The Third *Aspergillus* Meeting

University of Natural Resources and Applied Life Sciences
Vienna, Austria
April 8th, 2006

Organized by the
***Aspergillus* Genomes Research Policy Committee**

Aspergillus Genomes Research Policy Group (AGRPG)

In the *Aspergillus* Genomics workshop held at the March 2003 Asilomar Fungal Genetics meeting, a provisional *Aspergillus* Genomes Research Policy Committee (AGRPC) was conscripted and charged with creating a structure for community-wide coordination and organizing an annual meeting. The First *Aspergillus* Meeting, held in Copenhagen, April 21, 2004, as a satellite meeting of the European Congress on Fungal Genetics-7, was attended by 160 *Aspergillus* researchers. At this meeting statutes were adopted and committee members were elected. The Second *Aspergillus* Meeting, held March 13-15, 2005, at Asilomar as a satellite of the 23rd Fungal Genetics Meeting, was attended by 102 *Aspergillus* researchers. For more information on activities of the AGRPG including meeting programs and minutes see http://www.fgsc.net/Aspergillus/asperghome.html.

2005 AGRPC:

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**Special 2005 appointments.*

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Third *Aspergillus* Meeting
University of Natural Resources and Applied Life Sciences (BOKU)
Lecture Halö XX, Muthgase 18, 1190, Vienna, Austria
April 8th, 2006

8.00 Breakfast sponsored by DSM

Session 1 — AGRPC Chair, Michelle Momany, University of Georgia, USA

9.00 Welcome

9.10 Berl Oakley, Ohio State University, USA
Coordination of microtubule and actin functions is essential for rapid hyphal growth of *Aspergillus nidulans*

9.40 Arthur F.J. Ram, Leiden University, Netherlands
Multiple roles of the small GTPase RACA during the life cycle of *Aspergillus niger*

10.00 Ana Calvo, Northern Illinois University, USA
VeA subcellular localization is dependent on light in the filamentous fungus *Aspergillus nidulans*

10.20 Coffee Break

Session 2: Omics Updates — Chair, Paul Dyer, University of Nottingham, UK

10.40 Bill Nierman, The Institute for Genomic Research (TIGR), USA
Update on genome sequencing of *Aspergillus clavatus*, *A. terreus*, and *Neosartoya fischeri*

10:50 Jiujiang Yu USDA/ARS and The Institute for Genomic Research (TIGR), USA
Progress of genome sequencing of *Aspergillus flavus*

11:00 Scott Baker (Pacific Northwest National Laboratory, USA) and Igor Grigoriev (DOE Joint Genome Institute, USA)
Progress of DOE-JGI *Aspergillus niger* genome sequencing project and annotation update

11:20 Herman Pel, DSM, the Netherlands
Update on DSM *Aspergillus niger* genome sequencing project

11.35 Linda Lasure, Pacific Northwest National Lab, USA
Application of proteomics to *Aspergillus* research

11.55 Discussion and Elections — Michelle Momany

12:25 Lunch

Session 3: Talks from abstracts

Chair, Michael Hynes
University of Melbourne, Australia

1.30 Jens C. Frisvad, Denmark Technical University, Denmark
Aspergillus taxonomy updated

1.45 Hui-Lin Liu, The Ohio State University, USA
Affinity purification and mass spectrometric analysis identifies two novel proteins required to maintain the mitotic core of the *Aspergillus nidulans* nuclear pore complex

2.00 Katsuya Gomi, Tohoku University, Japan
Crawler, a novel transposable element of Tc1/mariner superfamily in *Aspergillus oryzae* transposes under stress conditions

2.15 Fatih Sari, Georg-August-University Göttingen
Analysis of the protein kinase ImeB in *Aspergillus nidulans*

2.30 Carrie Jacobus, North Carolina State University, USA
Trans-sensing mechanism in a diploid strain of *Aspergillus flavus*

2.45 Gethin Allen, University of Sheffield, UK
Functional analysis of essential genes in *Aspergillus fumigatus*

3.00 Session 4: Posters and reception sponsored by the British Mycological Society

4.15 Claudio Scazzocchio, Universite of Paris-Sud, France
Pontecorvo Lecture: Musings on *Aspergillus*
sponsored by Gilead

5.00 Meeting close: Michelle Momany
DSM Poster Prize and Election Results

Special thanks to meeting organizer Paul Dyer (University of Nottingham, UK) and local arrangements organizer Joseph Strauss (University of Natural Resources and Applied Life Sciences in Vienna).

1. FUNCTIONAL ANALYSIS OF ESSENTIAL GENES IN ASPERGILLUS FUMIGATUS

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Essential gene products are seen as potential targets for developing antifungal drugs, and genome wide screening methods to identify such genes have been devised in many laboratories. These have made use of random insertion approaches in diploids, Where failure to recover haploid segregants after haploidisation is considered evidence of essentiality.
Genes identified by such a procedure may also include those whose deletion leads to impaired growth, rather than no growth, since the latter could also result in failure to recover haploid mutant segregants.
In many cases bioinformatics can provide information about the probable function of such essential genes. However, often very little is known about apparent orthologuous genes, and orthologs may not have identical functionality in different species. Bioinformatics can be used to analyse the predicted primary sequences of gene products, but this may reveal very little about their functions.
Establishing or confirming gene function therefore requires experimental analysis, including mutation. Since haploid mutants are often inviable, other approaches are needed for functional analysis of essential genes.
These include recovery of haploid spores carrying lethal deletions from balanced heterokaryons, use of conditional promoters like the alcA promoter (Romero et al(2003) Fungal Genet Biol 40:103–114), and protein tagging.
A selection of putative essential genes have been analysed by downregulation after promoter exchange, and total deletion, the results and limitations of which will be discussed.

2. CHARACTERIZATION OF THE ASPERGILLUS NIDULANS PUTATIVE BLUE LIGHT RECEPTOR GENE CRYA ENCODING A CRYPTOCHROME

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Cryptochromes have important roles in the blue light response and circadian rthyms of filamentous fungi, plants, flies, or mammals. The Aspergillus nidulans genome revealed one copy of a putative cryptochrome-encoding gene, conclusively named cryA. The deduced amino acid sequence from this locus shows 29 % identity to the Arabidopsis thaliana cry1 gene product. The cryA coding sequence is 1.7 kb in length and consists of two exons interrupted by one short intronic region with the capacity to express a 567 amino acid protein that comprises highly conserved domains such as a photolyase domain as well as a FAD

binging domain. cryA locus has been knocked out in a wild-type genetic background. The resulting null mutant forms hülle cells in submerged culture and has a purple colour after prolonged incubation. This effect is rescued with the genomic fragment of the cryA gene. Additionally, double knock-out strains were created lacking established regulators of fruit body formation. Conditional growth and expression profiling experiments will reveal any influence of the putative cryA-encoded blue light receptor within the life cycle of the A. nidulans. The current state of the project will be presented.

3. GENOMICS APPROACHES TO STUDY THE REGULATION OF THE PROTEOLYTIC SYSTEM OF *ASPERGILLUS NIGER*

Machtelt Braaksma[1*], Felix G. Eikmeyer[1], Kees A.M.J.J. van den Hondel[1,2], Mariët J. van der Werf[1] and Peter J. Punt[1]
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The proteolytic system of *Aspergilli* is quite complex. A broad range of proteolytic activities has been identified from *A. niger* over the past years. Several of the genes involved have been characterized and studied in more detail. Random mutagenesis and targeted disruption of genes encoding proteases have resulted in mutants with significantly lower protease activities. However, even with the use of protease mutants as host strains for heterologous protein production, proteolytic degradation is still a major problem. Although different regulatory systems have been described, until now only a very small part of the (regulatory) components of the proteolytic system have been characterized in detail. It is the objective of our project to follow a rational approach for analysis of the regulation of the proteolytic system, by using a combination of various genomics tools in combination with extracellular protease profiling. For this purpose a protease assay has been developed.
For these genomics experiments a number of different growth conditions have been selected. These include variation in carbon sources, nitrogen sources and pH and limitation of phosphate and sulfate. These conditions are tested in batch fermentations for growth, production of protein and protease activity. For the final genomics experiments a selection of conditions will be made and samples from fermentations run under these conditions will be analyzed with metabolomics and transcriptomics tools. Correlations between the different data sets will be used to identify and understand the biological processes involved in the regulation of the fungal proteolytic system.
The final goal is to identify the strain and cultivation conditions that reduce proteolytic activities in order to further improve heterologous protein production.

