FINAL REPORT

Lactose induction of sterigmatocystin formation in the filamentous fungus *Aspergillus nidulans* (Project: NN 116519)

Levente Karaffa PhD, DSc
Department of Biochemical Engineering, University of Debrecen

Introduction, scientific background

Aflatoxins (AF) are among the most carcinogenic natural substances known to date and the most important mycotoxins. They are produced by a large diversity of ascomycetous fungal species, mainly from the genus *Aspergillus* (Houbraken *et al.* 2007; Varga *et al.* 2009; Rank *et al.* 2011). These fungi can contaminate cereal crops and other staple commodities before harvest or during storage, leading to huge economic losses worldwide, and occasional famine in tropical countries (Wilkinson *et al.* 2004). In addition, upon exposure, the toxins can cause acute hepatic failure in humans and animals (Wogan 1992). AF can even penetrate into the milk and the eggs of animals that are fed with contaminated feeds.

Due to safety considerations, hazardous compounds such as AF are complicated to deal with in the laboratory. Importantly, instead of AT, one can more safely study another fungal compound named sterigmatocystin (ST), which is structurally similar to AF, but less potent. In fact, ST is the penultimate intermediate in the biosynthesis of AF in *A. flavus*; however, in several fungi including the model fungus *A. nidulans*, it is the end product of the corresponding pathway, lacking the last two genes present in *A. flavus* and other AF producing fungi (Amaike and Keller 2011). ST therefore shares practically all the regulatory mechanisms with AF, making it an ideal experimental tool to study AF biosynthesis. Much of our understanding of the genetics and molecular mechanisms of AF regulation comes from ST studies performed in *A. nidulans* (Amaike *et al.* 2013).

AF is a member of a large and diverse class of compounds known as polyketides (Hopwood and Sherman 1990). The AF/ST biosynthetic pathway is well-characterized in *A. nidulans* with all the structural genes and intermediates described, but many of the regulatory aspects including those related to the carbon source available for the fungus are still enigmatic (Maggio-Hall *et al.* 2005). This is particularly true for the heterodisaccharide lactose (milk sugar; 1,4-O-β-D-galactopyranosyl-D-glucose), the main carbohydrate in cheese whey, which is traditionally considered a cheap and abundant industrial growth substrate for micro-organisms, fungi in particular.

The principal investigator of the foreign matching proposal of this collaborative research project, Professor Nancy Keller (Department of Medical Microbiology and Immunology and Department of Bacteriology, University of Wisconsin-Madison, WI, USA) had described that while several metabolic steps and pathways in *A. nidulans* were apparently essential for ST biosynthesis on D-glucose, ST formation restored upon growth on lactose (Tsitsigiannis *et al.* 2005). The potential in this scientific problem and the complementary expertise in Madison and Debrecen prompted us to initiate a collaboration, formalized in this outgoing reserach project. This final report contains the results of our joint efforts from the past four years.

Method for the determination of ST concentration during submerged fermentation

Because of the economic and clinical importance of mycotoxins, lots of of studies have dealt with the development of a quantative method to measure ST. The easiest (and therefore most widespread) one to detect ST is thin layer chromatography, but this method is semi-quantitative at best, thus we opted to avoid it.

At the onset of the project we therefore developed a rapid, easy and reliable HPLC–UV method to isolate and detect ST from submerged fungal cultures. Because the concentration of ST in liquid cultures (even in complex media) is typically low (a few mg per litre), ST had to be concentrated in an organic phase. Several organic solvents have been tested with ethyl-acetate found as the most suitable one (partition coefficient of ST in water-ethyl-acetate mixture is q = 5.94 while in e.g. water-chloroform mixture, it was only q = 2.58). The sample has been extracted thrice with one and a half volume of ethyl-acetate, and then the organic phase was collected and evaporated. Samples were then resuspended in 1 ml of aceto-nitrile. This method is suitable to extract ST with an efficiency of 98.5% from water and an efficiency of up to 70 % from fungal cultures. The 30 percent loss could likely be attributed to the fact that ST binds strongly to the cell wall; however, the extraction efficiency could be successfully reproduced, thereby generating a standard error only.

Reverse-phase high performance liquid chromatography coupled to ultra-violet detection (RP-HPLC-UV) has been employed to determine ST concentrations. The mobile phase applied was a water : acetonitrile (4 : 6) mixture buffered with acetic-acid and Na-acetate (pH 4.76), at a flow rate of 0.5 ml/min with isocratic elution. The acidic solvent was necessary to separate ST from other products of fungal metabolism such as proteins and nucleotides. Since ST is a stable molecule (pK_a= 9.58), pH of the mobile phase did not modify structure and thus the elution properties. Temperature of the column was kept at T = 55 C°, detection occurred at λ = 245nm. Under these conditions the retention time of ST is 11.9 minutes, while the detection limit is 0.1 mg L^{-1}, which is safely over the concentration range *A. nidulans* can produce under submerged conditions, even in minimal medium. Accordingly, concentration of ST was measured as being between 0.4-0.7 mg L^{-1} in minimal medium and up to 2.5 mg L^{-1} in complex medium. However, like I noted above, actual final ST-concentrations could be some 30 percent higher.

