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PHYSIOLOGY AND
METABOLISM

Chair:

Jens Nielsen

& Matthias Brock



Vlo-1

The trehalose 6 phosphate synthase gene of *Magnaporthe grisea* plays an integrative role in regulation of carbon and nitrogen metabolism and is essential for plant pathogenicity

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Deletion of the trehalose-6-phosphate synthase gene of *M. grisea*, *tps1*, leads to abolishment of trehalose biosynthesis, a concomitant reduction in asexual spore production, loss of plant-pathogenicity, and an inability to grow on glucose minimal media. This last effect is reminiscent of the glucose non-utilising phenotype of *Saccharomyces cerevisiae* $\Delta tps1$, where loss of Tps1 protein causes a catastrophic and lethal decline in ATP due to the phenomenon of glycolytic mis-regulation. We considered the glucose non-utilising phenotype of *M. grisea* also to be a consequence of similar unregulated influx of glucose into glycolysis, and wanted to separate and study this aspect of the *M. grisea* $\Delta tps1$ phenotype from that of loss of plant pathogenesis. In addition, we wished to use functional analysis of the Tps1 protein, and deletion of the Tps3-encoding gene, to determine the role of trehalose biosynthesis in the plant-pathogenic process. In yeast, deletion of the hexokinase gene in $\Delta tps1$ slows entry of glucose into glycolysis and remedies growth of the mutant on glucose. The homologous *M. grisea* hexokinase gene was identified and disrupted in wild type and $\Delta tps1$ strains with the expectation that growth and development of the mutant on glucose minimal media would be similarly improved. However, while some remediation of the $\Delta tps1$ phenotype was seen in a hexokinase null background, our observations led us to conclude that, in contrast to the situation in yeast, deletion of the *tps1* gene of *M. grisea* does not affect glycolytic flux. Rather, the growth defect of the $\Delta tps1$ mutant on glucose minimal media can be explained by the inability of $\Delta tps1$ to utilise nitrate, present in our minimal media salts, as sole nitrogen source and demonstrates an absolute requirement of Tps1 for nitrate reductase activity. Subsequent plate growth tests and enzyme activity assays have determined a novel interaction of Tps1 and nitrate reductase that is mediated via glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH levels and is not described in other fungi. Moreover, our functional studies suggest this effect of the $\Delta tps1$ mutant on nitrate utilisation, via NADPH levels, and on plant-pathogenicity occurs through a sugar signalling pathway involving glucose-6-phosphate, the substrate of Tps1. Interestingly, we also show the trehalose product is dispensable for both nitrate reductase activity and plant-pathogenicity. Therefore, Tps1 in *M. grisea* plays a central role in integrating sugar signalling and nitrogen metabolism and is essential for plant-pathogenicity.

An endophyte nonribosomal peptide synthetase in siderophore biosynthesis is essential for mutualistic interactions with grasses

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Siderophores are low molecular weight, iron-chelating agents that solubilize iron (III) and control intracellular iron levels. We have identified a novel siderophore from symbiotic fungal endophytes (genera *Epichloë* and *Neotyphodium*) of cool-season grasses. In nature, these fungi are never free-living, but form mutualistic associations where they are confined to the intercellular spaces (apoplast) of leaf sheaths and blades. We undertook a degenerate PCR approach to identify NRPS genes from endophytes with an aim to functionally characterise their role in symbiosis. One of these from *E. festucae* has high amino acid identity to other NRPSs involved in siderophore biosynthesis. Functional analysis of this gene (termed *sidEf*) by targeted gene replacement eliminated biosynthesis of an extracellular siderophore that was induced under iron-depleted conditions. Structural characterisation by LC-MS-MS showed clear similarities to hydroxamate-type siderophores of the fusarinine family. To our knowledge this is the first siderophore detected from grass endophytes and from our studies appears to be the only one synthesised. Surprisingly, plants inoculated with strains carrying a targeted deletion in *sidEf* have a dramatic phenotype. Siderophore loss consistently caused de-regulation of fungal hyphal growth, plant stunting and sometimes even tiller death. Transmission electron microscopy revealed an altered hyphal ultrastructure, including uncharacteristic vacuolation. Additionally, in these plants, we also found significantly higher levels of the endophyte alkaloids ergovaline and lolitrem. These alkaloids are only produced *in planta*, but the mechanism for regulation is unknown. From our results, we hypothesise that competition for iron is a critical factor in keeping the grass-endophyte interaction mutualistic and that siderophore loss upsets the carefully controlled process of iron homeostasis in the whole symbiotum. We are now investigating the molecular mechanisms controlling global regulation of iron in the symbiotum, and how this influences endophyte secondary metabolism by using Affymetrix GeneChips. In addition to the transcriptomic analysis of plants infected with the siderophore knock-out, a detailed metabolomics analysis is also underway.