4. VeA SUBCELLULAR LOCALIZATION IS DEPENDENT ON LIGHT IN THE FILAMENTOUS FUNGUS *Aspergillus nidulans*

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The *veA* gene is a light-dependent regulator that governs development and secondary metabolism in *Aspergillus nidulans.* We have previously reported a putative bipartite NLS motif in the *A. nidulans* VeA deduced amino acid sequence and demonstrated that this NLS is functional when expressed in a yeast system. Furthermore, we showed that the migration of VeA to the nucleus is dependent on an alpha-importin protein. We have recently demonstrated the functionality of this bipartite NLS when VeA is expressed in *A. nidulans*. Interestingly, we found that VeA migration to the nucleus is light-dependent in *A. nidulans*. While in the dark VeA is located mainly in the nuclei, under the light VeA is found abundantly in the cytoplasm. On the other hand, the VeA1 mutant protein form (lacking the first 37 amino acids at the N-terminal region) was found predominantly in the cytoplasm independent of the illumination regimen, indicating that the truncated bipartite NLS in VeA1 is not functional and fails to respond to light. These results explain the lack of the morphological light-dependent response in strains carrying the *veA1* allele. We also evaluated the effect of light on the production of the mycotoxin sterigmatocystin in a *veA* wild-type strain and compared it with that of a *veA1* mutant strain. In our studies we saw that the highest amount of toxin was produced by the *veA+* strain growing in the dark, a condition that favored the accumulation of VeA protein in the nucleus.

5. IDENTIFICATION OF NOVEL GENES ENCODING SPECIFIC PLANT CELL WALL DEGRADING ENZYMES

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The potential of a genome sequence for the rapid identification of genes encoding specific enzymes was evaluated using the *Aspergillus nidulans* genome and plant cell wall polysaccharide degrading enzymes as an example. These enzymes are used in many industrial applications and many genes encoding these enzymes have already be identified in *Aspergillus*. Detailed in silica analysis of the ORFs assigned to the relevant families of the Carbohydrate Active enzyme database (CAZY, http://afmb.cnrs-mrs.fr/CAZY/index.html), using the Blast and Clustal programs, resulted in a reliable assignment of enzymatic function for most ORFs. This analysis demonstrated that approximately two-third of the *A. nidulans* ORFs do not yet have a characterised *Aspergillus* paralogue and also identified some ORFs

encoding enzyme functions that have not been cloned previously in *Aspergillus*. A comparison of the biochemical characteristics of previously purified enzymes from *A. nidulans* to the *A. nidulans* ORFs did not result in the identification of the ORF corresponding to the enzyme. However, using an elimination strategy the number of candidate ORFs could be reduced to 2-5.
The analysis also revealed that the *A. nidulans* genome contains at least 33 ORFs that encode putative intracellular oligosaccharides degrading enzymes as well as ORFs with homology to oligosaccharides transporters of other organisms. This suggests that oligosaccharides are not exclusively degraded extracellularly, but can also be imported and degraded inside the cell.

6. MCMA, A TRANSCRIPTIONAL REGULATOR FROM A MADS – BOX FAMILY PARTICIPATES IN REGULATION OF ARGININE CATABOLISM IN ASPERGILLUS NIDULANS.

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The arginine catabolism genes agaA and otaA are specifically induced by arginine. This induction depends on Zn2Cys6 transcriptional activator ARCA. We have shown that MCMA, a transcriptional regulator from a MADS-box family also participates in this process. In $mcmA_{I70A}$ mutant, the induced levels of arginase and ornithine transaminase activities are lower than in a wild type. We have isolated a cDNA clone of mcmA (Gene Bank Acc. No. AY957455). MCMA is a homologue of Saccharomyces cerevisiae Mcm1p transcriptional regulator. Similarly as the yeast protein, MCMA posses a MADS domain and glutamine rich region.

7. *ASPERGILLUS* TAXONOMY UPDATED

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Since the publication of Raper and Fennells The genus Aspergillus from 1965, there has been no full treatment of the genus. The nomenclature, sectional classification, several names and the placement of species in sections has been changed considerably since 1965. The new taxonomy reflects both ecology and phylogeny and ideally both extensive phenotypic characterization and sequencing of several genes is necessary to obtain a stable and predictive taxonomy and phylogeny. Fortunately a classification of Aspergillus species based on morphology, extrolites (secondary metabolites and other outwards directed metabolites) and physiology point to the same species as do multi gene cladifications. Furthermore several Aspergillus species have been full genome sequenced. Thus PCR based and nucleotide sequence based identification and bar coding is one way of dealing with identification of Aspergillus, while electrospray MS or HPLC-DAD of extracts of Aspergilli (Larsen et al., 2005) is another way of quickly identifying these fungi. Morphological and colony colour and texture based identification is also a possibility, either in the traditional way or by image analysis, but morphology based identification often require expert knowledge, so certain combinations of these techniques are required in a polyphasic taxonomy (Hong et al., 2005). Here we report on the state of the art Aspergillus taxonomy with examples of the use of different modern methods of characterization. An overview of mycotoxin and drug lead production by Aspergillus is given, together with an overview of the latest proposal of an overall classification of all Aspergillus species.

References
Hong, S-B., Go, S, Shin, H., Frisvad, J.C. & Samson R. A. 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* 97 (6) in press.
Larsen, T.O., Smedsgaard, J., Nielsen, K.F., Hansen, M.E. and Frisvad, J.C. 2005. Phenotypic taxonomy and metabolite profiling in microbial drug discovery. Natural Product Reports 22: 672-695.

8. Crawler, a novel transposable element of Tc1/mariner superfamily in Aspergillus oryzae transposes under stress conditions

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We have recently identified a novel Tc1/mariner-type transposable element, named Crawler, in an Aspergillus oryzae industrial strain OSI1013 as an insertion in the niaD gene. Crawler is 1.3 kb in length with terminal inverted repeats of 22 bp and has an ORF encoding a putative transposase of 357 amino acids. The transposase shows overall sequence similarity to that encoded by impala of Fusarium oxysporum (31% identity). The element is present in multiple copies (>16) in the strain OSI1013, but is found as a single defective copy with RIP-like mutations in the strain RIB40 that was used for genome analysis.
Expression of the transposase-encoding gene (aotA) was found in standard growth medium, and was stimulated slightly by stress treatments, such as heat shock and $CuSO_4$. When

conidiospores of OSI1013 were screened for mutations by chlorate resistance under stress conditions, a number of mutant strains harboring insertion of Crawler in the crnA gene or in the niaD gene could be isolated. In contrast, insertion event was seldom detected in the mutants obtained without stress. In addition, judged from a result of selection of revertants capable of assimilating nitrate from a resultant crnA mutant, excision events of Crawler occurred at a frequency of 10^{-4} - 10^{-5} by treatment of heat shock or $CuSO_4$. Excision sites of the revertants contained the footprint of 5 nucleotides. To our knowledge, this is the first observation that the resident transposable element is able to transpose under stress conditions in Aspergillus species.

9. Dissection of the Unfolded Protein Response pathway in *Aspergillus niger*.

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Filamentous fungi like *Aspergillus niger* are renowned for their high secretion capacity of homologous secreted enzymes. Unfortunately the expression of heterologous secreted proteins by these filamentous fungi results mostly in mediocre product recovery. In most cases, this expression is accompanied by triggering of the Unfolded Protein Response (UPR). UPR is a universal reaction of eukaryotic cells to protein folding stress in the endoplasmic reticulum (ER).
UPR results in induction of expression of genes that allow the cell to cope with the surplus of protein- folding intermediates. Our goal is to improve the folding capacity, and thus the secretion performance of *A. niger*. Towards this goal we are studying the ER-stress responsive genes and their regulatory circuits. Here, we will report on three of the topics of our research:

1. Analysis of UPR in very young mycelium, induced by treatment with tunicamycin. Genome wide expression analysis has been performed on Affymetrix arrays and up- and down regulated genes have been identified.

2. The development of an improved genetic screen for the isolation of regulatory mutants with altered UPR characteristics. A first strain, in which selection is based on the HacA (transcription regulator, involved in up regulating UPR genes) responsive *cyp*B promoter, has been constructed. Additional strains will also be constructed which contain more optimal UPR-responsive promoters based on the results from the transcriptomics experiments.

3. Induction of UPR in tightly controlled fermentor cultivations of *A. niger* strains by regulated expression of a poorly folded protein (scFv). First, a steady-state culture under repressing conditions for the *gla*A promoter is established which is then switched to *gla*A inducing conditions until steady-state is reached again. Cultivations are performed at different dilution rates. An isogenic control strain, not expressing scFv, is grown at identical conditions. The samples collected throughout these fermentations are used for transcriptomic and protein analysis.

