* gene 3′flank
PLku80-3r	ACGACGGCCAGTCTTAAGCTCGGGCCCGTGTCTCCATCACGCCATTTC	
SbfI5F-PLku80	GGCCAGTGCCAAGCTTGCATGCCTGCAGGGCGTTGTTGAAACCCTGAA	For amplification of the *PlKu80* gene to construct the complement plasmid
XbaI3R-PLku80	TCCAAGTGTCTACTGCTGGCCTCTAGAGCGAGCACCCAGTGTAAAT	
GFP-5F	TTCACCTTGATGCCGTTCT	Validation primers of *PlKu80* transformants
GFP-3R	CCACAAGTTCAGCGTGTCC	
ku80-F	ACGACACCAAGTCCGAGAT	Validation primers of *PlKu80* transformants
ku80-R	CGTTCAGGGAAGGAGCAT	
ku80-5	AACTGAAGGCGGGAAAC	Validation primers of *PlKu80* complete transformants
ku80-3	TGACTTGAAGCGGAGGA	
P-ku80-5F	GATTAGCCGAACGAAATGTG	Southern blot probe primer of *PlKu80* gene
P-ku80-3R	CCCAAGTGATGTTTGCCTC	
brlA-15-5f	GGGGACAGCTTTCTTGTACAAAGTGGAAGCATCCGAGTGGACCTGA	For amplification of the *PlBrlA* gene 5′ flank(1500bp)
brlA-15-5r	GGGGACTGCTTTTTTGTACAAACTTGTCCCAACGGGCAAAGAAT	
brlA-15-3f	GGGGACAACTTTGTATAGAAAAGTTGTTGGGGCGGCTAGTTATAGGA	For amplification of the *PlBrlA* gene 3′ flank(1500bp)
brlA-15-3r	GGGGACAACTTTGTATAATAAAGTTGT CTGAGTCGCAAGCAAGGAA	
brlA-10-5f	GGGGACAGCTTTCTTGTACAAAGTGGAAGCATCCGAGTGGACCTGA	For amplification of the *PlBrlA* gene 5′ flank(1000bp)
brlA-10-5r	GGGGACTGCTTTTTTGTACAAACTTGTACCTTACGCTTACCTTTACCTT	
brlA-10-3f	GGGGACAACTTTGTATAGAAAAGTTGTTGGGGCGGCTAGTTATAGGA	For amplification of the *PlBrlA* gene 3′ flank(1000bp)
brlA-10-3r	GGGGACAACTTTGTATAATAAAGTTGTGGCGAGGTACTGGGTATGTT	
brlA-20-5f	GGGGACAGCTTTCTTGTACAAAGTGGAACCACCTCCTTCTCGATCTTC	For amplification of the *PlBrlA* gene 5′ flank(2000bp)
brlA-20-5r	GGGGACTGCTTTTTTGTACAAACTTGT G CAAACGGACGCACGACT	
brlA-20-3f	GGGGACAACTTTGTATAGAAAAGTTGTT GGGGCGGCTAGTTATAGGA	For amplification of the *PlBrlA* gene 3′ flank(2000bp)
brlA-20-3r	GGGGACAACTTTGTATAATAAAGTTGTAGGACTTGGTCGGCGGTCGT	
sur5	TGACTCCACGATTCACAGC	Validation primers of *PlBrlA* transformants
sur3	CCATTGGGTCACCATTCC	
brlA-5	CGGTAATACTCCCGCAAAT	Validation primers of *PlBrlA* transformants
brlA-3	CACAAATCCGAGCAACAAG	
wetA-5f	GGGGACAGCTTTCTTGTACAAAGTGGAATGTACTTCGCACATTTTAGTTGC	For amplification of the *PlWetA* gene 5′ flank
wetA-5r	GGGGACTGCTTTTTTGTACAAACTTGTGTATTGGGGTTGGTGTTTGTTC	
wetA-3f	GGGGACAACTTTGTATAGAAAAGTTGTTCGCGTAACTGTGCTATATGTCCT	For amplification of the *PlWetA* gene 3′ flank
wetA-3r	GGGGACAACTTTGTATAATAAAGTTGT GGATAGAATGCGTGAGAGGTTT	
abaA-5f	GGGGACAGCTTTCTTGTACAAAGTGGAACTCTTACCTTGGATGTGTCC	For amplification of the *PlAbaA* gene 5′ flank
abaA-5r	GGGGACTGCTTTTTTGTACAAACTTGT TCAAACACATGAAACAACGG	
abaA-3f	GGGGACAACTTTGTATAGAAAAGTTGTTAAATGTATGCTCTGAAGGGG	For amplification of the *PlAbaA* gene 3′ flank
abaA-3r	GGGGACAACTTTGTATAATAAAGTTGT CCAAGCAAGCATGAACTATG	
PlPr1D-5f	GGGGACAGCTTTCTTGTACAAAGTGGAATACGAGGACAAACGCAAGC	For amplification of the *PlPr1D* gene 5′ flank
PlPr1D-5r	GGGGACTGCTTTTTTGTACAAACTTGT ATGATGAGGATGGAAGGGC	
PlPr1D-3f	GGGGACAACTTTGTATAGAAAAGTTGTTAGAGCCCATTGATTCCTGGA	For amplification of the *PlPr1D* gene 3′ flank
PlPr1D-3r	GGGGACAACTTTGTATAATAAAGTTGT CACGAGTCAGGCATGATTCG	
PlPr1D-RT-f	CGTCAACGACATCGTCAA	Real-time PCR primers for *PlPr1D*
PlPr1D-RT-r	GCAGGGGGCCTCCAACAA	
tubulin-RT-f	GGAAGTAAAAAAGTGTAACAAGGT	Real-time PCR primers for beta-tubulin (inner control)
tubulin-RT-r	GCGTAATGCAAAAGAGTTTTG	

