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PROTEIN TARGETING AND SECRETION

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Genomic analysis of the secretion stress response in the enzyme-producing cell factory *Aspergillus niger*

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Aspergillus spp., and in particular *A. niger*, are filamentous fungi that have a high capacity secretory system to support their saprophytic lifestyles. Many *Aspergillus* enzymes are prepared commercially for a wide range of applications. In addition, *A. niger* has proved to be a useful cell factory for achieving acceptable commercial yields of some heterologous proteins. However, the large flux of proteins being translocated into the endoplasmic reticulum (ER), and the difficulties in assisting the folding of a high throughput of heterologous proteins, leads to the induction of stress responses that are collectively called protein secretion stress or ER stress. In this study, we have used the *A. niger* genome sequence, made available to us by DSM, and Affymetrix gene chips to explore the secretion stress responses. We induced ER stress either by chemical treatment of the wild-type cells with dithiothreitol (DTT) or tunicamycin, or by expressing the human protein, tissue plasminogen activator (t-PA). As expected, all of these treatments triggered the unfolded protein response (UPR), as shown by the expression levels of several well-known UPR target genes. The cellular responses to each stress were compared and the overlaps common to these conditions led to the identification of robust sets of induced or repressed genes. The predicted proteins encoded by most of the up-regulated genes function as part of the secretory system including chaperones, foldases, glycosylation enzymes, vesicle transport proteins, and ER-associated degradation proteins. We use these data to provide insight into both the molecular basis of protein secretion and secretion-related stress in an effective protein-secreting fungus, and to identify target genes for manipulation in strain improvement strategies.

The transcription factor Hac1p and it's functions in *Candida albicans*

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The unfolded protein response (UPR) and the mediating transcription factor (Hac1p in *Saccharomyces cerevisiae*) are known to be regulate the level of chaperones and foldases in the lumen of the endoplasmic reticulum (ER) and to sense nitrogen levels and mediate cellular responses to low nitrogen. We were therefore interested in whether the UPR had a role in nitrogen- dependent processes in *Candida albicans*, such as the yeast-mycelial transition. Here we report the cloning of the gene encoding Hac1p in *C. albicans* and the construction of a *hac1/hac1* null mutant strain.

In *S. cerevisiae*,the UPR induces the dimerization of a ER-resident type I transmembrane protein, Ire1p which activates splicing of *HAC1*^{U} to *HAC1*^{i} mRNA by excising a 252-bp intron at the 3'end. This activates transcription via direct binding to the *cis*-acting UPR element (UPRE) present in the promoter regions of its target genes. In *Aspergillus niger*, a similar mechanism is observed, but the intron is limited to 20-bp. In addition to this unconventional splicing in *A. niger*, a 5'-UTR truncation occurs which affects the size of the transcripts.

In this study, the UPR was induced by treating *C. albicans* yeast cells with dithiothreitol (DTT) and tunicamycin. The analysis of cDNA made from *HAC1* mRNA shows that there is a difference between the *HAC1*^{i} and *HAC1*^{U} towards the 3' end of the mRNA; *HAC1*^{i} mRNA lacks a 19-base intron that is present in *HAC1*^{U} showing close resemblance to *hacA* in *A. niger.* The resulting transcripts differ in their carboxy terminal with an added 10 amino acids to produce functional Hac1p in *C. albicans*. Further analysis will be done to establish if this is the only difference in size between the *HAC1*^{u} and *HAC1*^{i} mRNA using RACE-PCR. Furthermore, we have also deleted both copies of the *HAC1* gene in *C. albicans* to create a null mutant strain which we are studying now in relation to the UPR and morphogenesis.