10. THE NUCLEAR MIGRATION PROTEIN NUDF ASSOCIATES WITH NUDC AND BNFA IN ASPERGILLUS NIDULANS

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In filamentous fungi, migration of nuclei is established through the interplay of the molecular motor dynein in combination with dynactin and microtubuli. Several proteins like NUDF, NUDE, and NUDC had been identified as regulators of dynein-mediated movement, which are not part of the dynein/dynactin motor complex itself (Xiang et al, 1995; Efimov & Morris, 2000). We intended to identifiy interaction partners of these regulatory proteins in Aspergillus nidulans in order to clarify the regulatory pathway leading to dynein activation. Applying tandem affinity purification, we isolated ten proteins binding to NUDF during vegetative growth. Among these was a protein unique to A. nidulans, which we named BNFA (Binding of nudF) and characterized in more detail. We confirmed this interaction by yeast two-hybrid analysis and constructed an A. nidulans knock-out strain. We also analyzed the potential interaction between NUDF and NUDC by yeast two-hybrid analysis. Furthermore, the putative phosphorylation of NUDF was investigated to decipher its role in protein complex formation and localization of NUDF.

11. A MUTATION IN A PROTEIN KINASE C HOMOLOGUE CAUSES CALCOFLUOR HYPERSENSITIVITY IN ASPERGILLUS NIDULANS

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We have identified a set of mutant strains in Aspergillus nidulans, which show elevated sensitivity to the wall compromising agent Calcofluor White (CFW), indicating a probable defect in cell wall integrity. One of these, designated calC, shows several secondary phenotypes also frequently observed in wall mutants, including sensitivities to SDS, Caspofungin acetate, and Congo Red. In addition, Nikkomycin, Tunicamycin and Caspofungin induce excessive spore swelling in the calC, strain during spore germination at 30C. Sporulation is depressed at 42C, but can be osmotically remediated. We have complemented CFW hypersensitivity from an A. nidulans, plasmid genomic library, leading to the identification of AN0106.2 (identified in the Broad Institute database as "KPC1_ASPNG Protein kinase C-like") as the complementing sequence. This sequence is located in the same region of chromosome VIII to which the calC mutation has been mapped. The calC, PKC contains a predicted glycine-to-arginine substitution at position 564. The cloned wild type gene reduces sensitivity of the calC strain to CFW, SDS, Caspofungin, and Congo red, while reducing temperature-induced suppression of sporulation and drug-induced swelling of spores. Resistance of the calC strain to CFW was slightly improved by the PKC activator phorbol 12,13-diacetate. Interestingly, CFW resistance was even more strongly improved by the protein kinase inhibitors chelerythrine chloride and bisindolylmaleimide I HCl. These drugs had no effect on the CFW resistance/sensitivity of the wild type. This suggests that the possibility that multiple protein kinases participate in controlling cell wall integrity in A. nidulans.

12. TRANS-SENSING MECHANISM IN A DIPLOID STRAIN OF ASPERGILLUS FLAVUS

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Aspergillus flavus is an asexual filamentous fungus that produces the toxic and carcinogenic compound aflatoxin. The parasexual cycle can be induced in this fungus for genetic analyses, including assessment of gene dominance in stable diploids. All known mutations in genes for aflatoxin biosynthesis are recessive in diploids except for afl-1 in strain 649. Diploids between 649 and 86 (wild type) lack transcripts for the aflatoxin biosynthetic genes and fail to produce aflatoxin. We are characterizing this mutant to understand the mechanism of inhibition of aflatoxin biosynthesis. Loss of aflatoxin production in 649 is due to a deletion that includes the aflatoxin gene cluster. Failure to produce aflatoxin in 649 x 86 diploids does not appear to be due to a repressor of the transcriptional regulator AflR as diploids between 86 and a strain of 649 carrying ectopic copies of aflR produce aflatoxin. These data suggest that the location of aflR in the genome dictates whether it is functional in the 649 x 86 diploid. One explanation is that some form of transvection or trans-sensing mechanism is preventing aflatoxin production in the diploid strains. Investigations are underway to characterize the trans-sensing phenomenon.

13. BIOCHEMICAL EVIDENCE FOR THE PRESENCE OF A HIS-ASP PHOSPHORELAY IN FILAMENTOUS FUNGI

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His-Asp phosphorelays are the widespred signal transduction mechanisms in both prokaryotes and eukaryotes. The system consists of three types of common signal transducers: a sensor with histidine kinase activity, a response regulator containing a phospho-accepting receiver and often a histidine containing phosho-transmitter (HPt). A computer-aided similarity search has identified the 15 ORFs as a sensory histidine kinase, one ORF as an Hpt, and four ORFs as a response regulator in the genome of A. nidulans. In eukaryotic microorganisms, Saccharomyces cerevisiae and Shizosaccharomyces pombe, the His-Asp phosphorelay systems are well characterized. These organisms have only a small number of the common signal transducers: one histidine kinase, one HPt, and two response regulators in S. cervisiae, while three histidine kinase, one HPt and two response regulators in S. pombe. Therefore, the His-Asp phoshorelay systems in A. nidulans could respond more complicate environmental stimuli. At the same time, a simple question whether all the histidine kinases can transfer the phosphate group to the HPt has arisen. To address the question, we have attempted to examine the in vitro phospho-transfer with the all recombinant transducers. As a first step to construct the in vitro phospho-transfer network with all the components of A. nidulans, we purified the recombinant HPt and a response regulator SrrA and subjected to the in vitro phospho-transfer system using an Escherichia coli histidine kinase ArcB as a phosphate-donor. We found the phospho-transfer from ArcB to A. nidulans HPt and the subsequent transfer from HPt to SrrA, which is the first direct biochemical evidence for the presence of phospho-transfer system in filamentous fungi.

14. TARGETING MITOCHONDRIAL RESPIRATORY AND OXIDATIVE STRESS RESPONSE SYSTEMS FOR CONTROL OF ASPERGILLI

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Signal transduction and stress-response genes of fungal pathogens play important roles for exerting pathogenesis and, in some cases, biosynthesis of mycotoxins. As such, they should serve as potentially viable targets for antifungal compounds. Results of our research show

that targeting the mitochondrial respiratory chain, MAPK or vacuolar H(+)-ATPase systems using safe, natural compounds can significantly elevate the sensitivity of fungi to commercial fungicides or antifungal drugs. The use of such compounds can result in lowering effective dosages, costs of treatment and potential for development of resistance.

Our rationale is based on the fact that cellular targets of several conventional antifungal compounds are already known. Examples of these targets include macromolecular synthesis, cell division, signal transduction and respiration. We theorize that disruption of cellular redox homeostasis using phenolics may inhibit fungal development and invasiveness. Targeting these systems with drugs and additional safe, natural compounds leads to cellular oxidative stress responses, with a resultant decrease in cell viability. We illustrate the use of this target-based strategy to significantly improve control of fungi such as aspergilli. Combined treatments with these phenolics and conventional fungicides, that are inhibitors of the mitochondrial respiratory chain, have a synergistic fungicidal effect due to disruption of respiration and inhibiting the oxidative stress-response of the fungus.

We conclude that natural compounds (i.e. phenolics or alkaloids) can be developed as useful antifungal agents when the molecular target is identified. The potential use of this approach to effectively control a broad spectrum of fungal pathogens is discussed.

15. IDENTIFICATION OF AN SKN7 HOMOLOGUE IN ASPERGILLUS FUMIGATUS : MUTANT CONSTRUCTION AND PHENOTYPE ANALYSIS

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Among the various transcription factors contributing to the oxidative stress response, SKN7 encodes a response regulator containing a receiver domain typical of two-component signal transduction systems and similarity to the DNA-binding domain of heat shock factor. However, the oxidative stress function of Skn7p has been shown to be independent of the aspartyl residue of the receiver domain. In the present study, in order to examine the contribution of the A. fumigatus homologue to SKN7 for in vitro susceptibility to different stress, and pathogenesis of fungal infections, we created a mutant via targeted disruption, and established the phenotype associated to this mutation. The skn7 mutant had no significant morphological or growth differences associated to thermic and hyper-osmotic stresses, but was sensitive to hydrogen peroxide and tert-butyl hydroperoxide. No significant virulence differences were however observed between wild type, mutant and reconstituted strains in a murine model of pulmonary aspergillosis.

16. THE ROLE OF BEMA IN THE GENETIC CONTROL OF A. NIDULANS HYPHAL MORPHOLOGY

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The publication of the A. nidulans genome sequence has given us the opportunity to identify homologues of genes from other species that have been shown to be involved in the process of polar growth. Although there will be gaps in the data, which is inevitable because the organisms grow in different ways, it is expected that many of the fundamental mechanisms will be similar.