Vlo-3

Protein-protein interactions in the beta-lactam producer *Acremonium chrysogenum*: effects on secondary metabolism and morphology

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The filamentous fungus *Acremonium chrysogenum* is used industrially to produce the β -lactam antibiotic cephalosporin C. A complex regulatory network of transcription factors controls the expression of at least seven cephalosporin C biosynthesis genes. One of these proteins is the RFX transcription factor CPCR1 which has been used in a yeast two hybrid screen to identify potential interaction partners. A cDNA was identified encoding a novel forkhead transcription factor, so called AcFKH1, which is the first characterized member of the forkhead gene family in filamentous fungi. We have verified this protein-protein interaction *in vitro* by GST pull down assays and in the living fungal cells by the bimolecular fluorescence complementation (BiFC) approach [1, 2].

Using gel retardation analysis, the DNA binding properties of the fungal forkhead protein AcFKH1 were investigated. AcFKH1 recognizes two forkhead consensus binding sites within the promoter region of the cephalosporin C biosynthesis genes *pcbAB* and *pcbC* [2]. Furthermore, HPLC and Western blot analyses of *Acremonium* transformants with multiple copies of the *Acfkh1* gene and a knockout strain support the idea of AcFKH1 being a regulator of the early cephalosporin C biosynthesis genes.

In a second approach, the role of CPCR1 and AcFKH1 on fungal morphogenesis was analyzed. The filamentous fungus *A. chrysogenum* forms arthrospores which can be understood as vegetative spores appeared during prolonged cultivation under limited nutrient supply. Interestingly, it was reported that the phase of hyphal differentiation into arthrospores coincides with the maximum rate of cephalosporin C biosynthesis; however the molecular determinants connecting both processes remain largely undefined. Light microscopic analyses of a disruption strain, multicopy strains as well as several recombinant control strains revealed that CPCR1 is required for hyphal fragmentation, and thus the formation of arthrospores. In contrast to CPCR1, the AcFKH1 factor is not directly involved in the fragmentation of hyphae. Instead, the presence of AcFKH1 seems to be necessary for CPCR1 function in *A. chrysogenum* morphogenesis [3]. From the sum of our investigations we conclude that morphogenesis and cephalosporin C biosynthesis are dependent on the same transcription factors in *A. chrysogenum*.

[1] Schmitt EK, Hoff B, Kück U (2004) Gene 342: 269-281

[2] Hoff B, Kück U (2005) Curr Genet 47: 132-138

[3] Hoff B, Schmitt EK, Kück U (2005) Mol Microbiol 56: 1220-1233

Specific growth rate – and elusive but crucial factor in the investigation of regulation of fungal metabolism

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The specific growth rate ($\text{g/g.h} = \text{h}^{-1}$) has a strong impact on fungal metabolism, but its role in the observation of regulatory phenomena has received only little attention. Researchers studying the regulation of fungal metabolism mostly use plates, batch cultures (shake-flasks) or replacement cultures (washed cells). In such experiments, carbon sources are classified as "repressing", or "non-repressing", or "inducing" vs. "non-inducing". Here we will provide examples that this classification is arbitrary, and virtually a consequence of the growth rate only: one is CreA-dependent carbon catabolite repression in *Aspergillus nidulans*, using beta-galactosidase as a model system. It is repressed by D-glucose in batch cultures. However, in glucose-limited chemostats, beta-galactosidase formation was repressed only at high and moderate specific dilution rates, but derepressed at low dilution rates ($D = 0.015 \text{ h}^{-1}$). Chemostat cultures with a carbon catabolite derepressed *A. nidulans* mutant strain revealed a dilution rate-independent constant beta-galactosidase activity of the same range as that found in the wild-type strain at low dilution rate. Thus the creA-dependent carbon catabolite regulation is a growth rate dependent process in *A. nidulans*. Induction of beta-galactosidase activity, in contrast, was influenced by the inducer (lactose and D-galactose) in the contrary way: in both *Trichoderma reesei* and *A. nidulans* it peaked during the phase of fast growth and declined thereafter. In carbon-limited chemostat cultivations, beta-galactosidase activity formation paralleled to the dilution rate applied, with respective values being higher on d-galactose than on lactose. Finally, *T. reesei* cellulase formation is an example where the inducer remains undetected during batch growth. When grown on D-galactose in batch cultures, *T. reesei* does not produce cellulases. However, D-galactose induces cellulase gene transcription at $D = 0.015 \text{ h}^{-1}$ but not at higher dilution rates, and controls show that this is not due to carbon catabolite derepression. Our results imply that in batch cultures the specific growth rate significantly influences the effect observed for a given carbon source, and studies on regulatory mechanisms should make use of chemostat cultivations to correctly assess the underlying phenomena.

