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Short communication

Highly efficient gene targeting in the *Aspergillus niger kusA* mutant

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Abstract

Gene targeting frequencies in *Aspergillus niger* are often very low and hamper efficient functional genomics in this biotechnologically important fungus. Deletion of the *A. niger kusA* gene encoding the ortholog of the Ku70 protein in other eukaryotes, dramatically improved homologous integration efficiency and reached more than 80% compared to 7% in the wild-type background, when 500 bp homologous flanks were used. Furthermore, the use of the *ΔkusA* strain resulted in a high frequency of heterokaryon formation (70%) in primary transformants in the case disrupting an essential gene. Deletion of *kusA* had no obvious effect on the growth of the fungus, but renders the *ΔkusA* strain 10 times more sensitive to X-ray irradiation and two to three times more sensitive to UV exposure. The highly efficient gene targeting in combination with the *A. niger* genome sequence allows a systematic approach to generate gene knockouts and will help in improving the capacities of *A. niger* as producer of commercially interesting proteins and metabolites.

Keywords: Filamentous fungi; Functional genomics; Gene targeting; *ku70*; Gene knock outs; Strain improvement

The rapid increase in available fungal genomes requires the development of efficient tools for the functional characterization of genes in filamentous fungi, for example by an efficient method to construct gene knock-outs. Preferably, knock-out mutants are made via the construction of a gene replacement cassette and subsequent integration of such a cassette at the target site. In *S. cerevisiae,* it has been well established that

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integration of a DNA fragment into the genome can be mediated by two pathways. The homologous recombination (HR) pathway depends on the Rad52 epistasis group and results in the targeted integration of the DNA (Krogh and Symington., 2004). Integration can also be mediated via the non-homologous end joining pathway (NHEJ) which depends on the Ku70/Ku80-protein complex (Dudasova et al., 2004). In filamentous fungi, the NHEJ-pathway seems to be dominant over the HR-pathway as gene targeting efficiencies are low compared to those observed for *S. cerevisiae*. Recent studies in both yeasts and filamentous fungi revealed that by deleting components of the NHEJ-pathway, the random integration of DNA fragments is strongly reduced. Therefore, in those NHEJ-pathway defective mutants, DNA integrates mainly via the HR-pathway giving rise to high homologous recombination frequencies (Kooistra et al., 2004; Ninomiya et al., 2004; Nayak et al., 2006; Takahashi et al., 2006b; Goins et al., 2006; Pöggeler and Kück, 2006; Krappmann et al., 2006; Silva Ferreira et al., 2006).

The Ku70 homolog present in the *A. niger* genome (Dr. C. Sagt, personal communication) was identified by BlastP searches using other fungal Ku70 proteins sequences. The gene identified was named *kusA* (ku seventy; GenBank accession number EF061656). The *A. niger* KusA protein consists of 648 amino acids and is highly identical (over 80% amino acid identity) to the orthologs in *A. nidulans*, *A. fumigatus*, *A. oryzae* and *A. sojae* (Nayak et al., 2006; Takahashi et al., 2006a; Krappmann et al., 2006). A deletion construct (*kusA::amdS)* consisting of each 1.5 kb of 5′ and 3′ flanking regions of *kusA* and the *amdS* selection marker (Kelly and Hynes, 1985) was made and transformed into strain AB4.1 (*pyrG*$^{-}$) (van Hartingsveldt et al., 1987). Transformant MA70.15 (*kusA::amdS*, *pyrG*$^{-}$) with a deleted *kusA* gene was selected via Southern analysis (data not shown) and used for further analysis.