BemA, the orthologue of the S. cerevisiae protein Bem1p was identified through genome sequence comparison and we have shown that it plays a similar role to in yeast, acting as a scaffold protein in cell polarity establishment. BemA appears to be essential for the establishment of cell polarity as well as the maintenance of polarity once an axis has been established. We have shown that the first SH3 domain of the protein is not essential and have evidence to suggest alternate transcriptional start sites.

The BemA protein was tagged with RFP in order to visualise its location in the cell. BemA is first observed around the nucleus and it is also seen around the cell periphery. In early germlings the protein begins to accumulate at the hyphal tip, eventually forming a well-defined cap. These caps can also be seen at the tips of branches.

BemA-RFP has been introduced into several mutant backgrounds to observe its location when these strains are exhibiting defects in polarity. This may give us an insight into the role of these proteins in hyphal development and the interactions between them.

17. GENETIC CONTROL OF HYPHAL MORPHOLOGY IN A. NIDULANS

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The molecular biology behind the development of A. nidulans hyphae has been studied for many years and it has been shown that there is strong genetic control of the process.

HbrB was identified in a screen for temperature sensitive mutants. It has no known domains and orthologues are only detectable in closely related fungal species. When grown at restrictive temperatures cells produce many lateral branches and exhibit hyperseptation. In an alcA-hbrB strain made previously, growth is stunted and the cells are very swollen under promoter repressing conditions. A strain containing HbrB-GFP has been created and localisation of the protein studied.

The A. nidulans homologue of N. crassa cot-1, cotA, a member of the NDR protein kinase family, has previously been expressed under the control of alcA(p). Depletion of CotA led to a severe growth defect accompanied by loss of polarity. A CotA-GFP

fusion protein has been created and punctate regions of fluorescence were found similar to the distribution reported for human NDR2.
Co-localisation studies of CotA and HbrB with other tagged proteins may aid identification of the compartments in which they reside. The A. nidulans homologue of Vps52 (AN4014.2), a member of the Golgi Associated Retrograde Protein (GARP) complex has now been tagged. This protein has been shown to localise to Golgi in Saccharomyces and can now be used as a marker for Golgi in A. nidulans.
These new strains will facilitate further characterisation of the two proteins and indicate their role in the control of hyphal development in A. nidulans.

18. AFFINITY PURIFICATION AND MASS SPECTROMETRIC ANALYSIS IDENTIFIES TWO NOVEL PROTEINS REQUIRED TO MAINTAIN THE MITOTIC CORE OF THE ASPERGILLUS NIDULANS NUCLEAR PORE COMPLEX

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The Aspergillus nidulans nuclear pore complex (NPC) undergoes partial disassembly during mitosis but a conserved minimal NPC core structure, containing the An-Nup96 complex, remains in the nuclear envelope throughout mitosis. How disassembly and reassembly of the NPC is regulated isn't clear. By defining the structural components of the A. nidulans core NPC we hope to provide keys to understanding the mechanism of this regulation.

We report the identification of two novel NPC proteins, termed NupA and NupB, using mass spectrometric analysis of proteins that co-purify with endogenously S-tagged An-Nup96. NupA and NupB are highly conserved in filamentous fungi but are absent from Saccharomyces cerevisiae. By reciprocal affinity purifications, NupA-S-Tag or NupB-S-Tag also brings down a similar stoichiometry of the An-Nup96 sub complex. Confirming NupA and NupB to be nucleoporins, endogenously GFP-tagged versions were found to locate to the NPC throughout mitosis as do the other components of this complex.

nupA and nupB are not essential, however, their deletions show synthetic lethality with deletions of components in the Nup96 sub complex. Moreover, nupA+nupB double deleted cells are temperature sensitive at 42 degrees. Most interestingly, in nupA deleted cells, An-Nup96 and An-Nup133 no longer exclusively stay at the NPC during mitosis but instead become partially dispersed throughout the cell.

In conclusion, we have identified two new fungal specific nucleoporins that are part of the Nup96 sub complex. The results suggest that NupA and NupB represent evolutionarily specialized nucleoporins involved in maintaining the core structure of the NPC during fungal mitosis.

19. IDENTIFICATION OF GENES INVOLVED IN A. NIDULANS HYPHAL DEVELOPMENT

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Filamentous fungi, such as Aspergillus niger and Aspergillus oryzae have been used secrete large amounts of native proteins for many industries. More recently the production of heterologous proteins has been attempted with limited success, as production yields obtained were often low. The link between the hyphal growth and protein secretion has been noted and several studies to improve protein secretion capacities through the production of hyperbranching mutants have been carried out.

With the availability of genome sequences for the 3 species of Aspergillus and microarray technology, the identification of genes involved in morphological development can be established by transcriptome analysis of hyperbranching mutants and using bioinformatics to identify Aspergillus homologues of known genes.

The temperature sensitive *A. nidulans* mutant HbrB3 exhibits hyperseptation and shows a marked increase in hyphal branching at the restrictive temperature 40°C. RNA was isolated from the mutant strain HbrB3 and a wild type strain, R153, grown under continuous culture conditions at both 30°C and 40°C, and reverse transcribed with cy3 and cy5. Analysis of microarrays probed with fluorescently labelled cDNA has been used to identify genes whose expression differs significantly in the mutant strain relative to the wild type. Functional analysis of genes using the *alcA* (alcohol dehydrogenase) conditional promoter is currently in progress. The ORF AN6796.2 when down-regulated showed a hyperbranching phenotype. Initial investigation of AN5624.2 shows enlarged vacuoles when the gene is down regulated. A KU 70 knockout strain is now available which will provide a quicker method of promoter exchange, allowing many genes to be functionally investigated.

20. ENZYME, INTRA AND EXTRA CELLULAR METABOLITE ANALYSIS IN OXYGEN LIMITED CULTIVATIONS OF ASPERGILLUS NIGER

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Aspergillus niger is known to be a strictly aerobic organism, meaning it is not using the reductive part of the TCA cycle to produce organic acids like fumarate and succinate. In the present study, our aim was to investigate the effect of different oxygen levels on the physiology and especially the organic acid production of Aspergillus niger. The hypothesis is that under strict oxygen levels Aspergillus niger might need an additional electron acceptor next to oxygen leading to more organic acid production in the form of fumarate or succinate

by the reductive part of the TCA cycle. Five batch cultivations were performed under different aeration rates (0.01, 0.02, 0.05, 0.1, and 1 vvm). Intra and extra cellular metabolites were analysed together with the quantification of enzyme activities in the glycolysis, gluconeogenesis and TCA cycle. We measured 10 different enzymes: glucose-6P dehydrogenase, pyruvate dehydrogenase, pyruvate decarboxylase, pyruvate carboxylase, malic enzyme, malic dehydrogenase, fumarase, succinate dehydrogenase, isocitrate lyase and citrate synthase. The results indicate that the different metabolic pathways are drastically influenced by the oxygen availability. Furthermore, by a detailed analysis of the metabolite fingerprint of Aspergillus niger we expect to verify the aforementioned conclusion and reveal more information for the regulation of the different pathways by oxygen.

21. TWO APPROACHES FOR THE IDENTIFICATION OF ASPERGILLUS NIGER GENES INVOLVED IN POLAR GROWTH AND BRANCH INITIATION

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Analysis of protein secretion in *Aspergillus niger* revealed that proteins are mainly secreted from hyphae at actively growing tips, suggesting that protein secretion and hyphal tip growth are coupled processes. This has led to the suggestion that hyperbranching mutants displaying an increased apical surface have the potential to significantly secrete more protein of interest.
To modify and optimize the morphology of *A. niger* by rational genetic engineering, it is important to know which regulatory networks control polarized growth and determine morphological characteristics of *A. niger*.
One approach in which those networks can be identified, is to study the genome-wide transcriptional changes in *A. niger* germlings following exposure to morphology-affecting compounds. We have therefore screened a series of compounds and followed their effect on germ tube elongation and branching by microscopy. In response to the antifungal drugs caspofungin, fenpropimorph and AFP, tip splitting as well as subapical branching were observed, suggesting that these compounds affect polarity establishment and maintenance in *A. niger*. Thus, measurement of changes in gene expression upon exposure to those drugs can help to discover key genes involved in regulation of polar growth.
Another strategy we embark on focusses on a temperature-sensitive hyperbranching mutant of *A. niger* (*ramosa-1;* Reynaga-Pena and Bartnicki-Garcia, 1997). We could show that germlings of *ramosa-1* grown at permissive temperature display a wild-type morphology, but branch apically and subapically when shifted to the restrictive temperature. Hence, with the comparison of the transcriptome of *ramosa-1* with the transcriptome of the parental strain it will also be possible to uncover the network of genes involved in polarity establishment and maintenance.