brlA knockout plasmid. Two pairs of primers were designed to amplify approximately 1Kb/1.5Kb/2 Kb of the 5′and 3′ flanking sequences, respectively. Primers are described in Table 1 and each primer had a unique attB sequence at its 5′ end. The PCR products, the donor plasmid (pA-SUR-OSCAR), the recipient plasmid (pPK2-OSCAR-GFP) (Xu et al., 2014) and BP Clonase (Life Technologies, Shanghai China) were prepared as described by Paz et al. (2011). After a 16-h incubation at 25 °C, the mixture was transformed into *E. coli* (DH5α). When the constructs were completely assembled, they were transformed into *A. tumefaciens* (AGL-1). Subsequently, they were transformed into *P. lavendulum* wild-type, ku80 knockout and *ku80* complemented strains, respectively (de Groot et al., 1998). The transformants selected from M100 media containing 50 μg/ml chlorimuron were confirmed by PCR using primers described in Table 1 to obtain *ΔPlBrlA* strains.

In addition, we used the same plasmid to construct the *wetA* and the *abaA* knockout plasmids, including about 1 kb upstream and downstream of the target genes. Vector constructions and methods of verification of transformants were the same as those for *ΔPlBrlA* (Primers used are described in Table 1).

2.7. Identify and knock out of a serine protease

A local BLASTp search against *P. lavendulum* genome was carried out using the amino acid sequence of the cuticle-degrading protease PII from the nematophagous fungus *Arthrobotrys oligospora*

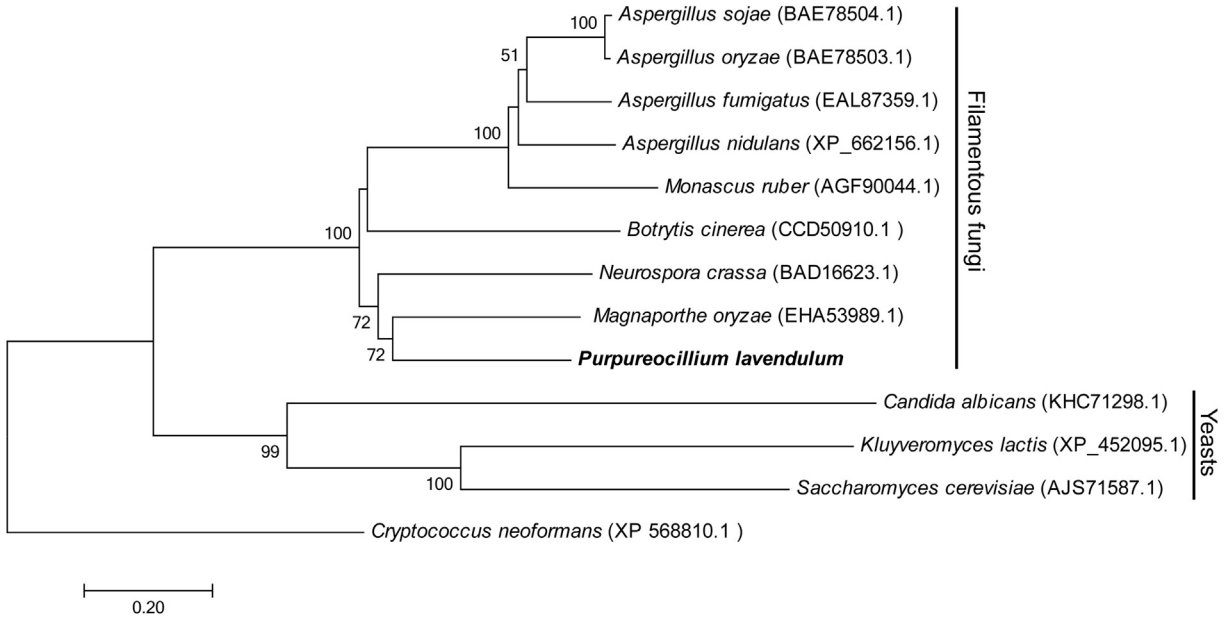

Fig. 1. Phylogenetic tree of fungal Ku80 proteins. The GenBank accession numbers of the proteins are shown in the square brackets after the species names. The KU80 sequence from *C. neoformans* was set as an outgroup.

(Åhman et al., 2002) as query sequence. The expression level of these proteases were examined by real-time PCR as described previously (Li et al., 2017), using total RNA isolated from eight-day infected nematodes. The knockout of the protease genes were carried out as described above. The ku80 and knockout strains were inoculated in PL-4 liquid media (Ye et al., 2009), culturing on a shaker at 28 °C, 150 rpm, for ten d. The protease activity were measured using a fungus PRSS ELISA kit (mlbio, Shanghai, China). The nematicidal activity of ku80 and the knockout strains were measured as above described.

2.8. Genbank accession numbers

The newly identified genes from *P. lavendulum* in this study has been deposited into GenBank under the following accession numbers: *PlKu80*: MG729631; *PlBrlA*: MG729632; *PlAbaA:* MG729633; *PlWetA*: MG729634; *PlPr1D*: MH824511.