The importance of VeA in ST biosynthesis

One of the genes important for ST production is the so-called “velvet” gene (*veA*). VeA is involved in the regulation of a variety of cellular processes such as asexual and sexual development as well as secondary metabolism. The wild type allele is *veA* while *veA1* is the mutation and one can miss important metabolic connections and identify artifacts using mutants with veA1 background. In *A. nidulans*, VeA has been shown to control the AF/ST regulatory gene aflR and, subsequently, ST production. Unfortunately, the lactose/D-galactose mutants in our strain collection as well as several widely used reference (wild-type) strains of *A. nidulans* were apparently all *veA1*, as vegetative growth stage events including primary metabolism in general are likely not influenced by *veA1*, since they are not directly involved in differentiation. Still, the issue of *veA* had to be settled. Therefore, to address the relative importance of VeA and its interaction with carbon source, we tested ST production in *A. nidulans* in both *veA* and *veA1* background. Two *veA1* mutant strains were tested. On both carbon sources, biomass-specified ST production was 10-15 times higher in the strain containing the wild-type allele, and maximal ST concentrations in the *veA1* mutant cultures were just slightly above the detection limit of our system (**Figure 1.**). While maximal ST concentration values (given in mg

L^{-1}) were higher on D-glucose than on lactose, biomass-specific ($\mu g_{ST}\ g_{DCW}^{-1}$) ST production was significantly ($p<1\%$) higher on lactose, due to the comparatively reduced growth on this carbon source. Data also revealed that even in a *veA* (wild-type) background, the ratio of ST produced to biomass formed is low, approximately one to ten-thousand.

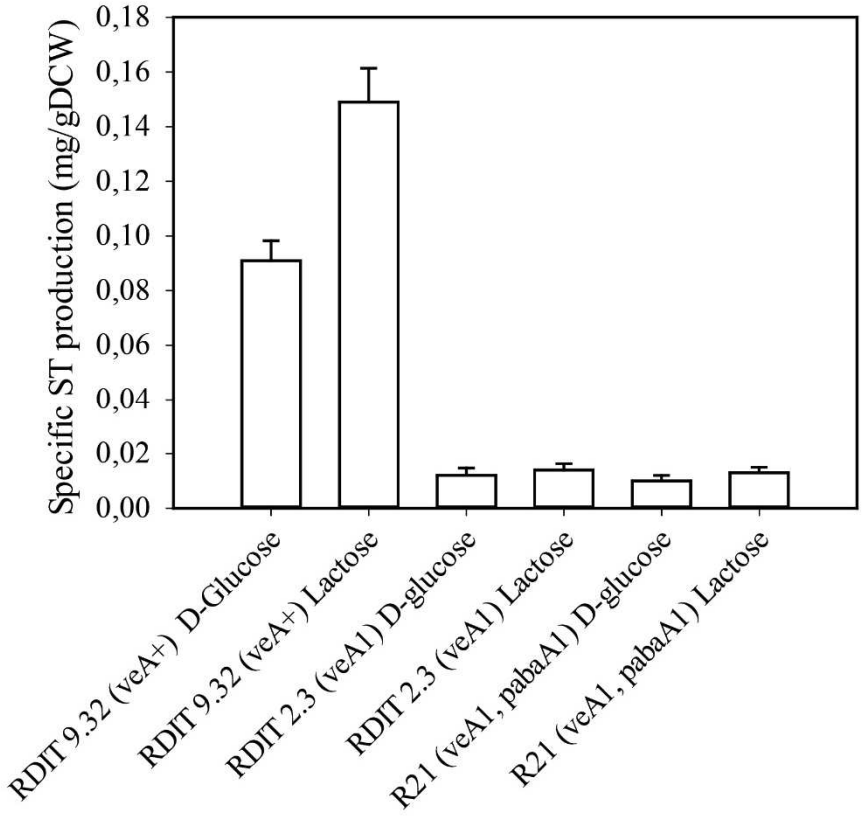

Figure 1. Maximal specific sterigmatocystin (ST) production of *A. nidulans* strains carrying the *veA* (wild-type) or the *veA1* (mutant) allele in liquid minimal medium initially containing 15 g/L D-glucose or lactose as sole carbon sources.

Kinetics of wild-type ST formation on D-glucose and lactose

The experimental setup we employed allowed us to investigate the ST production of *A. nidulans* in liquid cultures, which – due to its more homogenous nature – are more suitable for quantitative kinetic studies than the agar-solidified medium. While production rate of ST under liquid (= submerged, batch) conditions is four to five times lower than on solidified medium, the optimized extraction and analytical protocol (see above) still allowed us to determine the actual ST concentration in a reproducible way.

