Construction of Ubiqutin C-terminal Hydrolase (creB) Gene Deletion Mutant Via split-Marker Strategy*

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ABSTRACT Objective: To construct a mutant of A.fumigatus that lacks the ubiqutin C-terminal hydrolase (creB) gene gene. Methods: The methods of ClusterW alignment was applied to multiple alignments of amino acid sequences. We constructed disruptant fragments for creB via split-marker recombination strategy and used PEG-mediated protoplast transformation. PCR and seqenceing were used to screening and identification of homologous recombination. Results: Alignment of the proteins indicated that CreB had the typical six UBP domains. We constructed two fragments to delete creB gene and transformation, yielded 25 hygromycin-resistant colonies. 20 hygromycin-resistant transformants were identified by PCR analysis. Further analysis, one strain transformant was identified to be creB gene disruptant by sequencing. Conclusion: Split-marker recombination for rapid and efficient targeted deletion of A.fumigatus creB gene. The successfully constructed creB gene deletion mutant of A.fumigatus was provided the possibilities to further analysis of the gene function.

Key words: Aspergillus fumigatus; CreB; Split-Marker recombination

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Introduction

Aspergillus fumigatus is the most frequent cause of invasive fungal infection in immunosuppressed individuals [1]. Studies showed that extracellular proteases might play an important role in its infection [2].

Deubiquitinating enzymes (DUBs) belong to the superfamily of proteases, of which an estimated 561 members are present in the human genome [3,4]. The deubiquitinating enzymes mediate the removal and processing of ubiquitin, and have been implicated in regulating various critical cellular processes such as endocytosis and regulation of chromatin structure [5]. The USP12 (UBH1) as a member of human deubiquitinating enzyme is linked with cytoplasmic phosphorylation-based signaling systems to RNA processing [6]. And the ubiquitin-proteasome pathway has been implicated in several genetic diseases (including cystic fibrosis, Angelman's syndrome, and Liddle syndrome)[7].

CreB, a member of ubiquitin-processing protease (USP or UBP) family defined by the human homologue UBH1, is involved in the system that regulates carbon catabolite repression and resistance to molybdate in A.nidulans [8]. Mutation in creB increases extracellular protease levels in medium lacking a carbon source in A.nidulans [9]. Its A.fumigatus homologue ubiquitin C-terminal hydrolase gene (creB) is located on Af293 Chromosome 4.

Efficient targeted gene deletion with split-marker strategy was reported in Cochliobolus heterostrophus by Natalie.L et.al [10]. In order to analyze gene function in A.fumigatus, several mutagenesis methods were applied to the construction of several gene knockouts, such as rasB [11], pkaR [12,13], rhbA [14], StuAp (APSES) gene [15] and two erg3 genes (erg3A and erg3B) [16].

This study constructed a mutant of A.fumigatus that lacks the creB gene via split-marker recombination strategy.

1 Materials and methods

1.1 Strains and growth conditions

Aspergillus fumigatus wild type strain H237 (WT) used in this study was a clinical isolate. Aspergillus fumigatus ΔpkaC1 was a mutant in which the pkaC1 gene was replaced with the hygromycin resistance cassette from pan7-1[17]. WT and C1 were gifts kindly supplied by Dr JR Rhodes. All strains were grown at 37℃ in Aspergillus minimal medium (AMM) [18] agar plates containing 10 mM ammonium tartrate as the nitrogen source. Conidia were harvested and resuspended in double-distilled water (DDW) and counted with a hemacytometer. Conidia were inoculated into YG (1% yeast extract, 2% glucose) medium, grown at 37℃ for 12 h with shaking at 200 rpm; mycelia were then harvested and used to extract genomic DNA.

1.2 Multiple sequence alignment

The alignments of amino acid sequences were generated using the methods of Lipman-person alignment and ClusterW programme of DNASTAR software.

