Transient disruption of the *kusA* gene in *Aspergillus niger* facilitates high throughput gene deletion analysis

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Abstract

Biomass conversion by filamentous fungi is a promising resource for the production of enzymes and organic compounds that can offset our dependence on petroleum. Organic acid production by *Aspergillus niger* is a highly efficient model system studied in order to aid in process development for all fungi (Dai et al, 2004). We use genome sequence information from the DOE Joint Genome Institute for gene deletion analyses in *A. niger*. Deleting a gene requires the integration of a genetic marker at the precise locus of the gene through homologous recombination; however, the innately low rate of homologous recombination slows this analysis. Recent studies have shown that the rate of homologous recombination increases in other fungi via the deletion of *kusA*, a gene required for non-homologous end-joining of DNA (Ninomiya et al., 2004). A *kusA*⁻ strain was created in a citric acid producing strain of *A. niger* by inserting *pyrG*, a counter-selectable marker, at the *kusA* locus (Nielsen et al., 2008). We tested for improved homologous recombination by creating an *ArgB* knockout using polymerase chain reaction (PCR). Screening of transformants revealed the rate of homologous recombination was significantly higher in the *kusA*⁻ background (95%) than in the wildtype background (18%). This improvement is of great use in gene deletion analysis; however, strains lacking a functional *kusA* gene cannot repair double-stranded breaks in DNA, making them significantly more susceptible to mutagenesis. Therefore we designed the *kusA* mutant with direct repeats flanking the *pyrG* insertion to allow spontaneous reversion of the functional *kusA* gene. Spores from the wildtype, *kusA*, and reverted *kusA*⁺ strains were exposed to gamma radiation ranging from 50 to 400 Gy. The data shows that the ability to recover from radiation-induced mutagenesis was restored in the *kusA* revertant strain. Preliminary mutant screens utilizing the *kusA*⁻ strain have demonstrated that we have effectively shortened the time for analysis from several weeks to days. This capability will rapidly advance the study of organic acid production and morphology in *A. niger*, leading to expanded utility of all fungi in biomass conversion.

Background

- *Aspergillus niger* is a filamentous fungi that is studied as a model organism for organic acid production.
- Gene deletion analysis is used to discover key genes involved in citric acid production in *A. niger*.
- Gene deletion occurs when knockout constructs are integrated into the genome precisely at the gene locus via homologous recombination.
- Currently, gene deletion analysis is slowed by the inherently low rate of homologous recombination.
- Deletion of the *kusA* gene has been shown to increase the rate of homologous recombination in other filamentous fungi (Ninomiya et al., 2004).
- The *kusA* gene facilitates non-homologous end-joining (NHEJ), a process that randomly joins double-stranded DNA fragments. NHEJ is required for non-homologous recombination of knockout constructs and for DNA repair.

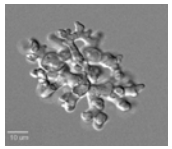


Figure 1: *A. niger* grown in citric acid production (CAP) media.

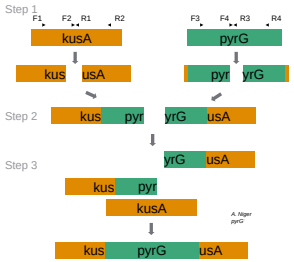
Figure 2: (a) Homologous recombination of the knockout construct results in the elimination of targeted gene. (b) Non-homologous integration of the knockout construct via NHEJ results in an unsuccessful gene deletion.

Goals

- Demonstrate that disrupting the *kusA* gene will lead to higher rates of homologous integration of knockout constructs in *A. niger*.
- Demonstrate that restoration of *kusA* gene will restore the ability to repair double-stranded breaks in DNA, making the strain more resistant to mutagenesis.

Methods

A *kusA*⁻ strain was made by inserting the *pyrG* gene at the *kusA* locus, disabling the function of the *kusA* gene product (Nielsen et al., 2008).



***pyrG*: A Counter-selectable Marker**
The *pyrG* gene is a counter-selectable marker required for the synthesis of uracil; cells lacking *pyrG* require the addition of uracil to the media, and cells with a functional *pyrG* gene cannot grow in the presence of 5-fluoroorotic acid (FOA), a fluorinated precursor to uracil.

Transformations: Integrating DNA
DNA fragments made with PCR were introduced into the fungal genome via transformations into *A. niger* protoplasts. Protoplasts are fungal cells without cell walls, and the transformation procedure causes the protoplasts to take up DNA and integrate the DNA into their chromosomes.

Figure 3: Method for disrupting *kusA* gene using counter-selectable marker *pyrG*. Fragments of *kusA* and *pyrG* genes containing overlapping regions were amplified using polymerase chain reaction (PCR) with forward (F) and reverse (R) primers (Step 1). Left fragments and right fragments of the two genes were fused together with PCR (Step 2). Selection on media lacking uracil gave cells with fragments integrated into the genome via homologous recombination at the *kusA* locus, resulting in a functional *pyrG* gene and a disrupted *kusA* gene (Step 3).

