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Purification of the MCAP 3-halogenase from pyrrolnitrin biosynthesis in P. fluorescens BL915

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Pyrrolnitrin is an antifungal compound first isolated from Pseudomonas pyrrocinia. The gene cluster responsible for pyrrolnitrin biosynthesis was identified in Pseudomonas fluorescens (BL915) and other pyrrolnitrin producing bacteria. Four conserved enzymes are involved in pyrrolnitrin biosynthesis namely PrnA, PrnB, PrnC, and PrnD, according to their order in catalysis. The third enzyme, monodechloroaminopyrrolnitrin (MCAP) 3-halogenase (PrnC), catalyses the regioselective chlorination of MCAP in the 3-position of the pyrrole ring. The reaction mechanism of PrnC is suggested to be very similar to that of other flavin-dependent halogenases.

PrnC is, besides the tryptophan halogenases, one of the very few flavin-dependent halogenases that catalyse the halogenation of a free substrate. Since the amino acid sequence of PrnC differs significantly from those of tryptophan halogenases, the 3-D structure of PrnC is of high importance to understand how substrate specificity and regioselectivity are regulated in flavin-dependent halogenases.

So far, purification of PrnC in its active form has not been achieved satisfactorily, precluding further analysis. We now report a novel purification strategy leading to purified, active PrnC. Using the GST-fusion protein strategy it is possible to obtain pure PrnC from recombinant Escherichia coli cells. Purity level of the eluted fusion protein depends on the growth temperature of the E. coli strain used for expression. MALDI-TOF-MS analysis revealed that the chaperonin GroEL and other proteins are co-purified by glutathione affinity chromatography when the growth temperature is not reduced to 20 °C. Both, the fusion protein as well as purified PrnC obtained after thrombin digestion showed good halogenating activity.

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Approaches for directed strain improvement targeting enhanced biosynthesis of gibberellic acid in Fusarium fujikuroi

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The filamentous fungus Fusarium fujikuroi is known to produce high amounts of different secondary metabolites such as the red pigment bikaverin, the mycotoxin fusarin C and gibberellic acids (GAs), a family of phytohormones. Particularly GAs exhibit a great biotechnological impact as their application induces early flower bud formation and shoot elongation as well as an increased fruit size in higher plants. Also enhanced yields of crops such as seedless grapes and corn can be gained. Therefore, each year about ten tons of biotechnologically produced GAs are consumed by the agricultural industry as plant growth regulators. Since the knowledge about fungal GA biosynthetic pathway and its regulation is increasing, the rice pathogen F. fujikuroi constitutes a capable model species for strain improvement by molecular techniques.

To increase GA yields directed genetic modifications of GA pathway genes, e.g. the overexpression of ggs2 encoding the first GA-specific enzyme, the geranyl-geranyl pyrophosphate synthase, were performed resulting in enhanced GA production rates compared to the reference strain. In addition, regulation of key enzymes of the precursor-providing primary metabolism, such as the hydroxy-methyl-glutaryl-Co-enzyme A reductase (HmgR) and the farnesyl-pyrophosphate synthase (FppS), has to be investigated to circumvent negative feedback regulation by different intermediates or end products. Thus, overexpression of hmgR led to a significant downregulation of GA genes. On the other hand, truncation of the N terminal transmembrane domains of the HmgR, which is most likely responsible for a negative feedback regulation led to a threefold higher growth rate, and subsequently to threefold higher GA amounts compared to the reference strain. By further investigating regulation mechanisms on transcriptional and protein levels, e.g. by identification of positively or negatively acting transcription factors, GA production shall be further enhanced.

Another approach is the down-regulation of sterol biosynthesis to channel the common precursor, FPP, into the GA biosynthetic pathway.

To finally combine single modifications in one production strain new selection markers or possibilities for re-using common markers have to be established for F. fujikuroi.

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Beyond Identification of Antimicrobial Compounds: Elucidating Antibiotic Mechanisms of Action
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The search for peptide-based antibiotics has turned up a number of antibacterial compounds and more such compounds are waiting to be discovered. We are currently synthesizing and screening small peptides for antimicrobial activity. Peptide-based antibiotics can differ widely in their mechanisms of actions, which is not surprising given the different physicochemical properties of their building blocks. We have established a 2D gel-based reference library of proteomic response patterns of Bacillus subtilis to different antibiotics with known mechanisms of action. The proteomic response mirrors the physiological disturbance in the cell and therefore correlates with the mechanism of action [1]. This platform is available to quickly sort structurally novel antibiotics into those with known and those with unprecedented mechanisms. Investigations of the mechanism of action of small peptide antibiotics (5 to 6 amino acids) revealed complex response patterns likely reflecting more than one mechanism of action. For instance, depending on the antibiotic concentration, the linear cationic peptide with the sequence RWRWRW [2, 3] elicits response patterns overlapping with the cyclic polypeptide bacitracin targeting cell wall biosynthesis or the cyclic dodecadepsipeptide valinomycin known to act as potassium ionophor [4]. Detailed mechanism of action studies are now being performed to gain a thorough understanding of the potential of such small peptide antibiotics.

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Using whole genome sequencing to identify genes and pathways of interest
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The basis for synthetic biology is knowledge of the genetic sequence of the gene(s) of interest. Going just a few years back obtaining genome sequence data from new organisms was difficult, time consuming and expensive. The recent appearence of massive parallel sequencing technologies have completely changed this situation. A bacterial genome can be sequenced for around $400 and a fungal genome for around $4000. However a series of other challenges have arisen, such as how do we check for cross-contamination of DNA samples, how do we analyse millions of short reads for genetic content.
In the present work, we will describe the bioinformatics needed for making quality control, de novo assembly of Illumina sequence data, gene finding, -annotation and identification of pathways of interest using the commercially available software tools "Genomic Workbench" and IOGMA.

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Rapid cloning and engineering of unknown natural product biosynthetic pathway via Red/ET recombineering, Xiaoying Bian1, Jun Fu2, Alberto Plaza1, Rolf Mϋller1, Youming Zhang3,
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Red/ET recombineering is an in vivo homologous recombination-based genetic engineering method employed primarily in E. coli that used short homologies, which is catalyzed by two equivalent phage protein pairs, Redα/Redβ from λ phage and RecE/RecT from Rac prophage in E. coli K12 chromosome. It not only allows precise and efficient modification of pre-existing replication-competent molecules, which has greatly facilitated the genetic engineering of complex biosynthetic pathways, but also can be applied to clone and subclone DNA regions from a DNA source into a plasmid. In addition, the genome-sequencing project has revealed that a large number of secondary metabolite biosynthetic gene clusters presented in bacterial genomes are uncharacterized or silent. To access the unexploited majority of natural products, genome mining and microbial metagenomic approaches are proving effective. When Red/ET recombineering meet these strategies, cryptic biosynthetic pathways in the native host can be efficiently cloned, engineered, and expressed in a suitable heterologous system. We describe a straightforward synthetic biology approach involving direct cloning and genetic engineering of unknown biosynthetic pathway via Red/ET recombineering followed by heterologous expression to investigate the products or function of cryptic biosynthetic pathways. An unknown biosynthetic pathway, encoding a nonribosomal peptide synthetase (NRPS) in the chromosome of Photorhabdus luminescens TT01, was directly cloned from digested genomic DNA by ET cloning without time-consuming construction and screening of genomic library. Heterologous expression in Escherichia coli resulted in the discovery of a group of cyclic pentapeptides and theirs detailed structures were elucidated using 1D, 2D-NMR, HR-MS, advanced Marfey's method, and bioinformatic analysis of the biosynthesis gene cluster. Then, according to the selectivity-conferring code of NRPS adenylation (A) domain, site-directed point mutation using ccdB counter-selection in combination with Red recombination was applied to rationally alter the substrate specificity of the third module that lead to significant changing of relative abundances of different derivatives. Recombineering mediated direct cloning and genetic engineering of complex biosynthetic pathway in E. coli coupling with efficient heterologous expression in a plug-and-play system would promote the discovery of new biologically active compounds from microorganisms, especially for the slow-growing, genetically hard-to-manipulate, or not-yet-cultivable sources, for drug discovery.

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antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences

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Secondary metabolites such as antibiotics are synthetised by a complex enzymatic machinery. Although there is an immense structural diversity of these metabolites the basic principles and thus the enzymes involved in biosynthesis are highly conserved. Over the last decades many secondary metabolite gene clusters have been isolated, sequenced and analyzed experimentally in great detail. The recent enhancements in sequencing technology now have provided many novel biosynthetic gene clusters even from organisms that have not been known to produce secondary metabolites. Thus, software tools are required to evaluate these sequence data and to predict the putative products of such clusters which are identified in silico.
Here we present antiSMASH[1], a modular collection of tools for sophisticated analysis and annotation of secondary metabolite biosynthesis genes. antiSMASH allows the rapid genome-wide identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genomes. The results are presented in an interactive, user friendly web interface as well as collected in a downloadable archive file for further use with genome browsers like Artemis.
antiSMASH can be found at http://antismash.secondarymetabolites.org/ for a web-based secondary metabolite prediction or can be downloaded and run on a local machine.

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Engineering a penicillin biosynthesis pathway into yeast to elucidate the effects of 3D positioning in the genome on transcription. B A Blount and T Ellis, Centre for Synthetic Biology and Innovation, Imperial College London, UK

The genome has traditionally been viewed in a very linear manner but it is becoming clear that both intra and inter-chromosomal interactions within the three dimensional space of the nucleus have important roles in transcriptional regulation. A modular system that allows DNA sequences to be inserted cleanly into the genome at user-defined chromosomal loci in Saccharomyces cerevisiae was developed to allow synthetic sequences encoding components of the benzylpenicillin biosynthesis pathway to be selectively integrated at various chromosomal locations. By comparing the interaction frequencies of chromosomal locations with transcription levels of sets of genes integrated at these locations, the importance of three dimensional placement of genes within a pathway can be investigated.

Selective pressure can be placed upon engineered antibiotic producing yeast via co-culture with Bacillus subtilis. In rich growth media B. subtilis will outcompete S. cerevisiae leading to a decline in S. cerevisiae cfu concentrations. Therefore, by co-culturing yeast strains engineered with an antibiotic production pathway with a bacterial competitor, selective pressure is in place to promote the optimisation of the pathway. By isolating cells that are able to outcompete B. subtilis it is hoped that recombination events can be discovered which have enhanced the efficiency of the benzylpenicillin production pathway either due to favourable re-localisation of the genes relative to each other in three dimensional space or by other means.