1.3 DNA extraction and Primers

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The genomic DNA was extracted with phenol-chloroform and precipitated with ethanol in accordance with modified procedures [19]. DNA was purified by using the Gel extraction kit (Omega, USA) and Cycle-pure kit (Omega, USA). PCR primers were designed to amplify the corresponding genomic fragments from H237 (Table 1). All primers were synthesized by Sangon Biotech (Shanghai, China).

Table 1 Primers used in this study

Primers	Primer sequences(5'-3')
P1	cgccagggttttcccagtcacgacAAGTGGAAAGGCTGGTGTGC
P2	CTCCATACAAGCCAACCACGG
P3	agcggataacaatttcacacaggaTCGCGTGGAGCCAAGAGCGG
P4	CGTTGCAAGACCTGCCTGAA
P5	TTCCGTTGGTTGTACTGTTG
P6	gtcgtgactgggaaaaccctggcgTTCCATCCTCCCTAATCG
P7	tcctgtgtgaaattgttaatccgctTCAGCATTCTGTCATAGCG
P8	AGGATTTGGGTCATCGTAA
P9	TGCTTCGTCAACGTCCTC
P10	GATTCCTGGCACGAAGTAC
P11	GGATGGGCCAATTAGGGA

In Table 1 Primers P5, P6, P7, and P8 are gene specific primers designed for the deletion of the A.fumigatus creB gene. For P1 and P3 primers, lowercase bold portions are M13F and M13R sequences; For P6 and P7 primers, lowercase bold portions are complementary to M13F and M13R sequences, respectively. P11, a sequencing primer.

1.4 PCR procedure

As shown in Fig.1A, the selectable marker and the gene flanks were amplified through four PCR reactions. The 5' and 3' flank sequences of creB (5'-creB and 3'-creB) and the HYG fragments (HY and YG) were amplified with oligonucleotide primers, respectively. In 2nd round fusion PCR (Fig.1B), two separate PCR reactions with primers P2&P5 and P4&P8 fused the flank sequences to get YG&5'-creB and HY&3'-creB. A split marker strategy were used to generate two fusion PCR fragments (Fig.1B). PCR products were purified using Gel extraction kit (Omega, USA) or Cycle-pure kit (Omega, USA).

Amplification of 5' and 3' flank sequences of creB and the HYG fragments were performed by using 100ng genomic DNA with 2 pmol primers in 50μl total volume, respectively. The PCR conditions were as follows: 95℃ for 2 min; 72℃ for 1min; Then Taq DNA polymerase (Promega,shanghai) was added; 52℃ for 1min; 72℃ for 3min; 35 cycles of 95℃ for 1min; 52℃ for 1min; 72℃for 2min30s(the second fusion PCR for 4min);95℃for 1min; 52℃ for 1min; 72℃for 10min.

1.5 Transformation

Transformation was performed by using a modified protocol, which has been successfully employed for A.fumigatus [12-14]. To obtain protoplasts of A.fumigatus, the mycelium was exposed to lysing enzymes from Trichoderma harzianum (Sigma; catalog no. L1412). For transformation, 10μg DNA fragments were added to 200μl protoplast suspension (approximately 1.0×10^7 protoplasts). The transformants were grown in osmotically stabilized medium (OSM) plates, which consisted of AMM supplemented with 1.2 M sorbitol (Shanghai, China) and 267 mg of hygromycin/ml (InvivoGen, USA). For each transformation experiment, a negative control of A.fumigatus protoplasts treated as described above but in the absence of DNA was prepared.