3. Results

3.1. Characterization of the Ku80 gene in P. lavendulum

Our genome sequencing showed that the *P. lavendulum* Ku80 protein was 827 amino acids long. From the phylogenic tree shown in Fig. 1, the Ku80 sequences from ascomycetes were clustered in two subclades. One subclade includes three related ascomycete yeasts and the other includes 11 filamentous ascomycete fungi. The *P. lavendulum* Ku80 protein was most closely related to the homolog in *Magnaporthe oryzae* and *Neurospora*.

3.2. The ku80 knockout strain did not show defects in growth or nematicidal activity

We constructed a *PlKu80* disruption plasmid using the In-Fusion methodology with *neo* as the selection marker, and disrupted *PlKu80* through homologous recombination. Two disruption mutants (*ΔPlKu80*) were obtained from 308 transformants with G418 resistance. Because the knockout plasmid is an *Agrobacterium* Ti plasmid, PCR amplification of the GFP gene which were located in the T-DNA but outside the recombination cassette could exclude the transformants containing multiple copies of T-DNA that had inserted in the genome (Xu et al., 2014). *ΔPlKu80* was complemented by a genomic clone of *PlKu80*. The details of the disruption of *PlKu80* and the complementation of *ΔPlKu80* are described in Fig. 2.

We compared the colony morphology and growth rate of *ΔPlKu80* with the wild-type strain and the *ΔPlKu80::PlKu80*. As shown in Fig. 3, the three strains showed similar growth rates, and the same purple color indicating the production of spores (Fig. 3A). The growth curves of the three strains could not be distinguished either (Fig. 3B).

The virulence of the three strains against the model nematode *C. elegans* were also tested and compared. In this bio-assay, we found that *C. elegans* could swallow the spores of *P. lavendulum*, along with its food, *E. coli*. The ingested spores were intact in the intestinal tract. (Fig. 4A-1, 12–24 h). Some of the ingested spores then germinated inside the intestinal tract, most of which were just anterior to the anus (Fig. 4A-2, ~4–5 d). The hyphae firstly extended throughout the intestinal tract (Fig. 4A-3) and then penetrated other tissues (Fig. 4A-4). Finally, the fungus killed the hosts and grew out from the worm body (Fig. 4A-5). Infection of nematodes by ingested spores has previously been reported by the nematophagous fungus *Podocrella peltata* (Chaverri et al., 2005) and this manner of infection by *P. lavendulum* was not discovered previously. As shown in Fig. 4B, the killing process was slow and that the survival rate decreased at day 11 compared with the OP50 control. The knockout and complement strains showed the same nematicidal activities as the wild-type strain.

These results indicate that the disruption of *PlKu80* did not affect mycelial growth, sporulation or the virulence of *P. lavendulum*. Therefore, we proceeded to use *ΔPlKu80* as the model strain for subsequent gene knockouts.

3.3. Deletion of PlKu80 increased gene disruption efficiency in P. lavendulum

In order to test the gene disruption efficiency in *ΔPlKu80*, the homolog of the *Aspergillus nidulans* major regulatory transcription

