

Ku80 Gene is Related to Non-Homologous End-Joining and Genome Stability in *Aspergillus niger*

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Abstract In this study, the *ku70* and *ku80* homologs from the *Aspergillus niger* genome were identified and their function was analyzed using targeted mutagenesis. The role of the *ku80* gene in non-homologous end-joining (NHEJ) was investigated by calculating the frequency of homologous recombination. The transformation test verified that the frequency of homologous recombination significantly increased, from 1.78 to 65.6% in *ku80* single deletion strains and to 100% in *ku70/ku80* double deletion strains. These results suggest that the *ku80* gene is important for non-homologous end-joining. Although the morphology of the *ku* deletion strains colonies was similar to that of the wildtype strain, mutants were more sensitive to the mutagen phleomycin. Furthermore, the purified *ku80* deletion strain produced some sectorized colonies on hygromycin

B-containing plates. This result suggests that the *ku80* gene deletion leads to genomic instability in *A. niger*.

Introduction

There are two main pathways for DNA repair or DNA recombination: homologous recombination (HR) and non-homologous DNA end-joining (NHEJ). The primary pathway in unicellular eukaryotes (e.g., *Saccharomyces cerevisiae*) is HR, but in multicellular eukaryotes (e.g., mammals) NHEJ is more common [11]. The model for NHEJ is generally conserved between *S. cerevisiae* and mammals. The NHEJ process is mediated by the Ku heterodimer, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and the DNA ligase IV-Xrcc4 complex [15]. The Ku heterodimer, which contains the Ku70 and Ku80 proteins, binds tightly to the DNA ends for kinase and ligase to repair DNA double-strand breaks, a key step in NHEJ repair [4, 7]. Eliminating the NHEJ pathway significantly increases the HR pathway. Mutants deficient in Ku proteins have significantly increased HR compared with Xrcc4 and DNA-PKcs mutants [17].

The filamentous fungus *Aspergillus niger* (*A. niger*) is a commercially important organism for production of industrial enzymes and biochemicals. The complete genome of *A. niger* has been sequenced and published, and development of an efficient gene targeting system would facilitate the analysis of gene function. Although gene targeting is possible, homologous recombination is not efficient in *A. niger*. Most transformed DNA integrates into chromosomes heterologously through the NHEJ pathway. Although the Ku protein homologs in various organisms have diverged substantially in primary sequence, they are similar in overall size and subunit structure [16]. Genes homologous to human *ku70* and *ku80* have been identified and assessed in

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filamentous fungi. Deletions of the *ku70* and/or *ku80* genes have been shown to significantly increase the frequency of HR within the genera *Aspergillus*, including *A. nidulans* [14], *A. fumigatus* [5, 10], *A. niger* [12], *A. sojae*, *A. oryzae* [18, 19], and *Neurospora* strains [15].

In *A. niger*, deletion of the *ku70* gene increased the efficiency of homologous recombination to 95% [12]. Here, the authors report that deletion of the *ku80* gene alone and the double deletion of *ku70/ku80* genes significantly increased the frequency of gene targeting in *A. niger*. In addition, *ku* deletion strains were sensitive to the mutagen phleomycin. These results validate the hypothesis that the rate of homologous recombination can be increased by blocking NHEJ function.

Materials and Methods

Strains and Growth Media

Strain GICC2773 was used as the parental strain for deletion experiments of target genes. The growth GMP media and culture conditions for strains were described previously by Wang [20]. AMD medium (15 mmol/l acetamide, 0.1% K₂HPO₄, 0.05% KCl, 20 mmol/l CsCl, 0.05% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 2% glucose, and 1.0% agar) was used for growth of Amd⁺ strains.