Reynaga-Pena CG and Bartnicki-Garcia S (1997) Apical branching in a temperature-sensitive mutant of *Aspergillus niger*. Fung Genet Biol 22: 153-167

22. LOCALISATION OF THE PUTATIVE POLARISOMAL COMPONENT SPA2 IN ASPERGILLUS NIGER

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The ability of cells to polarize is essential during morphogenesis in yeasts and filamentous fungi. Cytoskeletal components, including actin, are thought to play an important role in polarized growth. It has been shown in yeast that the actin-organizing polarisomal component Spa2 localizes to sites of polarized growth, demonstrating that Spa2 can be used as a marker for polarization in yeast. Having such a marker for the filamentous fungus *Aspergillus niger*, would allow us to examine the localisation of polarisomal components in growing hyphae.
We searched the *A. niger* genome and identified a *spa2* homologue that encodes a protein of 908 amino acids. This protein is the only one in the genome that shares significant homology with the yeast homologues and we thus designated it Spa2. In order to examine its cellular localisation, Spa2 was tagged with eCFP at its C-terminus and integrated into the genome of the *A. niger* strain via a gene-replacement with the endogenous *spa2* gene. Thus, the fusion protein is expressed under the control of its own promoter.
Transformants, carrying one, two or three copies of Spa2::eCFP were indistinguishable from the recipient strain with respect to morphology and growth rates, suggesting that the fusion protein is functional. Using fluorescence microscopy, we could localize Spa2::eCFP in germ tubes and hyphae of *A. niger* at sites of polarized growth. The fusion protein typically appeared as a crescent, a localisation pattern conforming to that found in yeasts, suggesting that the *A. niger* Spa2 may play a similar role in polarised growth.
However, the copy number of *spa2::ecfp* was critically important for detecting the fusion protein. Whereas a single copy of *spa2::ecfp* resulted in poor brightness, strains carrying two copies expressed Spa2::eCFP at a level sufficient for imaging. In strains harboring at least three copies, Spa2::eCFP was increasingly detected within the cytoplasm, indicating that Spa2 becomes aberrantly localised when overexpressed.

23. PERTURBATION OF CELL WALL CAUSED BY DEFECT OF THE KexB IN *Aspergillus Oryzae*.

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Disruption of the *kexB* gene encoding a subtilisin-like processing porotease in *Aspergillus oryzae* led to remarkable morphological defects on Czapek-Dox (CD) agar plates. We found that upregulation of transcription levels of *mpkA* and cell wall biogenesis genes in the *kexB* disruptant (delta-*kexB*) is autoregulated by phosphorylated MpkA as the active form through cell integrity signaling. In the present study, to reveal how the constitutive activation of the cell integrity signaling caused by the delta-*kexB* mutation affects structures and biogenesis of cell wall structures, we analyzed the organization and components of cell wall of *A. oryzae* delta-*kexB*. Contents of glucose and hexosamine in the cell wall of the delta-*kexB* grown on CD agar plates decreased and increased those in the cell wall of the wild-type strain, respectively. To analyze the structure of cell wall in the delta-*kexB* strain, cell wall was fractionated by alkaline extraction. The delta-*kexB* cells showed a significant decrease of the amount of alkali-soluble fraction compared with that of wild-type cells, whereas the amount of alkali-insoluble fraction prepared from the delta-*kexB* cells was slightly larger than that from wild-type. The alkali-insoluble fraction prepared from delta-*kexB* cells contained a larger amount of hexosamne than that from wild-type cells. Chitin synthase activity of delta-*kexB* cells was also higher than that of the wild-type cells. From these results, we speculated that the decrease of the alkali-soluble fraction in the delta-*kexB* was compensated with the increased chitin content by the high activity of chitin synthase through the activated cell integrity signaling.

24. MOLECULAR CHARACTERIZATION OF THE MANNOSYLTRANSFERASE PMT4 OF ASPERGILLUS FUMIGATUS INVOLVED IN CELL WALL MORPHOGENESIS

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O-glycosylation is a major post-translational modification of proteins. O-glycosylated proteins play major roles in eukaryotic cells from fungi to humans. The initial reaction of mannose transfer to serine and threonine residues is catalysed by protein O-mannosyltransferase in the endoplasmic reticulum. Seven PMT encoding genes (PMT1-7) has been characterized in S.cerevisiae. Disruption of three different types of PMT genes resulted in death of the yeast cells. In the filamentous fungus Aspergillus fumigatus, 3 orthologs are present in the genome. PMT2 has been the only gene of the PMT family characterized in Aspergillus. The role of PMT4 has been now investigated in A. fumigatus. This gene encoded for a transmembrane protein of 780 amino acids. ∆PMT4 of A. fumigatus has been constructed by gene replacement. The mutant show several morphological defects : reduced growth, altered mycelium, higher sensitivity to Calcofuor, very reduced conidiation.

25. DENEDDYLATION ACTIVITY OF THE COP9 SIGNALOSOME IS ESSENTIAL FOR *ASPERGILLUS NIDULANS* SEXUAL DEVELOPMENT

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The COP9 signalosome (CSN) is a conserved multiprotein complex, playing an essential role in development of eukaryotes through regulation of protein ubiquitination and degradation. All CSN subunits have been identified in the filamentous fungus *Aspergillus nidulans*. Considering the conservation of the proteasome-related machinery in this organism, it provides an easily amenable model for studying the broad array of CSN regulatory functions.

Deletion of *csnE* results in pleiotropic phenotypes affecting formation of fruit bodies during sexual development, sensitivity to oxidative stress and secondary metabolism. CSNE promotes cleavage of ubiquitin-like protein Nedd8 from the CULA subunit of the SCF ubiquitin ligase. We have examined the role of deneddylation by point mutagenesis of the JAMM metalloprotease motif in the *csnE* subunit. Analysis of mutant phenotypes indicates that intact JAMM motif is necessary to complete sexual development and its loss leads to the full mutant phenotype. Furthermore, as shown by Western blot, a modified (presumably neddylated) form of CULA is enriched in all *csn* mutant strains in early stages of *A. nidulans* development, indicating that CSNE acts as deneddylase in the fungus and that this function is required around the time of developmental competence. The role of the Nedd8 protein homologue and neddylation in *A. nidulans* are currently under investigation.

26. GENOME-WIDE MUTATION LIBRARY IN ASPERGILLUS NIDULANS

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For decades the filamentous fungus, Aspergillus nidulans, has served as a model organism for numerous industrially and medically significant Aspergilli. One of the main advantages of A. nidulans compared to most other Aspergillus species is that it has a sexual cycle, allowing mutant strains to be crossed. The release of the genome sequences for several important Aspergilli has created potential for systematic genome-wide modifications such as gene deletion, promoter replacements, fluorescent protein tags and allele replacements.

We have recently developed an efficient PCR based genome manipulation system for A. nidulans, which improves targeted integration of exogenous DNA while reducing ectopic integration. Inspired by the success of the gene deletion library from Saccharomyces cerevisiae, we intend to use this technology to initiate a genome-wide mutation library in A. nidulans. We here present our progress in the improvement and scale up of our current gene targeting capabilities, with the aim of providing a high-throughput genome-wide manipulation system that will be transferable to other fungal species.

27. GENETIC STABILITY OF MULTIPLE GENE INSERTIONS IN ASPERGILLUS NIDULANS

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In many filamentous fungi, including Aspergillus nidulans, transformation of recombinant DNA often leads to the integration of multiple copies of this DNA either as inverted or direct repeats. For many industrial applications transformants with multiple gene copies are obtained from screening because such strains often have high gene expression levels. However, multiple gene insertions often result in genetic instabilities due to homologous recombination between the repeated sequences. We have developed a conidiospore color assay in A. nidulans that allows the detection of spontaneous or induced recombination events within a fungal colony and also determines whether the event occurred as a loop-out or a gene conversion event. The assay provides a practical way to measure the recombinogenic effects of different configurations of repetitive sequences and also a means to compare the effect of various gene mutations on recombination. The assay system includes a unique restriction site that can be cleaved in vivo by induction of an endonuclease. We have expanded the assay to include a hetero-allelic diploid that allows the study of inter-chromosomal recombination. The assays can be used to screen for genes involved in genomic stability as well as favorable insertion points and repeat configurations of heterologously expressed genes.