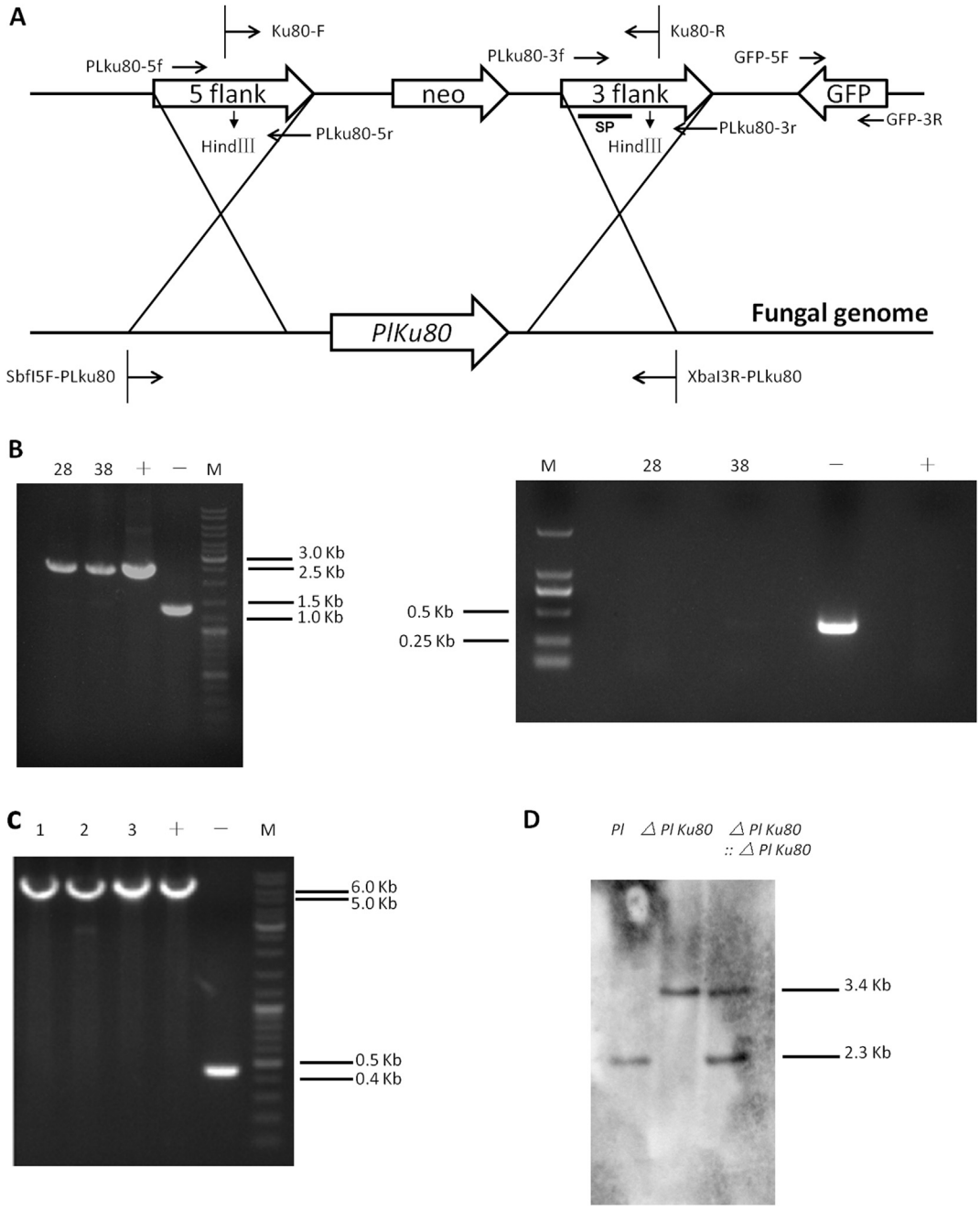

Fig. 2. Disruption and complementation of *ΔPlKu80* in *P. lavendulum*. (A) The disruption plasmid of *PlKu80* and the relative position of *PlKu80* in the wild-type strain. The relative positions of primers used for the construction of knockout and complement plasmids and the verification of transformants were indicated. neo, the G418 resistant gene; SP, the position of probe for Southern blot. (B) Confirmation of the disruption of *PlKu80* by PCR in the mutants with G418 resistance was achieved with primer pair Ku80-F/Ku80-R (Left, +, the knockout plasmid; -, the wild-type strain) and that for without a GFP sequence was achieved with primer pair GFP-5F/GFP-3R (Right, -, the knockout plasmid; +, the wild-type strain). 28 and 38 were two positive strains. (C) Confirmation of successful complementation of *ΔPlKu80* was achieved by PCR using primers Ku80-5 and Ku80-3. Three different transformants in the *ΔPlKu80* complementation experiment were selected for the confirmation. +, the complement plasmid; -, the plasmid pPK2-bml-GFP. (D) Southern blot analysis of *ku80* gene of wild-type *P. lavendulum* and transformant strains. The genomic DNA of each strain was cut by Hind III. The probe was amplified by the primers P-ku80-5F/P-ku80-3R.

factors, BrlA, AbaA and WetA, in the central regulation pathway of conidiation, named PlBrlA, PlAbaA and PlWetA, were selected as targets for disruption. The deletion of *PlbrlA* resulted in inability in conidiation, but higher hyphal growth rate compared with the wild-type strain (Supplementary Fig. 1 and Fig. 5). These visible phenotype changes allowed us to readily evaluate the efficiency of gene disruption. These disruption plasmids which had different lengths of the homologous arm for brlA were generated using the modified OSCAR methodology with *sur* as the selection marker. No disruption mutant was obtained from the wild-type strain or the *ΔPlKu80*::*PlKu80* strain, while 4.8 %, 1.6 % and 3.8 % true disruptants were obtained from the *ΔPlKu80* strain background using 1, 1.5 and 2 kb flanking regions, respectively. Subsequently, we disrupted other two genes in the central pathway of conidiation (*PlWetA*, *PlAbaA*) and both deletion mutants showed obvious phenotype defects in *ΔPlKu*80 strain background. From Table 2, we can see that the homologous recombination rate of *PlWetA* increased dramatically from 8.0 % to 100 %. Similarly, the *PlAbaA* gene disruption

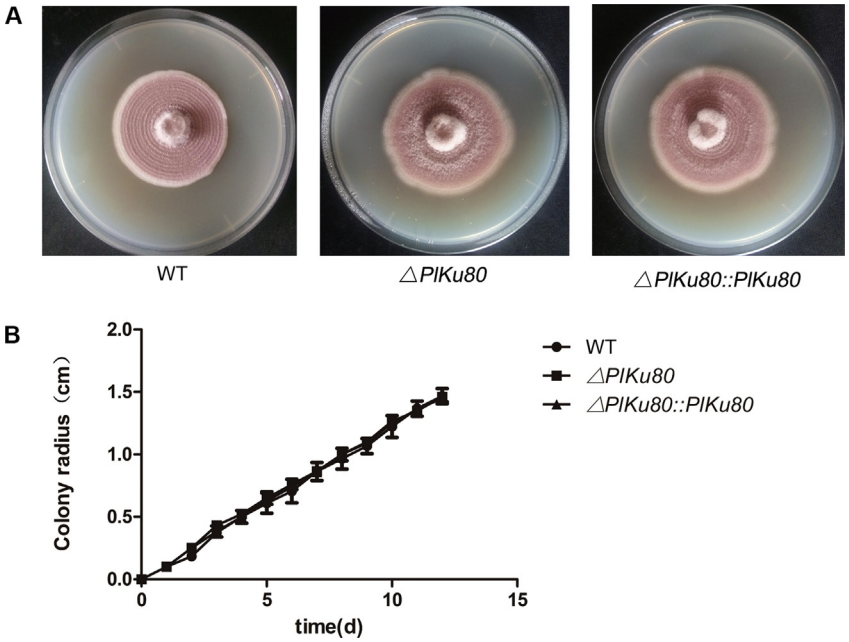

Fig. 3. Comparison of the colony morphology and growth rate of *ΔPlKu80* with the wild-type strain and *ΔPlKu80*::*PlKu80*. (A) The colonies of each strain grown on PDA for 5 d. (B) The growth curve of each strain in 12d.