Construction of Plasmids for Replacement of *ku80*, *ku70*, and *htmA*

Plasmids pBS-ku80-hph, pBS-ku70-amds, pUC-htmA-amds, and pUC-htmA-ble were used to construct gene deletion strains. Each contained two homologous fragments flanking the target region in addition to a selective marker (Fig. 1). For plasmid pBS-ku80-hph, a pair of PCR primers (Pku80-1, 5'-TTGTAACTTTGATCGGTGTTCTTGG-3' and Pku80-2, 5'-TTGTAAACAGTTGCAGAAGCAGATGATG-3') was designed to amplify the 2.9 kb *ku80* gene fragment. The *hph* expression cassette from plasmid pMW1 [9] was inserted into the *ku80* fragment at the *Sna*BI/*Bg*II sites (Fig. 1a). A pair of PCR primers Pku70-1 (5'-GGCAGCTGTTTTTGAATCTTGGGTGTG-3') and Pku70-2 (5'-TTCAGCTGCTCATCAGGCGAAAAAGTTAC-3') was used to amplify the 2.0 kb *ku70* gene fragment, which was inserted using the *amdS* expression cassette from p3SR2 [8] at the *Eco*47III site (Fig. 1b). For *htmA*, the 4.6 kb *htmA* gene fragment was amplified using primers Phtm1 (5'-ATACCAGTGGGTCAATCGCAGA-3') and Phtm2 (5'-GACGTAACGAGGTGGCT TTCTT-3'). The selective markers *amdS* from p3SR2 and *SH-ble* from pUT737 [13] were inserted, generating pUC-htmA-amds (Fig. 1c) and pUC-htmA-ble (Fig. 1d), respectively.

Construction of the Gene Deletion Strains Δku80 (Hyg^r), Δku70 (Amd⁺), and Δku70/ku80 (Hyg^r, Amd⁺)

Protoplast preparation and transformation were performed according to established protocols [3, 21]. The mutant strain was obtained by plasmid transformation and validated by PCR. Transformants with the targeted gene deletions were determined by PCR with a pair of primers located on the selective marker and chromosomal sequences described previously by Wang [20]. For the *ku80* gene, hygromycin resistant colonies were isolated and tested by PCR using primers Phph (5'-GGCTGTGTAGAAGTACTCGCCGATAGTG-3') and Pku80-out (5'-GATTCAGGATAAGGAGCGTTGATG-3') to determine whether replacement occurred at the target gene. For gene *ku70*, the primers used were Pku70-out (5'-TTCCAGTCTAGACACGTATAACGGC-3') and PamdS3 (5'-TCTTGTCCAGCAGCTTGACATTTCC-3'). The double deletion strain was obtained by transforming plasmid pBS-ku70-amds into the Δku80 (Hyg^r) strain.

Function Analysis of *ku* Genes in *A. niger*

The function of the *ku80* gene was investigated by examining the effect of the Δku80 strain on HR frequency in the target gene and measuring sensitivity to the mutagen phleomycin. HR frequencies were calculated by counting the number of PCR-validated specific gene targeting events. The phleomycin sensitivity test of the *ku* deletion strains was carried out on GMP agar medium containing 20 μg/ml phleomycin. The spore suspensions with gradient concentration of each strain tested were spotted onto the agar plate.