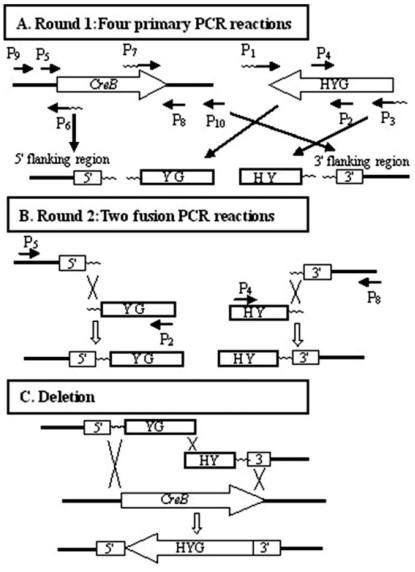

Fig.1 Overlap PCR and disruption of creB via split-marker strategy. A: First round PCR: amplification of the components using the specific and chimeric primers. The target gene fragment of 5' flanking sequence (~600bp) was amplified with primers P5& P6 , and the fragment of 3' flanking sequence(~1.0kb) was amplified with primers P7& P8. The two fragments of a selectable marker (HYG) were amplified. YG (~1.5kb) is amplified with primers P1&P2, HY (~1.2kb) is amplified with primers P3&P4. B: Second round PCR: Two separate PCR reactions (P2/P5) and (P4/P8) fused the flank sequences to the 5'(HY) or 3'(YG) portions of HYG. C: Deletion of CreB would occur when CreB is replaced by HYG cassette through homologous recombination.

2 Results

2.1 Domain analysis of A.fumigatus CreB

Multiple sequence alignment using Cluster W showed that the CreB proteins (A.fumigatus Af293 and A1163) are a ubiquitin carboxy-terminal hydrolase conserved with a sequence, CreB, of A.nidulans (CreB : 70.6% identity), H.sapiens (UBH1:49.5% identity), C.elegans (CAB54286:41.5% identity), A.thalania (UBP3: 47.7% identity), S.pombe (CAB66456:39.4% identity), D. melanogaster (AAF56066.1:35.2% identity), P.anomala (UBP1: 36.1%identity), S.cerevisiae (UBP13: 32.8% identity).

Alignment of the proteins also indicated that the typical six UBP domains of deubiquitinating enzymes [20] were present(Fig.2): DH-I amino acids 56-73; DH-II amino acids 214-228; DH-III amino acids 272-392 and DH-IV amino acids335-366; DH-V 402-442; DH-VI amino acids 445-454. These proteins display structural similarity within the catalytic core domains containing conserved Cys, Asp, and His residues (DH-I, DH-II, and DH-V, respectively). By domain analysis, it was found that A. fumigatus ubiqutin C-terminal hydrolase CreB was a member of UBP Family.

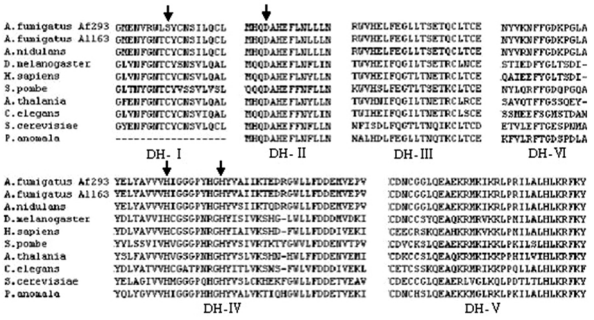

Fig. 2 Homology domains among members of UBP subfamily. The sequences are: A.fumigatus Af293 CreB (GenBank accession No. XP_751541.2), A.fumigatus A1163 CreB (GenBank accession No. EDP50644.1), A.nidulans CreB protein (GenBank accession No. AAL04454.1), D.melanogaster protein (GenBank accession No. AAF56066.1), H. sapiens UBH1 (GenBank accession No.O75317), S. pombe (GenBank accession No.CAB66456), A.thalania UBP3 protein (GenBank accession No.CAB80654.1), C.elegans protein (GenBank accession No.CAB54286), S.cerevisiae UBP13 (GenBank accession No. P38187), P.anomala UBP1 protein (GenBank accession No.BAA90762.1). Six conserved DUB homology domains from DH-1 to DH-VI are depicted. The functionally important Cys, Asp and His residues are indicated with " ↓ ".

