Int J Clin Exp Med 2019;12(4):3492-3499
www.ijcem.com /ISSN:1940-5901/IJCEM0081181

Original Article

Molecular cloning and stable knockout strain construction of the *AcuE gene in Talaromyces marneffei*

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Received June 11, 2018; Accepted December 9, 2018; Epub April 15, 2019; Published April 30, 2019

Abstract: Aims: To clone the malate synthase (MS)-encoding gene *AcuE* and to construct stable *AcuE* gene-knockout strains of *Talaromyces marneffei.* Methods: The *T. marneffei AcuE* gene sequence was identified utilizing a bioinformatics approach, and then it was amplified by the Q5 high-fidelity enzyme system using a polymerase chain reaction (PCR). The gene knockout vectors were constructed by In-Fusion seamless cloning technology. Stable gene knockout strains were constructed via protoplast-mediated transformation, and phenotypic assays of the confirmed transformants were conducted on a solid medium with glucose or acetate as the sole carbon source at both 26°C and 37°C. Results: The AcuE-knockout vector pGE AcuE KO was successfully constructed using pGEM-T Easy as the backbone, fusing the 5'-end and the 3'-end of the *AcuE* gene counterclockwise, and the *N. pyrG* cassette inserted clockwise in the middle. Through protoplast-mediated transformation, we obtained 29 positive transformants lacking the *AcuE* gene, and the knockout strains were proven to be correctly recombined and stably inherited. The confirmed knockout strains were unable to grow on a medium with acetate as the only carbon source at either 25°C (7 days) or 37°C (7 days), but no difference in growth compared with the wild-type strain was observed on the glucose medium. Conclusions: The MS-encoding gene *AcuE* was cloned, and stable *AcuE* gene-knockout strains of *T. marneffei* were successfully constructed. These strains may provide a basis for mechanistic studies of the glyoxylate cycle involved in the pathogenesis of *T. marneffei*.

Keywords: *Talaromyces marneffei*, gene knockout, glyoxylate cycle, malate synthase, In-Fusion assay

Introduction

Talaromyces marneffei (T. marneffei), previously known as *Penicillium marneffei*, is the only dimorphic species in its genus that causes a fatal systemic mycosis, named penicilliosis marneffei [1, 2]. *T. marneffei* mainly occurs in patients with impaired immune function, such as HIV/AIDS patients, cancer patients undergoing radiation therapy or chemotherapy, organ transplantation patients, and leukemia patients, with HIV/AIDS patients being the most predominant. In recent years, with the increasing incidence of AIDS and the extensive use of glucocorticoids, immunosuppressive agents, and tumor chemotherapy drugs, the incidence of penicilliosis marneffei has been climbing every year. In fact, after tuberculosis and cryptococcosis, it is the third most common opportunistic infection in patients who live in endemic regions [3, 4]. Therefore, understanding the pathogenic mechanism of *T. marneffei* is essential for the prevention and treatment of this disease.

The ability of *T. marneffei* to survive in the host macrophages is considered to be its key pathogenic factor, given that the internal environment of the host macrophage lacks glucose so that microorganisms cannot use the normal Krebs cycle to maintain energy metabolism, which is vital for survival. Therefore, the microorganism must fully mobilize various metabolic pathways for breeding and persisting in host cells with poor nutrition. It has been reported that most of the bacteria and fungi in macrophages obtain their carbon source mainly through the glyoxylate cycle [5, 6]. The glyoxylate cycle is also one of the metabolic branches of the Krebs cycle, in which the key enzymes are isocitrate lyase (ICL) and malate synthase (MS) [7-9]. We have studied the ICL coding gene

Table 1. Oligonucleotide primers used in this study

Primer name	Sequence (5'-3')
AcuE-5	TACATCGAGACGGTGTAGGTGGG
AcuE-5 R	GCGATAGGAAGGAGGATCATC
AcuE-3F	TATCGCCTACTATTCGTCTTG
AcuE-3	GCATGGCCTGTGACTGAATTAGG
P-5AcuE F	GAATTCACTAGTGATTACATCGAGACGGTGTAGGTGGG
P-5AcuE R	TGTAACGGTATTGACGCGATAGGAAGGAGGATCATC
P-3AcuE F	AGAGCATTGTCTGCGTATCGCCTACTATTCGTCTTG
P-3AcuE R	CCGCGGGAATTCGATGCATGGCCTGTGACTGAATTAGG
N. pyrG F	CGCAGACAATGCTCTCTATCC
N. pyrG R	GTCAATACCGTTACACATTTCCA
AcuE-pyrG F	GACGAAAGGACAGGTTAGGTTT
AcuE-pyrG R	CTATGGCCCAACAGTAACGAC

AcuD previously [6], but information regarding the MS-coding gene *AcuE* is lacking.