Saprophytic fungi such as *A. nidulans* never encounter lactose in their natural habitats, which renders it a poor carbon substrate that results in slow growth (i.e., low specific growth rates). In order to analyse the relationship between carbon utilization rate and ST formation in *A. nidulans*, we cultivated the wild-type strain on minimal medium with D-glucose – which is rapidly metabolized – and lactose. Time-profiles of ST formation were markedly different: on D-glucose, ST could be detected only after D-glucose was completely depleted from the medium, while on lactose ST already appeared in the growth phase, when the majority of the carbon source was still available. Although growth stopped as a result of D-glucose depletion,

ST production continued for an additional 70 hours, ultimately reaching a concentration of 2.5 mg L^{-1}. The final ST titer in lactose medium was even higher (2.8 mg L^{-1}), resulting in a significant difference regarding biomass-specified ST production (**Figure 2.**).

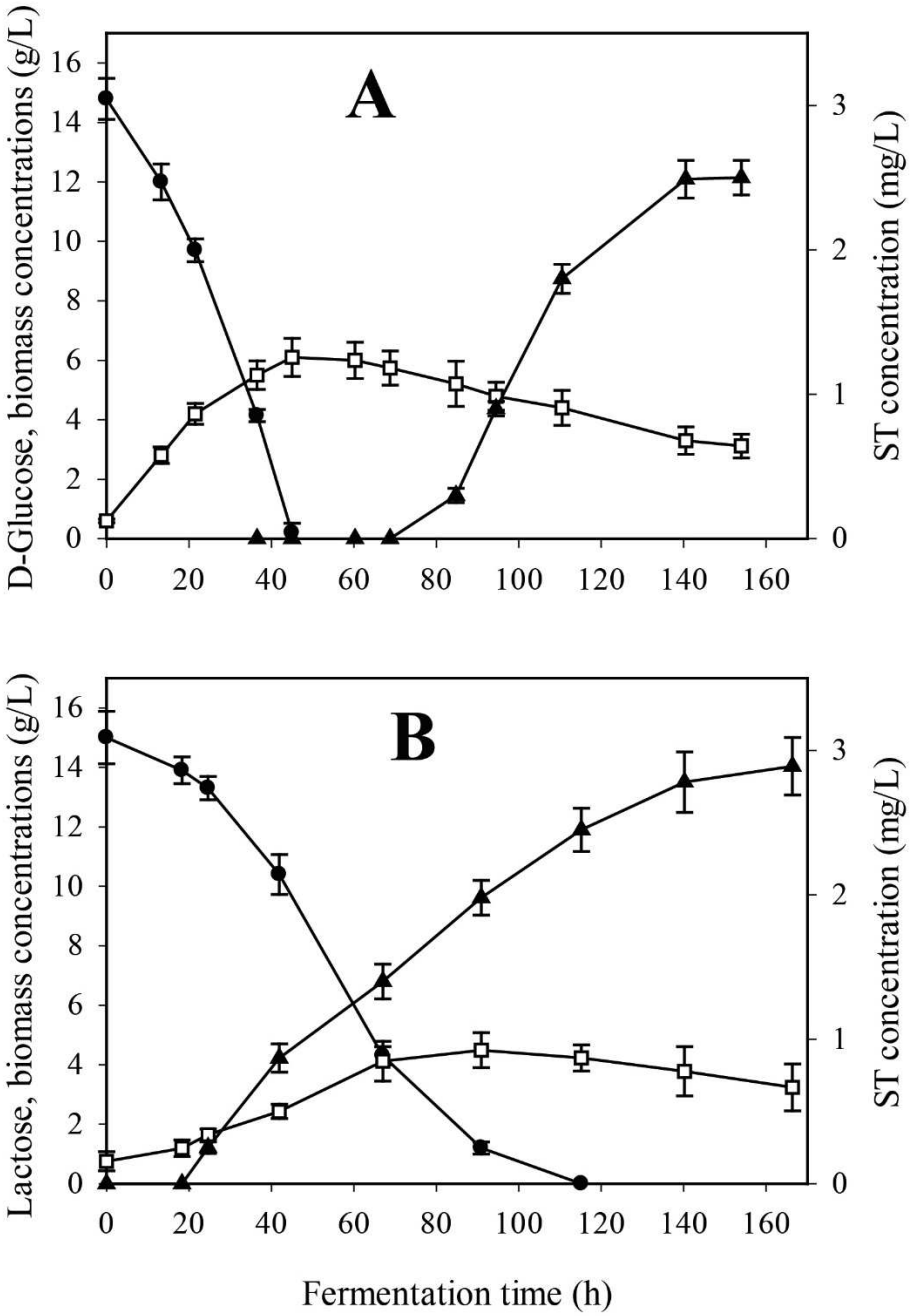

Figure 2. Time-profile of growth (□), residual carbon source concentrations (●) as well as sterigmatocystin (ST) production (▲) in batch fermentations of an *A. nidulans* wild-type strain in minimal media initially containing 15 g/L sole carbon substrate. (A): D-glucose, (B): lactose.