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Characterization of alpha-synuclein in *Aspergillus nidulans*

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Fungal model systems are increasingly used to study the pathophysiology of human diseases due to various advantages like the availability of complete genome sequences, short life cycles and well established genetic tools. We have recently started to study Parkinson's disease (PD) including Lewy body (LB) pathology in the filamentous fungus *Aspergillus nidulans*. LBs are intraneuronal aggregations found in PD patients consisting mainly of the pre-synaptic protein alpha-synuclein. In this study, we constucted strains carrying genomic integrations of one or more copies of either the wild-type *alpha-synuclein* gene or mutant genes encoding the A53T and A30P alpha-synuclein variants, respectively. These strains were analyzed regarding growth rate and asexual and sexual development. We also fused these genes to GFP to investigate Lewy body formation in *Aspergillus*. We found that *A. nidulans* is able to tolerate the expression of *alpha-synuclein* showing normal growth and asexual and sexual development. We observed aggregation only for wt alpha-synuclein in strains which harboured two copies of the gene. These results differ from findings in *Saccharomyces cerevisiae* in which the expression in some cases resulted in toxicity in a dosage dependent manner and aggregation also for the A53T variant could be observed.

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.

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Characterisation of the *Trichoderma reesei* proteasome

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The filamentous fungus *Trichoderma reesei* is one of the most efficient eukaryotic cell factories available. Considering its extraordinary secretion capacity, this species can be characterised as “a professional” protein secretor. Protein quality control is a crucial cellular function. Proteins that are not folded correctly or not fully assembled are recognised in the early secretory pathway and subjected to degradation by the ubiquitin-proteasome pathway featuring a large (approximately 2.5 MDa) multicatalytic protease, the proteasome. The proteolysis of cellular proteins is a highly complex, temporally controlled and tightly regulated process.

An isolation method for the 20S proteasome of *T. reesei* and a 2D master map of the fungal proteasome have been established. From the map, a number of proteasome subunit proteins as well as proteasome-interacting proteins have been identified. We are also in the process of creating a series of mutant forms of the main cellobiohydrolase enzyme CBHI to trace its secretion and presumed degradation in the proteasome.

Imaging the last stages of protein secretion in the filmentous fungus *Trichoderma reesei*.

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Available data suggest that the basic principles of secretion obtained from studies on *S. cerevisiae* and animal cells also apply to filamentous fungi. Although considerable research has been performed on the secretion process in filamentous fungi, little is known about their secretory machinery. The transport of proteins between the cellular compartments occurs in membrane-bounded vesicles that bud from the donor and fuse with the acceptor compartment. Membrane bound proteins called SNAREs (Soluble N-ethylaleimide-sensitive factor Attachment protein REceptors) play a key role in membrane fusion. SNAREs have been found to be involved in most membrane fusion events in the cell. This study describes the cloning and characterization of a putative exocytotic t-SNARE (*sso1*) from *Trichoderma reesei*. We cloned the *T. reesei* homologue of *Neurospora crassa nsyn2*. This protein was labelled with the Cerulean fluorescent protein mCer and the fusion protein expressed in *T. reesei*. Transformants expressing the fusion protein exhibit a clear labelling of the plasma membrane. The fusion protein is uniformly distributed along the hyphae with no tip-focused gradient. A v-SNARE SNCI was labelled with mRFP to analyze its interaction with the SSOI protein using fluorescence resonance energy transfer (FRET) imaging.

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Heterologous expression of *Aspergillus fumigatus* alpha-galactosidase in *Aspergillus sojae*

Betul Soyler*

unknown

Fungi belonging to the genus *Aspergillus* are ubiquitous in the surrounding. *Aspergillus fumigatus* is regularly encountered as contaminant in the clinical environment, and the effect of infection caused by this organism may be devastating. This organism is a human pathogen mainly affecting immunocompromised patients. *A. fumigatus* is a thermotolerant fungus and can efficiently produce thermostable alpha-galactosidase. Alpha-galactosidase is of medical and industrial importance. The enzyme has a proven activity of transgalactosylation in addition to its original galactose liberating acitivity. The aim of this study is to express alphagalactosidases of *A. fumigatus* in a safe host, *A. sojae*. Previously, two alpha-galactosidase genes with different characteristics were cloned from *A. fumigatus*. One of the gene that is responsible from degrading polymeric substrates with a high efficieny is cloned on an expression vector pAN52-4. The vector contains glyceraldeyhde 3-phosphate dehydrogenase promoter of *A. nidulans* and signal peptide region of the *A. niger* glucoamylase gene. Heterologus expression in *A. sojae* will be followed by the analysis of the physico-chemical properties of the expressed enzymes.