28. COORDINATION OF MICROTUBULE AND ACTIN FUNCTIONS IS ESSENTIAL FOR RAPID HYPHAL GROWTH OF *ASPERGILLUS NIDULANS*. Tetsuya Horio,[1] Xiaowei Dou[2,3] Stephen Osmani[2] and Berl R. Oakley[2]

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We have previously reported that microtubules play an essential role in rapid hyphal growth of *Aspergillus nidulans* (Molecular Biology of the Cell, 2005, 16, 918-926). The actin cytoskeleton is known to play an essential role in tip growth as well. To investigate the coordination of actin and microtubules in this process, we observed a strain expressing GFP-labeled actin by time-lapse microscopy. In rapidly growing hyphae, actin localized to a ring of plaques immediately behind the hyphal tip. Treatment with cytochalasin A caused an immediate cessation of tip growth and disassembly of the actin plaques. Treatment with the anti-microtubule agent benomyl resulted in a dramatic reduction of the growth rate. The actin plaques remained intact but dispersed through the cytoplasm as growth slowed. In about 40% of the hyphae, actin plaques failed to reorganize to any specific region throughout the observation period. However, in other hyphae, actin plaques re-accumulated either at the tip or in a medial region of the hyphal wall and slow growth resumed at the site of actin accumulation. When benomyl was washed out, the rate of tip growth began to increase prior to the reorganization of actin plaques. The actin plaques became reorganized, however, as the tip growth rate accelerated. Our results confirm that functional actin is essential for the tip growth and indicate that microtubules play a role in the organization of subapical actin plaques that are involved in rapid and steady hyphal tip growth. Supported by grants from the National Institutes of Health.

29. VEGETATIVE INCOMPATIBILITY IN ASPERGILLUS NIGER

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In filamentous fungi, anastomosis between hyphal filaments is essential to fungal growth and occurs both within and between individual colonies. As a consequence of the latter, heterokaryotic cells may arise, the viability of which is determined by heterokaryon incompatibility (*het-)* genes. For some of the ascomycetes (e.g. *Aspergillus nidulans*, *Neurospora crassa* and *Podospora anserina*) genetic analysis revealed the number of unlinked *het*-loci involved in the vegetative self/non-self recognition to vary between species from 5 to 11. In addition, there are downstream effectors of death during heterokaryon incompatibility.

For *A. niger* heterokaryon incompatibility was observed among natural isolates as the inability to complement recessive nuclear mutations in a heterokaryon and to transfer a mitochondrial marker. In addition, heterokaryon incompatibility prevented the spread of dsRNA viruses that were found in approximately 10% of the natural isolates of black Aspergilli. However, genetic analysis in this asexual fungus is impossible by definition because parasexual analysis requires heterokaryon formation. As a result it is formally not known whether heterokaryon incompatibility in this asexual fungus is under a similar genetic control to that found in related sexual species. Moreover, in the absence of recombination the maintenance of heterokaryon incompatibility depends on mutation to compensate for loss of incompatibility types by random drift.

In this study we investigated the *A. niger* database for the main proteins involved in the heterokaryon incompatibility reaction in either of the following sexual species: *A. nidulans*, *A. fumigatus* (recently found to be sexual), *N. crassa* or *P. anserina*.

30. GENOMIC COMPARISONS BETWEEN A. FLAVUS AND A. ORYZAE REVEAL UNIQUE GENES

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Aspergillus flavus and A. oryzae are closely related fungi that inhabit different ecological niches. A. flavus is plant and animal pathogen that produces the carcinogen aflatoxin. In contrast, A. oryzae is the major fungus used in food fermentation. Whole genome sequences are available for both of these fungi, which allows for a careful comparison of the two species. Overall, these two fungi are very similar in genome size, gene organization and nucleotide identity. However our initial studies show small differences in genome organization due to small-scale insertion-deletion events and transversions, as well as evidence of a translocation event in A. flavus between chromosomes II and VI. The translocation break sites and many of the indels are associated with families of uncharacterized repeat elements. Analysis of these repeat elements is ongoing, but amongst those studied to date three putative types of transposable element have been predicted. These transposable elements appear to be present in larger copy numbers in A. oryzae than in A. flavus. Interestingly, each species has approximately 350 genes unique to that species. Most of the genes are of unknown function, but within this group are genes for secondary metabolism, including polyketide synthases and non-ribosomal peptide synthases.

31. Proteolysis and protein processing in filamentous fungi

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Filamentous fungi have a well established use as production organisms for fungal proteins and, more recently, also for non-fungal proteins. Although significant production levels were obtained for several non-fungal proteins using a secretion-carrier approach, in most cases the yields were still significantly lower than those obtained for fungal proteins. We focused part of our research on a further understanding of processes underlying (efficient) protein production, in particular secretion-related proteolysis and protein processing, with the final

aim to improve protein production. Three different areas of research have been addressed: (i) isolation of protease deficient mutants, (ii) protein processing in the secretion pathway, (iii) vacuolar proteases

Protease mutants. Already in the beginning of fungal molecular biotechnology, protease production was addressed as a possible bottleneck for high levels of heterologous protein production. Several approaches to isolate strains with reduced protease production will be discussed. These include a new direct selection approach and options for functional genomics and HTS-type of approaches. Analysis of several of the obtained mutants will be discussed.

Protein processing. From previous research in several laboratories it was clear that the "secretion carrier" approach results in increased levels of secreted heterologous protein. In particular, in the absence of a processing site in the fusion-protein largely increased levels of fusion-protein were observed. To analyze the role of the protein processing protease on protein secretion, we have isolated fungal KEX2/furin homologues (*kexB/pclA*) and analyzed the effect of gene-disruption on protein secretion. The resulting mutant strains, which have a distinct aberrant morphology, showed significantly increased levels of glucoamylase-interleukin 6 fusion protein. Analysis of secretion of other fusion-proteins in these mutant strains revealed the presence of alternative, PclA- independent, protein processing pathways.

Vacuolar proteases . Based on research carried out in *S. cerevisiae* also protein targeting to the vacuole and release of vacuolar proteases is identified as a possible reason for obtaining low levels of secreted heterologous proteins also in filamentous fungi. To study this further we have isolated fungal mutants strains in which the major vacuolar processing protease (*pepE*) is deleted and have begun studying proteolysis and protein processing in this mutant strain.

32. MULTIPLE ROLES OF THE SMALL GTPASE RACA DURING THE LIFE CYCLE OF ASPERGILLUS NIGER.

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RacA encodes a small GTPase that belongs to the sub-family of Rho-like GTPases. Rac proteins are highly conserved among eukaryotic cells, but are absent in yeasts. One of the important functions of RacA, both in filamentous fungi and in other eukaryotic cells, is that it is involved in controlling and organizing actin polymerization and thereby controlling cellular morphology.

Analysis of the function of RacA in *A. niger* has indeed implicated a role for RacA during vegetative growth. Deletion of the racA gene resulted in abnormal branching at the hyphal tip. Overexpression of the dominant active form of RacA (RacA-G12V) has a dramatic effect on fungal morphology and results in loss of polarized cell growth. Whereas the wild-type strain and the strain overexpressing the wild type form of RacA form long hyphae, characteristic for filamentous fungi, cells expressing RacA(G12V) grow isotropically, resulting in round cells. Transcriptomic studies using Affymetrix microarrays have been performed to identify genes that are differentially expressed between polarised and isotropical cell growth.

To identify proteins that interact with the activated form of RacA (RacAG12V), a yeast two hybrid screen was performed. One of the two RacA(G12V) interacting proteins identified, is highly homologous to the mammalian p67phox protein. P67phox is part of the NADPH oxidase complex which is responsible for the production of reactive oxygen species (ROS). Our results suggest that RacA, like in mammalian cells, is controlling the production of ROS. The finding that yeast transformants containing the p67phox homolog as a RacA(G12V) interacting protein were exclusively obtained from the conidiation library, together with finding that deletion of RacA resulted in a conidiation defect suggest that the RacA regulated production of ROS is involved in the regulation of a-sexual development in A. niger.

33. ANALYSIS OF FOUR PUTATIVE BETA-OXIDATION GENES IN aspergillus nidulans

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Filamentous fungi are able to use fatty acids as sole carbon sources via β-oxidation. The enzymes required are present in both peroxisomes and mitochondria (Maggio-Hall and Keller 2004 Mol. Microbiol. 54:1173–1185).