One of the most direct methods to study a specific gene is to knock it out or replace it from the genome and then to observe the phenotypic changes. Therefore, in this study, we cloned the MS-encoding gene *AcuE* and constructed stable *AcuE* gene-knockout strains in order to provide a basis for mechanistic studies of the glyoxylate cycle involved in the pathogenesis of *T. marneffei*. The results of this study may give us novel targets or clues for the treatment strategy of penicilliosis marneffei.

Materials and methods

Strains, plasmids, and reagents

The *T. marneffei FRR2161* strain (wild-type) and the *ΔligD* strain, which originated from *FRR2161* (Genotype: *ΔligD pyrG*$^-$), and the plasmid PALX223 were constructed by *Andrianopoulos et al.* [10]. pGEM-T Easy Vector was purchased from Promega (USA), and TOP10 competent cells were purchased from Tiangen Biotech (China). Unless otherwise stated, all the *T. marneffei strains* used in this study were incubated in SD medium containing 1% (w/v) glucose as the carbon source and 10 mM yeast nitrogen base without amino acids (YNB) as the nitrogen source. Meanwhile, 5 mM uridine and 5 mM uracil were added to the medium according to the requirements of the various genotypes studied; this medium was named +U medium. The liquid cultures were bubbled with air supplemented with 1% (v/v) CO_2, and the strains were incubated at both 37°C and 25°C. Q5 High-Fidelity DNA Polymerase was purchased from New England Biolabs (USA), a TIANprep Rapid Mini Plasmid Kit was purchased from Tiangen Biotech (China), a PureYield Plasmid Midiprep System was purchased from Promega (USA), and a MiniBESTAgarose Gel DNA Extraction kit was purchased from Takara (China).

Molecular cloning of AcuE cDNA: bioinformatics search and polymerase chain reaction (PCR) amplification

The whole-genome sequence of *T. marneffei* (accession number NW_002196661.1) and the *AcuE* mRNA sequence (accession number XM_002143694.1) were retrieved from the National Center for Biotechnology Information (NCBI) GenBank. According to the position of the *AcuE* mRNA sequence in the whole-genome sequence, we obtained the *AcuE* cDNA sequence and the flanking sequences at both sides. Similarly, the *pyrG* sequence of *Emericella nidulans* (*N. pyrG*: accession number M19132.1) was obtained [11].

The PCR primers that were used for amplifying the target sequence are listed in **Table 1**. The PCR was performed using the Q5 High-Fidelity DNA Polymerase with an initial denaturation step of 98°C for 30 s, 30 cycles of amplification at 98°C for 8 s, 56°C for 20 s, 72°C for 2 min, and a final extension step of 72°C for 2 min. Agarose gel electrophoresis and DNA sequencing were performed to confirm that all target fragments were successfully obtained. DNA sequencing was accomplished by Sangon Biotech (China) with the Sanger sequencing method.

Knockout vectors: design, construction, and screening

Genomic DNA of *T. marneffei* was extracted from frozen mycelia, as described previously [10]. The knockout vectors for polyethylene glycol-$CaCl_2$-mediated transformation were constructed with the pGEM-T Easy plasmid as the backbone.

According to the principle of In-Fusion cloning, an additional 15 bp must be included on both the upstream and downstream sides of the destination vector to enable ligation-indepen-