2.2 Construction of deletion fragments for creB

The first round PCRs was started with four pairs of primers (P1&P2,P3&P4,P5&P6,P7&P8) to get four fragments : the creB DNA 5' flanking fragment was amplified with oligonucleotide primers P5&P6 and 3' flanking fragment was amplified with oligonucleotide primers P7&P8; The fragment YG was amplified from the sequence of hygromycin resistance cassette with P1&P2 (P1 lowercase bold portions, complementary to primer P6) and HY was amplified with P3&P4 (P3 lowercase bold portions , complementary to primer P7) primers. Four PCR fragments were obtained: 5'-creB (0.6kb), 3'-creB (1.0kb) and the marker cassette HY (1.2kb), YG (1.5kb) (Fig.3A and B). The second round fusion PCRs was with two pairs of primers (P2&P5, P4&P8). For the 5' construct, templates were YG and 5'-creB from round 1, primers P2 and P5. For the 3' construct, templates were HY and 3'-creB, primers P4 and P8. Two fused PCR products were about 2.0kb (YG/5'-creB) and 2.1kb (HY/3'-creB), respectively (Fig.3C). These two fragments shared an approximately 450 bp overlap sequence within the HYG cassette. The two PCR products from the second round fusion PCRs were sequenced (data not shown).

2.3 Transformation efficiency and identification of creB mutants

Transformation of 1.0×10^7 protoplasts with 10μg of DNA fragments yielded 25 hygromycin-resistant colonies (data not shown). Colonies arising on plates containing hygromycin were transferred onto fresh hygromycin containing plates at low density, repeated once to isolate colonies derived from single conidia. The

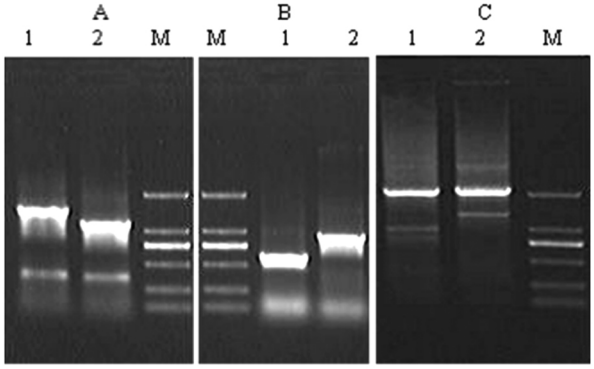

Fig.3 Construction of deletion fragments for creB. Electrophoresis results of PCR products. (A) PCR products YG (~1.5kb), HY (~1.2kb) fragments which were amplified with primers P1&P2 and P3&P4 are in lanes 1-2, respectively. (B) PCR products 5'-creB (0.6kb) and 3'-creB (1.0kb) fragments with primers P5&P6 and P7&P8 are in lanes 1-2, respectively. (C).The second round fusion PCR products. fragments YG/5'-creB(2.0kb) and HY/ 3'-creB (2.1kb) with primers (P2&P5 and P4&P8) are in lanes 1-2. Lane M is the molecular size marker 2000bp. The sizes of bands from above to bottom are: 2000bp, 1000bp, 750bp, 500bp, 250bp, 100bp.

transformants were screened for the desired integration event by PCR using upstream and downstream primers which would amplify the specific fragments of creB gene only if the deletion construct integrated into the chromosomal DNA by homologous recombination. For this, the first primer pairs (P1&P3 in Table 1) were used to screen for the presence of hygromycin resistance cassette. The second primer pairs (P2&P9 in Table 1) were used to screen for homologous integration. Primer P9 was 332bp upstream of P5, and P10 was 424bp downstream of P8. The PCR product from a third

primer pairs (P9&P10 in Table 1) was sequenced to confirm the replacement of creB with hygromycin resistance cassette.