Results

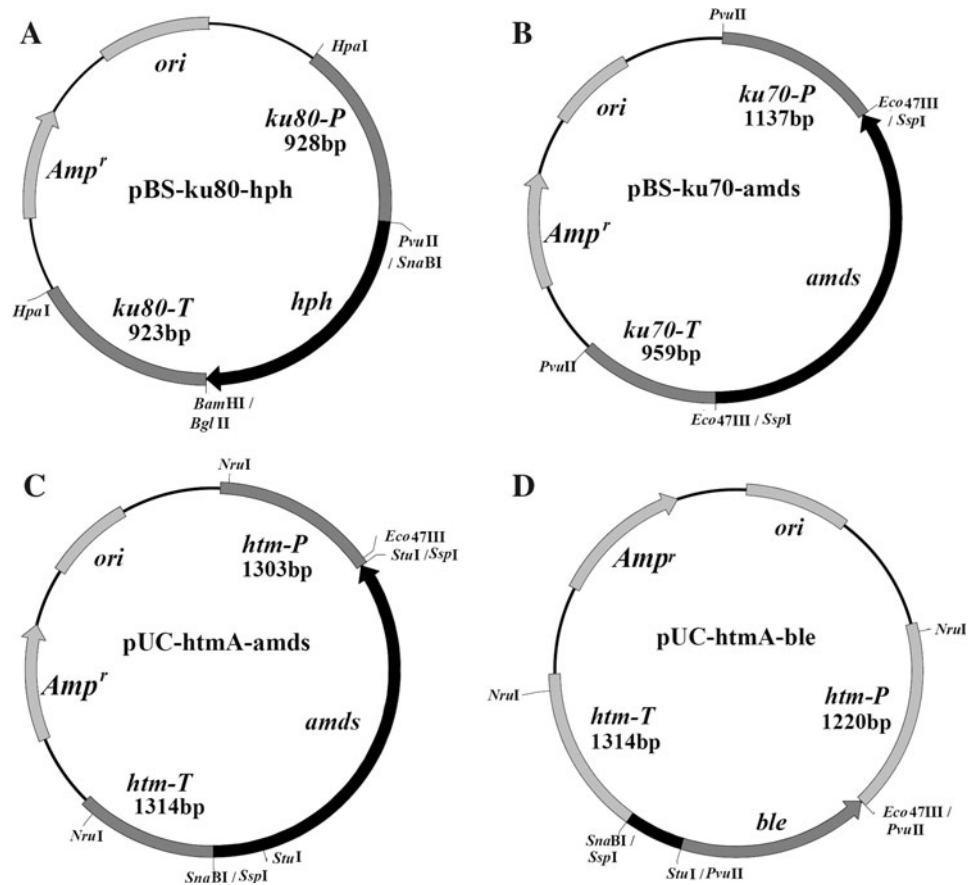
Deletion of the *ku80* and *ku70/ku80* genes in *A. niger*

The allele exchange cassettes of *ku70* and *ku80*, generated by the corresponding plasmids and consisting of a selective marker and ~1 kb homologous fragments on each side of target gene, were transformed into strain GICC2773. The *ku80* single deletion strain was characterized by PCR using two pairs of primers using a previously described PCR strategy [20]. The *Ku70/ku80* double deletion strain (Δku70/Δku80) was obtained by deleting the *ku70* gene using the *ku80* deletion strain as recipient.

Phenotypic Characterization of Δku70, Δku80, and Δku70/Δku80 Strains

The DNA-damaging chemical phleomycin was used to examine the growth sensitivity of various mutants on GMP

Fig. 1 Maps of the plasmids for gene targeting. **a** Plasmid pBS-*ku80*-*hph* for *ku80* gene deletion with the *hph* selective marker, **b** pBS-*ku70*-*amds* for *ku70* gene deletion with the *amds* selective marker, **c** pUC-*htmA*-*amds* for *htmA* gene deletion with the *amds* selective marker and **d** pUC-*htmA*-*ble* for *htmA* gene deletion with the SH-*ble* selective marker



medium (20 μ g/ml phleomycin). *Ku80* deletion strains were more sensitive than the wild type strain (GICC2773) to phleomycin (Fig. 2a). The double deletion strain had greater sensitivity than the single deletion, and the order of decreasing sensitivity was $\Delta ku70/\Delta ku80 > \Delta ku70 > \Delta \varpi ku80 > GICC2773$ (Fig. 2b). However, no differences were observed with respect the appearance of the colony, the rate of growth, and sporulation on GMP agar (data not shown). However, occasionally when the $\Delta ku80$ strain was cultured on plates containing 200 μ g/ml of hygromycin B, a few sectored colonies were present (Fig. 3), indicating the colony contained heterokaryotic cells. This result suggests that the *ku80* gene is important for retaining genomic stability.

