Workshop VIII

Fungal Cell Factories
Chair: Cees van den Hondel

VIIIo-1

Improvements infilamentous fungal cell factories in the area of genomics

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Abstract not available at time of print.

VIIIo-2

GENOMIC APPROACHES TO THE RELATIONSHIP BETWEEN HYPHAL DEVELOPMENT AND PROTEIN SECRETION IN THE ASPERGILLI-GAPSIA

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Filamentous fungi, such as the Aspergilli, are very proficient at secreting native proteins. While this ability makes them potential hosts for the production of heterologous proteins, the yield of secreted foreign proteins is frequently much less than that for native proteins. The Aspergillus secretory pathway is complex and appears to be optimised for the secretion of native enzymes. Therefore it is necessary to identify and overcome major bottlenecks in the secretion machinery that reduces the efficiency of the secretion of recombinant proteins. A limited number of Aspergillus mutants are known to affect hyphal morphology, growth and polarity, including cotA and hbr mutants. In order to compare different strains (wild type, recombinant and morphological mutants) in terms of gene expression levels and protein production, we are using chemostat cultivations to compare them under equivalent, tightly controlled, physiological conditions.
The temperature sensitive mutant of A.nidulans hbrB3 exhibits hyperseptation and increased hyphal branching, when grown at 42°C. hbrB3 and wild type R153 were grown in chemostats both at 30°C and 42°C and DNA microarray analysis was carried out using cy3/cy5 labelled cDNA to compare the effect of hyphal alterations. Initial data show a set of genes, linked to the cell cycle and cytoskeleton that appear upregulated in the hbr mutant when compared to parental strain R153 (both grown at 42°C).
With the availability of the genome sequences of three Aspergillus species, microarray technology and proteome analysis, we hope to identify common genes/proteins which play a crucial role in protein secretion and stress responses), which occurs when misfolded proteins accumulate in the Endoplasmatic Reticulum (ER) and hyphal development.

DIFFERENTIAL PROTEIN SECRETION IN ASPERGILLUS NIGER

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Aspergillus niger is considered an excellent system for protein production due to its ability to secrete large amounts of enzymes into the medium. Secretion of certain enzymes of industrial relevance, such as glucoamylase, has been studied in detail and improved very successfully in some cases. Strain improvement strategies included creation of multicopy transformants, improved fermentations and/or mutagenesis and screening. Currently we are studying secretion from a mycelial point of view.
Previous studies demonstrated that glucoamylase is exclusively secreted at the periphery of the colony. In this zone only few hyphae secrete the protein. From these observations we believe that the production of a certain enzyme can be improved by increasing the number of hyphae that secrete it. This can be done by expressing glucoamylase behind promoters that are active in hyphae normally not expressing this enzyme. We have designed and optimised a ring plate system, which allows taking concentrical liquid samples within the colony. Enzymes secreted in different zones of the colony can be visualized by SDS-PAGE and their activity measured by assays using p-nitrophenol substrates. Several zone specific proteins were detected using five different carbon sources and two different strains. These proteins are being identified using N-terminal amino acid sequencing, allowing us to clone the corresponding genes. By cloning the glucoamylase coding sequence behind the promoters of these genes it is expected that glucoamylase secretion will mimic the zone specific secretion pattern of the selected proteins. In this way we will be able to see whether glucoamylase can be produced by hyphae that normally do not secrete the enzyme.

SISOPENICILLIN N EPIMERASE SYSTEM IN *Acremonium chrysogenum*: THE DISCOVERY OF THE MISSING PIECE OF THE PUZZLE.

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The epimerization step that converts isopenicillin N into penicillin N during cephalosporin biosynthesis has remained uncharacterised for many years in spite of its industrial relevance. A transcriptional analysis of a 9 kb region located downstream of the *pcb*C gene revealed the presence of two transcripts that correspond to the genes named *cef*D1 and *cef*D2 encoding proteins with high similarity to long chain acyl-CoA synthetases and acyl-CoA racemases from *Mus musculus, Homo sapiens and Rattus norvergicus*. Targeted inactivation of *cef*D1 and *cef*D2 was achieved by the two marker gene replacement procedure. Disrupted strains lacked isopenicillin N epimerase activity, were blocked in cephalosporin C production and accumulated isopenicillin N. Complementation *in trans* of the disrupted non-producer mutant with both genes restored cephalosporin production indicating that the proteins encoded by *cef*D1 and *cef*D2 are required for epimerisation of isopenicillin N into penicillin N in the cephalosporin biosynthesis. This epimerisation system occurs in eukaryotic cells and is entirely different from the known epimerisation systems involved in the biosynthesis of bacterial β-lactam antibiotics. To determine if the IPN-epimerization is a rate-limiting step in cephalosporin biosynthesis, the copy number of *cef*D1 and *cef*D2 genes has been increased. We obtained two types of transformants TMCD26, TMCD53, TMCD242 and TMCD474, have 2 to 7 extra copies of *cef*D1 and *cef*D2 genes and showed double IPN-epimerase activity than *A. chrysogenum* C10 and overproduce cephalosporin C. On the other hand, tranformants TMCD2, TMCD32 and TMCD39, have 3 to 5 extracopies of *cef*D1 and *cef*D2 do not have detectable IPN-epimerase and do not produce HPLC detectable cephalosporin C. These transformants seem to have a problem in mycelia differentiation

VIIIo-5

APPLICATION OF RIBOZYME TECHNOLOGY IN ASPERGILLUS GIGANTEUS IN ORDER TO INFLUENCE GENE EXPRESSION

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Metabolic engineering in filamentous fungi is very promising since many fungi produce high value secondary metabolites (e.g. antibiotics). Ribozyme technology as a tool for metabolic engineering can be used in order to influence metabolic pathways more broadly than other methods available such as mutagenesis or gene disruption. For example, reduction in expression of essential genes is possible when inducible ribozymes are used. Since there are only reports on ribozyme application in bacteria, yeast and mammalian cells, we tested whether this technology is functional in filamentous fungi as well.
A model system was established in the ascomycete Aspergillus giganteus consisting of different ribozymes targeting a constitutively expressed reporter gene (beta-glucuronidase; uidA). Inducible expression of ribozymes is mediated by the alcA promoter of A. nidulans. Assays for GUS activity in different clones are under investigation and will reveal whether ribozyme technology represent a useful tool in fungal metabolic engineering.

VIIIo-6

MUSHROOM MOLECULAR PHARMING

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There is a pressing need for alternative systems for the production of heterologous proteins of high value and mushroom growing provides an unexploited opportunity for biomanufacturing. There is also potential for enhancing the production of bioactive compounds already present in mushrooms. Constraints on exploiting mushrooms for the production of bioactive compounds have been a lack of rigorous characterisation of these compounds and good nutritional and/or clinical data for their efficacy. Strategies for enhancing their production are therefore probably premature but evidence of real clinical worth could emerge at any time. Recent advances in the transformation of the cultivated Agaricus bisporus have enabled facile introduction of novel genes and means the ability to produce high value proteins is now a reality for this major crop species. Transformation using Agrobacterium tumefaciens is particularly effective and we have used this technology to introduce transgenes for five proteins of commercial interest into A. bisporus. Progress in heterologous protein expression in A. bisporus and development of a new commercial enterprise will be discussed.