By elucidating relationships between genomic loci, future attempts to engineer more complicated pathways into S. cerevisiae can be more effectively designed and implemented.

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Production of Pravastatin by metabolically engineered Penicillium chrysogenum cells, R.Boer, M.Hans, B.Meijrink

The filamentous fungus Penicillium chrysogenum is widely used in industry for the fermentative production of antibiotics such as penicillins and cephalosporins. By employing decades of classical strain improvement, strains were identified which showed increasingly higher productivities as well as beneficial fermentation behaviors. Given the often lengthy and therefore costly strain development programs for new products, it would be desirable to be able to benefit from such improved strain lineages by using them to create high-productivity industrial strains for novel engineering approaches. Such an approach would lead to shorter and therefore less expensive R&D programs. At DSM, work towards that goal focused on the generation of Penicillium chrysogenum strains in which “unwanted” metabolites such as beta-lactams were abolished, but still harbored the beneficial features leading to high fermentation productivities. Such “empty strains” were harnessed for the strain development of novel fermentation processes of substances different from beta-lactams and unknown to the Penicillium chrysogenum metabolism. As a main example, construction of a high productivity strain for the cholesterol lowering drug Pravastatin is described. Currently, Pravastatin is industrially produced by Penicillium citrinum fermentation of the natural product precursor compactin followed by a bioconversion with Streptomyces carbophilus, yielding the hydroxylated product pravastatin. DSM’s breakthrough technology resulted in a Penicillium chrysogenum one-step fermentation ensuring an environmentally and economically advantageous process. Towards this goal, the whole compactin biosynthetic gene cluster from Penicillium citrinum was split into three parts, and heterologously expressed in Penicillium chrysogenum, yielding high productivities of compactin. To get to this high productivity, an esterase that was shown to be responsible for degradation of compactin was knocked out by homologous recombination.
Subsequently, a P450 compactin hydroxylase was discovered from Amycolatopsis orientalis. Unfortunately, this enzyme produced the wrong epimer, so-called epi-pravastatin. Two rounds of directed evolution in E.coli led to a mutated P450 that produced 98 % of the correct molecule. For expression and good activity in Penicillium chrysogenum, the P450 mutant was fused to the reductase domain from Rhodococcus sp. and heterologously expressed in a compactin producing Penicillium chrysogenum. The resulting pravastatin producing strains were further optimized by improving the transcription activator in the compactin biosynthetic cluster and deletion of the compaction transporter.
Further strain and process improvements including up-scaling to several cubic meter fermentation volumes were carried out.

Keywords
Penicillium chrysogenum, Beta-Lactam, Fermentation, Metabolic Engineering, Compactin, Pravastatin, P450 oxygenase

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Resolution of gene regulatory conflicts caused by combinations of antibiotics

Regulatory conflicts occur when two signals, which individually trigger opposite cellular responses, are present simultaneously. Here, we investigate how such gene regulation conflicts are resolved in the bacterial response to antibiotic combinations. We used an Escherichia coli promoter-GFP reporter library to study the transcriptional response of many different promoters to either additive or antagonistic drug pairs at fine two-dimensional resolution of drug concentration. Surprisingly, we find that this complete dataset can be almost fully characterized as a linear sum of only two principal components. The first principal component, accounting for over 70% of the response, represents the response to the net effectiveness of the drug combination in inhibiting growth. The second principal component describes how regulatory conflicts are resolved for promoters that respond differently to each of the individual drugs. For the non-interacting drug pair conflicts are resolved by linearly interpolating the two single drug responses, while for the antagonistic drug pair, the drug that has the stronger impact on growth dominates the transcriptional response. Importantly, for a given drug pair, the same strategy of conflict resolution is used for almost all genes. These results therefore provide a recipe for predicting gene expression responses to antibiotic combinations, which may lead to a more rational design of combination treatments.

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Destruction of silence: protein stabilization uncovers silenced biosynthetic gene clusters in the fungus Aspergillus nidulans

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Destruction of silence: protein stabilization uncovers silenced biosynthetic gene clusters in the fungus Aspergillus nidulans

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Antimicrobial resistance is spreading but the number of newly discovered antibiotics is declining. The genomes of filamentous fungi comprise numerous putative genes and gene clusters for chemically and structurally diverse secondary metabolites which are never expressed under laboratory conditions. Previous approaches to activate these genes were primarily based on artificially targeting the cellular protein synthesis apparatus. Here we applied successfully the alternative approach of genetically impairing the protein degradation apparatus of the cell. We identified novel secondary metabolites of the model fungus Aspergillus nidulans by deleting the conserved eukaryotic csnE/CSN5 deneddylase subunit of the COP9 signalosome, This defect in protein degradation results in an altered secondary metabolism where a normally silenced novel gene cluster comprising a polyketide synthase gene is expressed. We identified its product as 2,4-dihydroxy-3-methyl-6-(2-oxopropyl)benzaldehyde (DHMBA). Comparison of the wild type and the mutant resulted in the identification of 3,3-(2,3-dihydroxypropyl)diindole (DHPDI) as a second compound which had previously not been found in Aspergilli. We propose the deletion of the highly conserved csn5 as a novel approach for identification of new secondary metabolites in filamentous fungi.

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Exploring the role of A9 conserved motif in the adenylation domain of tyrocidine synthetase 1 from Bacillus brevis

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Nonribosomal peptide synthetases (NRPSs) are modular proteins responsible for the production of peptide natural products, many of which have antibiotic activity. The modular nature of NRPS makes them suitable targets for engineering through domain or module rearrangements. In practice, the ability to obtain functional enzymes depends on the extent to which the protein-protein interactions necessary for catalytic activity of the engineered NRPS assembly have been preserved. High-resolution NMR and crystal structures of NRPS domains, didomains and even an entire module obtained during last several years greatly expanded our understanding of intra- and intermodular protein interactions in NRPS systems. Distinct protein-interaction surfaces were reported mainly for the peptidyl carrier protein (PCP) domain; however, several attempts to map protein interaction surfaces on adenylation (A) domain failed to pinpoint a particular region of the protein.

Our search for the putative surface on the A domain, involved in protein interaction with the PCP domain was based on sequence analysis and homology modeling. We examined all the available structural data not only from NRPS A domains, but also of the other members of adenylate-forming superfamily, and hypothesized that the region of A domain assigned as A9 core motif could be of importance for A and PCP domain interaction. When analyzing homology models build for the protein conformation in the first half-reaction (amino acid activation) and during the second half-reaction (transfer of the activated amino acid to the 4'-phosphopantetheine arm of the adjacent PCP domain), this region seems to position itself favorably for the interaction with PCP domain during the second half-reaction. To test this hypothesis, we created a set of mutant proteins, in which single amino acid exchanges were introduced into the A9 core motif of A domain from tyrocidine synthetase 1, 484LPAYMLP. The mutant proteins were expressed and purified from E. coli as His-tagged proteins. The influence of mutation in A9 motif was examined by assaying enzymatic activity (with or without the acceptor of activated amino acid in the second-half reaction), susceptibility to proteolysis, and fluorescence properties of the mutant enzymes. The results obtained indicate that, while the A9 core motif is not indispensable for the activation of amino acid, it contributes to the structural stabilization of the protein and might play an important role for the transfer of activated amino acid from the adenylation onto the PCP domain.

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Domain substitution in Pseudomonas aeruginosa PAO1 to create novel pyoverdines

Non-ribosomal peptide synthetases (NRPSs) are large multi-modular enzymes that function as assembly lines for small peptide-derived products. Typically, each module selects an amino acid substrate that is subsequently condensed with the substrate of the downstream module. The growing peptide chain is passed down the modules and released at the final module. This mode of synthesis potentially allows for creation of new products by replacing domains or modules with ones specifying alternative substrates.

Attempts to create new products by modifying NRPS enzymes often cause impaired or non-functional enzymes. This results in low product yield, which can make screening for new products difficult. We have carried out domain substitution in PvdD to use as a model system for studying hybrid NRPS function. PvdD is a two module NRPS where each module specifies L-threonine and incorporates these residues at the C-terminus of pyoverdine, a fluorescent siderophore of Pseudomonas aeruginosa PAO1. The fluorescence of pyoverdine allows rapid and easy detection of functional hybrid enzymes.

To test the utility of this system we substituted domains within the second threonine-activating module of pyoverdine. First, the adenylation (A) domain of this module was substituted for alternative A-domains. Two A-domains that activate threonine in their native context had a high level of activity. In contrast, the function of seven A-domains that activated other substrates was severely inhibited. These results were not unexpected, with condensation (C) domains known to exhibit a high degree of specificity for the downstream substrate in a condensation reaction. To overcome this “acceptor residue proof-reading” limitation, we instead substituted cognate CA-domain partners into the same PvdD module. This time, the function of one of the substituted threonine activating CA-domains was severely impaired whereas the other was not. Furthermore, two out of seven CA-domain substitutions that specified alternative substrates were now functional, leading to production of novel pyoverdines. This is the first time novel residues have been incorporated into pyoverdine by domain substitution and demonstrates the utility of PvdD as a tool for studying NRPS function.

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A pipeline for organism engineering at Ginkgo BioWorks

Existing applications of bio-based manufacturing rely mainly on natural or minimally engineered microbes because of the intense effort and uncertain outcomes associated with traditional approaches to strain engineering. Ginkgo BioWorks is developing a high-throughput, automated, scalable pipeline for the forward design and engineering of microbes. Whereas previously engineered microbes tend to harbor a small number of recombinant genes or gene deletions, the Ginkgo pipeline is capable of rapidly introducing tens to hundreds of modifications. Important components of the pipeline are (i) robot automation of laboratory DNA manipulations; (ii) a unique DNA assembly approach that allows re-use and combinatorial re-configuration of previously engineered pathway components; and (iii) software and CAD tools to guide the process of organism creation from the design phase to the strain testing phase. We expect this pipeline to be well suited to the production of antibiotics and other small molecules. We are deliberately using this pipeline to engineer a diverse range of microbes for the production of a diverse range of small molecules to ensure the pipeline is as general-purpose as possible.