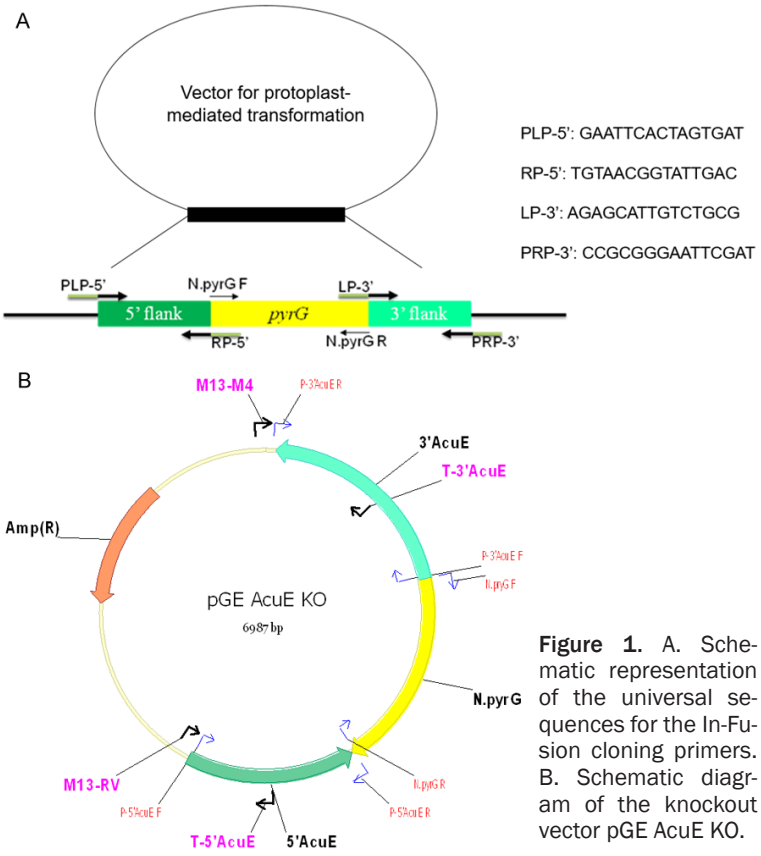

Figure 1. A. Schematic representation of the universal sequences for the In-Fusion cloning primers. B. Schematic diagram of the knockout vector pGE AcuE KO.

dent In-Fusion cloning (Clontech). Based on this principle, we designed universal sequences for the In-Fusion cloning primers, which are listed in **Table 1**. The schematic representation of the universal sequences for the In-Fusion cloning primers is shown in **Figure 1A**. The PCR amplification conditions were the same as those of the target gene. The PCR-amplified products and enzyme-digested products were purified and diluted to 60 ng/μL. A 10-μL volume of premix consisting of three inserts and a vector (molar ratio of 1:1:1:2) was added to the In-Fusion reaction system. The culture of *E.coli* transformants was spread on the lysogeny broth plate supplemented with ampicillin (100 μg/mL). After overnight incubation at 37°C, the transformants were obtained. The positive clones were screened by colony PCR and were randomly sequenced.

Knockout strains: genetic transformation, mitotic stability test, and molecular confirmation

The protoplast transformation procedure of *T. marneiffei* was based on a previously proposed approach [12], with some parameters adjusted so that it could be applied under our laboratory conditions [13]. The sole nitrogen source of the *ΔligD* strains was the YNB contained in the osmotically stabilized selection plates (selecting for *pyrG* complementation). Circular vectors of 500 ng were transformed into *ΔligD* strains, and the obtained transformants were selected for complementation of the uridine/uracil auxotrophy [12]. The mitotic stability of the *T. marneffei* transformants was determined by analyzing the stability of the *pyrG* gene. All transformants were subcultured more than five times before we conducted subsequent experiments to ensure that the knock out cassette was stably integrated into the host genome. Finally, the knockout screening primers AcuE-pyrG F and AcuE-pyrG R, located on both homologous arms of the knockout genes, were designed (**Table 1**; **Figure 2C**) to confirm that the target genes were correctly knocked out. The electrophoretic band of each knockout strain was compared with that of the wild-type one (positive fragment); if the band was in the same position as that of the positive fragment, it proved to be a false-positive transformant; if the electrophoretic band and the positive fragment were not in the same position and the former was consistent with the knockout strain target fragment, it proved to be a true-positive transformant; if both bands were visible at the same time, the colonies might not be pure, and a single-spore isolation was required to obtain a true-positive transformant. All the PCR products of the knockout strains were sequenced to guarantee accurate results.

Phenotype assay of the confirmed transformants

Solid YNB containing acetate (1%) as the sole carbon source (YA) was used to test the ability of the transformants to assimilate carbon sources, and YNB with glucose (1%) was used as a control. The cultures were then incu-