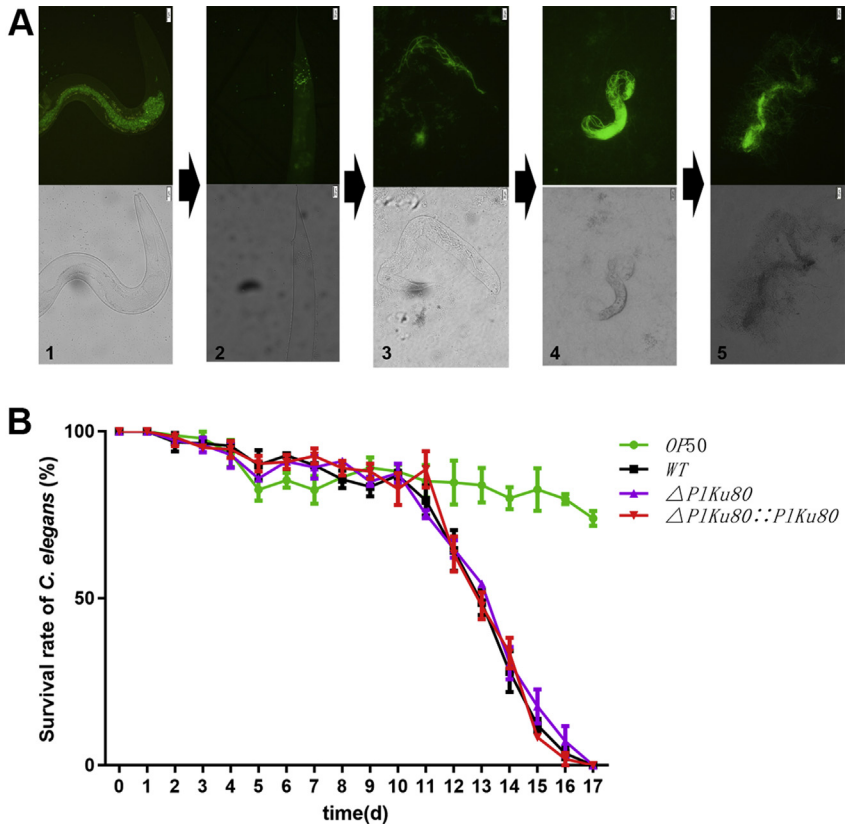

Fig. 4. Comparison of the nematicidal activities among strains. (A), the infection of *C. elegans* by *P. lavendulum*. The spores of *P. lavendulum* could be ingested by *C. elegans*, and then germinated and killed the host from the intestine tract. (B) The bioassay of each strain against *C. elegans* was conducted as described in the Materials and Methods. OP50, the control group, in which *C. elegans* was feed by *E. coli* OP50 only. Wild-Type, the wild-type strain; *ΔPLKu80*, the *PLKu80* knockout train; *ΔPLKu80::PLKu80*, the *PLKu80* complementation strain.

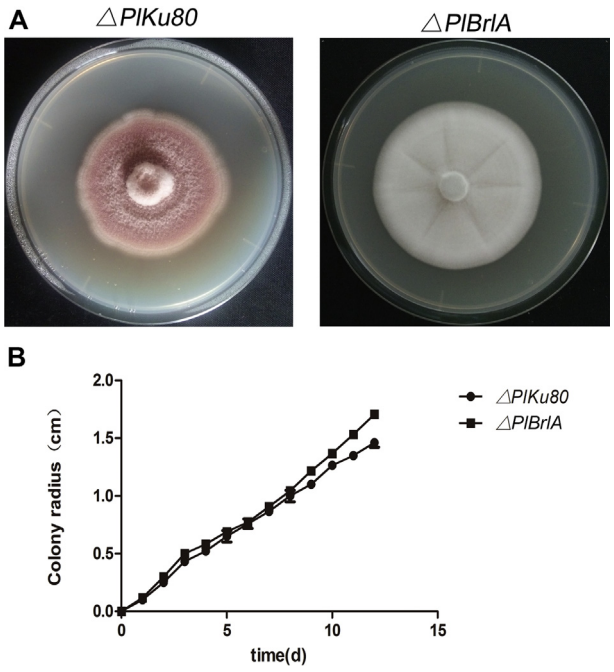

Fig. 5. Comparison of the growth and conidiation rates between *ΔPlKu80* with *ΔPlBrlA*. (A) The colonies of the two strains. (B) The colony radius of the two strains.