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Cell host design for the production of malonyl-CoA based compounds.
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Polyketides comprise one of the major families of natural bioactive products, which include a large number of medically important compounds. They are typically assembled by condensation of several units of short-chain acil-CoA molecules.
Streptomyces are widely known as polyketides producers. In these organisms particularly, malonyl-CoA has a pivotal role: It is the precursor of fatty acids and phospholipids necessary for cell growth, but it is also involved in the biosynthesis of both secondary metabolites and triglyceride (TAG) storage lipid. According to this, reducing TAG content in the model bacteria Streptomyces coelicolor, by mutating key genes of its biosynthetic pathway does increase the production of the polyketide actinorhodin (Act), suggesting that TAG actually competes with Act for same substrates. In this sense, the carbon flux of malonyl-CoA from primary metabolism to secondary metabolism and the availability of this molecule is a key factor that determines the yield of polyketide antibiotics production.
Here we describe the design and construction of a S. coelicolor host strain suitable for the production of malonyl-CoA based compounds. To achieve this end, we engineered the cell by deleting genes of competitive pathways and inserting novel enzymatic activities, thus redirecting the malonyl-CoA flux to the desired product. We employed the CH999 strain (act cluster deficient mutant), a widely used host for in vivo production of engineered natural products; and a mutant strain deficient in TAG biosynthesis. In these genetic background, we also overexpressed the S. coelicolor Acetyl-CoA Carboxylase (ACC) complex. As a straight proof of concept we overproduce free fatty acid (a malonyl-CoA demanding product) in this recombinant microorganism by expressing the thioesterase tesA from Escherichia coli.
These expressions were achieved using ad hoc customized vectors that consist in modular-combinable DNA features with BioBricks format. Using this approach, we constructed several plasmids carrying the genes coding for the target enzymes under different endogenous Streptomyces promoters, which are transcriptionally active in late exponential or stationary phase of growth. Thus, were able to exert a temporal control of genes expression in order to minimize the interference with cell chassis and optimize the timing of gene induction accordingly to the metabolic state of the cell. Then, we evaluate the production of fatty acid as an indicator of malonyl-CoA availability.
This recombinant strain could represent a novel optimized platform for systematic and combinatorial high yield production of different polyketides, and a new starting point for further chassis improvement for malonyl-CoA based compounds.

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New chemical keys to unlock the production of novel microbial antibiotics

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New antibiotics are urgently required. While multi-drug-resistant "Superbugs" have been proliferating at an alarming rate, the number of new antibiotics approved for clinical use during the last 30 years has decreased dramatically.[1] Members of the bacterial genus Streptomyces are the main producers of medically useful compounds but, surprisingly, the details of the regulation of antibiotic biosynthesis in these bacteria are still poorly understood.[2, 3]

Interestingly, genome sequencing has revealed the presence of many "cryptic" secondary metabolic gene clusters in streptomycetes.[4, 5] However, many of these gene clusters are not expressed in the laboratory environment. We have recently discovered a novel family of signalling molecules (or microbial hormones), named AHFCAs, that regulate the production of the methylenomycin antibiotics in Streptomyces coelicolor A3(2).[6] By understanding, in detail, the mechanisms whereby AHFCAs induce antibiotic production in Streptomyces species, new strategies to discover antibiotic biosynthesis are currently being developed.

In this poster, two new strategies for natural product discovery that involve either genetic manipulations or media supplementations both in combination with comparative metabolic profiling analyses will be described. Resulting metabolic changes in Streptomyces species will also be presented. These approaches rely on state-of-the-art analytical instrumentation, such as ultra high resolution-mass spectrometry, in order to prevent the rediscovery of known natural products.

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See also website at: http://www2.warwick.ac.uk/fac/sci/chemistry/research/chemicalbiology/corre/

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Reprogramming Fungal Polyketide Synthases

The tenellin PKS-NRPS consists of a fungal highly-reducing polyketide synthase fused to a single module non-ribosomal peptide synthetase. The PKS is an iterative Type I system with a cryptic programme capable of controlling chain length, methylation pattern and reduction pattern. We have deployed molecular methods to dissect the tenellin PKS and begin to unravel the cryptic programme.

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Small Molecule Perturbation of Secondary Metabolism
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The streptomycetes are famed for their secondary metabolites; many of which have found clinical use as antibiotics, immunosuppressants, anti-cancer agents and other drugs. To advance the field of natural product drug discovery, we have taken a chemical biological approach to identify small molecules capable of altering Streptomyces`s secondary metabolism. We screened 30,000 small molecules for their ability to alter the pigmented secondary metabolites, actinorhodin (blue) and undecylprodigiosin (red) produced by Streptomyces coelicolor. 19 molecules were found to increase actinorhodin production. Strikingly, four molecules: ARC2, 3, 4 and 5 (the ARC2 series) exhibited similar structures, suggesting that they might interact with the similar targets.

The initial ARC2 series resulted in increased actinorhodin production and reduced undecylprodigiosin production compared to untreated cells. Through structure activity analysis, we have increased the potency of this series and found a perfect correlation between enhancement of actinorhodin production and reduction of undecylprodigiosin production. This suggests that the same target pathway is leading to both alterations.

The structure activity analysis also revealed similarities of the ARC2 series to the antibiotic triclosan. Triclosan inhibits FabI, an enoyl reductase which catalyses the last step in fatty acid biosynthesis, the reduction of the enoyl-ACP. FabI is an attractive possibility for the target of the ARC2 series as secondary metabolite production shares many similarities with fatty acid biosynthesis including the same precursors. For example, undecylprodigiosin biosynthesis borrows the fatty acid machinery to synthesize the lipid portion of the molecule. FabI inhibition would be a likely explanation for this reduction in undecylprodigiosin production. Importantly, actinorhodin biosynthesis does not require FabI.

To test the possibility of FabI as the target of ARC2 series, we have expressed a triclosan resistant enoyl reductase, FabV from Pseudomonas aeruginosa, in S. coelicolor. Over-expression of FabV reversed the effects of both triclosan and the ARC2 series strongly suggesting that FabI is the target of the ARC2 series. Our hypothesis therefore is that the ARC2 series functions by reducing the turnover of FabI, slowing down fatty acid and undecylprodigiosin biosynthesis and resulting in actinorhodin production via the liberation of precursors such as acetyl-CoA and malonyl-CoA.

The ARC2 series is an attractive set of molecules for screening other Streptomycetes for new metabolites as these molecules target primary metabolism. We are currently working to screen other streptomycetes for changes in their secondary metabolites due to perturbation by the ARC2 series.

Abstract received: 30/06/2011 19:40:17 *********

Amit Tatyasaheb DESHMUKH
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"α-Aminoadipate is not the limiting precursor aminoacid in the penicillin biosynthetic pathway in a high producing P.chrysogenum strain"
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The first step of the penicillin biosynthesis pathway is the condensation of the three precursor amino acids, L-α-amino adipate, L-cysteine and L-valine to form L-α-aminoadipoyl-L-cysteinyl-D-valine (ACV) by multifunctional enzyme ACV synthetase (ACVS). ACV is further oxidized to isopenicillin N (IPN) by Isopenicillin N synthetase and upon addition of phenylacetic acid (PAA), its activated form along with IPN gets converted to penicillin G (PenG) by Acetvl-CoA: lsopenicillin N Acyltransferase (IAT).

In previous studies it has been found that the intracellular concentration of α-AAA, an intermediate of the lysine biosynthesis pathway, limits the synthesis of the tripeptide ACV, and thereby the overall penicillin biosynthesis rate in producing strains [1, 2, 3]. As part of an attempt to elucidate the in-vivo kinetic properties of the β-lactam biosynthesis pathway, we have investigated whether α-AAA is limiting penicillin-G production in the high producing Penicillium chrysogenum strain DS17690. Therefore the strain was grown in a glucose limited chemostat, with addition of the pen-G side chain precursor PAA. After a steady state was reached, 2 mM of DL-α-AAA was supplied to the feed while at the same time injecting DL-α-AAA into the culture vessel to instantaneously obtain a concentration of 2 mM in the culture. Subsequently the dynamics of the intra- and extra- cellular concentrations of all compounds related to the penicillin biosynthesis pathway were measured within a time frame of 1 h and further followed until a new steady state was reached.
After addition of the α-AAA the intracellular concentration showed a rapid 60 fold increase, indicating that it was readily transported into the cell. However, no significant increase of the intracellular ACV level was observed. Also the intracellular pen-G level was not affected and no increase of the rate of pen-G production was measured. Possibly the current strain has lost the α-AAA limitation during the prolonged strain improvement process. Instead of being converted to ACV, part of the added α-AAA was converted to lysine, which was observed from a significant increase of the intracellular lysine concentration.

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Abstract received: 21/06/2011 16:57:45 *********

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Application of Synthetic Promoter Library for modulation of actinorhodin production in Streptomyces coelicolor

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Actinorhodin biosynthetic genes occur as an operon in the S. coelicolor genome. The cluster encodes a pathway specific positive regulator actII orf4. The aim of this study was to investigate how modulation of the expression level of this positive regulator affects ACT production in S. coelicolor. The expression was enhanced by constitutive overexpression (oxp) from the promoter ermE*. In addition, the native actII orf4 promoter was replaced with synthetic promoters and a S. coelicolor library with a broad range of expression levels of actII orf4 was generated. The resultant library was screened based on the yield of actinorhodin on biomass and characterized for physiological behavior. One of the strains from the library showed considerably higher yields of actinorhodin and final actinorhodin titers, when compared to S. coelicolor wild type and S. coelicolor with actII orf4 expressed from the ermE* promoter. ScoSPL20 showed exceptional productivity despite having a comparatively weak promoter. Moreover, a transcriptome analysis was undertaken for the high-yielding strain to gain a deeper understanding for how metabolism and regulation was affected due to the replacement of the native promoter.

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The Induction of Nitrate Reductase 3 Biosynthesis Correlates with the Onset of Secondary Metabolism in Streptomyces coelicolor
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Streptomyces coelicolor is an obligate aerobic, high GC Gram-positive actinobacterium found predominantly in soil and on decaying vegetation. Its developmental program is characterised by undergoing a complex life cycle with stages that include growth as vegetative hyphae, generation of hydrophobic aerial hyphae and the production of exospores. Streptomyces species are characterized by the ability to produce a variety of secondary metabolites, which are synthesized after a metabolic switch (transition phase) from primary metabolism during exponential growth to secondary metabolism in the stationary growth stage. During secondary metabolism S. coelicolor, for example, synthesizes the antibiotics actinorhodin and undecylprodigiosin, amongst other compounds (1). The onset of secondary metabolism requires energy and reducing power to synthesize these complex compounds and the use of alternative electron acceptors when oxygen becomes limiting is one means by which a membrane potential and thus an energy-conserving proton gradient can be maintained. Recent studies revealed that despite being an obligate aerobe S. coelicolor is able to reduce nitrate to nitrite as a means of partially overcoming oxygen limitation. The enzyme responsible is the membrane-associated, energy conserving nitrate reductase (NAR). The genome of S. coelicolor has three copies of the narGHJI operon, each encoding a NAR (2). The synthesis and activity of one of these, NAR3, correlates with the onset of secondary metabolism (2). Mutants unable to synthesize NAR3 still make both undecylprodigiosin and actinorhodin but synthesis appears somewhat delayed in comparison to wildtype. Surprisingly, imposing aerobic salt stress by increasing for example the NaCl concentration, revealed a negative correlation between antibiotic production and the activity of NAR3. These findings will be discussed in terms of a requirement for NAR3 for energy generation during secondary metabolism in the absence of oxygen.

