NADH oxidase, *nadA*, and its involvement in oxidative stress in *Aspergillus flavus*

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Abstract

nadA, which encodes a predicted NADH oxidase, was identified as part of a sugar utilization cluster that lies adjacent to the aflatoxin biosynthetic cluster in several species of *Aspergillus*. NADH oxidases convert NADH to NAD+, which is a possible coenzyme needed for reactions in the aflatoxin biochemical pathway. In a microarray experiment comparing gene expression between a wild type strain of *A. parasiticus* and a deletion mutant for the pathway regulatory gene *aflR*, *nadA* expression was significantly decreased in the mutant background. Although *nadA* is transcriptionally controlled by AflR, aflatoxin levels were unaffected in *A. flavus nadA* deletion strains under several conditions. NADH oxidases can also be a source of reactive oxygen species formation. Previous reports have shown a relationship between oxidative stress and aflatoxin production in *Aspergillus sp.* The activity of several antioxidant enzymes were examined in *nadA* deletion strains and peak activity was delayed when compared to wild type. Lower levels of lipoperoxide accumulation were also seen in *nadA* deletion strains. These data suggest that *nadA* plays a role in the oxidative stress response in *A. flavus*. Investigations are underway to characterize additional phenotypes of *nadA* deletion mutants.

aflR, the transcriptional regulator of aflatoxin biosynthesis, was knocked out in the *Aspergillus parasiticus* wild-type strain SU1. The Δ*aflR* strain is unable to produce aflatoxin because the aflatoxin biosynthesis genes are not transcriptionally activated. Microarray studies were performed comparing transcript levels of Δ*aflR* and SU1 to see if AflR controlled genes outside of the aflatoxin gene cluster (Price *et al.* 2006).

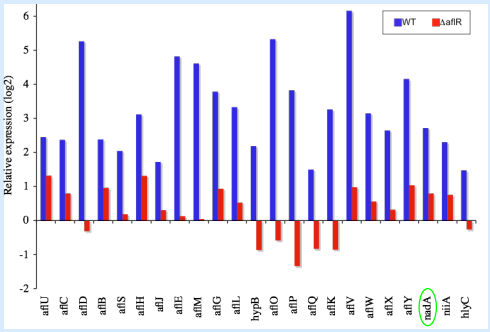

Figure 1 - *nadA* is transcriptionally controlled by AflR.

Genes that were significantly upregulated in SU1 vs. Δ*aflR* are shown. AflR controls three genes outside of the aflatoxin gene cluster: *nadA*, a putative NADH oxidase, *niiA*, encoding nitrate reductase, and *hlyC*, encoding a homolog of a α-hemolysin from *Aeromonas hydrophila*.

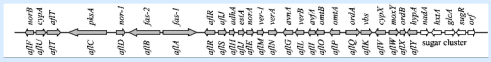

Figure 2 - *nadA* is located directly adjacent to the aflatoxin gene cluster.

nadA was first bioinformatically characterized as part of a sugar utilization gene cluster (Yu *et al*. 2000). It encodes a putative NADH oxidase, which has the capability of oxidizing NADH to form NAD+. Dehydrogenase enzymes involved in aflatoxin biosynthesis are thought to use NAD+ as a cofactor. We hypothesized that *nadA* is needed for aflatoxin biosynthesis based upon its genomic location, its transcriptional regulation, and its putative enzyme activity. A gene replacement construct was designed and three Δ*nadA* strains of *Aspergillus flavus* were obtained. These strains along with the wild type strain 3357 were grown in media containing either glucose, fructose, or sucrose as a carbon source. The media were analyzed for aflatoxin production by TLC. All strains produced similar amounts of aflatoxin (data not shown). One Δ*nadA* strain and the wild type strain were further analyzed for aflatoxin by LC-MS in a time course study.

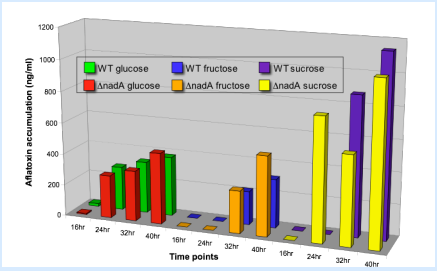

Figure 3 - Aflatoxin production is unaffected in Δ*nadA* strains.

The putative NADH oxidase, encoded by *nadA*, has homology to NADH oxidases in anaerobic bacteria. These enzymes protect the organism against oxygen exposure by either oxidizing O_2 to H_2O_2 or to H_2O. *nadA* contains an old yellow enzyme-like FMN binding domain, which is often found in the protein family NADH:flavin oxidoreductase/NADH oxidase (Interpro). Many proteins in this family have been associated with oxidative stress. Several studies have demonstrated a link between aflatoxin biosynthesis and oxidative stress in *Aspergillus parasiticus* (Jayashree *et al.* 2000; Narasaiah *et al.* 2006; Reverberi *et al.* 2005; Zjalic *et al.* 2006). We hypothesized that *nadA* may play a role in oxidative stress. The activities of several antioxidant enzymes were analyzed at multiple time points during the growth of a Δ*nadA* strain and the wild type strain NRRL 3357.

