An efficient marker recycling system for sequential gene deletion in a deep sea-derived fungus *Acremonium* sp. HDN16-126

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1. Introduction

*Acremonium* sp. is a group of filamentous fungi widely distributed in soil, sea water and on decaying plant material in nature [1,2]. As the marked [beta]-lactam antibiotic of cephalosporin C was first discovered from *Acremonium* species, this genus has been regarded as one of the most pharmaceutically important filamentous fungi following *Penicillium* species [3-5].

Chemical and pharmaceutical studies on *Acremonium* spp. have proven that they are also prolific producers of other secondary metabolites including steroids, terpenoids, metroerterpenoids, polyketides, alkaloids and peptides [6-8]. However, the vast majority of the biosynthetic machinery of those metabolites as well as physiological and biochemical effectors of this genus are still obscure. This is partially due to the lack of efficient genetic manipulation system and the limited availability of selection markers for transformation.

As far marker recycling system and scarless deletion methods are widely adopted to circumvent the limitation of selection markers [9,10], among which, the pyrG-based marker recycling system is often used in eukaryotic microorganism [11]. *PyrG/URA3* orthologues are genes that...
encode orotidine-5'-monophosphate decarboxylases (OMPdecase) or orotidylate decarboxylase that catalyze the decarboxylation of orotidine-5'-monophosphate to form uridine monophosphate [12,13]. This family of enzymes also could convert 5-fluoroorotic acid (5-FOA), an analogue of the uracil precursor, to 5-flourouracil, a toxic compound that inhibits DNA and RNA synthesis [13,14]. Therefore, a pyrG or URA3 gene could be used as a feasible counter-selection marker when it is positioned between homologous sequences, excision of pyrG/URA3 cassette by homologous recombination can be selected with 5-FOA added in medium. Indeed, the pyrG/URA3-based counter-selection system has been successfully applied in Saccharomyces cerevisiae [15], Aspergillus spp. [16,17], Candida albicans [18], Neurospora crassa [19], Talaromyces versantis [20], Mucoircrinelloides [21] and Colletotrichum orbiculare [22]. However, this system has never been applied to the genus of Acremonium species.

During our continued work in searching pharmaceutical fungal strains from Deep-Sea environments, one fungal strain Acremonium sp. HDN16-126 was obtained from surface sediment sample collected from Mariana Trench. Chemical studies on this strain afforded several anti-azines and peptaibols (data not published), which indicated talented metabolic capacity of HDN16-126. To facilitate further biochemistry studies on the strain HDN16-126, we established a uridine auxotrophy based scarless gene deletion system. In this method, the native pyrG (pyrG-A1) in HDN16-126 was first deleted from chromosome using a hyg cassette to generate the ΔpyrG strain, which enables the employment of pyrG-A1 gene as a counter-selection marker. This ΔpyrG strain was then subjected to take up a gene knock out cassette carrying pyrG-A1 flanked by three homologous arm fragments. After two rounds of screening on uridine free medium (pyrG-A1 worked as complementary for the uridine auxotrophy) and MM medium supplemented with 5-FOA (pyrG-A1 worked as counter-selection marker), the cassette was first knocked-into and then knocked-out from the chromosome, resulting in the recycling of the pyrG-A1 marker and scarless deletion of target genes. Here we will describe the details of the marker recycling system together with its successful application in disrupting two target genes pepL and pepM, which are proposed to be related to 2-aminoisobutyric acid metabolism in fungi.

2. Materials and methods

2.1. Strains and culture conditions

The fungal strain HDN16-126 was isolated from Mariana Trench sediment sample (∼8000 m) and identified as Acremonium sp. based on the internal transcribed spacer (ITS) region of rDNA sequence (GenBank no. MKS43173). The strain was cultivated on PDA solid medium and incubated at 28 °C for 5 days, the spores were collected and stored at −80 °C in 20% glycerol.

Saccharomyces cerevisiae BJ5464 [23] was used for yeast homologous recombinant, Escherichia coli DH10B was used for standard cloning (pursuaded from Solarbio, Beijing) (Table 1).

2.2. Bioinformatic analysis of target genes

The genome of Acremonium sp. HDN16-126 was sequenced at Beijing Genomics Institute (BGI) Co. Ltd (Qingdao, China). OMPdecase (Gen-Bank: P14017.1) from the strain Acremonium chrysogenum [24] was selected as a query to identify pyrG gene from the genome of Acremonium sp. HDN16-126 using NCBI local blastp tool (https://www.ncbi.nlm.nih.gov/books/NBK52637/). Similarly, two target genes pepL and pepM which are postulated to be 2-aminoisobutyric acid metabolism relating genes were identified through local blastp tool by using the amino acid sequence of TqAL and TqAM as queries from Pencillium aethiopicum [25].