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Abstract received: 29/06/2011 17:06:30 *********

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Involvement of intra- and extracellular beta-galactosidases in lactose catabolism in Penicillium chrysogenum

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Penicillium chrysogenum is an economically important fungus used as industrial producer of penicillin. Apart from traits related to penicillin biosynthesis, general aspects of its carbon metabolism have received little attention. We investigated the catabolism of lactose, an abundant component of the diary residue whey that has been used extensively in pencillin fermentation, comparing the type strain NRRL 1951 as a wild-type reference with the industrial penicillin-producer ASP-78.

Both strains grew similarly on lactose as the sole carbon source under batch conditions, consuming 15 g/L lactose in about 84 hours. The time-profile of sugar depletion concurred with the presence of both intra- and extracellular beta-1,4-D-galactosidase (bGal) activities, measured as ortho-nitrophenyl beta-D-galactopyranoside (ONPG) hydrolases. Upon growth on commonly occuring monosaccharides, such as D-glucose, D-fructose, D-xylose or D-galactose, as well as on glycerol, neither extra- nor intracellular bGal could be detected. However, L-arabinose induced ONPG-hydrolysing activity to about half the values measured with lactose-grown material. Similar results were obtained with mycelia pregrown on glycerol and transferred to fresh minimal medium with the various carbon sources tested. The measured bGal activities were similar for the two investigated strains.

In silico analysis of the genome sequences revealed that P. chrysogenum features at least five putative bGal-encoding genes at the annotated loci Pc22g14540, Pc12g11750, Pc16g12750, Pc14g01510 and Pc06g00600. The first two proteins appear to be orthologs of the Aspergillus nidulans intracellular family 2 glycosyl hydrolases specified by neighboring loci AN3201 and AN3200 that cluster with a lactose permease-encoding gene (AN3199). The latter three P. chrysogenum proteins feature an N-terminal secretion signal and appear distinct paralogs related to the extracellular bGal from Aspergillus niger, LacA, a family 35 glycosyl hydrolase.

Transcript analysis of Pc22g14540 and Pc12g11750 showed that they were expressed exclusively in response to lactose but completely repressed on the mixed growth substrate glucose/lactose. Pc16g12750 was seemingly co-expressed with the two putative intracellular bGal genes, while its two paralog genes were apparently not transcribed under any condition tested. This expression profile is distinct from those in other ascomycetes, like Trichoderma reesei or A. nidulans, where bGal genes are induced by the monosaccharides D-galactose and/or L-arabinose. Our results indicate that the L-arabinose-induced ONPG-hydrolase observed in P. chrysogenum may correspond to a side activity of glycosyl hydrolases that are not classed in families 2 or 35. Nevertheless, it is likely that various of three transcribed bGal genes described here are involved in lactose catabolism in P. chrysogenum.

Abstract received: 28/05/2011 10:19:46 *********

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INDUSTRIAL CONTRIBUTIONS TO EXPLOIT ACTINOMYCETES AS HIGH VALUED RESOURCES FOR THE DISCOVERY OF NOVEL BIOACTIVE NATURAL PRODUCTS

In the last decade natural products screening programs have been gradually abandoned from the small molecule discovery approaches in big pharma, finding the refuge in alterative business model. These include public private partnerships that focuse on ensuring the continuity of research in the field, and the expertise transfer, as well as the discovery of novel bioactive molecules from microbial natural products that potentially could be developed as new drugs.

Fundación MEDINA is a new not-for-profit public-private partnership between Merck Sharp and Dohme de España, the Government of South Spain and the University of Granada, that was created in response to this need, from the transfer of all the research programs and know how of the former Center of Basic Research of Merck in Spain.

MEDINA leverages today the extended experience from the Merck research group in the discovery of new strains of actinomycetes as producers of novel bioactive compounds. MEDINA scientists continue to focus in the development of new approaches to further exploit the microbial biosynthetic potential as the source for the production of novel metabolites. Examples will be presented about different approaches applied to better exploit the resource in our microbial collections and the new challenges that are faced today to ensure the evolution of these strategies into the framework of the new model that is being built to address the discovery of new bioactive compounds

Abstract received: 26/06/2011 01:32:49 *********

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Engineering Streptomyces coelicolor for the expression of heterologous gene clusters,
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We have developed Streptomyces coelicolor as a host for the efficient expression of heterologous secondary metabolite gene clusters.

To provide a clean genetic background for activity and metabolite screening, and to eliminate unwanted carbon and nitrogen sinks, four secondary metabolism gene clusters (those for actinorhodin, prodiginines, PKSI (cpk) and CDA) have been removed from S. coelicolor M145 by homologous recombination.

To further increase productivity from cloned gene clusters, mutations known to pleiotropically enhance secondary metabolite gene expression (Appl Environ Microbiol. 74,2834-40) were introduced into the genes encoding the β subunit of RNA polymerase (rpoB) and ribosomal protein S12 (rpsL).

By analyzing actinorhodin production from a reintroduced act gene cluster in a variety of mutant strains and using several culture media, we concluded that a quadruply deleted strain carrying a single rpoB mutation plus a single rpsL mutation, M1154 [Δact Δred Δcpk Δcda rpoB(C1298T) rpsL(A262G)] is the most suitable host for expressing secondary metabolite gene clusters.

Here we demonstrate the use of these strains for the expression of distinct heterologous gene clusters and believe that these strains will be of considerable value in the application of Synthetic Biology to streptomycetes.

Abstract received: 15/06/2011 10:36:31 *********

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Molecular fingerprint in actinomycetes

Actinomycetes are a group of Gram-positive bacteria and one of the most important producers of bioactive metabolites. Particularly, the reduced polyketides (PKs) and non-ribosomal peptides (NRPs) are produced by a set of multifunctional enzymes containing several modules and domains with multiple enzymatic activities. Based on this information it is possible to estimete the metabolic potential of a strain using PCR probes as it directs the access to metabolic production.
The methodology consists in a molecular fingerprint based on the assessment of the metabolic potential of strains to produce secondary metabolites, effective to facilitate the process of dereplication. This type of study allows us to focus our efforts on screening bioactive metabolites in the most promising microorganisms, which is essential for drug discovery. The primers used in this study were already described in the literature and target conserved domains of ketosynthase (KS) systems of PKS-I (1200 bp) and adenilation domains of NRPS (700) bp.
We studied actinomycetes' strains isolated from Citrus spp and observed the amplification of fragments related to the biosynthesis of NRPs and PKs in high frequencies: 87% for PKS and 92% for NRPS. The PCR products were cloned and sequenced. Guided by the information of biological tests, the sequences of the strains named A19, A23, A30 and B1 were selected for cluster analysis. Analyses were performed for clusters of five clones of each strain and the sequences were compared to those available in the NCBI database resulting in molecular taxonomic trees.
Clones from A19 and A30 led to high similarities with sequences coding for quite general PKs. Three clones from A23 showed high similarity with the protein FscD from Streptomyces FR008 translated from the gene cluster responsible for biosynthesis of Candicidin, a polyene macrolide that has antibiotic activity. In the same clade we observed additional enzymes involved in the biosynthesis of this class of metabolite. There were also high similarities to sequences of KS domains involved in biosynthesis of polyene to clones A19 and A30.
The cluster analysis of NRPS for strains A23 and B1, led to similarities of the sequences with the gramicidin synthetase, a non ribosomal peptide produced by Streptomyces albus. For microorganism B1 we also observed similarity to the gene involved in the biosynthesis of indigoidine, a blue pigment which was interestingly observed in solid and liquid cultures of this microorganism.
These results demonstrate the possibility of screening the metabolic potential of actinomycetes without the need of full sequencing, to predict the class of metabolite produced and to select the most talented strains for further studies.

Abstract received: 01/07/2011 03:33:34 *********

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Elucidating and Exploiting Biosynthesis: Synthetic Biology Approaches to Access Novel Uridyl Peptide Antibioics

A new approach toward generating natural product analogues has been developed by the Goss group: using synthetic biology to enable chemistry. Applying this new approach named “ChemoGenetics”, a gene is introduced to complement an existing biosynthetic pathway and installing a selectably functionalisable handle such as a halogen. The resultant chemical handle that is installed into the natural product enables subsequent selective modification to generate libraries of natural product analogues; this may all be achieved in the aqueous microbial fermentation broth.

This and other approaches to making analogues of the uridyl peptide antibiotics (a new structural class of antibiotics with a clinically unexploited mode of action) through mixing and matching genes as well as through chemical synthesis will be described.

Scheme 1. ChemoGenetics: a new paradigm in natural product analogue synthesis.The introduction of prnA, a gene encoding a halogenase, into the pacidamycin producer results in the generation of chlorinated pacidamycins. The chlorine may be used as a selectively functionalisable handle enabling further synthetic diversification.

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Chemically coded two-step synthesis of lactones

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The megasynthase protein family includes fatty acid synthases (FAS) and polyketide synthases (PKS), representing the machineries responsible for the biosynthesis of fatty acids and the bioactive polyketides. Exploiting the high synthetic potential of megasynthases for directed biosynthesis of polyketide compounds is an ambitious project in modern synthetic chemistry.
Based on structural and functional data collected on 2.6 MDa type-I FAS from Saccharomyces cerevisiae, we engineer FAS do not synthesize fatty acids, but lactones. A first module, modified in fatty acid chain length control, produces short-length fatty acids, which are elongated by a second module to 4-hydroxy-pyran-2-ones. By adapting the priming reaction in the second module, 4-hydroxy-pyran-2-ones can be produced by a chemically coded reaction sequence in one-pot. This proof-of-principle approach reflects the concept of our research focus in biosynthetic chemistry, which is extending the catalytic versatility of megasynthases by interfering in the working mode of selected proteins. Our focus can be seen as complementary to general efforts in the PKS-field to increase the biosynthetic spectrum of megasynthases by increasing the number of available proteins.