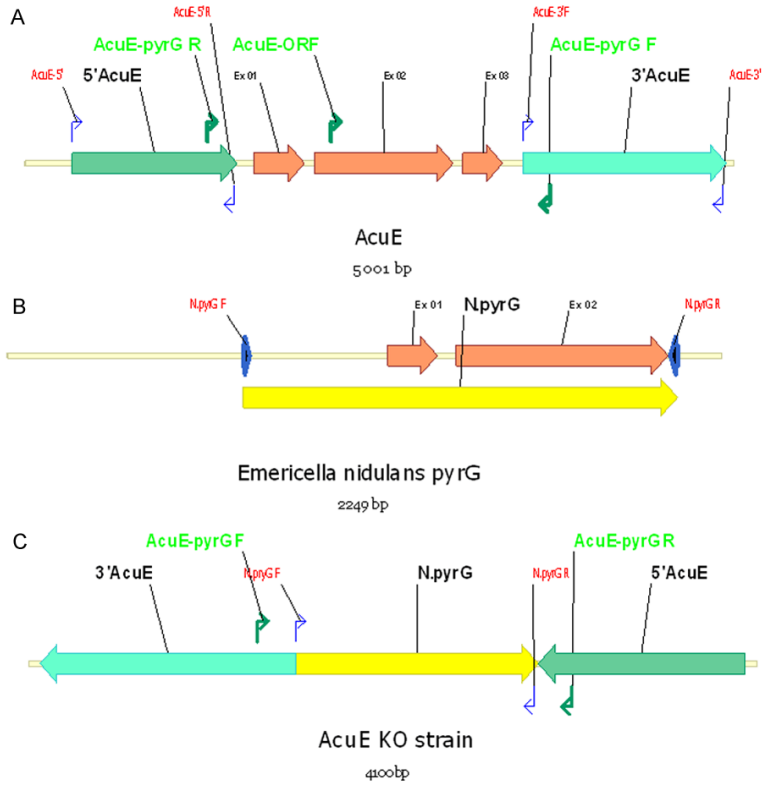

Figure 2. Gene structure diagrams. (A) *AcuE*, (B) *Emericella nidulans pyrG*. Ex: exon; F: forward primer; R: reverse primer; ORF: open reading frame. (C) Gene structure of the knockout strains.

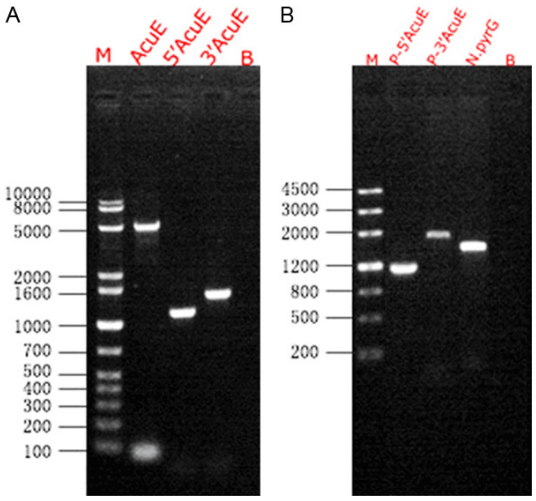

Figure 3. Electrophoresis results. A. The *AcuE* target gene and its flanking sequences. Band sizes: 4626 bp, 1167 bp, and 1438 bp (from left to right). M: 1 kb plus DNA ladder; B: Blank control. B. Insert fragments for pGE AcuE KO knockout vector construction. P-5'AcuE, 1197 bp; P-3'AcuE, 1468 bp, *N. pyrG*, 1369 bp. M: Marker III, B: Blank control.

bated at 25°C or 37°C for 14 days. Photographs were taken using a Nikon digital camera.

Results

Bioinformatics search and molecular cloning of the AcuE gene and its flanking sequences

The whole-genome sequences of *T. marneffei* and *AcuE* mRNA were retrieved from NCBI GenBank. The *AcuE* open reading frame spans 1761 bp and consists of three exons and two introns (**Figure 2A**), and the *pyrG* sequence of *E. nidulans* spans 2249 bp and consists of two exons and one intron (**Figure 2B**). PCR amplification was performed using the Q5 High-Fidelity DNA Polymerase. The sizes of the *AcuE* +, 5'-*AcuE*, and 3' *AcuE* flanking sequences were 4626 bp, 1167 bp, and 1438 bp, respectively (**Figure 3A**). The electrophoresis and sequencing results confirmed that all the target fragments were successfully obtained.

Knockout vectors: design and construction

The *AcuE* gene knockout vector, which we named pGE AcuE KO (**Figure 1B**), contained the pGEM-T Easy plasmid as the backbone, fusing the 5'-end and the 3'-end of the *AcuE* gene counterclockwise, and the *N. pyrG* cassette inserted clockwise in the middle. The positive recombinants identified by colony-PCR were further sequenced, and the alignment results revealed that the sequences of the constructed recombinant vector and the knockout cassette were corrected, indicating that the knockout vector pGE AcuE KO was successfully constructed.

Knockout strains: genetic transformation, mitotic stability test, and molecular confirmation

By protoplast-mediated transformation, we obtained 29 positive transformants lacking the *AcuE* gene (**Figure 4**). All positive transformants were segregated after five transfers on SD selective plates without uridine or uracil, indicating that pyrG was stably maintained in all of the transformants. By comparison with the