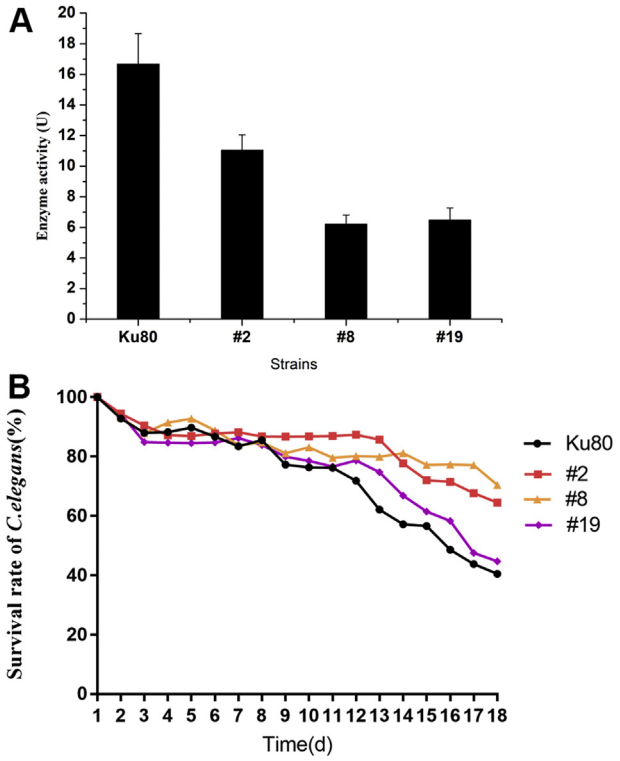

Fig. 6. Knockout of a serine protease *PlPr1D* affect the virulence of *P. lavendulum* against *C. elegans*. (A), The protease activities were reduced in the *PlPr1D* knockout strains. These results are mean ± SD of three independent experiments performed in triplicate. *P < 0.05 versus *ΔPlKu80* (B), knockout of *PlPr1D* in *P. lavendulum* decreased the virulence in infection of nematodes. Ku80, the *ΔPlKu80* strain, #2, #8 and #19, three *PlPr1D* knockout strains.

efficiency was also raised, from 2.9 % to 60 %. The knockout efficiencies of both genes declined when the experiments were performed in the *PlKu80* complementation strain.

3.4. The serine protease PlPr1D was involved in the infection of C. elegans by P. lavendulum

Extracellular serine proteases (or cuticle-degrading proteases) in nematophagous fungi were identified as key virulent factors during fungal infection (Yang et al., 2007). The cuticle-degrading protease PII played important role in infection of the model nematophagous fungus, *A. oligospora* (Åhman et al., 2002). We identified PII homologs in the *P. lavendulum* genome by local BLASTp search. Six putative serine proteases were isolated. All of them have serine protease S8 domain. The expression profile were examined by real-time quantitative PCR, and one gene were upregulated mostly, for almost 50 folds after 4 d infection, while the others were not upregulated significantly (data not shown). This protease showed 40 % identity with PII, and showed 72 % identity with the serine protease Pr1D in *Metarhizium anisopliae* (Bagga et al., 2004), and thus we named it PlPr1D. To further confirm its role in the infection, knockout strains of *PlPr1D* were made from the ku80 strain of *P. lavendulum*. The knockout ratio of *PlPr1D* was 43 % (nine mutants out of 21 transformants, Supplementary Fig. 2). The protease activities of *ΔPlKu80* and *ΔPlPr1D* strains were measured in liquid media. as shown in Fig. 6A, the protease activities were reduced for 33.71 %–62.80 % in the three knockout strains. The nematode activities of *ΔPlku80* and *ΔPlPr1D* strains were compared, as shown in Fig. 6B, all the three knockout strains showed reduced nematicidal activities compared with the *ΔPlKu80* strain. These results revealed that the serine protease PlPr1D was required for full virulence of *P. lavendulum* in the infection of *C. elegans*.

4. Discussion

There have been growing concerns about excessive use of chemical pesticides that have caused significant environmental problems (Elwakeil, 2013). As a result, biocontrol of nematodes and insects by microbial agents has attracted increasing attention of both scientists and the general public. The nematophagous fungus *P. lilacinus* has been used as a bionematicide for many years. However, two main issues have restricted the utilization of this fungus. One is due to fungistasis probably caused by volatiles of microbial origins, the effects of this fungus to control plant-parasite nematodes in field were not stable (Li et al., 2007; Fang et al., 2011). The other reason is that the fungus can cause opportunistic

Table 2
Effect of *ku80* disruption on gene targeting frequency.

Targeted gene	5′/3′ flanking regions (bp)	Knockout efficiency of *WT* (%)	Knockout efficiency of *ΔPlKu80* (%)	Knockout efficiency of *ΔPlKu80::PlKu80* (%)
PlWetA	942bp/1074bp	8.0 (6/79)	100 (82/82)	38 (35/91)
PlAbaA	1211bp/1308bp	2.9 (27/94)	60 (57/95)	25 (22/86)
PlBrlA	1082bp/1053bp	0 (0/117)	4.8 (5/104)	0 (0/92)
PlBrlA	1526bp/1463bp	0 (0/95)	1.6 (2/125)	0 (0/88)
PlBrlA	2188bp/1841bp	0 (0/82)	3.8 (4/105)	0 (0/97)

infections in humans. The newly defined species *P. lavendulum* cannot grow at 35 °C, a feature that differs from its sister species, *P. lilacinum* (Perdomo et al., 2013). This feature would make *P. lavendulum* much safer to humans than *P. lilacinum* during biocontrol applications in agricultural fields. Therefore, *P. lavendulum* should have a great potential as a source of nematicides and a comprehensive understanding the infection mechanism of *P. lavendulum* at the molecular level is needed.