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Regulation of pristinamycin biosynthesis in S. Pristinaespiralis

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The streptogramin antibiotic pristinamycin, produced by Streptomyces pristinaespiralis, is a mixture of two types of chemically unrelated compounds: pristinamycin PI and PII, which are produced in a ratio of 30:70. Pristinamycin PI is a cyclic hexadepsipeptide, belonging to the B-group of streptogramins, while pristinamycin PII has the structure of a polyunsaturated macrolactone of the A-group of streptogramins.Both compounds alone inhibit the protein biosynthesis by binding to the peptidyl transferase domain of the 50S subunit of the ribosome and are bacteriostatic. The A-group prevents the binding of the aminoacyl-tRNA to the 50S subunit of the ribosome. In contrast, the B-group facilitates the release of the peptidyl-tRNA from the ribosome. Together they show a strong synergistic bactericidal activity, which can reach 100 times of the separate components. The pristinamycin biosynthetic gene cluster is partially characterized. It covers a region of about 210 kb where genes for PI and PII biosynthesis are interspersed. Moreover, the pristinamycin coding region is interrupted by a cryptic secondary metabolite gene cluster which probably encodes for an actinorhodin-like compound. Seven regulatory genes were identified within the 210 kb region: spbR, papR1, papR2, papR3, papR4, papR5 and papR6. SpbR (S. pristinaespiralis butyrolactone-responsive transcriptional repressor) is a specific receptor protein for γ-butyrolactones and the global regulator of pristinamycin biosynthesis. papR1, papR2 and papR4 encode proteins that are homologous to SARPs which are pathway-specific transcriptional activator proteins, whereas papR3 and papR5 code both for proteins that belong to the family of TetR repressors. papR6 encodes a protein belonging to the class of response regulators.On the basis of RT-PCR, bandshift and mutant analysis, a preliminary model of the regulation mechanism of pristinamycin biosynthesis was established: SpbR is global regulator with a γ-butyrolactone binding function. PapR2 is a hierarchical superior regulatory protein for the transcription of papR1, whereas PapR1 is the direct activator of the pristinamycin structural genes. As another SARP homologue, PapR4 could be a further superior regulatory protein for the transcription of papR2. PapR3, as a TetR repressor protein, may temporarily retard the expression of papR1 to ensure that the cells are able to gain self-resistance against pristinamycin. This repressing function of PapR3 could be abolished by the function of another TetR repressor, which might be the role of PapR5. PapR6 might control the transcription of papR5.

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Substrate specificity of the trans-acting acyltransferases of the Mupirocin biosynthetic cluster.

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Acyltransferases (ATs) play a vital role in polyketide biosynthesis by delivering both the starter and the extender units to the polyketide synthase. While classical type I polyketide synthases have an AT domain as an integral part of each module an increasingly common type of synthase uses trans-acting ATs. The mupirocin polyketide synthase from Pseudomonas fluorescens NCIMB 10586 is of this type, with the MmpC protein incorporating two trans-ATs. Sequence analysis indicates that both of the Mupirocin ATs cluster with malonyl-specific ATs, and a review of the literature indicates malonate as a common substrate among trans-AT PKSs. AT2 was expressed in E. coli and purified by nickel-affinity chromatography in preparation for use in enzymatic assays. An assay using Ellman's reagent to measure the release of Co-enzyme A after transfer of the substrate to the active site of the AT was utilised to determine the specificity of AT2. Autoradiography was used to track the transfer of malonate between AT2 and ACPs within the cluster. We have shown that AT2 prefers malonyl-CoA exclusively as a substrate, and that it increases the rate of integration of malonate to select ACPs from the mupirocin cluster. This work has raised some important questions about the role of trans-ATs.

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title: Studies of the biosynthesis of gabaculine

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Gabaculine ((S)-5-aminocyclohexa-1,3-dienecarbonic acid) is a gamma-amino acid produced by Streptomyces toyocaensis sp. 1039. This bioactive compound was first isolated and characterized by Kobayashi et al. in 1976 (1). Gabaculine is well known as an irreversible inhibitor of the gamma-aminobutyrate aminotransferase, due to its covalent binding to the cofactor pyridoxal phosphate. The natural product occurs in the S-configuration, this enantiomer shows twice the activity of the racemate in the inhibition of the aminotransferase.
In addition, a gabaculine derivative serves as a precursor in the sythesis of the antiviral compound oseltamivir. With the availability of gabaculine the synthesis of oseltamivir could be reduced to four steps.
There are several total syntheses described for gabaculine, however its biosynthetic pathway is elusive so far.
The structure suggests that a not yet described biosynthetic pathway, showing similarities to the shikimic acid and the amino shikimic acid pathway, is responsible for the production of gabaculine. Ganem et al. postulated a pathway starting from isochorismate (2). This assumption could not be proven yet.
The possibility to find new antibiotic compounds in the cell extract of Streptomyces toyocaensis sp. 1039 is very high, because the mC7N-moieties, like gabaculine, serve as starter units to polyketide-synthase clusters as can be seen amongst others in the biosynthesis of rifamycin B, ansamitocin, pactamycin or geldanamycin (3). The starter molecule is derived from the amino shikimic acid pathway or in the case of pactamycin from transamination of 5-deoxy-3-dehydroshikimic acid. All of these compounds show a high antibiotic activity.
Here, we present feeding experiments with labelled glucose as carbon precursor. We could show that the labelled glucose is incorporated into gabaculine.
In order to identify the gene cluster responsible and to characterize the biosynthetic enzymes the genome of Streptomyces toyocaensis sp. 1039 will be elucidated. Putative gene clusters responsible for the biosynthesis of gabaculine containing shikimate pathway – related enzymes will be tested by gene knock – out experiments.

Abstract received: 30/06/2011 18:50:23 *********

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Mining the cinnabaramide biosynthetic pathway and biochemical and structural characterization of the involved octenoyl-CoA reductase/carboxylase, Liujie Huo, Nick Quade+, Shwan Rachid*, Jennifer Herrman*, Marc stadler#, Bärbel Köpcke#, Jens Bitzer#, Dirk W. Heinz+ and Rolf Müller*. *:Department of Microbial Natural Products (MINS), Helmholz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infection Research (HZI), Saarland University, Saarbrücken, Germany. +:Department of Molecular Structural Biology, Helmholtz Centre for Infection Research,Braunschweig, Germany. #: InterMed Discovery GmbH, Dortmund, Germany.*

The cinnabaramides are mixed polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS)-derived natural products isolated from a terrestrial Streptomyces sp.. They interfere with the proteasome and thus potentially inhibit the growth of the cancer cells. Exhibiting a significant structural similarity to salinosporamide isolated from a marine Streptomyces sp., the compounds contain a γ-lactam-b-lacton bicyclic ring core structure attached to a non-proteinogenic amino acid cyclohexenyl-alanine and an unusual hexyl side chain. Both cinnabaramides and salinosporamides exhibit potent cancer cell cytotoxicity and exert their effects via binding to the 20S proteasome core particle, thereby inducing cell-cycle arrest and programmed cell death (apoptosis). To improve anti-cancer activity and permit genetic approaches to novel analogs, we have firstly cloned and characterized the cinnabaramide biosynthetic gene cluster from Streptomyces sp. JS360. Next to the expected PKS/NRPS encoding genes, the pathway also comprises additional tailoring genes shown to be involved in the biosynthesis by gene inactivation studies.

The putative reductase/carboxylase CinF was further investigated. Structural analysis and biochemical characterization of the enzyme, including active site mutations, are reported. In vitro experiments has confirmed its function in the generation of the unusual polyketide extender unit 2-Carboxyoctanoyl-CoA. Our analysis reveals how primary metabolic CCRs can evolve to produce various dicarboxylic acid building blocks, thereby setting the stage to employ CCRs for the production of novel extender units and consequently altered polyketides.

Precursor directed biosynthesis studies demonstrated that CinF exhibits a relaxed substrate-specificity towards a number of synthesized precursors enabling the production of novel chlorinated cinnabaramides. A similar strategy (mutasynthesis) led to the generation of a further derivative, cyclopentylcinnabaramide. Compared to the native cinnabaramide A, the chlorinated derivatives show improved inhibitory activity on the proteasome’s proteolytic subunits (especially the trypsin and chymotrypsin units) and higher cytotoxicity against certain human tumor cell lines.

Abstract received: 20/05/2011 12:06:36 *********

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Involvement of intra- and extracellular beta-galactosidases in lactose catabolism in Penicillium chrysogenum.

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Penicillium chrysogenum is an economically important fungus used as industrial producer of penicillin. Apart from traits related to penicillin biosynthesis, general aspects of its carbon metabolism have received little attention. We investigated the catabolism of lactose, an abundant component of the diary residue whey that has been used extensively in pencillin fermentation, comparing the type strain NRRL 1951 as a wild-type reference with the industrial penicillin-producer ASP-78.

Both strains grew similarly on lactose as the sole carbon source under batch conditions, consuming 15 g/L lactose in about 84 hours. The time-profile of sugar depletion concurred with the presence of both intra- and extracellular beta-1,4-D-galactosidase (bGal) activities, measured as ortho-nitrophenyl beta-D-galactopyranoside (ONPG) hydrolases. Upon growth on commonly occuring monosaccharides, such as D-glucose, D-fructose, D-xylose or D-galactose, as well as on glycerol, neither extra- nor intracellular bGal could be detected. However, L-arabinose induced ONPG-hydrolysing activity to about half the values measured with lactose-grown material. Similar results were obtained with mycelia pregrown on glycerol and transferred to fresh minimal medium with the various carbon sources tested. The measured bGal activities were similar for the two investigated strains.

In silico analysis of the genome sequences revealed that P. chrysogenum features at least five putative bGal-encoding genes at the annotated loci Pc22g14540, Pc12g11750, Pc16g12750, Pc14g01510 and Pc06g00600. The first two proteins appear to be orthologs of the Aspergillus nidulans intracellular family 2 glycosyl hydrolases specified by neighboring loci AN3201 and AN3200 that cluster with a lactose permease-encoding gene (AN3199). The latter three P. chrysogenum proteins feature an N-terminal secretion signal and appear distinct paralogs related to the extracellular bGal from Aspergillus niger, LacA, a family 35 glycosyl hydrolase.