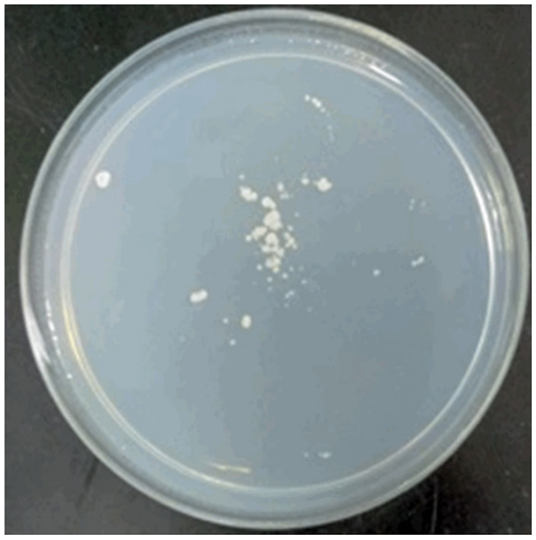

Figure 4. Positive transformants by uridine/uracil auxotrophy selection. Media: 2% glucose + yeast nitrogen base without uridine or uracil, 37°C, 5 days.

wildtype, the PCR and sequencing results revealed that all of the transformants lacked *AcuE* and obtained *pyrG* (**Figure 5**), confirming that the *AcuE* gene was targeted by the *pyrG* gene at that locus.

Phenotype assay of the confirmed transformants

The transformants were unable to grow on a medium with acetate as the only carbon source at either 25°C (7 days) or 37°C (7 days), but no difference in growth compared with the wild-type strain was observed on the glucose medium (**Figure 6**). This finding further indicated that the *AcuE* gene was deleted from the host chromosome and also proved that the *AcuE* gene plays an important role in the glyoxylate metabolism of *T. marneiffei*.

Discussion

The glyoxylate cycle is a ubiquitous metabolic branch in prokaryotes, lower eukaryotes, and plants, in addition to vertebrates. Like glycolysis, gluconeogenesis, and the Krebs cycle, it constitutes the basal metabolic network of microorganisms [14, 15]. Previously, our lab conducted the RNAi-mediated silencing of the *T. marneiffei Acu*D gene [6], confirming that the *AcuD* gene participates in the glyoxylate cycle. However, the function of the MS-encoding gene in this cycle remains elusive.

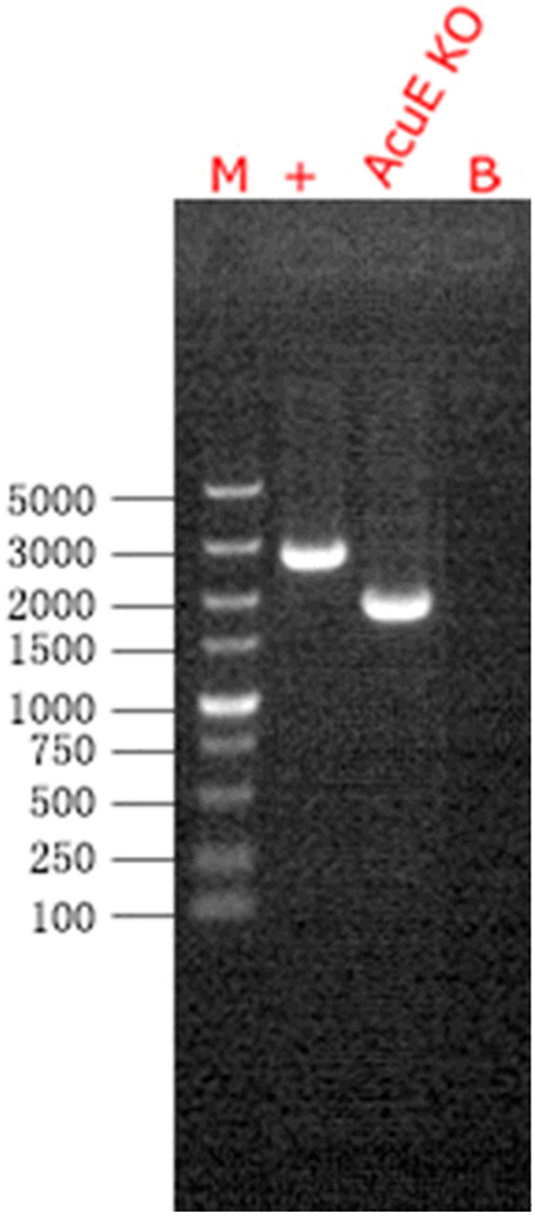

Figure 5. Validation of *AcuE* knockout strains by PCR. The results showed that the PCR-amplified band of the knockout strain was not in the same position as that of the wild-type strain. The band size was within the theoretical range and was a single band, demonstrating that the *AcuE* gene was successfully knocked out. Sizes of bands: AcuE +, 2445 bp; AcuE KO, 1793 bp. M: DL5000 DNA ladder; +: wild-type strain; B: blank control.