Deletion of *kusA* did not affect the growth behaviour of *A. niger*, as neither differences in hyphal growth rate nor in conidiation efficiency were observed between the *ΔkusA* strain and AB4.1 after cultivation on agar plates at 25, 30 and 37 °C (data not shown). Spore germination, septum formation, and subapical branching also occurred normally and biomass production in shake flask cultures was the same in both strains (data not shown). In addition, the *ΔkusA* mutant did not show altered sensitivity (neither resistance nor hypersensitivity) towards the DNA damaging agents phleomycin and methyl methanesulfonate (MMS) (data not shown). In *N. crassa,* deletion of the Ku70 homolog *mus-51* results in increased sensitivities towards phleomycin and MMS (Ninomiya et al., 2004). However, our results are similar to the results obtained for *A. nidulans* and *A. oryzae* mutants lacking Ku70, as those mutants did also not show increased sensitivities towards phleomycin and MMS (Nayak et al., 2006; Takahashi et al., 2006a). The *A. fumigatus* mutant lacking the Ku70 protein displays similar sensitivity towards phleomycin (Krappmann et al., 2006), but increased sensitivities towards MMS (Silva Ferreira et al., 2006). The similar sensitivity of the *ku70* mutants and the corresponding wild type strains towards phleomycin suggests that double-strand breaks reported to be induced by phleomycin (Povirk et al., 1977) are not the only cellular consequence of drug treatment but that other targets might be important for its antifungal action as well (e.g. oxidation of cellular RNAs; Hecht, 2000). To examine the role of the *A. niger* KusA protein in DNA double-strand break repair, the sensitivity towards X-ray irradiation, which specifically causes double strand breaks in DNA, was determined. As shown in Fig. 1A, the *ΔkusA* strain is much more sensitive to X-ray irradiation compared to the parental strain. Low dose intensities (100–200 Gy) resulted in the killing of 90–95% of all *ΔkusA* derived spores. The results clearly indicate that the NHEJ machinery is important to repair X-ray induced double strand breaks in *A. niger*. The sensitivity of both the *ΔkusA* and the parental strain towards UV exposure was also examined. As can be seen in Fig. 1B, the *ΔkusA* mutant displayed a higher sensitivity towards UV radiation, however, the effect was less pronounced than observed for the X-ray irradiation. This can be explained by the fact that UV induces less double strand breaks but induces mainly point mutations and single strand breaks which can be repaired by KusA independent repair mechanisms (Eckardt-Schupp and Klaus, 1999; Goldman et al., 2002). The finding that the *A. niger kusA* mutant is more sensitive to UV was somewhat surprising, as UV sensitivities of *ku70* mutants in *N. crassa* and *C. neoformans* were similar to the wild type strains (Ninomiya et al., 2004; Goins et al., 2006). This discrepancy might be explained by the use of different experimental conditions.

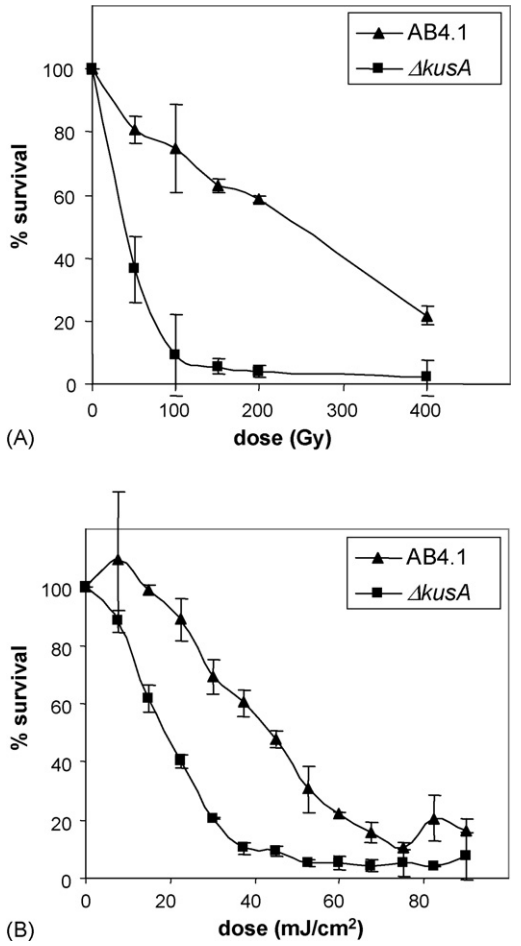

Fig. 1. Sensitivity of AB4.1 (*pyrG*$^-$) and MA70.15 (*ΔkusA*, *pyrG*$^-$) to X-ray irradiation and UV exposure. (A) Survival after X-ray irradiation. Four millilitres spore solution (1×10^7 spores/ml in 0.9% NaCl) of strains AB4.1 and MA70.15 were irradiated up to 400 Gy using a YXLON Y.TU225-DO2 X-ray Machine (Yxlon International). Serial dilutions of irradiated spores were plated in triplicate on plates containing complete medium (CM) plus uridine. After 2 days of growth at 30 °C, colonies were counted and survival was calculated by comparison to non-irradiated spores. The mean values of a duplicate experiment are given. (B) Survival after UV exposure. Spores of AB4.1 or MA70.15 were exposed to UV using a Ultraviolet crosslinker (Amersham Bioscience). 15 ml of resuspended spores (1×10^7 ml^{-1}) were transferred to a petri-dish and exposed to up to 11 doses of UV irradiation (each dose is equivalent to 7500 μJ/cm^2). After each dose, 1 ml samples were taken. Each sample was 1000-fold diluted in 0.9% NaCl and 50 μl of the dilution was plated on CM plates plus uridine in triplicate. Plates also contained Triton X 100 (0.05%) to get compact colonies that facilitate counting. Spores were kept in the dark to prevent a photo lyase repair reaction. Plates were incubated at 25 °C in the dark and counted after 5 days of growth. Values are derived from two independent experiments.

