VII
CELL-FACTORIES AND BIOTECHNOLOGY

Chair:

Cees van den Hondel

& David Archer

VIIo-1

Genetic breeding of edible mushrooms: from the genome to the production of new varieties of *Pleurotus ostreatus*

Lucía Ramírez*, Eneko Idareta, Javier Jurado, Jordi Castellón, Luis M. Larraya, Antonio G. Pisabarro

Department of Agrarian Production, Public University of Navarre, 31006 Pamplona, Spain

The breeding of new varieties of industrially cultivated edible mushrooms must proceed in the framework defined by the breeding objectives, the biological characteristics of the material and the legal and cultural constraints imposed to the breeding technology to be used. This last aspect is of the greatest importance in the case of a food that is considered in European countries as high quality and closer to nature than other industrially produced foods. This fact prevents the use of genetic-engineering based technologies for breeding, as the consumers would hardly accept genetically modified mushrooms. Consequently, mushroom breeding should be based on time-consuming processes of classic breeding. Molecular biology, however, can offer to the breeders useful tools for speeding up the selection process, for evaluating the new bred lines and, last but not least, to identify and eventually protect legally the outcome of their breeding programs. Here, we will review the use of the molecular-marker assited selection of parentals for the construction of new strains of the industrially produced edible basidiomycete *Pleurotus ostreatus* and the use of these markers for the legal protection of the new bred products

Transcriptional analysis of *Trichoderma reesei* bioprosesses with the novel TRAC method

Jari Rautio, Kari Kataja, Reetta Satokari, Merja Penttilä, Hans Söderlund, Markku Saloheimo*

VTT Biotechnology, P.O. Box 1500, 02044 VTT, Espoo, Finland

Moving of biological research to postgenomic era with a growing number of organisms including filamentous fungi has increased the interest in functional genomics, and the need for fast and reliable transcriptional profiling methods is thus growing. We have developed a rapid method for transcriptional profiling of microbial cultivations based on a novel technique called TRAC "transcriptional profiling with the aid of affinity capture". This method allows fast gene expression analysis for sets of mRNAs by solution hybridisation with a pool of target-specific oligonucleotide probes of distinct sizes that are identified and quantified by capillary electrophoresis. The assay procedure has been semi-automated for simultaneous treatment of 96 samples using magnetic bead particle processor. To further enhance the robustness of the method it was set up to work with crude cell lysates. TRAC has been shown to produce results highly consistent with mRNA quantification by Northern hybridisation. Computational methods have been applied for design of target-specific oligonucleotide probes and to assign them into minimal number of pools. The whole assay procedure can be performed in three hours, implying its usefulness in bioprocess monitoring and control.

The developed TRAC method application has been used for monitoring the levels of a set of mRNAs in the filamentous fungus *Trichoderma reesei* in fermentation conditions. Chosen gene markers for bioprocess monitoring are involved in various cellular pathways including unfolded protein response, protection against various stress conditions, oxygen and nutrient limitation responses, protein synthesis and growth. Data collected from different types of fermentations, batch, fed-batch and continuous cultures, shows the potential of the method for use in optimisation of production processes and provides novel information about regulation of various genes during different phases cultivations. We have also used TRAC successfully for assessment of steady states in chemostat cultures.

VIIo-3

Proteolysis and protein processing in filamentous fungi

Peter J Punt[1*], Margreet Heerikhuisen[1], Cora van Zeijl[1], B Christien Lokman[1], Machtelt Braaksma[1], Cees AMJJ van den Hondel[2]

[1] TNO Quality of Life, Department of Microbiology, P.O. Box 360, 3700 AJ Zeist, The Netherlands
[2] Leiden University, Institute of Biology Leiden, Fungal Genetics Research Group, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

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 fungalKEX2/furinhomologues (*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.

The peroxisomal hydrophobic penicillin cell factory: Two peroxisomal enzymes phenylacetyl-CoA ligase and isopenicillin N acyltransferase convert cytoplasmic IPN into hydrophobic penicillins.

J.F. Martín[1], M. Lamas-Maceiras[1], I. Vaca[2], C. García-Estrada[2]

[1] Área de Microbiología, Fac. CC. Biológicas y Ambientales, Universidad de León, Campus de Vegazana, s/n, 24071 León, Spain.
[2] Instituto de Biotecnología de León (INBIOTEC). Science Park, Av. Real 1. 2004 León. Spain.

The first two enzymes involved in penicillin biosynthesis (a non-ribosomal peptide synthetase named ACV synthetase) and isopenicillin N synthase) are cytoplasmic enzymes. Isopenicillin N (IPN) is formed in the cytoplasm and is partially secreted to the culture medium. A gene (*phl*) encoding the authentic phenylacetyl Co-A ligase of *P.chrysogenum*, which activates the aromatic side chain to aryl-CoA before its addition to IPN,was cloned and deleted. Deletion of the *phl* gene decreased production of benzylpenicillin and its amplification resulted in an 8-fold increase in phenylacetyl-CoA ligase activity, higher penicillin production and increased resistance of the strain to phenylacetic acid or phenoxyacetic acid. Evidence was obtained for the involvement of at least two aryl-CoA ligases in *P.chrysogenum*. (Lamas-Maceiras *et al.*, 2006). The Phl protein contains a canonical peroxisome targeting sequence (PTS1). A second enzyme IPN acyltransferase (IAT) containing also the PTS1 sequence and responsible for the addition of the activated aryl-CoA side chain to IPN, was confirmed to be located into peroxisomes by immunoelectron microscopy. IAT is a self-processable enzyme that splits the inactive 40 kDa proIAT into a functional a-b heterodimer (29 + 11 kDa). An unprocessable variant IAT^{C103S} (lacking enzyme activity) was also shown to be located into the peroxisomal lumen, suggesting that the acyltransferase is targeted and transported into the peroxisomes as proIAT (before processing).