Efficiency of Gene Targeting in $\Delta ku80$ and $\Delta ku70/\Delta Ku80$ Strains

To analyze the effect of *ku80* deletion on homologous recombination, the authors tested the HR efficiency of targeted deletion of *ku70*. The *ku70* fragment with insertion of the *amds* expression cassette (Fig. 1) was transformed into both the control strain GICC2773 and the *ku80* deletion strain. Transformants were identified by PCR, and the

homologous recombination frequencies were calculated accordingly. The data revealed that the frequency of homologous recombination increased more than 18-fold in the *ku80* deletion strain, from 3.6 to 65.6% (Table 1). The frequency of homologous recombination in the *ku70/ku80* double deletion strain was determined by targeted *htmA* deletion with the selective marker Sh-*ble* (Fig. 1). The frequency of HR reached an astonishing 100% (Table 1). This result suggests that the efficiency of gene targeting in the *ku70/ku80* double deletion strain was higher than in the single deletion strain.

Discussion

The HR frequency was increased in both the $\Delta ku80$ and the $\Delta ku70/\Delta ku80$ strains in this study. Deletion of the *ku80* gene increased the HR frequency from less than 4–66%, and in the double deletion $\Delta ku70/\Delta ku80$ strain the HR frequency reached 100%. Using *Agrobacterium*-mediated transformation, the HR frequency in the $\Delta ku70$ stain also increased dramatically (data not shown), which is in agreement with results reported by Meyer et al. [12]. Because of the similarity in the structure and sequence of

Fig. 2 **a** Hypersensitivity to phleomycin of $\Delta ku80$ strain in contrast to parental strain GICC2773 with three independent samples. **b** Each quarter of the agar plate contained one strain; a concentration gradient of spores was used for inoculation. The parental strain GICC2773 produced seven colonies of ordinary size. The other strains only produced one colony with fewer spores

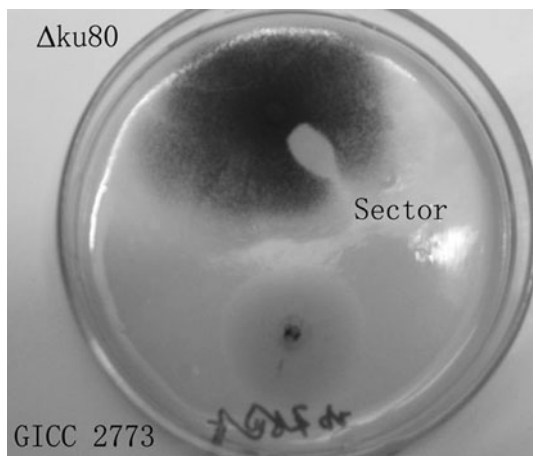
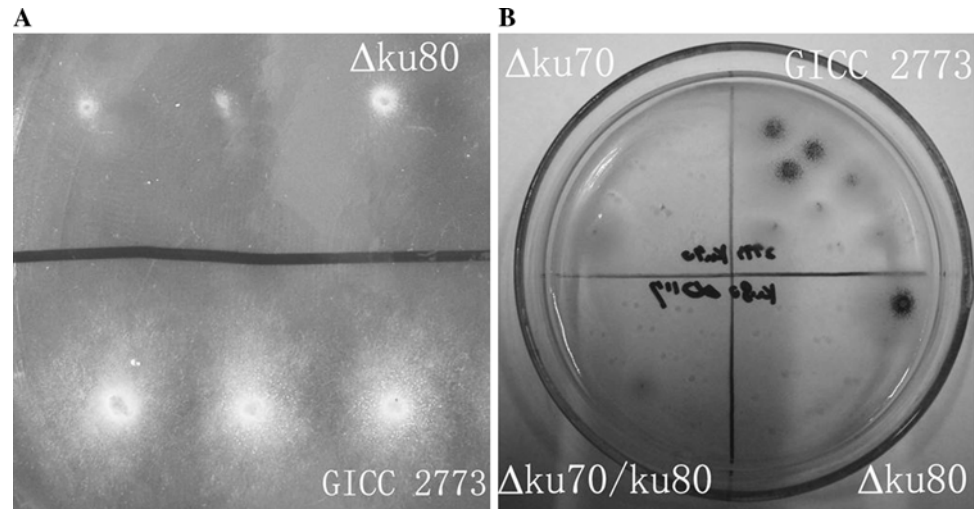


Fig. 3 Sectoring in the $\Delta ku80$ strain on GMP containing hygromycin B

the *ku* genes, this result suggested that the function of the *ku70* and *ku80* genes might complement each other in *A. niger*.