The effect of *kusA* deletion on homologous recombination efficiency was determined by transforming strains MA70.15 (*ΔkusA*, *pyrG*$^-$) and AB4.1 (*pyrG*$^-$) with PCR constructs containing the *A. oryzae pyrG* gene flanked on each side with either 100, 200, 500, 1000 or 1500 bp of *gfaA* 5′ and 3′ regions, respectively. *GfaA* encodes a glucosamine:fructose-6-phosphate amidotransferase which catalyzes the formation of glucosamine-6-phosphate from glutamine and fructose-6-phosphate. Deletion of the *gfaA* gene can directly be scored by phenotype analysis of primary transformants as *gfaA* deletion strains show defects in conidiation when the medium is supplemented with low concentrations of glucosamine. In addition, *ΔgfaA* strains are not viable on glucosamine-free medium (Ram et al., 2004). The respective PCR fragments were obtained by using plasmid pΔgfaA (Ram et al., 2004) as a template and suitable primer combinations. As shown in Table 1, deletion of the *kusA* gene in *A. niger* clearly increased HR frequencies. Frequencies between 88 and 95% were reached when flanking regions between 500 and 1500 bp were used. In the parental strain, however, HR frequencies were dramatically lower (7–29% for flanking regions between 500 and 1500 bp, respectively). The increase in HR frequency in the *ΔkusA* strain was not accompanied by a strong reduction in the total number of transformants obtained (Table 1). In addition to the results obtained for deleting the *gfaA* gene, several other genes have been successfully disrupted with an efficiency of over 80% using gene deletion constructs with 500 bp flanks (Ram, unpublished results). Shortening the flanks to 200 or 100 bp strongly reduced HR frequencies and also reduced the number of transformants (Table 1), especially in the *ΔkusA* background, indicating that efficient HR in *A. niger* requires a minimal length of the flanking regions. Apparently, the HR machinery (encoded by the *RAD52* epistasis group genes) is not able to efficiently mediate integration when the homologous flanks are only 200 bp in length. Similar results have been obtained in *N. crassa* and *A. sojae*. In these studies, flanking regions of 100 bp length were not sufficient to result in efficient gene targeting (Ninomiya et al., 2004; Takahashi et al., 2006b).

Southern analysis was performed with selected *pyrG*$^+$ transformants in the *ΔkusA* and AB4.1 background. In twenty glucosamine auxotrophic mutants that were examined in the *ΔkusA* background, the

Table 1
Comparison of homologous recombination frequencies (HRF) between the recipient strains AB4.1 (*pyrG*$^-$) and MA70.15 (*ΔkusA*, *pyrG*$^-$)

gfaA flanks (bp)	AB4.1			MA70.15		
	pyrG$^+$ transformants[a]	*ΔgfaA*[b]	HRF (%)[c]	*pyrG*$^+$ transformants[a]	*ΔgfaA*[b]	HRF (%)[c]
1500	94	26	29	484	473	98
1000	120	23	19	253	239	95
500	175	12	7	125	110	88
200	87	4	5	18	6	33
100	75	3	4	28	5	18

[a] The total number of transformants obtained after transforming ~5×10^7 protoplasts with 8 μg DNA.
[b] The *ΔgfaA* phenotype was determined by transferring spores from the transformation plate to plates with or without 10 mg/ml glucosamine. No growth on plates lacking glucosamine was scored as *ΔgfaA*.
[c] HRF is the number of *ΔgfaA* transformants divided by the total number of *pyrG*$^+$ transformants.

hybridization patterns were consistent with a double crossover event at the *gfaA* locus. No additional integrations had occurred (Fig. 2, lane 2 and data not shown). Six transformants in the *ΔkusA* background that were not glucosamine requiring were also examined by Southern blot analysis. Two of them (obtained with the 1.0 and 1.5 kb flanks) arose from a single crossover event at the *gfaA* locus, indicating that the linear PCR fragment was circularized before integration (data not shown). The four remaining transformants (obtained with the 100, 200, and 500 bp flanks) had an identical pattern of hybridization in which only the wild-type *gfaA* gene was visible (Fig. 2, lane 3 and data not shown). There were no indications of an ectopic integration of the *gfaA* gene deletion cassette. A possible explanation is that the *A. oryzae pyrG* gene, which shares significant DNA sequence identity with the *A. niger pyrG* gene, integrates at the *pyrG* locus of *A. niger* (possibly via a double cross over), resulting in a *pyrG*$^+$ phenotype. Alternatively, as the *ΔkusA* showed higher sensitivity towards UV, a higher mutation rate could result in a more frequent reversion of the *pyrG* mutant gene to a functional *pyrG* gene.

