Workshop VII

Secondary metabolism
Chair: Christian Hertweck

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THE MOLECULAR BASIS FOR POLYKETIDE BIOSYNTHESIS IN FILAMENTOUS FUNGI

Christian Hertweck
Hans-Knöll-Institute for Natural Products Research, Junior Research Group Bioorganic Synthesis, Beutenbergstr. 11a, 07745 Jena, Germany
Phone: INT+49(0)3641-656701, FAX: INT+49(0)3641-656705,
Email: christian.hertweck@hki-jena.de

Fungal polyketides constitute a diverse group of natural products that play a major role as therapeutic agents, including antibiotics, cancerostatica, and cholesterol-lowering agents. However, metabolites from the same family are also known as infamous foodstock-poisening mycotoxins.
In general, polyketides are constructed by repetitive Claisen condensations in a manner that closely parallels fatty acid biosynthesis. An enormous metabolic diversity is governed through a number of programmed events that are dictated by polyketide synthases (PKS), which involve the selection of starter units, control of carbon chain length, degree of reduction, and cyclization. While a large number of bacterial PKS have been cloned and investigated, yet relatively few fungal PKS have been studied, and their detailed programming during the elongation cycles still remains a mystery.
A detailed investigation of architecture and function of the PKS, as well as the tailoring enzymes, is needed to understand the complex biosynthetic pathways. This knowledge could ultimately set the basis for engineering novel “non-natural” fungal hybrid metabolites with altered biological activities.
This talk will provide a general survey on the status quo of fungal PKS research. In addition, our progress on the characterization of fungal (in relation to bacterial) polyketide biosynthesis will be presented.

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A SECOND POLYKETIDE SYNTHASE (PKS) INVOLVED IN COCHLIOBOLUS HETEROSTROPHUS T-TOXIN PRODUCTION

Scott E. Baker*, Thipa Asvarak **, Bi-Yu Li ***, Liang Shi****, Olen C. Yoder***, B.Gillian Turgeon**
*Pacific Northwest National Laboratory, 902 Battelle Blvd., Richland, WA 99352, USA. Phone: (509) 372-4759 Fax: (509) 372-4732 email: scott.baker@pnl.gov; **Cornell Univ. Department of Plant Pathology, Ithaca, NY 14853, USA; ***Diversa Corporation, 4955 Directors Place, San Diego, CA 92121, USA; ****Syngenta Biotechnology Inc., PO Box 12257, Research Triangle Park, North Carolina 27709-2257, USA

Cochliobolus heterostrophus race T, causal agent of Southern Corn Leaf Blight, requires the polyketide T-toxin for high virulence on T-cytoplasm corn. Production of T-toxin is controlled by two unlinked loci, Tox1A and Tox1B, carried on 1.2 Mb of DNA not found in race O, a mildly virulent form of the fungus that does not produce T-toxin, or in any other Cochliobolus spp. or relative. PKS1, a polyketide synthase (PKS)-encoding gene at Tox1A, has been proven necessary for T-toxin production. Although there is evidence that additional genes at Tox1A are required for T-toxin production, efforts to clone them have been frustrated because the locus contains repeated DNA and is highly A+T-rich. To overcome these difficulties, we applied Ligation specificity-based Expression Analysis Display (LEAD, a comparative AFLP/gel fractionation/capillary sequencing procedure coupled to software analysis) to cDNA from a near isogenic pair of Tox1+ and Tox1- strains. This led to discovery of PKS2, a new PKS-encoding gene that maps at Tox1A and is required for both T-toxin biosynthesis and high virulence. Further analysis of LEAD data combined with genomic sequence from C. heterostrophus showed that a dehydrogenase (LAM1) is located on the same contig as PKS2 and two previously uncharacterized reductases (RED2 and RED3) are found at the Tox1B locus. Deletion of LAM1 indicated that this gene is needed for wild type levels of T-toxin production while elimination of the reductase had no effect. The discovery of these genes has led us to a model of how T-toxin is produced by C. heterostrophus.

MOLECULAR ANALYSIS OF OCHRATOXIN A BIOSYNTHESIS IN ASPERGILLUS OCHRACEUS.