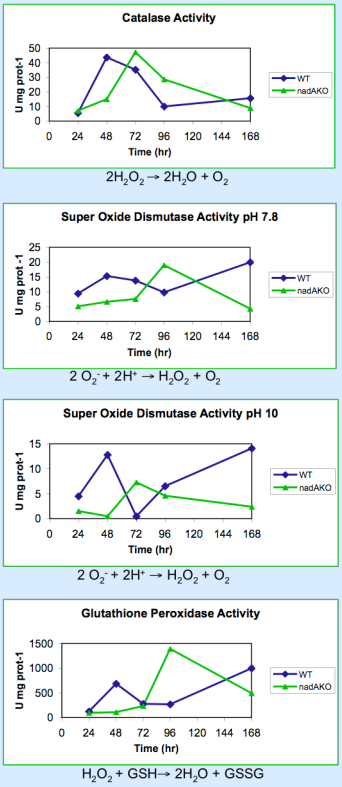

Figure 4 - Oxidative stress enzymes have delayed activity in a Δ*nadA* strain.

Lipoperoxides are also used as an indicator of oxidative stress. The accumulation of lipoperoxides over time in a Δ*nadA* strain and 3357 were analyzed.

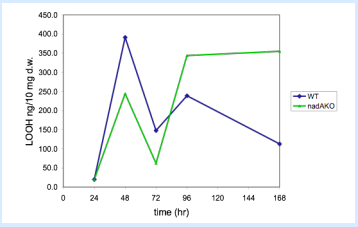

Figure 5 - Lipoperoxide accumulation is altered in a Δ*nadA* strain.

To further understand the function of *nadA*, *A. flavus* was cotransformed with an overexpression construct containing a *gpdA::nadA* cassette and a construct containing the *pyr4* gene from *Neurospora crassa*. Strains containing the overexpression construct (+C), transformed strains that did not contain the construct (-C), and a wild type strain were grown in A&M medium and analyzed for aflatoxin production by TLC. RNA was extracted and RT-PCR analysis was performed to test the expression of *nadA* in the strains containing the overexpression construct. The expression of the *gpdA* gene was also analyzed as a positive control for RT-PCR.

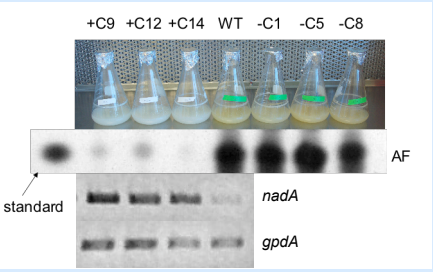

Figure 6 – Constitutive overexpression of *nadA* affects mycelial color and aflatoxin production.

In a wild type strain, we would expect to see *nadA* transcription after 12 hours of growth. Because *nadA* is constitutively expressed in the *nadA* overexpressing strains, *nadA* is expressed much earlier and the phenotype we are seeing may not really reflect the native role of *nadA*. However, the *nadA* overexpressing strains demonstrate that NadA is able to have a functional role in the cell. We hypothesize that the NADH/NAD+ ratio has been shifted in the overexpressing strains and this may have altered metabolism. Similarly when NADH oxidase was overexpressed in yeast, they observed an overall shift in the distribution of metabolic fluxes in the cell (Vermuri *et al.* 2007; Heux *et al* 2006a; Heux *et al*. 2006b).

Conclusions

- *nadA* is not required for aflatoxin biosynthesis.
- The oxidative status is altered in Δ*nadA* strains.
- Overexpression of *nadA* may change the redox status of the cell and shift metabolism.

References

•Heux, S., Cachon, R., and S. Dequin. Cofactor engineering in Saccharomyces cerevisiae: Expression of a H2O-forming NADH oxidase and impact on redox metabolism. Metab Eng. (2006) 8: 303-14.
•Heux, S., Sablayrolles, J. M., Cachon, R., and S. Dequin. Engineering a Saccharomyces cereisia wine yeast that exhibits reduced ethanol production during fermentation under controlled microoxygnation conditions. Appl Environ Microbiol. (2006) 72: 5822-8.
•Jayashree, T. and C. Subramanyam. Oxidative stress as a prerequisite for aflatoxin production by Aspergillus parasiticus. Free Radic Biol Med. (2000) 29: 981-5.
•Narasaiah, K. V., Sashidhar, R. B., and C. Subramanyam. Biochemcial analysis of oxidative stress in the production of aflatoxin and its precursor intermediates. Mycopathologia. (2006) 162: 179-89.
•Price, M. S., Yu, J., Nierman, W. C., Kim, H. S., Pritchard, B., Jacobus, C. A., Bhatnagar, D., Cleveland, T. E., and G. A. Payne. The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. FEMS Microbiol Lett. (2006) 2: 275-9.
•Reverberi, M., Fabbri, A. A., Zjalic, S., Ricelli, A., Punelli, F., and C. Fanelli. Antioxidant enzymes stimulation in Aspergillus parsicius by Lentinula edodes inhibits aflatoxin production. Appl Microbiol Biotechnol. (2005) 69:207-15.
•Vermuri, G. N., Eiteman, M. A., McEwen, J. E., Olsson, L., and J. Nielsen. Increasing NADH oxidation reduces overflow metabolism in Saccharomyces cerevisiae. Proc Natl Acad Sci. (2007) 104: 2402-7.
•Yu, J., Bhatnagar, D., and T. E. Cleveland. Completed sequence of aflatoxin pathway gene cluster in Aspergillus parasiticus. FEBS Lett. (2004) 564: 126-30.
•Zjalic, S., Reverberi, M., Ricelli, A., Mario Granito, V., Fanelli, C., and A. Adele Fabbri. Trametes versicolor: a possible tool for aflatoxin control. Int J Food Microbio. (2006) 107: 243-9.

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