The sequence relationship of those genes with their orthologues were further predicted and analyzed by ClustalW alignments [26] and cytoscape software [27,28].

3.1. Strains plasmids used in this study.

| Strains or plasmids | Characteristics | Sources |
|---------------------|-----------------|---------|
| **Strains** | | |
| Acremonium sp. HDN16-126 | Isolated from sediment sample collected from Mariana Trench | Preserved in our laboratory |
| Saccharomyces cerevisiae BJ5464 | Recombination host of the knock out fragments | Preserved in our laboratory |
| Escherichia coli DH10B | Cloning host | Solarbio life sciences |

| Plasmids | | |
|-----------------|-----------------|---------|
| pYEU | Recombination vector of the knock out fragments | Preserved in our laboratory |
| pHg | Hygromycin resistance gene carrier | Preserved in our laboratory |
| pYEU-pyrG | pYEU carrying pyrG-up, pyrG-da, hyg, pyrG gene fragments | This study |
| pYEU-pepL | pYEU carrying pepL-up, pepL-da, pyrG, pepL gene fragments | This study |
| pYEU-pepM | pYEU carrying pepM-up, pepM-da, pyrG, pepM gene fragments | This study |

HDN16-126 was cultured in PDA solid medium at 28 °C for 5 days, and the spores were collected and stored at −20 °C in 20% glycerol (4 × 10^7/mL). 1 mL spores were then inoculated into 40 mL PDB + YE liquid medium and incubated at 28 °C, 180 rpm for about 10 h. Mycelia was harvested by centrifugation at 4000 rpm for 15 min at 4 °C. The supernatant was removed and the mycelia was washed with 15 mL Osmotic buffer (1.2 M MgSO4·7H2O, 10 mM sodium phosphate, pH 5.8) and centrifuge at 4000 rpm for 15 min at 4 °C. Then the sediment was resuspended with 10 mL Osmotic buffer containing 0.04 g Yatalase and 0.04 g EDTA.
incubated at 28 °C, 80 rpm overnight, then the culture liquid was transferred into 50 mL sterile centrifuge tube and added isovolumetric trapping buffer (0.6 M sorbitol, 0.1 M Tris-HCl, pH 7.0) softly, the mixture was centrifuged at 4000 rpm at 4 °C for 15 min. Then the protoplast layer was obtained and re-suspended in STC buffer (1.2 M sorbitol, 10 mM CaCl₂, 10 mM Tris-HCl, pH 7.5) with 2x volume, followed by centrifuge at 4000 rpm at 4 °C for 15 min. The supernatant was discarded and the protoplast was harvested and stored in 1 mL STC buffer for transformation.

2.5. Generation of pyrG-A1 deletion mutant

Yeast homologous recombination [29] was used for the construction of pyrG knockout cassette (Fig. S2). The cassette was designed to carry four fragments: upstream (left flank sequence of pyrG-A1 gene’s promoter), downstream (right flank sequence of pyrG-A1 gene’s termina- tor), hyg (hygromycin resistance gene) and pyrG-A1 (partial sequence of pyrG-A1 expression cassette). For the construction, hyg gene fragments with its promoter and terminator were PCR amplified from the phyg plasmid using primer pair of hyg-F/ hyg-R and pyrG-A1 fragments together with its upstream and downstream flanking sequences amplified from genomic DNA of HDN16-126 using primer pairs of pyrG-F/pyrG-R, pyrG-up-F/pyrG-up-R, pyrG-dn-F/pyrG-dn-R (Table S1), respectively. To facilitate yeast homologous recombination and assemble these DNA fragments into one construct, 30-bp homology regions were added to primers thus generating short overlap sequences pyrG-F/pyrG-R, pyrG-up-F/pyrG-up-R, pyrG-dn-F/pyrG-dn-R (Table S1), respectively. To facilitate yeast homologous recombination and assemble these DNA fragments into one construct, 30-bp homology regions were added to primers thus generating short overlap sequences pyrG-F/pyrG-R, pyrG-up-F/pyrG-up-R, pyrG-dn-F/pyrG-dn-R (Table S1), respectively. To facilitate yeast homologous recombination and assemble these DNA fragments into one construct, 30-bp homology regions were added to primers thus generating short overlap sequences pyrG-F/pyrG-R, pyrG-up-F/pyrG-up-R, pyrG-dn-F/pyrG-dn-R (Table S1), respectively. To facilitate yeast homologous recombination and assemble these DNA fragments into one construct, 30-bp homology regions were added to primers thus generating short overlap sequences pyrG-F/pyrG-R, pyrG-up-F/pyrG-up-R, pyrG-dn-F/pyrG-dn-R (Table S1), respectively.