J. O'Callaghan, P.C. Stapleton and A.D.W. Dobson.
Department of Microbiology, University College, National University of Ireland, Cork, Ireland. Tel:+353-21-4902743, Fax: +353-21-4903101, email a.dobson@ucc.ie

Ochratoxin A (OTA) is a secondary metabolite produced by Aspergillus and Penicillium species. It is a potent nephrotoxin and is also classified as being carcinogenic and teratogenic. The biosynthetic pathway for OTA has not been characterised and prior to the commencement of this study none of the genes involved has been cloned. Suppression subtractive hybridisation-PCR (SSH-PCR) was employed to select for genes that are only expressed during OTA production. We have identified a polyketide synthase (pks) gene from A. ochraceus that is essential for OTA production as mutants with a disrupted pks gene are atoxigenic (1). Real-time PCR has been used to monitor expression of the pks gene which reaches a maximum level at 4 days of growth, corresponding to maximal OTA production in these cultures as assessed by HPLC. The pks gene is expressed at extremely low levels (<20 copies/μg total RNA) in growth media where little or no OTA (<0.1 μg/g mycelium) is produced and at much higher levels (>10,000 copies per μg total RNA) when high levels (>500μg/ g mycelim) of OTA are produced. Media that allow intermediate levels of OTA production (10-20 μg/g biomass) results in the production of intermediate levels of pks expression (2000 copies/ mg total RNA). Expression of the pks gene has also been measured during growth of A. ochraceus at low water activity, different pH values and on different nitrogen sources, where in all cases expression of the pks gene correlates strongly with OTA production. Thus the *pks* gene is differentially regulated and is a strong indicator of OTA production in A. ochraceus.
We have also cloned a number of other putative OTA biosynthetic genes and the expression pattern of two of these genes, an acyl coA dehydrogenase and a P450 monooxygenase which displays a high level of identity to a trichodiene oxygenase from Fusarium sporotrichioides correlates well with expression of the pks and with OTA production. The role of these genes in OTA biosynthesis in A. ochraceus will be discussed.

(1) O'Callaghan, J., Caddick, M.X. and Dobson, A.D.W. (2003). A polyketide synthase gene required for ochratoxin A biosynthesis in Aspergillus ochraceus. Microbiology ; 149: 3485-3491

REGULATION OF GIBBERELLIN BIOSYNTHESIS – CATCHING THE COMPONENTS OF A REGULATORY NETWORK

Sabine Teichert, Birgit Schönig, Bettina Tudzynski
Institut für Botanik, Westfälische Wilhelms-Universität Münster, Schlossgarten 3, D-48149 Münster, Germany, Phone: (0049) 251 8324801; Fax: (0049) 251 8323823; E-mail: teichert@uni-muenster.de

Gibberellin biosynthesis in Gibberella fujikuroi is highly depended on nitrogen availability: Nitrogen starvation and poor nitrogen sources, such as acetamide, induce the production of gibberellins (GAs), whereas most of the other nitrogen sources cause a strong inhibition. Recently we have shown that AREA, responsible for mediating nitrogen metabolite repression of many genes, is also required for the activation of the GA biosynthetic genes (1). However, so far it is not much known on the signaling pathways transducing the ammonium or amino acid signals from the cell surface to the corresponding target genes. Thus, it is not yet known if TOR signaling specifically responds in a yeast-like manner to the intracellular level of the key effectors glutamine and/or glutamate. Several putative members of the nitrogen regulation network with homology to known factors of nitrogen regulation in yeast were identified in Gibberella fujikuroi by different approaches.
One of the genes of our interest is the G. fujikuroi homologue of TOR, which in Saccharomyces is responsible for the phosphorylation/inactivation of the AREA homologue GLN3. The gene was cloned and overexpressed, gene replacement experiments are underway.
Furthermore, components of the nitrogen uptake system with putative sensing functions, such as ammonium and amino acid permeases and their proposed linkage factor to the TOR pathway, NPR were investigated for their tasks in nitrogen induced gene expression. The role of the glutamine synthetase (GS) as an enzyme with a proposed regulatory function is as well discussed. By differential screening of macroarrays with cDNA from the wild-type and a GS mutant, a homologue of the yeast MBF1 was found, which is a putative bridging factor between the cross pathway control protein, CPCA, and the TATA box binding protein.
The aim of this project is to understand the manner of AREA mediated regulation of the GA biosynthesis by isolating upstream and downstream factors of the regulatory network and to finally connecting the dots.