The aforementioned kinetic data suggested that ST production does not occur before the complete exhaustion of D-glucose. To investigate whether the reverse of this correlation also holds true – i.e., whether D-glucose inhibits already ongoing ST production – we employed fed-batch cultivations. By the time ST concentration in the wild-type *A. nidulans* culture

reached 1 mg L^{-1}, extra D-glucose was added to the medium. The extra D-glucose mainly went to renewed biomass production, whereas ST-production stopped immediately. The D-glucose was again rapidly exhausted, after which ST production resumed (**Figure 3.**).

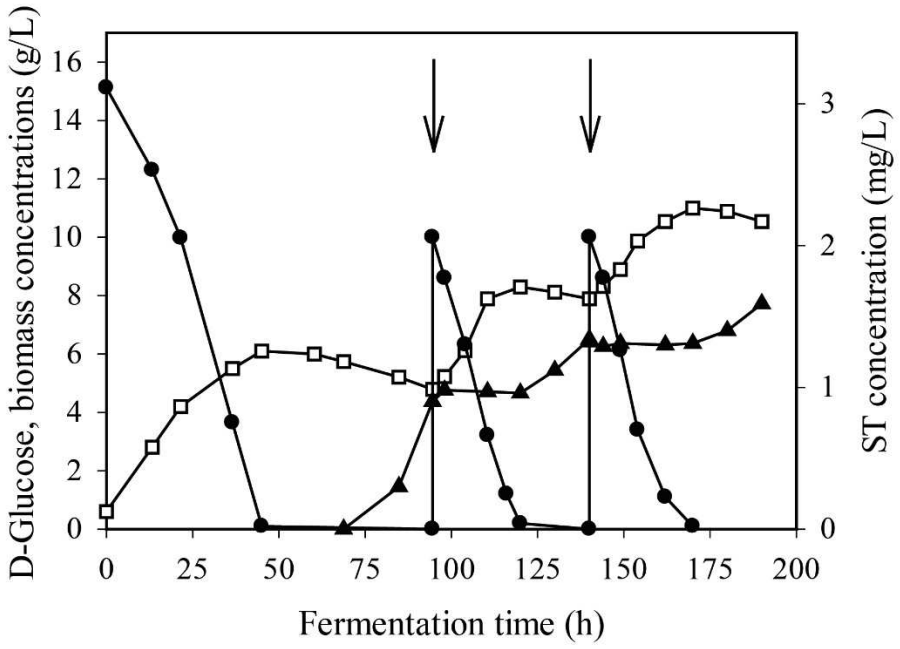

Figure 3. Time profiles of growth (□), residual D-glucose concentrations (●) as well as sterigmatocystin (ST) production (▲) in fed-batch fermentations of an *A. nidulans* wild-type strain in minimal media. Additional D-glucose (indicated by plain arrows) was added at 94 h and 140 h.

These results suggested that ST-formation in *A. nidulans* may either be mediated by a carbon catabolite regulatory mechanism prominent on D-glucose, or induced by the low specific growth rate typical on lactose and also under D-glucose limitation. To test this hypothesis, we repeated the submerged fermentations with a carbon catabolite derepressed CreA-mutant strain. Time-course of D-glucose uptake and biomass formation were similar to those of the wild-type strain, whereas lactose disappeared from the growth medium of the CreA-mutant almost as rapidly as D-glucose did from the wild-type culture. Most importantly, ST appeared only at the very late stages of the lactose fermentations, when mycelia were already starting to disintegrate, thereby resembling the time-profile of the wild-type strain on D-glucose, albeit with lower ST concentrations. Thus, it appears that carbon assimilation rate and the resulting biomass production rate inversely correlate with ST formation in *A. nidulans*.

Growth rate effect over sterigmatocystin formation

The above results suggest that ST formation is initiated either by the reduced growth rate or by the general relieve from carbon catabolite repression (CCR). Since one can not discriminate between these two effects in a batch or fed-batch culture, we turned to chemostat cultures where the specific growth rate was set according to the actual dilution rate. A set of constant-mass, D-glucose-limited continuous fermentations of the wild-type and the CreA-loss of function *A. nidulans* strain were performed at two different dilution rates (0.090 h^{-1} and 0.020 h^{-1}), representing a state of carbon catabolite repression and derepression, respectively, in *A. nidulans* (Ilyés *et al*. 2004). Analysis of ST production in these chemostat cultures showed that

the dilution rate (= specific growth rate) negatively correlates with ST production. At high growth rate, no ST formed. In contrast, low growth rate led to the formation of 0.5 mg L^{-1} ST. Similar results were obtained with the CreA-loss of function mutant strain, indicating that CreA does not regulate the formation of ST during growth on D-glucose. Essentially identical results to the wild-type reference strain were obtained with the CreA-loss of function mutant, providing evidence that CreA is not directly involved in the regulation of ST formation during growth on D-glucose. Since the residual D-glucose concentration was set close to zero in each chemostat culture irrespective of the dilution rate, we also concluded that the depletion of D-glucose itself is not an initiator but rather a prerequisite for ST biosynthesis through the cessation of growth.