The pyrG-A1 knock out cassette was PCR amplified from plasmid pYEUP-pyrG using primers of kz-pyrG-F/kz-pyrG-R and transformed into the protoplast by the polyethylene glycol (PEG)-CaCl₂ method as described before for homologous recombination. After one round of screening on uridine free medium and one round of screening on medium with 5-FOA, the mutant strain with scarless deletion of pepl (Δpepl) was obtained. The Δpepl mutant was generated by the same method as above, both mutants were verified by PCR primers (yz-L-F/yz-L-R for Δpepl and yz-M-F/yz-M-R for Δpepl).

3. Results

3.1. Bioinformatic analysis of pyrG, pepL and pepM

PyrG, pepL and pepM homologous sequences were identified by NCBI local blastp program. The 1104 bp open reading frame encoding a protein of 368 amino acids was identified from HDN16-126 and designated as pyrG-A1 based on the high similarity with OMpDecase from Acremonium chrysogenum (81.71% identity). Protein interaction analysis of OMpDecase was conducted using A. chrysogenum, Trichoderma sp., Fusarium sp. and other fungal species as references. As shown in Fig. 1A, OMpDecase-HDN16-126 clustered with homologues from A. chrysogenum and several other species, which showed a high possibility of the similarity in functions among those genes (E value: 50).

The gene of pepl encoding a putative 2OG-Fe (II) dioxygenase and pepM encoding a putative aldolase were identified based on their similarities to Penicillium aethiopicum derived Tqal. (57.95%) and TqAM (67.32%), respectively. The protein interaction network as shown in Fig. 1B and C denoted their functional similarities with the homologous genes from reference filamentous strains (E value: 50).

3.2. Antibiotic sensitivity of Acremonium sp.

Wild type Acremonium sp. (WT) was subjected to agar dilution test with hygromycin (0–300 μg/mL) and 5-FOA (0–2.5 mg/mL). Growth of WT could be totally inhibited on PDA medium with 50 μg/mL of hygromycin and MM medium with 0.5 mg/mL of 5-FOA, which showed the working concentration of 50 μg/mL for hygromycin and 0.5 mg/mL for 5-FOA in transformants and mutants selection (Fig. S6A).

3.3. Generation of pyrG-A1 mutant

A 6732 bp pyrG-A1 knock out cassette was amplified from plasmid pYEUP-pyrG (Fig. S4) and dissolved in 20 μL STC buffer with final concentration of 400 ng/μL. Protoplast harvested from 5-day culture of HDN16-126 was transformed with above pyrG-A1 knock out cassette solution. After the first round of recombination (Fig. 2B), 12 mutants were picked up from the PSA plus 50 μg/mL of hygromycin and 0.5 mg/mL of 5-FOA were verified by PCR to be the correct clones with the hyg gene cassette integrated into the correct locus by homolo- gous recombination (Fig. S7). Strikingly, the homologous recombination rate in this step was much higher than literature reports for other filamentous fungi where a big portion of wrong insertions were generated by non-homologous recombination [31]. Then the 12 mutants were further selected on MM plates plus uridine and 5-FOA (0.5 mg/mL), where the hyg gene and pyrG-A1 gene would be removed from the genome with 5-FOA working as a counter selection marker. After this second round of selection, one pyrG-A1 deficient strain HDN16-126-ΔpyrG was obtained and verified by PCR test. As shown in Fig. 2C, the length of the PCR product for the mutant strain was about 0.7 kb and for the wild type strain was about 1.8 kb, which confirmed the successful removal of pyrG-A1 from HDN16-126 chromosome.

The HDN16-126-ΔpyrG strain was unable to grow on MM, but grow on MM supplemented with 10 mM uridine (Fig. 2D). This strain also showed low sensitivity to 5-FOA treatment, which is consistent with the absence of the pyrG-A1 cassette. In addition, the morphology of mycelium showed no significant difference compared with that of the WT except for a slightly darker pigmentation on the mycelium (Fig. 2D).
Together, we concluded that by two rounds of selection with hygromycin and 5-FOA treatments, the pyrG-A1 gene could be scarlessly knocked out from HDN16-126 genome.