Transcript analysis of Pc22g14540 and Pc12g11750 showed that they were expressed exclusively in response to lactose but completely repressed on the mixed growth substrate glucose/lactose. Pc16g12750 was seemingly co-expressed with the two putative intracellular bGal genes, while its two paralog genes were apparently not transcribed under any condition tested. This expression profile is distinct from those in other ascomycetes, like Trichoderma reesei or A. nidulans, where bGal genes are induced by the monosaccharides D-galactose and/or L-arabinose. Our results indicate that the L-arabinose-induced ONPG-hydrolase observed in P. chrysogenum may correspond to a side activity of glycosyl hydrolases that are not classed in families 2 or 35. Nevertheless, it is likely that various of three transcribed bGal genes described here are involved in lactose catabolism in P. chrysogenum.

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Secondary metabolism in filamentous fungi: there is more to it than just the cytosol.

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Current synthetic biology approaches are performed by inserting complex biosynthetic pathways in organisms ranging from bacteria to filamentous fungi. Until now, it has not been sufficiently recognized that in eukaryotic systems many enzymatic reactions required for secondary metabolite formation may not take place in the cytosol, but rather in organelles like peroxisomes. Peroxisomes are ubiquitous organelles that consist of a protein-rich matrix surrounded by a single delimiting membrane. In filamentous fungi, peroxisomes are among others crucial for the primary metabolism of several unusual carbon sources used for growth (e.g. metabolism of fatty acids via β-oxidation). However, evidence is accumulating that these organelles also have a crucial role in the biosynthesis of a variety of secondary metabolites (e.g. penicillin and cephalosporin biosynthesis in Penicillium chrysogenum and Acremonium chrysogenum, respectively). For this peroxisomes either harbor specialized enzymes or utilize the existing pool of enzymes (e.g. β-oxidation enzymes) for product formation. Peroxisomes are dynamic organelles that are continuously formed and degraded dependent on the growth conditions of the cells. Therefore, regulating the interplay between biogenesis of these organelles and their autophagic degradation is crucial in obtaining high product yields. I will highlight the role of peroxisomes in secondary metabolite formation with emphasis on β-lactam formation in Penicillium chrysogenum.

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Dr. Gregor Kosec is the first author in the abstract (short talk) proposed by his supervisor Dr. Hrvoje Petkovic who is also applying to attend the conference - AFID: 58154

Title of the abstract: New insights into substrate supply and regulation of FK506 biosynthesis and their implications for bioprocess development and drug discovery

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Membrane protein CpkF from Streptomyces coelicolor A3(2) is a transporter of the yellow pigmented polyketide CPK.

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Over 50% of antibiotics of medical and veterinary importance are produced by bacteria from the genus Streptomyces. These organisms have the potential to produce dozens of secondary metabolites as shown by genome analysis, however many of the products remain unknown. One of such substances which remained cryptic until recently is the yellow pigment produced by a modular polyketide synthase Cpk from a model organism Streptomyces coelicolor A3(2).
The cpk gene clusters contains cpkF gene coding a protein homologous to membrane transport proteins such as QacA from Staphylococcus aureus, EmrB from Escherichia coli and SgcB from Streptomyces globisporus. All those proteins belong to the Major Faciliator Superfamily (MFS) of transporters. Sequence analysis suggests that CpkF belongs to the MFS and the 14 transmembrane segments (14TMS) family.
We have constructed deletion mutants of S. coelicolor A3(2) by PCR targeting. Secretion of the yellow pigment to the medium was not observed in mutated strains P112 and P113 were the cpkF gene was replaced with hygromycin and apramycyin resistance cassettes, respectively. After reintroducing the cpkF gene under strong constitutive promoter into the chromosome, secretion of the CPK polyketide was restored. Over-expression of the CpkF protein from a high copy number plasmid resulted in enhanced production of the yellow compound. As measured by microplate resazurin fluorescence assay, the strain P127 over-expressing the CpkF survived in about 50% higher concentrations of biocides (acriflavine and benzalconium), than the control strain P126 carrying the empty vector. We conclude that the CpkF protein is responsible for the efflux of the CPK polyketide and that it is a multidrug resistance protein similar to QacA and EmrB.

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Molecular systems development for studying and manipulating natural product biosynthesis.
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The recent explosion of data from microbial genome sequencing projects and their subsequent bioinformatic analysis has revealed a multitude of new secondary metabolite pathways. These have the potential to be exploited to produce new biologically active compounds. Reconstructing these pathways for heterologous expression in eukaryotic hosts can be difficult and time consuming or even impossible using conventional restriction/ligation cloning methods. We have developed several vectors that allow the rapid assembly of many genes in one multigene expression plasmid, using homologous recombination in yeast (Saccharomyces cerevisiae). Many natural product pathways, such as those for polyketide compounds, have 'megasynthase' enzymes that are encoded by large and unwieldy genes. To simplify the manipulation of such genes we have utilised the Gateway transfer technology marketed by Invitrogen, in conjunction with homologous recombination, to reassemble or modify very large genes and enable simple transfer to the multigene expression vectors. Three plasmids with alternative selectable markers currently provide the capacity to express up to twelve genes in Aspergillus oryzae, and possibly other filamentous fungi, while the principle behind their development is transferable to other heterologous hosts. These molecular systems should help to simplify the heterologous expression of biosynthetic pathways, and therefore help to further our understanding of natural product biosynthesis.
Much of our fundamental research is focused on the activities of hybrid polyketide synthase-non-ribosomal peptide synthases (PKS-NRPS). These include tenS and dmbS from Beauveria spp., which are involved in the synthesis of the dimethylated pentaketide tenellin and the monomethylated hexaketide desmethylbassianin, respectively. We have used our dual recombination system to exchange functional domains between tenS and dmbS. Expression of the chimaeric genes in A. oryzae has established that chain-length programming resides in the ketoreductase domain while methylation patterning is governed by the C-methyltransferase domain. This knowledge is now being used to construct hybrid enzymes designed to synthesise novel compounds of defined structure. We have also proved the concept of the multigene expression system by reconstructing the aspyridone pathway from A. nidulans, and the fusarin C pathway from Fusarium verticillioides. New combinations of megasynthases and downstream tailoring enzymes from diverse pathways are easily produced, and we are investigating this approach as a further means to generate "unnatural natural products" of predicted structure.

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Metabolic engineering and chemical strategies to the synthesis of novel aminoglycoside antibiotics

Aminoglycosides are natural products synthesized by actinomycete bacteria of the Streptomyces and Micromonospora genus. They can bind specifically to RNA scaffolds thus interfering with numerous key cellular processes such as translation, splicing, and expression regulation. For instance, they bind to the 16S ribosomal bacterial RNA and induce an error-prone behavior of the ribosome, eventually leading to bacterial cell death. Aminoglycosides have thus been extensively used as potent antibiotics in the treatment of severe nosocomial infections caused by multi-resistant pathogens. However, after fifty years of usage, totoresistant bacterial strains, able to inactivate all clinically used aminoglycosides, have recently emerged triggering the need to find new molecules.

We are developing original systemic methods combining microbiology and chemistry for synthesizing novel RNA binding compounds based on the aminoglycoside scaffold. The long term objective is twofold : obtaining new antibiotics which would escape the current resistance mechanisms but also designing original compounds able to bind specifically novel RNA targets.

This project is based on recent knowledge of aminoglycoside biosynthesis, which opens the way for biological engineering of the biosynthetic pathways. In particular, recent work on /Streptomyces fradiae, /the natural producer of Neomycin, has highlighted the role of most of the enzymes responsible for the antibiotic biosynthesis.

Using a mutasynthesis approach, we first knocked out a key enzyme involved in the biosynthesis of Desoxystreptamine, the core moeity of most of the aminoglycosides. It was then possible to feed genetically modified bacteria with original building blocks designed to act as analogs of natural metabolites. To quantitatively assess the production of aminoglycosides analogues, we developed an ion exchange purification strategy followed by HPLC MS analysis.

Our recent results show that we can alter the biosynthetic pathway efficiency and selectivity using small molecules. Current research aim at identifying and purifying unnatural aminoglycosides analogues, synthesized by the genetically modified bacteria from a synthetic building block.

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Automated Mining and Synthetic Biology Implementation of Secondary Metabolite Biosynthesis Gene Clusters

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Microbial secondary metabolites constitute a rich source of bioactive compounds with potential pharmaceutical applications as antibiotics, antitumor drugs or cholesterol-lowering drugs. In the genome of the actinomycete Streptomyces clavuligerus alone, we identified almost fifty secondary metabolite biosynthesis gene clusters, of which twenty-four on a giant 1.8 Mb-sized plasmid which may facilitate rapid horizontal transfer of these clusters. Most gene clusters appeared to be 'cryptic' clusters: they were not expressed under typical conditions. As hundreds of genomes with similar numbers of gene clusters are being sequenced at the moment, there is great need for automated tools to identify and analyze them. We have therefore launched antiSMASH (antibiotics & Secondary Metabolite Analysis Shell, http://antismash.secondarymetabolites.org), the first comprehensive pipeline capable of identifying biosynthetic loci covering the whole range of known secondary metabolite compound classes (polyketides, nonribosomal peptides, terpenes, aminoglycosides, aminocoumarins, indolocarbazoles, lantibiotics, bacteriocins, nucleosides, beta-lactams, butyrolactones, siderophores, melanins and others), predicting their core chemical structures of major classes, aligning them at the gene cluster level to their nearest relatives from a database containing all other known gene clusters, and integrating or cross-linking all previously available secondary-metabolite specific gene analysis methods in one interactive view. We have devised strategies to implement the identified gene clusters in high-throughput synthetic biology methodologies by constructing a specific chassis for each gene cluster type. Currently, we are developing further tools to facilitate this.

*Abstract received: 01/04/2011 13:00:33 *********

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Fungal survival strategies against antibiotics.
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The emergence and spread of pathogenic bacteria and fungi that are resistant to virtually all available antimicrobials represents a serious challenge for medicine and agriculture and has stepped up efforts to develop new antimicrobials. The use of smarter antibiotics, also called “dirty drugs” affecting multiple cellular targets is one discussed strategy to prevent the development of resistance mechanisms. Of special interest is the exploitation of antimicrobial peptides (AMPs), which are natural products of pro- and eukaryotic organisms and function as defense molecules to combat nutrient competitors, colonizers or invaders.
The activities of signaling pathways are critical for fungi to survive antifungal attack and to maintain cell integrity. However, little is known about how fungi respond to antifungals, particularly if these interact with multiple cellular targets. The antifungal protein AFP is a very potent inhibitor of chitin synthesis and membrane integrity in filamentous fungi and has so far not been reported to interfere with the viability of yeast strains. With the hypothesis that the susceptibility of fungi toward AFP is not merely dependent on the presence of an AFP-specific target at the cell surface but relies also on the cell’s capacity to counteract AFP, we used a genetic approach to decipher defense strategies of the naturally AFP-resistant strain Saccharomyces cerevisiae. The screening of selected strains from the yeast genomic deletion collection for AFP-sensitive phenotypes revealed that a concerted action of four signaling pathways is likely to safeguard S. cerevisiae against AFP. Our studies uncovered that the yeast cell wall gets fortified with chitin to defend against AFP and that this response is largely dependent on calcium/Crz1p signaling. Most importantly, we observed that stimulation of chitin synthesis is characteristic for AFP-resistant fungi but not for AFP-sensitive fungi, suggesting that this response is a successful strategy to protect against AFP. We thus propose the adoption of the damage-response framework of microbial pathogenesis for the interactions of antimicrobial drugs and microorganisms in order to comprehensively understand the outcome of antimicrobial treatments.

