

The NADH oxidase, NadA, and its Role in Aflatoxin Biosynthesis



Carrie Jacobus¹, Gary Payne², Niki Robertson^{1,3}
 Department of Genetics¹, Department of Plant Pathology², Department of Botany³
 North Carolina State University, Raleigh, NC, USA, 27606



Abstract

The *nadA* gene is part of a gene cluster for sugar metabolism that lies adjacent to the aflatoxin gene cluster in *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. The enzyme encoded by *nadA* converts NADH to NAD⁺, cofactors needed for certain reactions in the aflatoxin biochemical pathway. Microarray experiments comparing gene expression between a wild type strain of *A. parasiticus* and an *afIR* deletion mutant showed that *nadA* expression was significantly reduced in the mutant background. These results were confirmed by quantitative RT-PCR and are consistent with the presence of a putative AfIR binding site upstream of the coding region of *nadA*. We hypothesized that NadA may be needed to supply NAD⁺ cofactors for the aflatoxin biosynthetic pathway and hence is upregulated by AfIR. In order to investigate a connection between *nadA* expression and aflatoxin production, a gene replacement construct was used to knock out *nadA* expression in *A. flavus*. Aflatoxin production in the presence of sucrose, fructose, or glucose was investigated in three independent *nadA* mutants. In each case, aflatoxin levels in the mutant strains were similar to those produced by the wild type strain. This suggests that NADH oxidase activity is somehow compensated for in the mutants or that the NADH oxidase is not required for aflatoxin production. Investigations are currently underway to characterize additional phenotypes of these mutants to better understand the role of NAD⁺ in aflatoxin biosynthesis.

nadA is identified as part of a sugar cluster

A sugar utilization gene cluster that lies adjacent to the aflatoxin gene cluster is present in *A. flavus* and *A. parasiticus*. This cluster contains four genes: 1) *nadA*, a putative NADH oxidase, 2) *hxtA*, a gene with homology to hexose transporters, 3) *glaA*, a gene that has homology to genes encoding α -1,4 or α -1,6-glucosidases, and 4) *sugR*, a putative sugar utilization regulatory protein.



Figure 1 - The sugar gene cluster lies adjacent to the aflatoxin gene cluster in the genome of *A. parasiticus*, *A. flavus*, and *A. nomius*

AfIR regulates *nadA*

A microarray experiment comparing gene expression in *A. parasiticus* in an *afIR* deletion strain vs. a wild type strain has revealed that AfIR controls some genes outside of the aflatoxin gene cluster. One of these genes is *nadA*, which suggests that it may in fact be part of the aflatoxin cluster and not the sugar cluster. This data is supported by the presence of a putative AfIR binding site upstream of the *nadA* coding region that is conserved in the three species of *Aspergillus*.

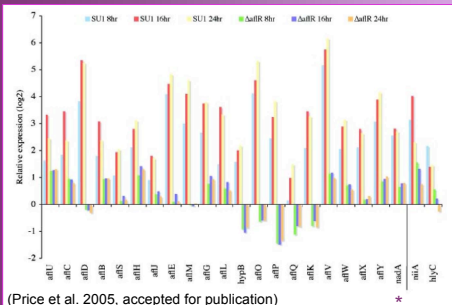


Figure 2 - In a $\Delta afIR$ background, *nadA* expression is significantly reduced at all three time points when compared to wild type

NAD⁺/NADH coenzymes in aflatoxin production

NADH oxidase provides NAD⁺, which is a necessary cofactor for some dehydrogenase reactions. The gene *afIH* (formerly *adhA*) encodes an alcohol dehydrogenase that catalyzes the conversion of 5-hydroxyaverantin (HAVN) to oxoaverantin (OAVN). Another part of the aflatoxin pathway in which norsolorinic acid (NOR) is converted to averantin (AVN) mainly by the enzyme encoded by *afID* (formerly *nor-1*) is affected by NAD⁺(or NADP⁺)/NADH(or NADPH) ratios.