In summary, as opposed to CCR and carbon availability in the medium, it is the growth rate that seems to have a direct, causal relationship with ST formation. Lactose is a poor carbon source for *A. nidulans*, and we believe the low growth rate caused by its slow assimilation will trigger sustained ST formation. By the same token, faster growth means the fungus has to put more resources into primary metabolism by creating cell materials needed for growth, and has less ability to shunt to ST. When lactose utilization rate gets higher (as in the CreA loss-of-function mutants), ST production will not occur before its complete exhaustion, just like on D-glucose.

Regulation of ST formation by the alternative oxidase activity and by light

Alternative oxidase (AOX) has been reported to be present in many organisms, especially in higher plants and fungi. Responsible for this activity is the cyanide-resistant alternative oxidase (AOX), a quinol oxidase localized in the inner mitochondrial membrane (Del-Saz *et al.*, 2018). AOX is induced by stresses such as wounding, chilling, drought, osmotic stress and pathogen attack, in addition to treatment with salicylic acid, H_2O_2 or with inhibitors of the cytochrome-dependent respiratory chain. Unlike most of the cytochrome C oxidase (COX) subunits AOX is encoded in the nuclear genome. It functions as an 'alternative' for the electron flow opposite to the cytochrome-dependent pathway. The site of the branching point from the main chain is at the level of coenzyme-Q and then electrons are passed directly to oxygen without producing ATP. Therefore, the alternative path is resistant to the inhibitors of complex III and IV such as cyanide, nitric oxide, azide, and sulfide, but can be blocked selectively by aromatic hydroxamic acids like salicylic-hidroxamate (SHAM). However, unlike the COX respiratory pathway, the AOX pathway moves fewer protons across the inner mitochondrial membrane to generate a proton motive force that can be used to synthesise ATP. This pathway provides low levels of ATP that represent only 40% of the normal efficiency for energy conservation. This is due to the fact that complexes III and IV of the mitochondrial electron transport system are bypassed, and AOX lacks proton pumping activity (**Figure 4**).

Unlike many other related fungi, *A. nidulans* features only a single alternative oxidase gene named *aodA* (GenBank Accession number AB039832). Functionally, AOX is associated with metabolic conditions during which it enables the cell to uncouple NADH re-oxidization from ATP synthesis, allowing carbon catabolism to continue. This also occur during secondary metabolite production, like that of ST, under the prevalent low-energy conditions of the stationary growth phase. Within the research project we have studied possible relationships between AOX activity and ST production of *A. nidulans* liquid cultures, employing well-controlled cultivation conditions conductive to ST synthesis in bioreactors. In addition, *aodA* was both deleted (delta strain) and overexpressed (OE strain), in a *veA*+ background.

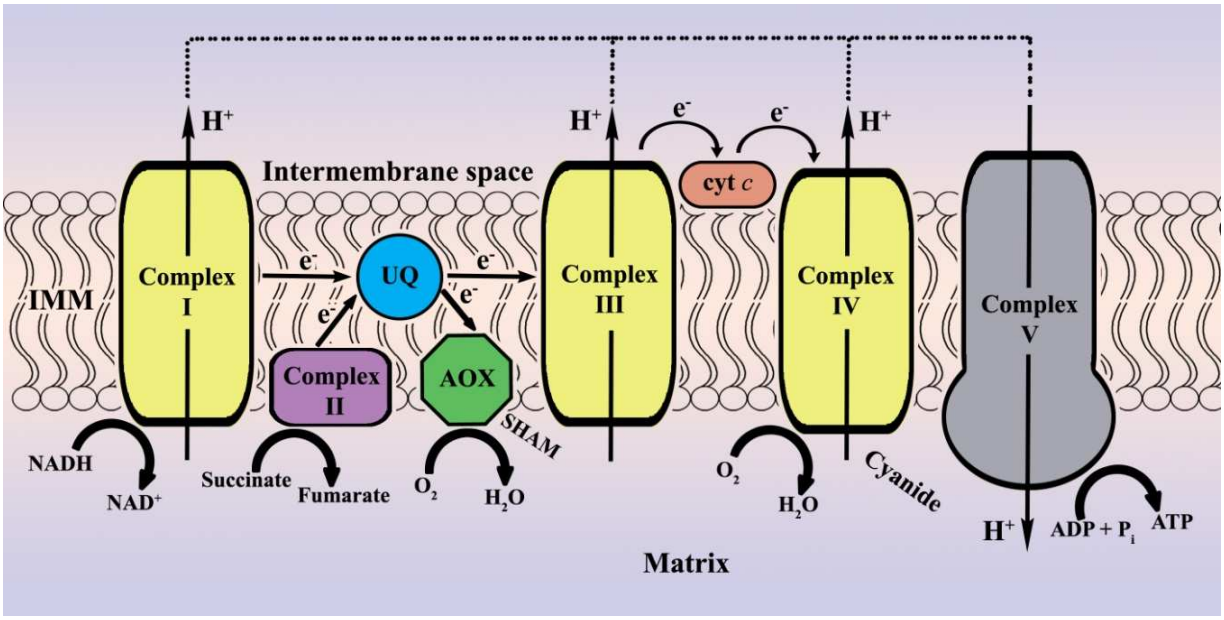

Figure 4. schematic overview of cytochrome-mediated- and alternative respiration. Modified from Figure 1 in the paper of McDonald *et al*. (J. Exp. Biol., 2009, 212, 2627-2634).