PCR with the first pair of primers (P1&P3) resulted in the amplification of 2.1kb fragment in some transformants (Fig.4A). Twenty transformants containing hygromycin resistance cassette were identified according to the amplification of the 2.1kb PCR product. PCR with primers (P2&P9) resulted in the amplification of 2.3kb fragment in transformants with hygromycin resistance cassette integrated into the creB gene (Fig.4B). The #15 mutant was selected for further analysis.

The PCR with #15 mutant genomic DNA as template and P9&P10 as primers resulted in the amplification of 4.3kb fragment. Sequencing results of this fragment showed that it contained both sequences from hygromycin resistance cassette and genomic DNA sequence of A. fumigatus (data not shown). The #15 mutant (Δ creB15) was identified as candidate which had undergone the desired homologous integration.

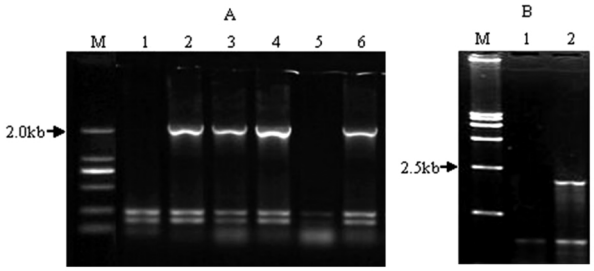

Fig.4 Screening and identification of homologous recombination. (A) PCR products with P1&P3 as primers, WT (lane 1) and transformants (Lanes 2-6) genomic DNA as templates were separated on agarose gel. Lane M is 2000bp molecular size marker. (B) PCR products with P2&P9 as primers, WT, Δ creB15 genomic DNA as templates were shown in lanes 1 and 2, respectively. Lane M is 15000bp molecular size marker

3 Discussion

This study prepared the A. fumigatus creB gene disruption constructs via split-marker recombination strategy. After transformation with these two flanks, the transformants were screened and identified by PCR using upstream and downstream primers which amplified the specific fragments of mutants only if the deletion construct integrated into the chromosomal DNA by homologous recombination, and confirmed by sequencing. These results show that we have successfully obtained A.fumigatus creB deletion mutant. This advanced method is easy, rapid and efficient, in addition to being applicable to any A.fumigatus genes in principle. An analysis of gene function using the artificial targeted gene disruptants will provide an sufficient opportunity to advance our understanding of the unkown genes.

Arst et al.first described mutations in creB those were selected as molybdate-resistant and affected in nitrate reductase and or xanthine dehydrogenase activity for A.nidulans [18]. A.fumigatus CreB is the ubiquitin-processing proteases (UBP), a member of a family of deubiquitinating enzymes. Further analysis of mutants in the control of protein ubiquitination and carbon catabolite repression will reveal multiple functions of CreB, which will provide information to elucidate the mechanism of proteases in A.fumigatus infection and human diseases such as cystic fibrosis[7].

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Split-marker 重组技术构建泛素 C 末端水解酶基因缺失菌株*

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摘要 **目的**:对烟曲霉(Aspergillus fumigatus)泛素末端水解酶(creB)基因进行敲除。**方法**:通过氨基酸序列分析软件初步分析烟曲霉 CreB 蛋白结构. 利用 split-marker 重组技术构建重组片段,并通过 PEG- 原生质体方法对烟曲霉野生菌株进行转化,采用 PCR 方法对转化子进行筛选,最后选取初步筛选的转化子进行测序鉴定。**结果**:结构分析显示烟曲霉 CreB 蛋白具有泛素特异蛋白酶(ubiquitin-processing protease)UBP 亚家族六个结构域。本实验构建了转化片段并转化,在抗性平板中获得了 25 个 Hyg 抗性转化子,进一步采用 PCR 方法筛选到 20 个转化子,最终通过测序分析获得一株 creB 基因缺失菌株。**结论**:Split-marker 重组技术是对烟曲霉 creB 基因进行敲除的快速有效的方法。获得的 creB 缺失菌株可用于基因功能研究。

关键词:烟曲霉;泛素 C 末端水解酶;Split-Marker 重组技术

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