One of the most direct methods for determining the function of a specific target gene is to knock it out or replace it. Therefore, gene knockout technology has become a key method for the study of hypothetical genes and hypothetical proteins. The main issues in knocking out fungal genes are target gene cloning, knockout

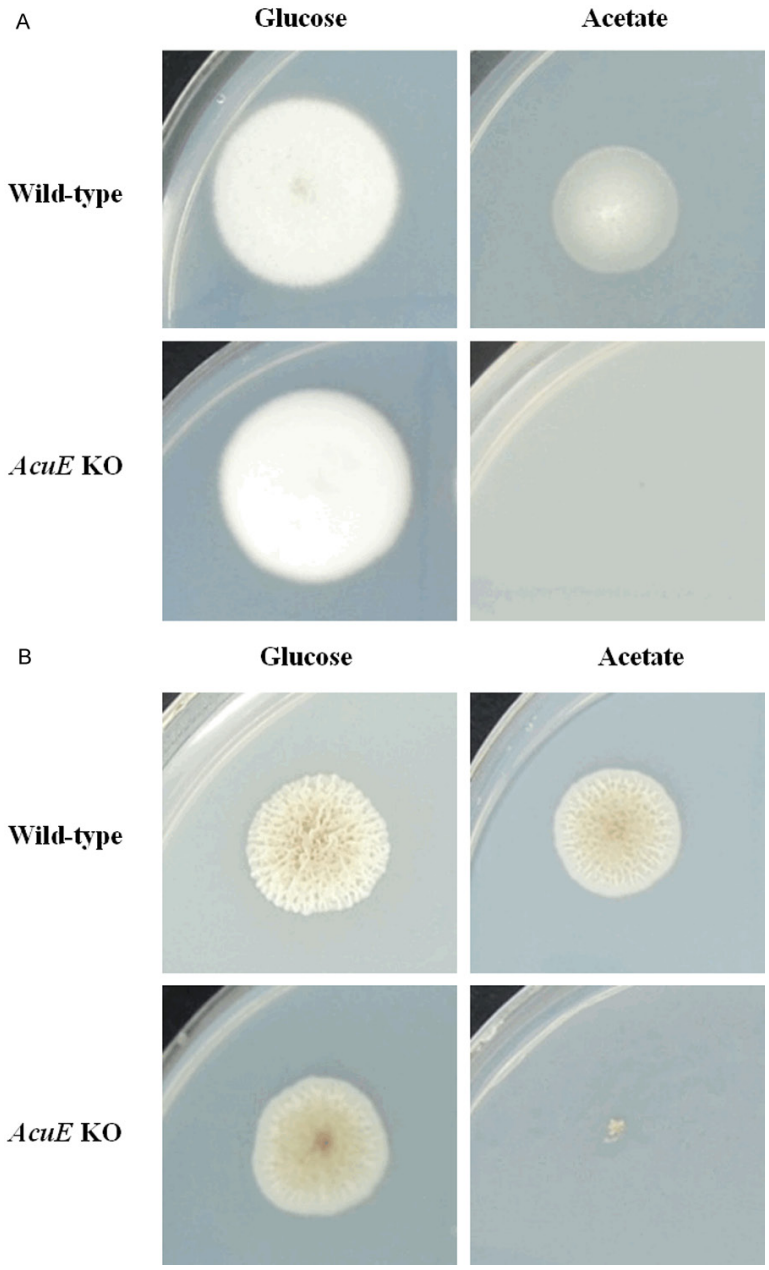

Figure 6. Growth of the wild-type and *AcuE*-knockout strains on yeast nitrogen base (YNB) containing different carbon sources. A: 25°C, 7 days. B: 37°C, 7 days. Glucose: 1% glucose + YNB; Acetate: 1% acetate + YNB.

vector construction, and fungal transformation. The *T. marneiffei AcuD* knockout strain has been successfully constructed [16], but there are no reports regarding the knockout strain construction of the MS-encoding gene *AcuE*.

According to the literature, the sequence of a specific gene to be studied is usually obtained through three procedures. First, the partial cDNA sequence is obtained by using either suppression subtractive hybridization or degenerate primers based on the conserved domain of other related species. Then, the complete cDNA sequence of the target gene can be obtained by 5'- and/or 3'-rapid amplification of cDNA ends. Finally, primers are designed according to the full-length cDNA sequence, and the complete target DNA sequence can be PCR-amplified from genomic DNA [17]. By employing a heterologous probe, *Bugeja et al.* screened the *T. marneffei* λ-based genomic DNA library at low stringency, and then the new homologous sequences were determined by southern blot hybridization analysis and sequencing [18]. In recent years, the rapid development of high-throughput sequencing technology has made it much easier to obtain the whole-genome sequences of any fungus, resulting in a massive gene database. In this study, a BLAST analysis was performed based on the whole-genome sequence of *T. marneffei*, and then the *AcuE* cDNA sequence and its flanking sequences on both sides were identified, which greatly reduced the workload of the experiments and shortened the study period.