Vlo-5

Secretion differentiation in *Aspergillus niger*: glucoamylase is only secreted by part of the hyphae in the exploring mycelium

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Mycelial fungi play a central role in element cycling in nature by degrading dead organic material such as wood. Fungal colonization of a substrate starts with the invasion of exploring hyphae. These hyphae secrete enzymes that convert the organic material into small molecules that can be taken up by the fungus to serve as nutrients. Using GFP as a reporter, we show for the first time that exploring hyphae of *Aspergillus niger* differentiate with respect to enzyme secretion; some strongly express the glucoamylase gene *glaA*, while others hardly express it at all. When a cytoplasmic GFP was used, 27% of the exploring hyphae of a 5-day-old colony belonged to the low expressing hyphae. By fusing GFP to glucoamylase and by introducing an ER retention signal, this number increased to 50%. This difference is due to cytoplasmic streaming of the reporter in the former case, as was shown by using a photoactivatable GFP. Our findings indicate that a fungal mycelium is highly differentiated, especially when taking into account that hyphae in the exploration zone were exposed to the same nutritional conditions. Future research should establish whether this differentiation operates during fungal pathogenesis and in industrial fermentations.

The roles of SC3 and SC15 of *Schizophyllum commune* during growth on wood

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The SC3 hydrophobin plays several roles in growth and development of *S. commune*. It lowers the surface tension of the aqueous substrate, enabling aerial growth, and it coats the aerial hyphae rendering them hydrophobic. SC3 also allows hyphae to attach to hydrophobic surfaces and has a role in the cell wall architecture. Next to SC3, the SC15 protein is abundantly secreted into the medium. This protein, which is not a hydrophobin, can also be found in the mucilage that binds aerial hyphae together. SC15 and SC3 were inactivated by disruption, which occurred at a frequency of 1-5%. SC15 was also inactivated by RNAi using a hairpin construct. Silencing occurred at a frequency of 80% and was also observed in dikaryons in which one of the partners contained a wild-type nucleus. These findings demonstrate the potential of RNA-based gene silencing for functional analysis of genes in *S. commune*. During growth on synthetic medium SC15 mediates formation of aerial hyphae in the absence of SC3. In contrast, during growth on birch wood both SC3 and SC15 are needed for abundant aerial growth. Biomass formation in the substrate, however, was not affected by the absence or presence of SC3 and SC15. Possibly, SC3 and SC15 form a mixed membrane at the water-air interface and at the surface of aerial hyphae during growth on wood. Uncolonized birch wood attracts aerial hyphae of monokaryotic but not dikaryotic strains of *S. commune*. No hyphae were attracted when the birch wood was colonized with a compatible or incompatible mating partner. Apparently, the sterile aerial hyphae of a monokaryon are not dedicated to find a mating partner but to find new wood resources. Future experiments should reveal which wood volatile is responsible for the tropic growth response.

Vlo-7

The fungal hydrophobin RoIA laterally moves on hydrophobic surfaces and recruits polyesterase

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When fungi grow on plant or insect surfaces coated with wax polyesters that protect against pathogens, the fungi generally form aerial hyphae to contact the surfaces. Aerial structures such as hyphae and conidiophores are coated with hydrophobins, which are surface-active proteins involved in adhesion to hydrophobic surfaces. Hydrophobins are small proteins containing eight conserved cysteine residues, which are ubiquitous among filamentous fungi. Hydrophobicity profiles of hydrophobins are used to categorize them into class I and class II, which seem to differ in the solubility of the aggregates or assembled forms. When the industrial fungus *Aspergillus oryzae* was cultivated in a liquid medium containing the biodegradable polyester polybutylene succinate-coadipate (PBSA), the *rolA* gene encoding class I hydrophobin RoIA was highly transcribed. High levels of RoIA and its localization on the cell surface in the presence of PBSA were confirmed by immunostaining. Under these conditions, *A. oryzae* simultaneously produced the cutinase CutL1, which hydrolyzes PBSA. Cutinases, which are well known to be produced by pathogenic fungi (including *Fusarium*), hydrolyze cutin, a heterologous complex of wax esters found on the surfaces of plants. Preincubation of PBSA with RoIA stimulated PBSA degradation by CutL1, suggesting that RoIA bound to the PBSA surface was required for the stimulation. Immunostaining revealed that PBSA films coated with RoIA specifically adsorbed CutL1. Quartz crystal microbalance analyses further demonstrated that RoIA attached to a hydrophobic sensor chip specifically adsorbed CutL1. CD spectra of soluble-state RoIA and bound RoIA suggested that RoIA underwent a conformational change after its adsorption to hydrophobic surfaces. These results suggest that RoIA adsorbed to the hydrophobic surface of PBSA recruits CutL1, resulting in condensation of CutL1 on the PBSA surface and consequent stimulation of PBSA hydrolysis. A fluorescence recovery after photobleaching experiment on PBSA films coated with FITC-labeled RoIA suggested that RoIA moves laterally on the film. We discuss the implications of both novel properties of hydrophobin RoIA in making contact to plants, insects, or animals prior to fungal infection, because the PBSA degradation process catalyzed by *A. oryzae* CutL1 in combination with RoIA likely mimics the early stage of fungal infection.