An effective genetic manipulation system will facilitate the molecular studies of this fungus. In this study, we created an efficient genetic manipulation system for the nematophagous fungus, a non-model filamentous fungus, *P. lavendulum*, with the integration of selective markers, an effective method to construct knockout plasmids, and the construction of a ku80 knockout strain for higher homologous recombination and gene integration rate. The *ΔPlKu80* strain showed the same morphological structure and insecticidal efficiency as the wild-type strain. Based on a series of tests with different genes and recombination arms with different lengths, our results confirmed that the *ΔPlKu80* gene knockout strain had significantly enhanced efficiency for gene knockout over the wild type strains. In our study, the knockout efficiency of *PlBrlA* was significantly lower than that of genes *PlWetA* and *PlAbaA* in all parental strains (wild-type, *ΔPlKu80* or *ΔPlKu80::PlKu80*). The flank length seemed not correlated to the knockout efficiency. The reason for the low yield of *PlBrlA* disruptants may be related to its key role in asexual reproduction and/or its unique sequences flanking the coding region. More studies are needed to understand the relatively low recombination efficiency of *PlBrlA*.

The infection of *C. elegans* by *P. lavendulum* from the intestinal tract was investigated in the present study. The conidia could be ingested by the nematode along with their food, *E. coli* OP50, likely due to its overall size is similar to the bacteria. Specifically, the size of a *P. lavendulum* conidium is 2–3 × 1–2 μm (Perdomo et al., 2013), while *E. coli* is 0.5 × 1–3 μm (Rogers and Kadner, 2017). Moreover, based on the gene knockout system, we identified a virulent factor, the serine proteases PlPr1D, which contributed in the infection procedure. The homologous proteases of PlPr1D are cuticle-degrading proteases, which could hydrolyze the portentous components of the nematode cuticle during fungal penetration. We speculated that PlPr1D could contributed to degrade the intestinal tract during *P. lavendulum* infection of *C. elegans*. Several issues we thought were worthy to elucidate about the infection in this manner. The bacteria cannot maintain their integrity and survive during nematode chewing. How can the spores survive the ingestion and host digestive tract and eventually germinate and kill the host? What factors affect the germination of the spores? The genetic manipulation system developed here made a solid base to facilitate such studies.

Acknowledgments

We are grateful to Professor Weiguo Fang, Institute of Microbiology and College of Life Sciences, Zhejiang University for providing plasmids pPK2-OSCAR-GFP, pA-SUR-OSCAR and for his technical guidance. This work was supported in part by grants from the National Natural Science Foundation of China (31572063 and 31770064), Yunnan Applied Basic Research Project (2016FB062) and Grants from the Young Academic and Technical Leader Raising Foundation of Yunnan Province (to Lian-Ming Liang).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2018.10.009.

References

Abad, P., Gouzy, J., Aury, J.M., Castagnone-Sereno, P., Danchin, E.G., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V.C., Caillaud, M.C., Coutinho, P.M., Dasilva, C., De Luca, F., Deau, F., Esquibet, M., Flutre, T., Goldstone, J.V., Hamamouch, N., Hewezi, T., Jaillon, O., Jubin, C., Leonetti, P., Magliano, M., Maier, T.R., Markov, G.V., McVeigh, P., Pesole, G., Poulain, J., Robinson-Rechavi, M., Sallet, E., Segurens, B., Steinbach, D., Tytgat, T., Ugarte, E., van Ghelder, C., Veronico, P., Baum, T.J., Blaxter, M., Bleve-Zacheo, T., Davis, E.L., Ewbank, J.J., Favery, B., Grenier, E., Henrissat, B., Jones, J.T., Laudet, V., Maule, A.G., Quesneville, H., Rosso, M.N., Schiex, T., Smant, G., Weissenbach, J., Wincker, P., 2008. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nat. Biotechnol. 26, 909–915.

Åhman, J., Johansson, T., Olsson, M., Punt, P.J., van den Hondel, C.A., Tunlid, A., 2002. Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. Appl. Environ. Microbiol. 68 (7), 3408–3415.

Bagga, S., Hu, G., Screen, S.E., Leger, R.J.S., 2004. Reconstructing the diversification of subtilisins in the pathogenic fungus Metarhizium anisopliae. Gene 324 (1), 159.

Carvalho, N.D.S.P., Mark, A., Jin, K.M., Vera, M., Ram, A.F.J., 2010. Expanding the ku70 toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses. Appl. Microbiol. Biotechnol. 87, 1463–1473.

Chaverri, P., Samuels, G.J., Hodge, K.T., 2005. The genus Podocrella and its nematode-killing anamorph Harposporium. Mycologia 97, 433–443.

de Groot, M.J.A., Bundock, P., Hooykaas, P.J.J., Beijersbergen, A.G.M., 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat. Biotechnol. 16, 839–842.

Duarte, R.T., Staats, C.C., Fungaro, M.H., Schrank, A., Vainsten, M.H., Furlanetomaia, L., Nakamura, C.V., De, S.W., Furlaneto, M.C., 2007. Development of a simple and rapid *Agrobacterium tumefaciens*-mediated transformation system for the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum*. Lett. Appl. Microbiol. 44, 248–254.

Elwakeil, N.E., 2013. Botanical pesticides and their mode of action. Gesunde Pflanz. 65, 125–149.