3.4. Generation of pepL and pepM mutant

With the uridine auxotroph mutant HDN16-126-△pyrG in hand, we continued to test whether the marker recycling system could be used for sequential disruption of multiple genes. Two genes of pepL and pepM which are proposed to be 2-aminobutyric acid metabolism relating genes were selected as targets for disruption. The pepL and pepM disruption cassettes were designed as shown in Fig. 3A. After first round of selection on MM-sorbital dropout uridine medium, the cassettes with pyrG-A1 gene flanked by three homologous fragments were integrated into the upstream next to pepL and pepM respectively. Each 12 single clones were picked up from pepL and pepM disruption mutants, within which the expression of pyrG-A1 from the cassette could complement the uridine auxotrophy and restore the cell growth. Then all the clones were transferred to MM plus uridine and 5-FOA medium for dropping out of pyrG-A1. 10 out of 12 clones for △pepL and 8 out of 12 clones for △pepM were grown. All the mutants were verified to be correct by PCR using primer yz-L-F/yz-L-R or yz-M-F/yz-M-R (Fig. 3B), where the length of PCR products from △pepL and △pepM mutants were 1105 bp and 1078 bp respectively, while as control, the PCR products from WT were 2460 bp and 2399 bp in correlation with pepL and pepM. The △pepL, △pepM mutants were then tested for their growth on MM with or without uridine (10 mM) and results showed they could only grow on uridine supplied MM medium and compared with WT strain, both △pepL and △pepM mutants showed less spore production and much darker pigmentation on mycelium (pink for mutants and pale for WT, Fig. 3C). The disruption efficiency for gene pepL and pepM were 83.3% and 66.7%, respectively, and these data demonstrate that the marker recycling system can be applied to genetic manipulation of Acremonium species for generating scarless gene deletion and replacement mutations.

4. Discussion

In general, traditional homologous recombination techniques are commonly used to generate gene disruption and replacement [32] in fungi. During this process, resistant marker will be retained on the chromosome after one round of recombination, making it impossible to use the same selection marker during the next cycle of gene manipulation, which means increasingly more selection markers are required for completing multiple gene disruptions. Consequently, the physiological and biochemical studies of many fungal strains are limited by the availability of suitable selection markers.

In this study, we report the establishment of a uridine auxotrophy based scarless gene deletion method in Acremonium sp. HDN16-126, a fungus collected from the Mariana Trench (~8000 m). Importantly, the pyrG-A1 marker was successfully recycled from the chromosome, which allows the use of the same selection marker in successive transformations. The pyrG-A1-based marker recycling system showed several advantages over other gene deletion systems, such as sit-directed recombination methods including Flp-FRT and Cre-loxP recombination, which have been widely used in prokaryotes and eukaryotes [22]. Those tools could easily conduct the excision of target DNA between two repeated specific sites, while for a legacy, one of the repeated sites is left on the chromosome, which makes it an unstable factor as the site specific sequences accumulated in the genome. Compared to above methods, the pyrG-A1-based marker recycling system could create much more stable multiple knock-out mutants.

Using this pyrG-A1 based marker recycling system, sequential disruption of two target genes pepL and pepM were achieved in HDN16-126-△pyrG strain. To our best knowledge, this is the first report of the application of uridine auxotrophy based marker recycling system in deep sea-derived Acremonium species. In addition, the reintroduction of pyrG-A1 could restore the growth of HDN16-126-△pyrG mutant to wild-type levels, which indicated the potential of this system to access disruption and function analysis of any gene of interest in filamentous fungi. With the help of this efficient gene manipulation system, more in depth physiological and biochemical investigations including the pepL and pepM
Fig. 2. Construction of markerless \(\Delta\text{pyrG}\) mutant. (A) Morphology of the WT spores with spores in dormancy period, spores in germination and protoplasts of the WT; (B) Flow chart of construction procedure of \(\Delta\text{pyrG}\) mutant; (C) PCR verification of the MT, the length of PCR product from MT was about 0.7 kb, and the length of PCR product from WT was about 1.8 kb; (D) Growth of \(\Delta\text{pyrG}\) on MM medium, MM plus uridine (10 mM) medium and MM plus 5-FOA and uridine medium, the WT on MM medium was used as a control.
functions in amino acids metabolism are at the right time in progress.

CRediT authorship contribution statement

Ruonan Sun: Experiment operation, Writing – original draft. Hangyi Xu: Experiment operation. Yanyan Feng: and Xuewen Hou: Formal analysis, and chemical study of strains. Tianjiao Zhu: and Qian Che: and Blaine Pfeifer: Checking and confirming all procedures. Guojian Zhang: and Dehai Li: Designed the study, supervised the laboratory work, and, contributed to the critical reading of the manuscript.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.05.001.

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