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The influence of the aconitase AcnA in the onset of secondary metabolism in Streptomyces viridochromogenes Tü494

In many organisms, aconitases have dual functions: they serve as primary metabolisms enzymes in the tricarboxylic acide cycle and as regulators of iron metabolism and oxidative stress response. Inactivation of the aconitase AcnA in Streptomyces viridochromogenes Tü494, the producer of herbicide antibiotic phosphinothricyl-alanyl-alanin (phosphinothricin tripeptide=PTT), leads to strong defects in physiological and morphological differentiation. This mutant (MacnA) fails in sporulation and antibiotic production which are characteristic secondary metabolism specific properties of sreptomyces. Furthemore, AcnA, in addition to its catalytic function, is capable of binding to iron responsive elements (IREs) thus altering the m-RNA stability in a similar mechanism described for the iron regulatory proteins (IRPs). A mutation preventing the formation of the [4Fe-4S] cluster of the aconitase (HisacnA1(C538A)) abolishes its catalytic activity, but does not inhibit its RNA-binding ability. In contrast, HisacnA2(∆125-129)in which 5 highly conserved aminoacids of AcnA are deleted shows an higher affinity to IREs than HisacnA. Furthermore, expression of hisacnA2(∆125-129) instead of native acnA gene results in a strain that sporulates earlier and has increaseg PTT production than wild type. This correlates with the improved RNA-binding ability of HisacnA2(∆125-129). In silico analysis of the S. viridochromogenes genome revealed several IRE-like structures e.g. upstream of recA gene, involved in the bacterial SOS response, ftsZ gene, required for the onset of sporulation in streptomyces. The binding of AcnA to these IREs is confirmed in gel shift assays. In conclusion, the demonstrated regulatory function of AcnA on the posttranscriptional level provides a new, so far unknown and unexploited form of regulation of secondary metabolism in streptomyces which might serve as possibility to optimize antibiotic production.

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Engineering a Natural Product Chemicopia

The rate of successful new drug discovery is sliding even as costs soar. One reason may be the neglect of Natural Products (NPs) in drug screens, a trend driven by the practical difficulties of working with NPs and by the perception that NP libraries have been played out. The convergence of next generation sequencing, informatics, mass spectroscopy and synthetic biology promises to rejuvenate NP drug discovery. Mindful of this opportunity, The Experimental Therapeutics Centre is embarking on a synthetic biology program to create a NP "Chemicopia" for drug discovery. The Chemicopia program integrates omics, informatics, natural product chemistry, high through-put screening and synthetic biology. In the first phase it will encompass the whole genome sequencing of ~ 100 Streptomycetes sp. strains chosen to represent the majority of secondary metabolite diversity in a large, existing strain collection, transcriptional analysis by "RNA seq" of these strains under various inducing conditions, bioinformatic identification of metabolic gene clusters and operons from the genomes, the construction of a physical library of genes, and the insertion of these genes into a streptomyces-based chassis organism to be designed and built as part of the project, and the assembly of a collection of purified NP compounds for deployment in drug screens.

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The discrete acyltransferases KirCI and KirCII involved in "Supramolecular Templating" of kirromycin biosynthesis

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The antibiotic kirromycin is produced by Streptomyces collinus Tü 365. This compound binds to the elongation factor Tu and blocks bacterial protein biosynthesis. The molecule backbone is synthesized by a large complex of type I polyketide synthases and non-ribosomal peptide synthetases (PKS I/NRPS complex), encoded by the genes kirAI-kirAVI and kirB.
The PKSs KirAI-KirAV have a “trans-AT”-architecture. These megaenzymes have no acyltransferase domains integrated into the PKS modules. In contrast, KirAVI belongs to the classical “cis-AT”-type PKS, where the ATs are part of the PKS protein.
In the gene cluster of kirromycin two separate genes, kirCI and kirCII, were identified, which are similar to acyltransferases.
To investigate the involvement of kirCI and kirCII in kirromycin biosynthesis, mutants were constructed and analyzed for kirromycin production. The inactivation of kirCI (ΔkirCI) resulted in a significant reduction of kirromycin production. In ΔkirCII kirromycin synthesis was completely abolished. To confirm the effects of the kirCI and kirCII deletions, both mutants were complemented with the wild type genes. The complemented strains produced the antibiotic at levels comparable with the parent strain S. collinus Tü 365. These data indicate that both genes are involved in kirromycin biosynthesis and the gene kirCII is essential for the production of this antibiotic.
For kirromycin assembly, a selective loading of ACPs with the building blocks malonyl-CoA and ethylmalonyl-CoA is required. This function is presumably carried out by KirCI and KirCII, respectively. To confirm this hypothesis and to determine the specificity of KirCI and KirCII an in vitro ACP loading assay was carried out.
Therefore KirCI, KirCII and two selected ACPs were expressed in E. coli and purified. The proteins were used in the in vitro assay and the loading of malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA to the ACPs was monitored by autoradiography and HPLC/ESI-MS. The experiments showed that KirCI loads specifically malonyl-CoA onto ACP4 and the second enzyme, KirCII, is the first biochemically characterized “trans-AT” with high specificity for ethylmalonyl-CoA and transfers this substrate to ACP5.
Thus, there is a specific recognition of the ACP of module 4 and 5 by KirCI and KirCII, respectively. To our knowledge, such interaction mechanism, where free-standing proteins that provide building blocks, dock site-specific to the “recipient”-protein to achieve structural diversity in polyketides was not characterized until now.

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Molecular and chemical characterization of secondary metabolite gene clusters in Fusarium fujikuroi: the fusarin gene cluster

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The filamentous fungus F. fujikuroi is known to produce a variety of structurally diverse secondary metabolites such as the plant hormones gibberellins, polyketide pigments like bikaverin and fusarubin, and mycotoxins such as beauvericin, fusarin C, fusaric acid and moniliformin which cause enormous economical losses in trade of crops.

In order to reduce the health risk of these mycotoxins in food, feed and biotechnologically produced gibberellin preparations, identification of the involved gene clusters is of great importance.

The recently sequenced genome of F. fujikuroi contains 17 polyketide synthases (PKS). So far only four of them can be linked to specific products: bikaverin, fusarin C, fumonisin and fusarubin. However, the biosynthetic pathways of these polyketides and their regulation are poorly understood.

Currently we are studying the biosynthetic pathway of fusarin C (which is mutagen in the ames test) by using deletion mutants of each single gene of the involved gene cluster including the hybrid polyketide synthase/non-ribosomal peptide synthase (NRPS)-encoding gene. In addition, we have created a deletion mutant missing all cluster genes except for the PKS/NRPS key enzyme gene to identify the first intermediate in the fusarin C pathway.

Besides, we study the regulation of gene expression for fusarin pathway genes by external signals, such as nitrogen availability and pH and the involvement of potential transcription factors such as AreA, AreB and PacC. Furthermore, we analyze the impact of global regulators on fusarin gene expression, e.g. Velvet, LaeA, and histone-modifying enzymes.

*Abstract received: 10/06/2011 17:17:36 *********

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Remodeling secondary metabolism with synthetic chemical signals.

The secondary metabolism of the actinomycetes has provided bioactive small molecules that are used as antibiotics, chemotherapeutic drugs, immune suppressants, and for numerous other pharmaceutical applications. While the genetics and biochemistry of secondary metabolism have been investigated for many years there are many gaps in our ability to explain and understand it. For example, we do not understand how secondary metabolism is linked to primary metabolism or what the biological triggers are for secondary metabolism to occur. This is important because most strains express only a small fraction of their secondary metabolites under laboratory conditions: most secondary metabolites encoded in the sequenced actinomycete genomes are of unknown structure and biological activity. Learning how to trigger the expression of these molecules could lead to the discovery of new drugs.

We have taken a novel approach to this question by screening a 30,000 compound library for synthetic small molecules that alter secondary metabolism in the model actinomycete Streptomyces coelicolor. We identified 19 molecules in this screen that significantly alter yields of the pigmented secondary metabolites actinorhodin and undecylprodigeosin. LC/MS analysis of secondary metabolite yields demonstrated that these molecules in fact influence many secondary metabolites in addition to these two well-studied pigmented molecules. Interestingly, four of our 19 hits, the ARC2 series, have related structures, we assumed meaning that they interact with the same target(s). We carried out in depth structure/function analysis of this class and defined the minimal active structure. This work suggested that these molecules inhibit fatty acid biosynthesis. Genetic experiments confirm this, identifying the enoyl reductase FabI as the primary target. We suggest therefore that the mechanism of action of the ARC2 series involves the liberation of precursors such as acetyl-CoA and malonyl-CoA from fatty acid biosynthesis for utilization by polyketide syntheases. Transcriptome analysis of the effects of the ARC2 series is consistent with this and furthermore reveals additional primary metabolic consequences of this ‘remodeling’ of secondary metabolism. Another class of molecules, the ARC6 series, function by a distinct mechanism because they alter yields of a different spectrum of secondary metabolites than the ARC2 series. Importantly, both ARC2 and ARC6 can remodel secondary metabolism in most streptomycetes. The effects of both classes are complex and distinct, consistent with distinct molecular targets in primary metabolism and/or the regulatory network. Importantly, we see the ‘awakening’ of cryptic metabolites by both classes of molecules.

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Exploring fungal-bacterial interactions for discovering new bioactive substances and their mechanisms of action.