Sakuno et al. (2003)

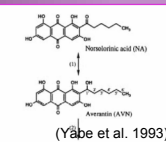
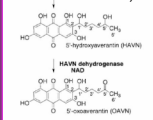


Figure 3 - Enzymatic reactions in aflatoxin biosynthesis in which NAD⁺ is implicated as a cofactor

nadA knockout

Because NAD⁺ is involved in aflatoxin production and *nadA* is controlled by AfIR, we hypothesized that *nadA* expression would be required for efficient aflatoxin production. To test if *nadA* is needed for aflatoxin production, a gene replacement construct was designed and used to knockout *nadA* expression.

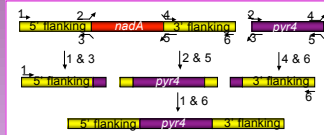


Figure 4 - Gene replacement construct

Three $\Delta nadA$ knockouts were obtained: $\Delta nadA146$, $\Delta nadA216$, and $\Delta nadA235$. All three mutants look phenotypically normal when grown on PDA media (only $\Delta nadA235$ is shown below).

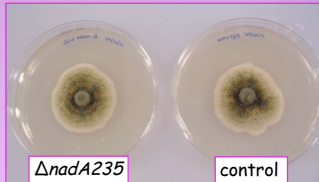


Figure 5 - No apparent growth defects $\Delta nadA$ on PDA medium

The absence of *nadA* does not significantly affect aflatoxin production on the tested media

Aflatoxin production in all three $\Delta nadA$ mutants was tested using sucrose, glucose, or fructose as carbon sources. Samples of media were taken from at 16 hrs, 24 hrs, 32 hrs, and 40 hrs and assayed for aflatoxin accumulation using HPLC analysis. The accumulation of aflatoxin in both the control and the mutant was similar for each carbon source.

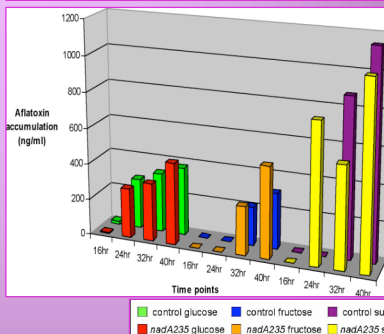


Figure 6 - Aflatoxin accumulation in the $\Delta nadA$ and wild type strains grown on glucose, fructose, or sucrose carbon sources.

Conclusion

Even though *nadA* expression is controlled by AfIR, NadA does not appear to be necessary for aflatoxin production when sucrose, fructose, or glucose is used as a carbon source. It is possible that the second NADH oxidase in the genome produces sufficient NAD⁺ to compensate for the *nadA* deletion or that NADH oxidase activity is not required for aflatoxin biosynthesis.

Future goals

- Identify other phenotypes of $\Delta nadA$
- Test on high salt medium
- Test on high sugar medium
- Test on range of pH
- Perform GCMS analysis to observe changes in metabolite production in $\Delta nadA$ mutant
- Microarray analysis of $\Delta nadA$ vs. wild type strains
- Knockout the second putative NADH oxidase gene separately and in parallel with *nadA*

References

Price, M.S., Yu, J., Nieman, W.C., Kim, S., Pritchard, B., Jacobus, C.A., Bhatnagar, D., Cleveland, T.E., and G.A. Payne. In press
 Sakuno, E., Yabe, K., and N. Hirotsu. 2003. Appl Environ Microbiol 69:6418-6426.
 Yabe, K., Matsuyama, Y., Ando, Y., Nakajima, H., and T. Hanasaki. 1993. Appl Environ Microbiol 59:2486-2492.
 Yu, J., Bhatnagar, D., and T.E. Cleveland. 2004. FEMS Lett 254:126-130.
 Yu, J., Chang, P., Bhatnagar, D., and T.E. Cleveland. 2000. Biochim Biophys 211:214.



Initiative for Future Agriculture and Food Systems



This work is supported by IFAFS/CISDA 2001-2003-12101-11-1107