Two putative fatty acyl-CoA dehydrogenases, AcdA and AcdB, and two putative fatty acyl-CoA oxidases, AoxA and AoxB, were identified in the genome of A. nidulans. Because of their homology to Fox1p of S. cerevisiae and a peroxisomal dehydrogenase of N. crassa, they are thought to be involved in the first step of β-oxidation. Homologues for each were found in other fungal species.

Three of the four proteins have a clear PTS1 (peroxisomal targeting sequence), while AcdA has a PTS1-like sequence. Hence, all four proteins are predicted to be peroxisomal. GFP and RFP fusion proteins are currently under construction to prove this hypothesis.

In the 5’ promoter region (1kb) of each gene, a six basepair sequence (CCGAGG/ CCTCGG) was found at least once. This sequence is predicted to be the core of a fatty acid depending regulation site (Hynes et al., unpublished) suggesting a positive regulation by fatty acids. Northern-blot analyses indicate that the genes are fatty acid inducible. Promoter-lacZ fusions are currently being constructed to elucidate the regulation of the genes by fatty acids.

Deletion of all four genes does not give a severe fatty acid growth phenotype. Only the ΔaoxA phenotype is clearly visible. This implies that there is great redundancy amongst peroxisomal proteins involved in the first step of β-oxidation.

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34. Analysis of the protein kinase ImeB in *Aspergillus nidulans*

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Protein kinases are central regulators of both the mitotic and the meiotic cell cycle. In the yeast, *Saccharomyces cerevisiae,* the *IME2* gene encoding a serin/threonin protein kinase is expressed only during meiosis. Ime2 is essential for meiotic cell cycle and it was shown that it can take over several functions of the cell cycle regulator Cdc28. A survey of the *Aspergillus nidulans* genome revealed a putative *IME2* homolog, conclusively named *imeB* showing 39% identities to the yeast *IME2*. Investigation of the 781 amino acid *imeB* protein sequence revealed a highly conserved protein kinase domain, suggesting a similar function to the yeast Ime2 in *Aspergillus nidulans*. To elucidate the role of *imeB* in *A. nidulans* a targeted deletion of the *imeB* locus in a wild-type genetic background was conducted. *ImeB* mutants show slower growth and form more sexual structures on plates. These results suggest that ImeB may be a negative regulator of sexual development in *A. nidulans*. We will discuss these unexpected findings, which indicate that ImeB and Ime2 have divergent roles in *A. nidulans* and yeast. The Current status of the ongoing project will be presented.

35. CAND1 OF ASPERGILLUS NIDULANS

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Targeted protein degradation by the 26S proteasome controls various cellular processes in eukaryotes. It requires protein ubiquitination by ubiquitin ligases (E3), like the SCF (Skp1-Cullin1-Fbox). Activity of the SCF is regulated by the Cand1 (cullin-associated and neddylation-dissociated), a regulator of assembly of cullin containing E3s that is lacking in bakers yeasts.
We identified an A. nidulans gene called cand1, with deduced aa identity of 20% to the human protein Cand1. To investigate the functions of Cand1 we constructed a cand1 deletion strain. This strain shows a red color phenotype, produces less conidia than the wild type and fruitbody formation is blocked at early nest stage. Cand1 interacts with the fungal counterparts of human Cul1 and Cul4 in the yeast two hybrid system, indicating a conserved molecular function to the human protein. Our results suggest that the system regulating targeted protein degradation at ubiquitination level is highly conserved from A. nidulans to humans. Therefore, A. nidulans can be used as a model organism to gain new insight into the biological function of the regulatory protein. First results on differential expression of Cand1 and localization at defined points of development will be presented.

36. PREMATURE POLYADENYLATION AND CONSEQUENT NONSTOP mRNA DEGRADATION REDUCE THE mRNA LEVEL OF HETEROLOGOUS GENE IN ASPERGILLUS ORYZAE

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Aspergillus oryzae has been paid an attention as a host organism for heterologous protein production. However, the secretion level of heterologous proteins is generally lower than that of the homologous proteins. In the previous study, using a mite allergen protein, Der f 7 as a model, we compared the expression levels of codon optimized Der f 7 (Der/opt) and of native codon Der f 7 (Der/ntv), and showed that codon optimization improved the secreted yield of Der f7 by increasing the mRNA level in A. oryzae. In this study, we analyze the effect of codon optimization on the mRNA level. We constructed chimeric Der f 7 genes, Der/opt-ntv and Der/ntv-opt, in which codons of 5' half or 3' half of the gene were optimized respectively. Interestingly, codon optimization of 3' half of the gene was sufficient for increasing the mRNA level. On the other hand, 3'-RACE analysis revealed that poly(A) tail was added at several positions within the 3' half of the transcripts of Der/ntv and Der/opt-ntv, whereas poly(A) tail was added only at the termination region of Der/ntv-opt and Der/opt. Taken together, these observations suggest that the 3' half of the native Der f 7 gene contains several potential poly(A) addition signals which could be eliminated by codon optimization. Moreover, insertion of termination codons upstream of Der f 7 resulted in the increased level of its mRNA, suggesting that premature polyadenylation and consequent mRNA degradation by the nonstop mRNA decay pathway reduce the Der f 7 mRNA level.

37. Functional analysis of the fumitremorgin gene cluster of Aspergillus fumigatus

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Aspergillus fumigatus strains have been reported to produce a variety of secondary metabolites, including toxic prenylated alkaloids such as fumitremorgin C, and many putative secondary metabolic gene clusters have been identified in the genome reference strain Af293 following the completion of the genome sequence. A gene ftmA encoding a dimodular non-ribosomal peptide syntetase (NRPS) was found within one of the putative secondary metabolic gene clusters. ftmA was overexpressed in strain Af293 by insertion of multiple copies, and in the naïve host Aspergillus nidulans, which lacks the equivalent gene cluster, under the control of the alcA promoter. Though neither fumitremorgins nor the dipeptide intermediate brevianamide F, cyclo-L-Trp- L-Pro, could be detected in wild-type strains, brevianamide F accumulated in liquid cultures of both species following increased expression

of the NRPS gene. The cyclic dipeptide brevianamide F is the precursor of a variety of prenylated alkaloids, including fumitremorgins A, B, C, tryprostatin B and verruculogen. RT-PCR indicated that some of the genes in this cluster are poorly transcribed in Af293. We are currently investigating a collection of A. fumigatus strains in order to identify fumitremorgin producers, and to understand the regulation of expression of this secondary metabolic cluster.

38. ISOLATION OF A POTENTIAL CELL CYCLE REGULATED TRANSCRIPTION FACTOR AS A COPY NUMBER SUPPRESSOR OF nimA1

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NimA is a cell cycle regulated protein kinase required for initiation of mitosis in Aspergillus nidulans. Levels of nimA mRNA become elevated as cells enter mitosis and drop at mitotic exit. While much is known about the role played by NimA during mitosis, less is know about its regulation.

To identify possible interacting genes of nimA, we performed a copy number suppressor screen of the temperature sensitive nimA1 mutation. The nimA1 mutation is a point mutation in the non-catalytic domain which restricts the protein to the cytoplasm instead of letting it accumulate in the nucleus during mitosis resulting in a reversible G2 arrest. On the other hand, the nimA5 mutation is in the catalytic domain and inactivates the kinase. There are two potential ways of suppressing the nimA1 mutation, one by making more NimA1 so that there is enough that diffuses into the nucleus or secondly by changing the transport properties of the nuclear envelope so that NimA1 can enter the nucleus. The screen identified mcnB which can suppress nimA1 but not nimA5. Elevated expression of mcnB causes up-regulation of NimA1 protein. McnB has homology to fork head domain containing transcription factors. Endogenously tagged McnB-GFP accumulates in nuclei in late G2 and disappears at the onset of mitosis before re-accumulating in the next G2.This demonstrates that McnB is regulated through the cell cycle.

In conclusion, we have identified a potential cell cycle specific transcription factor for nimA which can drive increased expression of NimA and is itself cell cycle regulated.

39. ISOLATION OF AN ASPERGILLUS SPECIFIC NUCLEOLAR PROTEIN AS A COPY NUMBER SUPPRESSOR OF nimA1

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The nimA gene in Aspergillus nidulans encodes a protein kinase that is required for chromosome condensation, mitotic spindle formation and nuclear pore complex disassembly.

Given the crucial role played by NimA in mitosis, we set a screen to identify copy number suppressors of nimA1 mutant and identified a novel Aspergillus specific gene mcnA.