To test the rate of non-homologous recombination in the *kusA*⁻ strain, a knockout construct was made with PCR to delete *ArgB*, a gene required for the synthesis of arginine. The knockout construct was transformed into *kusA*⁻ and wildtype protoplasts and spores from individual colonies were plated on media with and without arginine. When homologous recombination of the knockout construct occured, the cells required arginine to grow. When non-homologous recombination occurred, the cells were able to grow without arginine in the media.

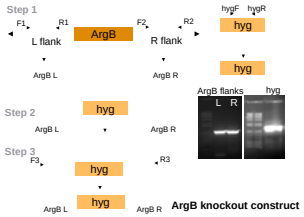


Figure 4: Method for making *ArgB* knockout construct using PCR. Hygromycin-resistance gene (hyg) and DNA sequences flanking *ArgB* were amplified (Step 1). Gel electrophoresis images were used to confirm that PCR products were obtained. Fragments were fused together with PCR using overlapping regions from primers R1, F2, and hyg primers (Step 2). The construct was amplified again to increase yield (Step 3).

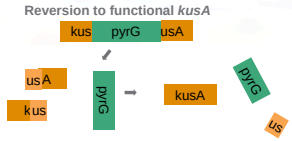


Figure 5: The *kusA/pyrG* construct (Figure 3) spontaneously reverts to the functional *kusA* gene due to the repeated segments of the *kusA* gene flanking *pyrG*. These sequences are depicted by "us" in the gene name "*kusA*" in this figure. Homologous recombination of these sequences causes the *pyrG* gene to be lost and the complete *kusA* gene to be restored. This event occurs at an approximate rate of 1 revertant/10^6 cells. Selection on media containing FOA was used to isolate *kusA*⁺ revertants.

Spores from *kusA*⁻, *kusA*⁺ revertants, and wildtype strains were exposed to gamma radiation at levels of 50, 100, 150, 200, and 400 Gy. Gamma radiation causes double-stranded breaks in DNA. It was expected that the restoration of the *kusA* gene would restore the cell's ability to repair DNA, enabling the *kusA*⁺ revertant spores to survive longer radiation exposures.

Figure 6: a) Vials containing *kusA*⁻, *kusA*⁺ revertant, and wildtype spores to be irradiated. b) Gamma irradiator with cobalt source used to irradiate spores.

Results

The observed rates of homologous recombination were:

- wildtype: 18% (σ = 4%)
- *kusA*⁻: 95% (σ = 0.3%)

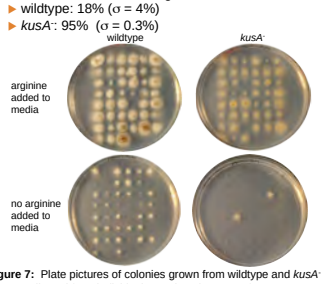


Figure 7: Plate pictures of colonies grown from wildtype and *kusA*⁻ spores collected from individual *ArgB* knockout transformants. Colonies in which homologous recombination occurred grew on media containing arginine but did not grow on media lacking arginine. These colonies are indicated by red circles in the figure.

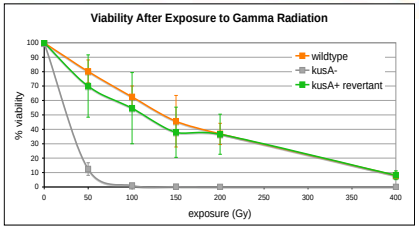


Figure 8: The percent viability of wildtype, *kusA*⁻, and *kusA*⁺ revertant spores exposed to gamma radiation. The percent viabilty of the *kusA*⁺ revertant is very similar to that of the wildtype and significantly higher than that of *kusA*⁻, indicating that the *kusA*⁺ revertant has restored ability to repair DNA.

Conclusions

- The high rate of homologous recombination achieved in the *kusA*⁻ strain will greatly advance efficiency of citric acid production analysis.
- The ability to repair double-stranded breaks in DNA can be restored in any mutant strain, making the strain less susceptible to mutagenesis.
  - Now in gene deletion analysis, the likelihood of obtaining a successful deletion is high (95%).
  - Mutants can be screened for unique phenotypes without performing time-consuming steps to confirm the gene deletion.
  - High numbers of gene knockouts can be screened rapidly, which will lead to the discovery of important genes involved in citric acid production.
  - After any gene is deleted using the *kusA*⁻ strain, *kusA* function can be restored.
  - This will eliminate the risk of significant mutagenesis occurring in the new strain over time as it is used in further studies.

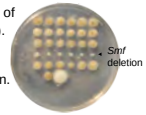


Figure 8: Phenotype screen of five mutants (six colonies each) on CAP media. The *Smf* mutant displays a unique phenotype in all six colonies, illustrating the high rate of homologous recombination in the *kusA*⁻ strain.

Acknowledgements

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Selected References

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