Esser, R., Gholl, N., 1993. *Paecilomyces lilacinus*, a fungus that parasitizes nematode eggs. Nematol. Circ. 203, 12–14.

Fang, L.Z., Xu, C.K., Song, Z.C., Qin, X.J., Qiu, H.Y., Duan, C.Q., He, M.M., 2011. Fungistatic intensity of agricultural soil against fungal agents and phylogenetic analysis on the actinobacteria involved. Curr. Microbiol. 62, 1152–1159.

He, Y., Liu, Q., Shao, Y., Chen, F., 2013. Ku70 and ku80 null mutants improve the gene targeting frequency in *Monascus ruber* M7. Appl. Microbiol. Biotechnol. 97, 4965–4976.

Koh, C.M.J., Liu, Y., Moehninsi, Du, M., Ji, L., 2014. Molecular characterization of KU70 and KU80 homologues and exploitation of a KU70-deficient mutant for improving gene deletion frequency in *Rhodosporidium toruloides*. BMC Microbiol. 14, 50.

Kooistra, R., Hooykaas, P.J., Steensma, H.Y., 2004. Efficient gene targeting in *Kluyveromyces lactis*. Yeast 21, 781–792.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870.

Li, L., Mo, M., Qu, Q., Luo, H., Zhang, K., 2007. Compounds inhibitory to nematophagous fungi produced by *Bacillus* sp. strain H6 isolated from fungistatic soil. Eur. J. Plant Pathol. 117, 329–340.

Li, X., Kang, Y.Q., Luo, Y.L., Zhang, K.Q., Zou, C.G., Liang, L.M., 2017. The NADPH oxidase AoNoxA in Arthrobotrys oligospora functions as an initial factor in the infection of Caenorhabditis elegans. J. Microbiol. 55 (11), 885.

Luangsaard, J., Houbraken, J., Doorn, T.V., Hong, S.B., Borman, A.M., Hyweljones, N.L., Samson, R.A., 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiol. Lett. 321, 141–149.

Morgan-jones, G., White, J.F., Rodriguez-Kabana, R., 1983. Phytonematode pathology: ultrastructural studies. II. Parasitism of Meloidogyne arenaria eggs and larvae by *Paecilomyces lilacinus*. Nematropica 14, 57–71.

Ninomiya, Y., Suzuki, K., Ishii, C., Inoue, H., 2004. Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc. Natl. Acad. Sci. U.S.A. 101, 12248–12253.

Orbach, M.J., Porro, E.B., Yanofsky, C., 1986. Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol. Cell Biol. 6, 2452–2461.

Paz, Z., Garcia-Pedrajas, M.D., Andrews, D.L., Klosterman, S.J., Baeza-Montanez, L., Gold, S.E., 2011. One step construction of Agrobacterium-Recombination-ready-plasmids (OSCAR), an efficient and robust tool for ATMT based gene deletion construction in fungi. Fungal Genet. Biol. 48, 677–684.

Pei, Z., Keese, C.R., Giaever, I., Kurzawa, H., Wilson, D.E., 1994. Effect of the pSV2-neo plasmid on NIH 3T3 cell motion detected electrically. Exp. Cell Res. 212, 225.

Perdomo, H., Cano, J., Gené, J., García, D., Hernández, M., Guarro, J., 2013. Polyphasic analysis of *Purpureocillium lilacinum* isolates from different origins and proposal of the new species *Purpureocillium lavendulum*. Mycologia 105, 151–161.

Rogers K, Kadner RJ. (2017). "Bacteria." from https://www.britannica.com/science/bacteria/Diversity-of-structure-of-bacteria.

Takahashi, T., Masuda, T., Koyama, Y., 2006. Enhanced gene targeting frequency in ku70 and ku80 disruption mutants of *Aspergillus sojae* and *Aspergillus oryzae*. Mol. Genet. Genom. 275, 460–470.

Villalba, F., Collemare, J., Landraud, P., Lambou, K., Brozek, V., Cirer, B., Morin, D., Bruel, C., Beffa, R., 2008. Improved gene targeting in *Magnaporthe grisea* by inactivation of MgKU80 required for non-homologous end joining. Fungal Genet. Biol. 45, 68.

Wang, M., Wu, W., Wu, W., Rosidi, B., Zhang, L., Wang, H., Iliakis, G., 2006. PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Res. 34, 6170–6182.

Xu, C., Zhang, X., Qian, Y., Chen, X., Liu, R., Zeng, G., Zhao, H., Fang, W., 2014. A high-throughput gene disruption methodology for the entomopathogenic fungus *Metarhizium robertsii*. PLoS One 9, e107657.

Yang, J., Tian, B., Liang, L., Zhang, K., 2007. Extracellular enzymes and the pathogenesis of nematophagous fungi. Appl. Microbiol. Biotechnol. 75 (1), 21–31.

Yang, F., Abdelnabby, H., Xiao, Y., 2015. The role of a phospholipase (PLD) in virulence of *Purpureocillium lilacinum* (*Paecilomyces lilacinum*). Microb. Pathog. 85, 11.

Ye, F., Liang, L., Mi, Q., Yang, J., Lou, Z., Sun, Y., Guo, Y., Meng, Z., Zhang, K., 2009. Preliminary crystallographic study of two cuticle-degrading proteases from the nematophagous fungi Lecanicillium psalliotae and Paecilomyces lilacinus. Acta Crystallogr. F: Struct. Biol. Cryst. Commun. 65 (Pt 3), 271–274.