As the above results demonstrated, ST formation only occurs at low specific growth rates. In a medium with D-glucose as growth substrate, ST production occurs only after carbon source exhaustion. Under the cultivation conditions used, maximal rate of ST formation occured 90 h after inoculation, while maximal ST concentrations were achieved at about 140 h.

Kinetics of growth in the D-glucose minimal medium was similar in every *A. nidulans* strain investigated, while D-glucose utilization rates as well as AOX activity – both early in the rapid growth stage and late in the carbon-depleted cultures – were proportional to the *aodA* copy number, regardless of the presence or absence of light. ST time-profiles were qualitatively similar in that ST production did not occur before the complete depletion of D-glucose and the cessation of growth. Quantitatively, however, statistically significant differences were found. ST volumetric yield (mg L^{-1}) of the *A. nidulans* aodA deletant strain was about half (50 %) of that of the wild-type reference when grown in the dark. By contrast, *aodA* OE mutants featured an 50 – 70 % increase in ST yield relative to the control strain, again in a copy-number dependent manner. Importantly, results were quite different when cultures were continuously illuminated: ST volumetric yields of all cultures went down to about a third of what the control strain produced when grown in the dark, and were statistically unvarying, regardless the presence, absence or the copy number of *aodA* (**Figure 5**). This effect of light on ST production was confirmed upon testing point-inoculated plate cultures. Although production rates of ST in submerged cultures were significantly (up to five times) lower than on solidified medium, the trends were similar: in the dark, the *aodA* deletant yielded some 60 % less, while the OE mutants produced more ($p < 0.1$) ST compared to the wild-type control, in positive correlation with the number of functional *aodA* genes. In the light, no such correlation existed, and all the strains investigated in this study produced statistically non-varying ($p < 1$) amounts of ST, that were approximately one-half of what the reference strain yielded on plates when grown in the dark. For the *ΔaodA* strain, i.e., in the absence of alternative respiration, the ST yield on plates held in the dark is only marginally different from that accumulated when plates were subjected to continuous light (**Figure 6**).

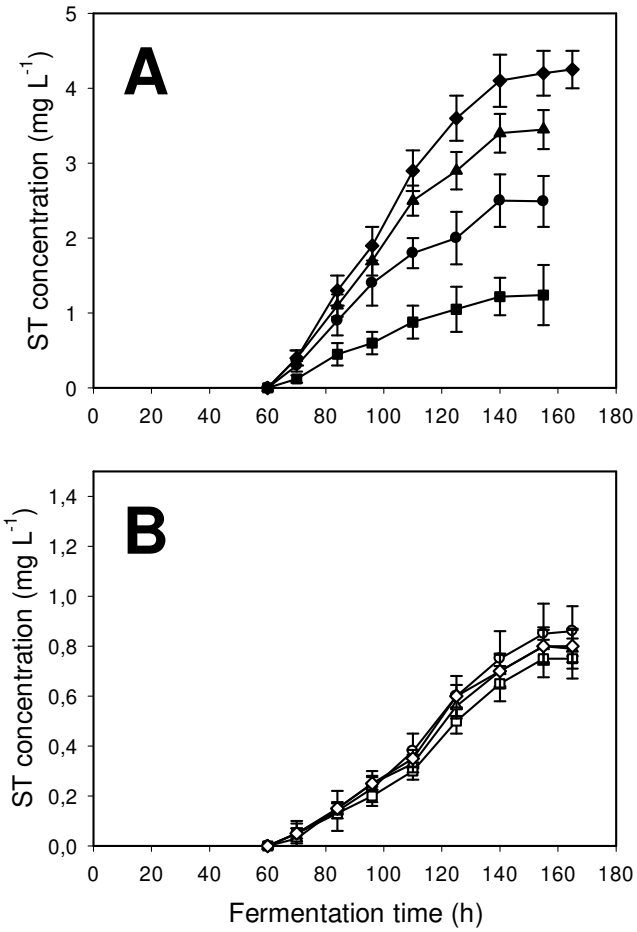

Figure 5: Time-profile of sterigmatocystin production in submerged fermentations of *A. nidulans* grown in the dark (black symbols, Panel A) and in the light (white or open symbols, Panel B) in minimal media initially containing 15 g L^{-1} D-glucose as the sole carbon substrate. The wild-type reference strain is indicated by circles [●,○], the *aodA* deletant strain by squares [■,□], the *aodA* 2-copy strain by triangles [▲,△], and the *aodA* 3-copy strain by diamonds [◆,◇].