(1) M. Mihlan, V. Homann, Ta-Wie D. Liu, and B. Tudzynski (2003) AREA directlymediates nitrogen regulation of gibberellin biosynthesis in Gibberella fujikuroi, but ist activity is not affected by NMR. Mol. Microbiol. 47: 975-991

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A MUTATION OF THE PHYTOENE DEHYDROGENASE GENE OF FUSARIUM FUJIKUROI LEADING TO THE ACCUMULATION OF GAMMA AND BETA-CAROTENE

Alfonso Prado-Cabrero and Javier Avalos
Departamento de Genética, Universidad de Sevilla.
Apartado 1095, E–41080 Sevilla, Spain.
Phone +34954557110 fax +34954557104 e-mail a_prado@yahoo.com

The fungus Fusarium fujikuroi (Gibberella fujikuroi, mating group C) is the biotechnological source of gibberellins, growth promoting plant hormones used in agriculture and brewing. When grown in the light, F. fujikuroi mycelia get an orange pigmentation due to the biosynthesis of carotenoids. The main end-product of the pathway is neurosporaxanthin, a xanthophyll synthesized from the uncolored precursor phytoene through seven enzymatic reactions: five dehydrogenations, a cyclization and an oxidative break. The dehydrogenations are carried out by a single enzyme, product of the gene carB. Deregulated mutants, affected in the gene carS, are deep orange and accumulate large amounts of carotenoids under any culture conditions.

After mutagenesis of a carS strain, we have identified a mutant (SF21) with an intense yellow pigmentation. Column chromatography, TLC and HPLC analyses show that SF21 accumulates beta-carotene and gamma-carotene as main products of the pathway. According to the chemical nature of the intermediate carotenes accumulated, the mutant lacks the ability to carry out the last dehydrogenation, while keeps the ability to produce the previous ones. Sequence analysis of this carB allele has confirmed the occurrence of a mutation changing an aminoacid conserved in the same enzyme of other fungi able to carry out five dehydrogenations. The mutation affects a domain of the protein different to the domain usually mutated in carB null mutants in other fungi. Additional carB alleles obtained from SF21 were analyzed. One of them contains an early stop mutation, producing the loss of the putative carotene binding domain of the protein. Unexpectedly, the mutant retains some dehydrogenase activity, as shown by the accumulation of minor amounts of zeta-carotene.

The concentration of carotenoids in SF21 duplicates at least the amount of neurosporaxanthin in the parental overproducing strain. Northern blot analysis of the structural genes carRA and carB indicates that this increase is not explained by an enhanced transcription. The mutation described should result in a similar phenotype in other Fusarium species or in other carotenogenic fungi containing a dehydrogenase able to carry out five dehydrogenation steps.

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FROM FUNGAL BIODIVERSITY TO NOVEL NATURAL PRODUCTS USING INTELLIGENT SCREENING METHODS BASED ON MASS AND UV SPECTROMETRIC METHODS

Thomas Ostenfeld Larsen, Michael Edberg Hansen, Jørn Smedsgaard*
BioCentrum, Technical University of Denmark, Søltoft Plads, 2800 Kgs. Lyngby, Denmark
Phone: +45 45252632
Fax: +45 45884922
Email: tol@biocentrum.dtu.dk; meh@biocentrum.dtu.dk; js@biocentrum.dtu.dk

In this presentation an integrated setup for the discovery of novel fungal metabolites such as polyketides and alkaloids will be presented. Initially fungal extracts are analyzed by direct injection mass spectrometry (DIMS). Subsequently mass profiles are clustered for the selection of few but representative chemotypes either for bioguided or spectroscophy guided isolation of novel compounds. For the latter we use a new algoritm (“Xhitting”) for automatic identification of different types of compounds based on their full UV characteristics. Initially two databases (DB’s) containing samples and known reference spectra are created. Next a relation between the the two DB’s based on the similarities between each of the reference spectra to the spectra contained by the samples is established. The use of this new algoritm and spectral library for automatic identification of both know structures (cross-hitting) but also for identifying possible new compounds similar to already known ones (new-hitting) will be demonstrated. E.g. we have found a number of new species producing already known polyketides such as statins by cross-hitting. Finally, two novel quinazolines and a novel benzodiazepine have been tracked, isolated and characterized proving the new-hitting concept of Xhitting.