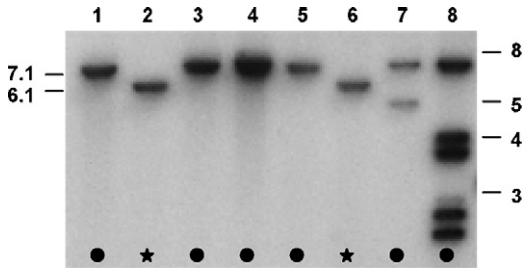

Fig. 2. Southern blot analysis of selected transformants obtained in AB4.1 (*pyrG*$^-$) and MA70.15 (*ΔkusA*, *pyrG*$^-$) background. Genomic DNA was restricted with *Pst*I and size fractioned by electrophoresis on a 0.7% agarose gel. For hybridisation, a ^{32}P-labelled probe targeting the *gfaA* promoter was used. A signal at 7.1 kb reflects the wild type *gfaA* gene, a signal at 6.1 kb reflects deletion of the *gfaA* ORF as a result of gene replacement with the *pyrG* gene. Lanes 1 and 4: genomic DNA of MA70.15 and AB4.1, respectively; lanes 2–3: genomic DNA of two transformants in the MA70.15 background. Lanes 5–8: genomic DNA of four transformants in the AB4.1 background. The molecular weights are indicated in kb, the results of phenotype screenings by a closed circle (*gfa*$^+$) and an asterisk (*gfaA*$^-$).

The results from a similar analysis of *gfaA*$^+$ transformants obtained with AB4.1 are quite different. Of 16 *gfaA*$^+$ transformants (a collection of transformants obtained with all gene deletion cassettes), only one transformant (obtained with the 0.5 kb flanks) displayed an integration pattern that can be explained by integration of the construct at the *pyrG* locus or as an reversion of the *pyrG* mutation, as only the wild type *gfaA* fragment was visible (Fig. 2, lane 5). In the other 15 transformants, the pattern is consistent with single or multiple ectopic integrations of the gene deletion cassette (Fig. 2, lanes 7 and 8 and data not shown).

A. niger is a haploid, asexual fungus which makes the determination and confirmation of essential genes time consuming. A useful approach for the analysis of essential genes in filamentous fungi is to screen for the presence of balanced heterokaryons in primary transformants (e.g. Osmani et al., 1988; Martin et al., 1997; Nayak et al., 2006). Heterokaryotic strains are able to grow under selective conditions, but the two types of uninuclear conidiospores produced from the heterokaryotic mycelium will not germinate on selective medium. Conidia from untransformed nuclei

will not grow because they lack the selection marker, and conidia from transformed nuclei will not grow because the essential gene has been disrupted. Thus, the inability to subculture conidiospores from primary transformants after a gene deletion experiment on selective medium indicates that the target gene is essential.

The efficiency of heterokaryon rescue in the *ΔkusA* strain was examined by deleting *rmsA*, a gene that is potentially essential in *A. niger*. The *rmsA* gene was isolated after complementation of the apical branching mutant *ramosa-1* of *A. niger* (Reynaga-Pena and Bartnicki-Garcia, 1997, Ram, unpublished results). Previous attempts to disrupt the *rmsA* gene failed, indicating that the gene might be essential and that heterokaryon formation was inefficient. We used in this work the same deletion construct and transformed it into the *ΔkusA* strain. A single transformation (15 μg of DNA) yielded in 23 primary transformants. Seventeen of these transformants are likely to be heterokaryons as conidiospores could not be subcultured after single spore isolation on selective medium plates, although subculturing of mycelial fragments was possible. Southern blot analysis of selected primary transformants indeed confirmed their heterokaryotic nature as the hybridization pattern indicated the presence of wild-type and *rmsA* disruptant nuclei (data not shown). Thus, deletion of *kusA* does not only improve the gene targeting efficiency in *A. niger*, it also facilitates the identification of essential genes.

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