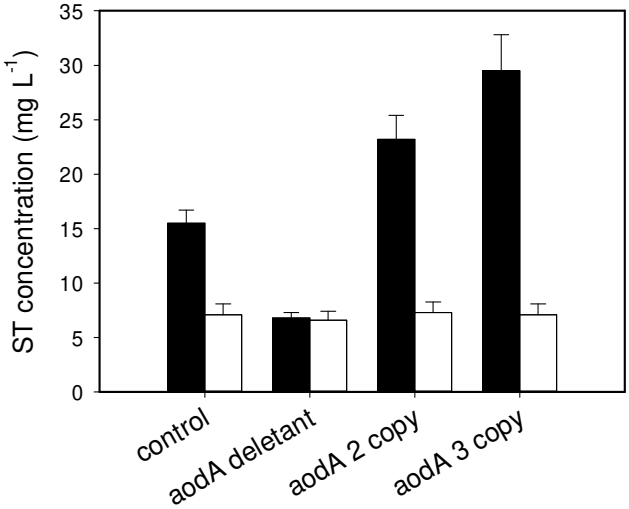

Figure 6: Sterigmatocystin production in point-inoculated ("single colony") plate cultures of *A. nidulans* strains grown in the dark (black bars ■) or in continuous light (white or open bars □) on agar-solidified minimal medium with 15 g L^{-1} D-glucose as the sole source of carbon.

By which mechanism(s) could AOX contribute to ST production? ST production takes place at low growth rate and requires considerable amounts of acetyl-CoA, ATP and NADPH, so in the late stationary growth phase, this anabolic effort is serious. Recycling of the catabolic reducing equivalents (NADH) released during the mobilization of reserve carbon sources by COX would result in high ATP formation. However, in the absence of growth, mycelia do not require high energy levels, and oxidative phosphorylation under such conditions inhibits further carbon catabolism. A switch from COX to AOX would enable the fungus to reoxidize NADH and maintain redox homeostasis without concomitant ATP production under low energy requiring conditions. Indeed, at the higher ST yields when mycelia were grown in the dark, ST production was positively correlated with AOX activity both in solid and submerged cultures.

Irrespective of the mechanisms by which AOX activity stimulates ST synthesis, it is clearly subordinate to antagonistic effects associated with the presence of light. The velvet transcription factor, of which ST synthesis depends, is concentrated in the nucleus when the fungus grows in the darkness on a minimal D-glucose medium, but resides in the cytosol when the fungus grows in the light (Stinnett *et al*., 2007). In our experiments, a velvet wild type strain produced over twice as much ST when grown in the dark, regardless whether the cultivation was on agar plates or in fermenters. Our results with the *aodA* mutant backgrounds imply that a basal level production under illumination ("stage 1") is essentially unchanged regardless the absence or presence of AOX, or its exact amount. Intriguingly, essentially the same levels of ST were detected in cultures of the *ΔaodA* deletion strain when cultivated protected from light, both in liquid and on plates. Our results further suggest that any increase in ST synthesis over the basal level ("stage 2") can only realized in the darkness in genetic backgrounds that express AOX, i.e., in strains that allow varying the ratio between alternative and cytochrome-mediated respiration. We therefore speculate that only "stage 2" expression is mediated by the Velvet Complex, and that "stage 1" (basal level) expression may not require the velvet protein.

The multi-cluster regulator (mcrA) story

A few years ago a new transcription factor gene was isolated and characterized from *A. nidulans* (Oakley *et al.*, 2017). The geneproduct was described as a usually (but not ultimately) negative regulator of the biosynthesis of various metabolites, including that of ST. Formation of ST was reported to increase in the delta and to decrease in the overexpresssing mutant. However, neither the growth of the mutant strains, nor the carbon source-dependent regulation of ST biosynthesis was tested. We foresaw possibilities in this area and thus requested a year-long extension of the grant proposal.

We first created a delta *mcrA* mutant in veA background to analyze phenotype in veA+ as a prototroph. This was necessary because the original publication used a *veA+* mutant strain. As we described in this report earlier, VeA plays a major role in ST formation. Therefore, our results cannot be scientifically compared to those of Oakley *et al* (2017) by default.

In contrast to Oakley *et al*. (2017), the *veA+ mcrA* delta mutant did not show increased ST formation in terms of final maximal concentration. However, the growth rate of the mutant was significantly reduced relative to the WT. We had demonstrated the crucial role specific growth rate plays in ST formation (see above). Since slow growth (e.g. on lactose) prompts ST formation even in the presence of available carbon source, and rapid growth delays the onset of ST production, it was not surprising that ST appeared in the medium of the deletion strain much earlier. The ultimate ST yield was not statistically different, however, it just showed a different production kinetics. However, even at this preliminary stage, our results again appear to suggest the regulatory importance of the growth rate in ST formation.