Currently, the establishment of stable gene knockout strains of filamentous fungi is mostly based on the principle of homologous recombination [19]. Unlike mammalian or yeast cell research with various existing commercial carrier systems, there is no available universal vector system for filamentous fungi. Therefore, a new, specific vector needs to be constructed for each gene to be studied. The traditional standard method for the construction of a knockout vector involves the assembly of four segments (one vector backbone and three inserts), which is tedious and inefficient [19]. Despite the efforts we

made to build the knockout vector by traditional restriction enzyme II and ligation-dependent methods, no satisfactory results were obtained (unpublished data). Due to the difficulty in the vector construction for gene knockout in filamentous fungi, some researchers have used the double-plasmid co-transformation method, which greatly limits the progress of functional gene studies [19]. Fortunately, with the rapid development of molecular biology, many novel high-throughput technologies for *in vitro* DNA fragment assembly have been developed, such as Gateway cloning [18, 20], uracil-specific excision reagent cloning [21], and In-Fusion cloning technology, among others.

An important feature of In-Fusion technology that makes it different from traditional DNA fusion cloning methods is the seamless connection, i.e., several segments can be fused to the backbone vector without any point mutations. This technique can also support the fusion of multiple fragments with almost the same efficiency as that of a single fragment; in addition, the correct coding sequence is guaranteed. However, despite its advantages, there are few studies of the functional genetics of fungi using seamless cloning technology. In this study, the *AcuE* gene knockout vector pGE AcuE KO was obtained using the In-Fusion method. The results demonstrated that four components covering a wide range of sizes (inserts: 1.3 kb, on average; pGEM-T Easy: 3.0 kb) can be simultaneously assembled in a single reaction. The key point for In-Fusion technology is the correct primer design. In this study, according to the principle of vector construction and the backbone vector sequences, we designed universal sequences (**Figure 1A**). The backbone vector pGEM-T Easy and the screening label *E. nidulans pyrG* cassette used in this study are also applicable to other filamentous fungi. So, the 15-bp universal sequences used in this study are also applicable to construct other target gene knockout vectors of *T. marneffei* or other filamentous fungi; researchers simply need to add the universal sequences to the 5'-end of the primers used to amplify the targeted flanking sequences. In this manner, the primer design becomes much simpler, thus saving time and simplifying the experiments.

In this experiment, the *AcuE* knockout strains were inoculated on a medium containing glucose or acetate as the sole carbon source. The results showed that the growth state of the *AcuE* knockout strain on the medium with glucose as the carbon source was the same as the wildtype one, but it could not grow on the medium with acetate as the carbon source. Therefore, we believe that the *AcuE* gene is essential for *T. marneffei* to utilize the glyoxylate cycle. However, its pathogenic mechanism that is responsible for the fungi-host reaction remains to be further studied.

In conclusion, we successfully cloned the *T. marneffei AcuE* gene and constructed the knockout vector pGE AcuE KO using the In-Fusion method. The *T. marneffei AcuE* gene knockout strains were obtained through protoplast-mediated transformation. Morphological experiments further confirmed that the *AcuE* gene is involved in the glyoxylate cycle. The construction of stable *AcuE* gene-knockout strains lays the foundation for subsequent mechanistic studies of the glyoxylate cycle involved in the *in vitro* growth and the *in vivo* pathogenicity of *T. marneffei*.

Acknowledgements

This study was supported by the National Natural Science Foundation of China NSFC (81171545).

Disclosure of conflict of interest

None.

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References

[1] Yilmaz N, Visagie CM, Houbraken J, Frisvad JC and Samson RA. Polyphasic taxonomy of the genus talaromyces. Stud Mycol 2014; 78: 175-341.

[2] Castro-Lainez MT, Sierra-Hoffman M, LLompart-Zeno J, Adams R, Howell A, Hoffman-Roberts H, Fader R, Arroliga AC, Jinadatha C. Talaromyces marneffei infection in a non-HIV non-endemic population. ID Cases 2018; 12: 21-24.