Endogenously GFP-tagged McnA appears as a single dot in the nucleus. Co-localization studies of McnA with nucleolar markers show McnA locates to the nucleolus and has a unique pattern of segregation during mitosis. During G2, McnA is located in a sub domain of the nucleolus. At mitosis, McnA locates in a focal point in the cytoplasm whereas other nucleolar proteins are dispersed throughout the cell. Post mitosis McnA remains as a single dot outside newly formed nuclei as the dispersed nucleolar proteins re-locate to daughter nucleoli. It is only later in G1 that McnA begins to appear within new nucleoli. In lower eukaryotic closed mitosis, the nucleolus divides within nuclei. In higher eukaryotic open mitosis the nucleolus disassembles and then undergoes a stepwise reassembly. Our studies of McnA and other nucleolar proteins, indicate that A. nidulans also undergoes disassembly then reassembly of its nucleolus.

Affinity purification of S-tagged McnA and SDS PAGE indicates that McnA migrates as several specific molecular weight species. Mass spectrometry identified these different forms to be modified by phosphorylation.

This study has identified a novel phosphoprotein specific to the Aspergilli and shows A. nidulans nucleoli to undergo mitotic segregation similar to higher eukaryotes.

40. MITOTIC CROSSING-OVER IN CHROMOSOME III OF THE ASEXUAL FUNGUS ASPERGILLUS NIGER; HOW WELL DOES PARASEXUAL ANALYSIS CORRELATE TO THE PHYSICAL MAP?

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In Aspergillus niger has many uses in food applications, for example as a producer of organic acids and enzymes. Strain improvement has thus far mainly been achieved by subsequent rounds of mutations and selection, and in some cases by the recombination of independent mutations located on different linkage groups. Another use of the parasexual cycle is the construction of diploids with improved characteristics. A. niger is an asexual fungus, but using the parasexual cycle, strains can be combined into heterozygous diploids, and upon haploidization, different combinations of unlinked mutations can easily be obtained. However, the exchange of mutations positioned on the same chromosomes, requiring mitotic crossing-over or gene conversion in the diploid phase, is very rare.

In order to study crossing-over, we improved the genetic map of chromosome III using the physical map. Using 6 auxotrophic markers, we constructed linkage group III specific marker strains. In a diploid containing five of these markers, we tested various effectors that could influence recombination of linked markers during haploidization and compared our findings with the physical map of chromosome III. In addition, we studied the mitotic crossing-over frequencies by

selection of homozygous cnxD diploids from a heterozygous diploid on chlorate. Chromosomal locations with increased mitotic cross-overs were searched for patterns possibly involved in recombination.

41. MATING TYPE GENES IN ASPERGILLUS NIGER AND A. TUBINGENSIS ISOLATES

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Mating processes in fungi are governed by mating type genes. Such mating type genes have recently been identified in some Aspergillus species including A. fumigatus, A. nidulans and A. oryzae (1). We searched the A. niger ATCC 1015 draft genome sequence (sequencing was carried out by the US DOE Joint Genome Institute) for MAT homologs, and identified a MAT1-1 gene in it. Degenerate primers were designed based on available MAT1-1 and MAT1-2 sequences of aspergilli, and a large number of black aspergilli were screened for the presence of either MAT1-1 or MAT1-2 homologs. Our search resulted in the identification of both MAT1-1 and MAT1-2 mating type strains among black aspergilli. However, the distribution of the mating type alleles among the strains examined was unequal. Among black aspergilli, 124/151 of the isolates carried the MAT1-1 gene, while MAT1-2 was only detected in the rest of the isolates. This observation is in contrast with that found in A. fumigatus, where the proportion of isolates carrying either the MAT1-1 or MAT1-2 allele was about the same (43% and 57%, respectively)(2).Such a strong deviation from the expected ratio indicates that the examined isolates are reproducing asexually (2).
The black Aspergillus isolates were assigned to species based on their mtDNA profiles, or by using species-specific ITS-RFLP analysis. Regarding intraspecific distribution of the mating type alleles, 69/77 A. niger, 49/63 A. tubingensis, 4/9 A. japonicus, 1/1 A. carbonarius and 0/1 A. brasiliensis isolates carried MAT1-1. Mating type homologues have also been identified in other Aspergillus species including A. clavatus, A. flavus and A. terreus. Further studies are in progress to find some explanation to the uneven distribution of mating type strains among black aspergilli, and to examine the distribution of MAT genes in other aspergilli.

1 Galagan JE, Calvo SE, Cuomo C et al. (2005) Sequencing of Aspergillus nidulans and comparative analysis with A. fumigatus and A. oryzae. Nature 438, 1105-1115
2 Paoletti M, Rydholm C, Schwier EU et al. (2005) Evidence for sexuality in the opportunistic fungal pathogen Aspergillus fumigatus. Curr Biol 15, 1242-1248

42. TRYPTOPHAN'S EFFECTS ON AFLATOXIN BIOSYNTHESIS AND ITS REGULATION IN ASPERGILLUS FLAVUS

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Aflatoxins are secondary metabolites produced by the fungi Aspergillus flavus and A. parasiticus. These compounds are toxic and carcinogenic. Many nutritional and environmental factors are known to affect aflatoxin formation. In order to better understand the molecular mechanisms that control or regulate aflatoxin production changes in aflatoxin biosynthesis in Aspergillus flavus and A. parasticus grown in yeast extract sucrose media supplemented with 50 mM tryptophan was examined. A. flavus grown in the presence of 50 mM tryptophan was found to have significantly reduced aflatoxin B_1 and B_2 biosynthesis, but A. parasitiucs cultures had significantly increased B_1 and G_1 biosynthesis. To determine if the effects of tryptophan were due to decreased levels of biosynthesis, microarray analysis of the effects of tryptophan on regulation of aflatoxin biosynthesis was performed. Preliminary analysis by MeV (TIGR) revealed 1174 genes occurred in both A. flavus and A. parasticus. Nine genes were found to be significant across the experiments by T-test. Further investigation of these candidate genes may identify potential regulators involved in aflatoxin biosynthesis.

43. IDENTIFICATION OF GENES INVOLVED IN AFLATOXIN BIOSYNTHESIS IN RESPONSE TO CARBON SOURCES IN ASPERGILLUS PARASITICUS

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Abstract

Aflatoxins are toxic and carcinogenic secondary metabolites produced by the fungi Aspergillus flavus and A. parasiticus. Media with simple sugars are more favorable for aflatoxin biosynthesis, media containing 0.1 M, or higher, of a suitable carbohydrate source will support biosynthesis of aflatoxin. This research is aimed at identifying genes differentially expressed and regulated in response to changes of different carbon sources. RNA was purified from the fungal mycelia harvested at six time points post shifting and aflatoxin contents were measured. Aflatoxin production is detected as early as 16 hours after shifting from a low carbon medium, Yeast Extract (YE) to a high carbon medium, Yeast Extract Sucrose (YES). Aflatoxin levels were reduced from 3 hour up to 12 hours time points post-shift. Aflatoxin biosynthesis recovered to the initial baseline level after 18 hours post shifting to YES. At the 24 hours time point, the aflatoxin level was increased to as high as 10 fold of the initial levels. Gene expression profiling using microarray identified a total of 1958 expressed genes across all experiments as analyzed by TIGR MeV software program. These included most of the aflatoxin pathway genes. Analysis of the microarray data using one-way ANOVA determined 62 expressed genes were significant. Targeted investigation into the functions of these highly expressed genes could help to identify potential genes for signal transduction between carbon assimilation and secondary metabolite production and for regulation of aflatoxin formation.

44. ASPERGILLUS FLAVUS GENOME SEQUENCE AND COMPARATIVE ANALYSIS WITH ASPERGILLUS ORYZAE

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Aspergillus flavus attacks several crop species and produces the potent carcinogen aflatoxin in nuts and agricultural grains. A 5X genome sequence has been assembled into 79 scaffolds ranging in size from 4.5 Mb to 1.0 kb. The 36.3 Mb genome contains 13,071 genes, 99.6% of which reside in the 16 largest scaffolds. The sequence data have been deposited at NCBI and is also available at (http://www.aspergillusflavus.org) through a web browser that allows visualization of Blast matches to genes, proteins and genomic sequence of other Aspergillus species, alignments of A. flavus ESTs, and GO annotations. Fortunately, a whole genome sequence is also available for A. oryzae, a closely related fungus that does not produce aflatoxins and has lost the ability to infect plants, animals and humans. Comparative genomic analysis between the two species indicates that the genome of A. flavus is very similar to that of A. oryzae, sharing greater than 96% sequence identity. Further, these two fungi have a similar number of genes necessary for secondary metabolism. The predicted number of genes for polyketide synthases (34), non-ribosomal peptide synthases (22), and cytochrome P450s (122) is similar to that predicted for A. oryzae, 31, 24, and 151, respectively.

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