We agreed with our international collaborating partner Professor Keller that the role of *mcrA* in ST biosynthesis needs further analysis. Because the original paper that described *mcrA* cannot be used as a reference, we are still in the experimental stage of this particular sub-project.

Other research projects more remotely related to the grant proposal

Organic acid overflow in filamentous fungi

Citric acid production by *Aspergillus niger* and itaconic acid production by *Aspergillus terreus* has been investigated for several years now in our lab. We are particularly interested in the molcular mechanisms of the so-called „manganese effect", the inhibitory role Mn(II) ions play in the overflow of both acids. Several publications on this topic including a review paper were published over the past four years.

Spliceosomal twin introns (stwintrons) in filamentous fungi

Introns are sequences that interrupt open reading frames in RNA. Spliceosome introns are exclusive of eukaryotic nuclear gene transcripts. We earlier described a new type of intervening sequences in fungi called "stwintrons" for "spliceosomal twin introns" where the excision of an internal intron is obligatory for the splicing of the external intron in order to yield the appropriate open reading frame. The stwintron phenomenon is a novelty and a key aim of our proposal is discover as many new features as possible. We have published a few papers also on this topic, including one in the prestigeous journal Nucleic Acids Research.

Metabolism of lignocellulose monomers in filamentous fungi

This is one of the oldest topics of our lab. Due to the overlapping functions pentoses and D-galactose play in the nutrition of fungi, we hypothetized – and eventually demonstrated – that each catabolic gene of their catabolic pathways will be responding by increased levels of expression to the presence of either sugar, irrespective of whether the enzymes encoded are actually involved in the catabolism of the given sugar. Results were published in early 2019.

Community-wide comparative projects

Time after time we are invited to participate in dedicated community efforts, related mostly to the carbon metabolism of filamentous fungi. Within the framework of this grant, we took part in a genomics study of ten highly diverse *Aspergillus* species, and compared them to sister and more distant genera. While overall, genome evolution appears to follow taxonomy, deviations of this have been identified for specific gene systems, possibly due to environmental pressure.

References

Amaike S, Affeldt K, Keller NP (2013): Genetics, Biosynthesis and Regulation of Aflatoxins and Sterigmatocystin. Ed: F Kempken In: The Mycota XI, (Springer-Verlag Berlin Heidelberg) p. 59-74.

Amaike S, Keller NP (2011): Aspergillus flavus. Annu. Rev. Phytopathol. 49:107-133.

Del-Saz NF, Ribas-Carbo M, McDonald AE, Lambers H, Fernie AR, Florez-Sarasa I (2018): An *in vivo* perspective of the role(s) of the alternative oxidase. Trends Plant Sci. 23: 206–219.

Hopwood DA, Sherman DH (1990): Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Ann. Rev. Gen. 24: 37-66.

Houbraken J, Due M, Varga J, Meijer M, Frisvad JC, Samson RA (2007): Polyphasic taxonomy of Aspergillus section Usti. Stud. Mycol. 59: 107-128.

Ilyés H, Fekete E, Karaffa L, Fekete É, Sándor E, Szentirmai A, Kubicek CP (2004): CreA-mediated carbon catabolite repression of β-galactosidase formation in *Aspergillus nidulans* is growth rate dependent. FEMS Microbiol. Letts. 235: 147-151.

Maggio-Hall LA, Wilson RA, Keller NP (2005): Fundamental contribution of β-oxidation to polyketide mycotoxin production in planta. Mol. Plant-Microbe Interact. 18: 783-793.

Oakley CE, Ahuja M, Sun WW, Entwistle R, Akashi T, Yaegashi J, Guo CJ, Cerqueira GC, Russo Wortman J, Wang CC, Chiang YM, Oakley BR (2017): Discovery of McrA, a master regulator of Aspergillus secondary metabolism. Mol. Microbiol. 103: 347-365.

Rank C, Nielsen KF, Larsen TO, Varga J, Samson RA, Frisvad JC (2011): Distribution of sterigmatocystin in filamentous fungi. Fung. Biol. 115: 406-420.

Stinnett SM, Espeso EA, Cobeño L, Araújo-Bazán L, Calvo AM (2007): Aspergillus nidulans VeA subcellular localization is dependent on the importin alpha carrier and on light. Mol. Microbiol. 63: 242–255.

Tsitsigiannis DI, Kowieski TM, Zarnowski R, Keller NP (2005): Three putative oxylipin biosynthetic genes integrate sexual and asexual development in Aspergillus nidulans. Microbiol-SGM 151: 1809-1821.

Varga J, Frisvad JC, Samson RA (2009): A reappraisal of fungi producing aflatoxins. World Mycotox. J. 2: 263-277.

Wilkinson HH, Ramaswamy A, Sung CS, Keller NP (2004): Increased conidiation associated with progression along the sterigmatocystin biosynthetic pathway. Mycologia 96: 1190-1198.

Wogan GN (1992): Aflatoxin carcinogenesis: interspecies potency differences and relevance for human risk assessment. Prog. Clin. Biol. Res. 374: 123-137.