[3] Hu Y, Zhang J, Li X, Yang Y, Zhang Y, Ma J and Xi L. Penicillium marneffei infection: an emerg-

ing disease in mainland China. Mycopathologia 2013; 175: 57-67.
[4] Le T, Wolbers M, Chi NH, Quang VM, Chinh NT, Lan NP, Lam PS, Kozal MJ, Shikuma CM, Day JN and Farrar J. Epidemiology, seasonality, and predictors of outcome of AIDS-associated Penicillium marneffei infection in Ho Chi Minh City, Viet Nam. Clin Infect Dis 2011; 52: 945-952.
[5] Deng Y, Ma N, Zhu K, Mao Y, Wei X and Zhao Y. Balancing the carbon flux distributions between the TCA cycle and glyoxylate shunt to produce glycolate at high yield and titer in Escherichia coli. Metab Eng 2018; 46: 28-34.
[6] Sun J, Li X, Feng P, Zhang J, Xie Z, Song E and Xi L. RNAi-mediated silencing of fungal acuD gene attenuates the virulence of Penicillium marneffei. Med Mycol 2014; 52: 167-178.
[7] Yang J, Yang W, Feng J, Chen J, Jiang M and Zou X. Enhanced polymalic acid production from the glyoxylate shunt pathway under exogenous alcohol stress. J Biotechnol 2018; 275: 24-30.
[8] Zhao X, Wang Y, Zhao Y, Huang Y, Zhang KQ, Yang J. Malate synthase gene AoMls in the nematode-trapping fungus Arthrobotrys oligospora contributes to conidiation, trap formation, and pathogenicity. Appl Microbiol Biotechnol 2014; 98: 2555-63.
[9] Singh KS, Sharma R, Keshari D, Singh N, Singh SK. Down-regulation of malate synthase in Mycobacterium tuberculosis H37Ra leads to reduced stress tolerance, persistence and survival in macrophages. Tuberculosis (Edinb) 2017; 106: 73-81.
[10] Borneman AR, Hynes MJ and Andrianopoulos A. An STE12 homolog from the asexual, dimorphic fungus Penicillium marneffei complements the defect in sexual development of an Aspergillus nidulans steA mutant. Genetics 2001; 157: 1003-14.
[11] Oakley BR, Rinehart JE, Mitchell BL, Oakley CE, Carmona C, Gray GL ang May GS. Cloning, mapping and molecular analysis of the pyrG (orotidine-5'-phosphate decarboxylase) gene of Aspergillus nidulans. Gene 1987; 61: 385-99.
[12] Borneman AR, Hynes MJ and Andrianopoulos A. The abaA homologue of Penicillium marneffei participates in two developmental programmes: conidiation and dimorphic growth. Mol Microbiol 2000; 38: 1034-47.
[13] Chen CM, Feng J, Chen ZW, Xiao X, Jiang MM, Shi ML, HE LY, Cai WY, Xi LY and Li XQ. Optimization of the protoplast-mediated transformation system of Penicillium marneffei. Chinese Journal of Mycology 2016; 3: 140-144.
[14] Ma Z, Marsolais F, Bernards MA, Sumarah MW, Bykova NV, Igamberdiev AU. Glyoxylate cycle and metabolism of organic acids in the scutellum of barley seeds during germination. Plant Sci 2016; 248: 37-44.
[15] Cantelli BAM, Bitencourt TA, Komoto TT, Beleboni RO, Marins M, Fachin AL. Caffeic acid and licochalcone A interfere with the glyoxylate cycle of Trichophyton rubrum. Biomed Pharmacother 2017; 96: 1389-1394.
[16] Canovas D and Andrianopoulos A. Developmental regulation of the glyoxylate cycle in the human pathogen Penicillium marneffei. Mol Microbiol 2006; 62: 1725-38.
[17] Feng P, Xie Z, Sun J, Zhang J, Li X, Lu C and Xi L. Molecular cloning, characterization and expression of PmRsr1, a Ras-related gene from yeast form of Penicillium marneffei. Mol Biol Rep 2010; 37: 3533-40.
[18] Bugeja HE, Boyce KJ, Weerasinghe H, Beard S, Jeziorowski A, Pasricha S, Payne M, Schreider L and Andrianopoulos A. Tools for high efficiency genetic manipulation of the human pathogen Penicillium marneffei. Fungal Genet Biol 2012; 49: 772-8.
[19] Schoberle TJ, Nguyen-Coleman CK and May GS. Plasmids for increased efficiency of vector construction and genetic engineering in filamentous fungi. Fungal Genet Biol 2013; 58-59: 1-9.
[20] Park J, Throop AL and LaBaer J. Site-specific recombinational cloning using gateway and infusion cloning schemes. Curr Protoc Mol Biol 2015; 110: 3.20.1-23.
[21] Bitinaite J, Rubino M, Varma KH, Schildkraut I, Vaisvila R and Vaiskunaite R. USER friendly DNA engineering and cloning method by uracil excision. Nucleic Acids Res 2007; 35: 1992-2002.