The allele exchange cassettes, consisting of the 5' and 3' terminal homologous fragments and a selective marker, were constructed with homologous arms similar of length. The authors observed no difference between *ku70* and

htmA in the $\Delta ku80$ strain with regards to HR frequency of gene targeting. These results suggest that the homologous recombination in the $\Delta ku80$ strain is not sequence dependent.

Hypersensitivity to DNA-damaging agents (*e.g.*, MMS, bleomycin, and phleomycin) has been studied in *Neurospora* and *Aspergillus*, but the results were not consistent [5, 10, 12, 14, 15, 18, 19]. In particular, sensitivity to phleomycin had not been detected in the *Aspergillus* strains tested. In the experiment, *ku* deletion strains showed varying degrees of sensitivity to phleomycin. Sensitivity increased incrementally from the $\Delta ku80$ to the double deletion $\Delta ku70/\Delta ku80$ strains, which supports the hypothesis that the NHEJ pathway is *ku*-dependent in *A. niger*. However, because the deletion strains contained different selective markers (*amdS* and *SH-ble*), which may influence the efficiency of HR, the conclusion that double deletions perform less function in NHEJ than the single deletion cannot be drawn hastily. In addition, fewer transformants (13) were obtained from the *htmA-ble* cassette transformed into the $\Delta ku70/\Delta ku80$ strain. This reduced number of transformants was probably caused by phleomycin in the selection medium, which may have damaged the DNA in the *ku* deletion strain and decreased the regeneration frequency of the protoplast.

Table 1 HR frequency among the single deletion strain of *ku80* gene $\Delta ku80$, the double deletion strain of *ku70* and *ku80* genes $\Delta ku70/\Delta ku80$ and the control strain GICC2773

Recipient strain	Deletion fragment	Transformants detected	HR transformants	Frequency of HR (%)
A				
GICC2773	<i>ku70</i> -P- <i>amdS</i> - <i>ku70</i> -T	84	3	3.6
$\Delta ku80$	<i>ku70</i> -P- <i>amdS</i> - <i>ku70</i> -T	90	59	65.6
B				
GICC2773	<i>htmA</i> -P- <i>amdS</i> - <i>htmA</i> -T	56	1	1.78
$\Delta ku80$	<i>htmA</i> -P- <i>amdS</i> - <i>htmA</i> -T	32	21	65.6
$\Delta ku80/\Delta ku70$	<i>htmA</i> -P- <i>ble</i> - <i>htmA</i> -T	13	13	100

The *ku80* deletion strain was obtained after several rounds of isolating single spores, which are uninucleate in *A. niger* [22]. Therefore, the *ku80* deletion strain should be a homokaryon. However, *ku80* deletion strain colonies occasionally contained sectoring, which suggested that some cells had become a heterokaryon, although it is difficult to understand how the *ku80* deletion results in the sector formation. Difilippantonio et al. [6] had reported that 15% of *ku80* deletion mouse embryo fibroblasts displayed polyploidy and 83% had breaks or translocations, which may indicated that *ku80* deletion resulted in formation of heterokaryon. The heterokaryon with two different nuclei differentiates into two individual colonies generating sector. Deletion of *ku80* gene increased recombination of the genome [2], and overexpression of both yeast Ku70 and Ku80 proteins reduces chromosomal rearrangements formation [1]. Consistent with this, the sector phenomenon suggests instability in the *ku80* deletion strain and implies that the *ku80* gene is related to genome stability. The mechanism of this phenomenon warrants further investigation. In conclusion, the Ku80 proteins are important factors for the NHEJ pathway and deletion of *ku80* gene increased the HR frequency in *A. niger*.

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