The peroxisomal location of these two enzymes indicates that the last steps of hydrophobic penicillin biosynthesis is performed by a peroxisomal complex that converts hydrophilic IPN taken up from the cytoplasm into different hydrophobic penicillins depending on the availability of different side chain precursors insidethese organelles. Some of these precursors, e.g. pentenoic acid (forming pentenylpenicillin, PenF) or heptanoic acid (forming heptanoyl penicillin, PenK) are formed by the peroxisomal fatty acid b-keto oxidation pathway, whereas phenylacetic acid or phenoxyacetic acid are taken up from the culture medium into peroxisomes. These findings have important implications for the transport of intermediates and the final secretion of hydrophobic penicillins to the culture medium.

Lamas-Maceiras, M., Vaca, I., Rodríguez, E., Casqueiro, J., Martín, J.F. (2006) Amplification and disruption of the phenylacetyl-CoA ligase gene of *Penicillium chrysogenum* encoding an aryl-capping enzyme that supplies phenylacetic acid to the isopenicillin N acyltransferase. Biochem J. 2005 Dec 2; [Epub ahead of print]

VIIo-5

Development of *Trichoderma reesei* transformation technology with the aid of an episomal vector system

Nina Aro, Ann Westerholm-Parvinen, Merja Penttilä, Markku Saloheimo*

VTT Biotechnology, P.O. Box 1500, 02044 VTT, Espoo, Finland

In conventional transformation procedures of filamentous fungi the transformation frequencies have been modest and only integrative vectors have been available. In modern fungal molecular biology, however, screening approaches with the help episomal vectors have advanced the progress of the field greatly. For example, in enzyme discovery or protein engineering it is beneficial to be able to screen for new enzymes or improved mutant enzymes in the ultimate production host of the enzyme. Episomal vector systems have been developed for a few fungal species including the Aspergilli, but Trichoderma reesei has suffered from a lack of such a system.

We have developed *T. reesei* transformation technology for genetic and enzyme screening purposes. The transformation procedure was optimised to improve the transformation frequencies of any vectors. Episomal vectors were used, and with these we obtained transformation frequencies that are far above those obtained with integrative vectors and are feasible for genetic and enzyme screening purposes. Procedures for plasmid rescue, colony picking and microtiter plate cultivation were also developed. The use of the novel transformation system in enzyme and genetic screening experiments will be discussed.

The major secreted protein of *Hypocrea atroviridis* on glucose is a member of a strongly conserved family comprising plant defense elicitors and human allergens

Verena Seidl[1*], Martina Marchetti[2], Reingard Schandl[2], Günter Allmaier[2], Christian P Kubicek[1]

[1] Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, TU Vienna, Getreidemarkt 9/166-5, 1060 Vienna, Austria.
[2] Institute of Chemical Technologies and Analytics, TU Vienna, Getreidemarkt 9/164-AC, 1060 Vienna, Austria

We used a proteomic approach to identify constitutively formed extracellular proteins of *Hypocrea atroviridis* (*Trichoderma atroviride*), a known biocontrol agent. The fungus was cultivated on glucose as a carbon source, and the secretome during active growth was examined by examined by 2D gel electrophoresis. Three major proteins were detected. Two of them represented the dimer and monomer of the same protein (16 kDa, pI 5.5). Peptide mass fingerprints generated by MALDI-TOF-MS and peptide sequence tags obtained by PSD or high energy collision induced dissociation (MS/MS) experiments were used to screen a database of *Trichoderma* EST sequences and the genome database of *H. jecorina* (*T. reesei*). This resulted in the identification of respective genes in *H. jecorina*, *H. atroviridis*, *T. asperellum* and *H. lixii* (*T. harzianum*) and *in silico* analysis of the *H. jecorina* genome database detected two additional, similar hypothetical proteins. The gene from*H. atroviridis* was cloned and named *epr1* (eliciting plant response) because it encodes a protein that exhibits high similarity to the Cerato-platanin from *Ceratocystis fimbriata* and Snodprot1 of *Phaeosphaeria nodorum*, proteins described to be involved in plant pathogenesis and plant response elicitation. Those proteins are characterized by a small size (ca 120 aa) and a strictly conserved presence and position of four cysteine residues. Based on the similarity of the N-terminus of the *HypocreaTrichoderma* proteins, we conclude that a previously identified 18-kDa plant response elicitor isolated from *T. virens* is an orthologue of *epr1*. Consistent with the identification of *epr1* in *H. atroviridis* from cultures grown on glucose, its transcript could be detected under all growth conditions tested, including different carbon sources, stress and starvation.