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Fungal-bacterial interactions are found almost everywhere in nature and can be of many different types from neutralism to mutualism. These interactions are also central in biological control of plant pathogenic fungi. Of special interest should be hyphae associated bacteria that naturally accumulate around fungal hyphae. Using a new method for isolating hyphae-associated bacteria from Rhizoctonia solani hyphae and testing them in interactions show that the hyphae associated bacteria can have different effects on the fungus from stimulating to inhibiting. For the most inhibiting bacterial isolate we also show that a bioactive compound is responsible for the observed control effect in microcosms mimicking the natural environment. Environments containing large proportions of new genera/species as in Greenland can be expected to yield many new compounds involved in fungal bacterial interactions. A novel antifungal bacterium affiliated to Pseudomonas fluorescence was isolated from rhizosphere soils in Greenland. It shows high antifungal activity at low soil temperature and using transposon mutagenesis we could show that a novel non-ribosomal peptide synthetase was responsible for most of the antifungal effect. Additionally, for an antifungal isolate of Paenibacillus isolated from Greenland we find an antifungal compound that is only produced in the bacterial-fungal interaction and is not produced in pure culture.
In silico studies of whole genomes show that many bacteria and fungi contain cryptic pathways for production of unknown secondary metabolites never expressed in pure culture but might be expressed during direct interactions with other organisms. Thus fungal-bacterial interactions could be an ample source of new bioactive compounds including antibiotics against bacteria and fungi. High throughput transcriptomics of fungal-bacterial interactions combined with isolating compounds present during the interaction might reveal both new bioactive compounds, the genes responsible for their production and their probable target mechanisms. Projects in this line are just being started in our lab and will be presented shortly.

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Production of the antibiotic thiomarinol and new analogues by genetic manipulation and mutasynthesis

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Thiomarinol, is a hybrid antibiotic composed of a pseudomonic acid analogue joined to a pyrrothine, isolated from marine bacteria belonging to Pseudoalteromonas spp, but not yet used clinically. By contrast mupirocin, used particularly to control commensal methicillin resistant Staphylococcus aureus (MRSA) as well as infections by this bacterium, is produced as a mixture of pseudomonic acids by Pseudomonas fluorescens NCIMB10586. The biosynthetic cluster for thiomarinol and most of the putative domains were previously identified by DNA sequence analysis. The pyrrothine component of thiomarinol must be joined with the pseudomonic acid homologue, marinolic acid, before release from the bacteria. Deletion analysis implicated TmlU in joining marinolic acid with the pyrrothine by an amide bond. TmlU shows sequence similarity to SimL and NovL which are amide synthases in simocyclinone and novobiocin biosynthetic pathway respectively. The aim of my work is to investigate selected aspects of the biosynthesis of both mupirocin and thiomarinol to underpin optimisation of production and creation of novel molecules. The first aspect is to determine whether TmlU can be used to create novel hybrids by feeding Pseudomonas fluorescens different compounds that could be joined with mupirocin. To test the function of TmlU an expression plasmid encoding tmlU was introduced into P. fluorescens NCIMB10586 WT and mupU mutant since mupU encodes a possible homologue. HPLC in combination with LC-MS revealed that tmlU did not complement the Pseudomonas fluorescens mupU mutant. However, it did cause structural truncation of the 9HN part of pseudomonic acid and when expression was induced to higher levels also blocked processing to the final form (Pseudomonic acid A, PA-A). These findings reveal unexpected interactions between components of the thiomarinol and mupirocin pathways which could interfere with use of simple combinations of genes from the two pathways and which may need to be overcome before creating new hybrids by combining different pathways that may allow the productions of novel hybrid amides. The second aspect of the work covered aims to reduce the amount of minor pseudomonic acid derivatives (especially PA-B) by increasing the expression of rate-limiting genes that are hypothesised to control the flow to PA-A. Since the apparent equivalent of PA-B in the thiomarinol pathway is a much more minor product, we are carrying out genetic manipulation to identify how the thiomarinol pathway works and trying to engineer the mupirocin pathway to work in the same way. The latest results will be presented.

Abstract received: 07/06/2011 23:48:43 *********

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Biosynthesis and genetic engineering of goadsporin, one of thiazole and oxazole containing peptides (TOPs) produced by Streptomyces sp. TP-A0584., Hiroyasu Onaka, Department of Biotechnology, Toyama Prefectural University, Japan

With the progress of genome-mining techniques for secondary metabolite screening, many kinds of thiazole and oxazole containing peptides (TOPs) synthesized by ribosome were discovered from a wide variety of microorganisms. The common feature of TOPs is the peptide backbone containing thiazole, metylthiazole, and oxazole rings, which are derived from serine, threonine, and cycteine, respectively, and these heterocyles contribute for the bioactivity and the structure stability.

TOPs biosynthetic gene clusters consist of the structure gene, the post-translational-modificaion enzyme genes, transcriptional regulator genes, and the immunity genes. Interestingly, although the all of them contains enzymatic genes for thiazole or oxazole formation, these genes have very low similarities among these producing strains.

Goadsporin (GS) is a linear polypeptide antibiotic produced by Streptomyces sp. TP-A0584. Goadsporin consists of 19 amino acids, which included 8 unusual amino acids, oxazole, thiazole and dehydroalanine. GS promotes the secondary metabolite and morphogenesis at low concentrations, and induces growth inhibition at high concentrations in actinomycetes.

The GS biosynthetic gene cluster contains a structural gene, godA, and nine god (goadsporin) genes involved in post-translational modification, immunity, and transcriptional regulation spanning 20 kb. GS biosynthesis is initiated by the translation of 49 amino acid godA polypeptide. The subsequent cyclization, dehydration to form oxazole and thiazole rings are probably catalyzed by godD, godE, godF, and godG. Finally, it would be digested the N-terminal 30 amino acids leader sequence and acetylated by godH acetyltransferase homolog to afford GS

Over 50 GS analogs were produced by site-directed mutagenesis of godA, suggesting that this biosynthesis machinery is applied for heterocyclization of peptide. This approach will open the door to biosynthesize the new biological active peptides.

Abstract received: 28/06/2011 15:20:03 *********

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Exploring and exploiting moenomycin biosynthesis to develop new class of antibiotics: contributions of genomics

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Suzanne Walker, Harvard Medical School
Victor Fedorenko, Lviv University

The rise of resistance to common antibiotics over the last decade has resulted in reduced livelihood, lost lives and an increased healthcare burden. This is a worldwide problem, which has to be countered via the development of new classes of antibiotics. Moenomycins are a small family of phosphoglycolipid natural products that possess a number of remarkable features from the drug development point of view, such as a good spectrum of activity and a unique mode of action. Moenomycins are not used clinically because of poor pharmacokinetics, which for more than 30 years motivated chemical synthesis of analogs. Total synthesis of moenomycin A (MmA) has recently been realized, although it does not allow for a rapid access to a wide range of MmA analogs. We have identified the Streptomyces ghanaensis MmA biosynthetic pathway through genome scanning and deciphered the formation of MmA via combination of genetic and biochemical experiments. These studies revealed parsimonious and highly modular nature of MmA biosynthesis reflected already at the level of genetic organization of biosynthetic genes (moe). Rapid generation of moenomycin analogs is possible through heterologous expression of different sets of moe genes. More diversity is expected through harnessing of genes from silent moenomycin-like clusters uncovered in course of bacterial genome sequencing. Evolution of moenomycis biosynthetic pahtways and unusual features of the regulation of their production are discussed.

Abstract received: 08/04/2011 14:11:12 *********

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Cell Engineering via Reiterative Recombination in Yeast

The plasticity of the metabolic network makes microorganism-based biosynthesis of natural products a promising platform for rapid, efficient and highly versatile drug and commodity production. Reprogramming cells for these increasingly sophisticated applications requires the construction of customized multi-gene pathways and their introduction into host organisms. Moreover, the ability to create libraries of pathway variants, in order to optimize function using screening and directed evolution approaches, is essential. Our DNA assembly system, “Reiterative Recombination”, employs endonuclease-induced homologous recombination in a cyclical format that allows for stepwise elongation of the construct of interest. Here, we exploit Reiterative Recombination as a straightforward and general technology for combinatorial mutagenesis of metabolic flux. We demonstrate preliminary results for the construction of terpenoid pathway in S. cerevisiae using Reiterative Recombination. Furthermore, we present progress towards the application of Reiterative Recombination to optimize metabolic flux for terpenoid production. In addition, we develop Reiterative recombination on a vector to enable shuttling of large gene assemblies between organisms. Together these results establish Reiterative Recombination as a simple and powerful library mutagenesis technique and advance our efforts to engineer the cell for fully in vivo directed evolution. More broadly, our network-oriented experimental approach expands the toolkit available for engineering living cells toward the routine production of valuable natural products in yeast.

Abstract received: 24/06/2011 21:11:10 *********

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Design, synthesis, assembly technologies for synthetic biology engineering

With the completion of myriad genome sequencing projects and the emergence of metagenomics, the genome engineering field has expanded into many applications including the integrated analysis of complex pathways, the construction of new biological parts and the re-design of existing, natural biological systems. These design and construction and validation areas require intelligent metabolic and genetic systems engineering, sequence verified, cost-effective gene synthesis and precise, concerted assembly of multiple DNA fragments of various size ranges, up to and including chromosomes. Current commercial cloning products are generally not scalable or robust enough to support the assembly of very large or very small genetic elements or a combination of both. In addition current strategies are not flexible enough to allow iterative modifications to the original design without complicated cloning strategies.

We present a set of integrated technologies that provide state-of-the-art rational genetic design and simulation, gene synthesis and seamless, simultaneous, flexible, and highly efficient assembly and transfer of genetic elements, designed for a wide size range (10s to 100,000s base pairs). The paradigm of next generation deep sequencing to build a foundational understanding at the genomic and transcriptome levels upon which to build metabolic models to guide directed engineering will be presented. Scaled, cost-effective gene synthesis methods and process considerations will be discussed. Vector construction and higher order HTP assembly of genetic elements in vitro and in yeast cells to precisely join DNA fragments with or without terminal homology will be described including “bridging” to join or edit non-homologous part junctions. Desk-top and web-based assembly/design software tool and resources to design and manipulate large DNA constructs in silico will be presented in the context of research products and enhanced development of synthetic biology engineering capabilities

Abstract received: 06/06/2011 21:44:21 *********

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New insights into substrate supply and regulation of FK506 biosynthesis and their implications for bioprocess development and drug discovery

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FK506 (tacrolimus) is a secondary metabolite with a potent immunosuppressive activity, currently registered for use as immunosuppressant after organ transplantation. FK506 and its biogenetically related natural product FK520 are synthesized by combined polyketide synthase / non-ribosomal peptide synthetase systems in several species of Streptomyces. We have recently sequenced the entire FK506 biosynthetic gene cluster from Streptomyces tsukubaensis NRRL18488. We have discovered a previously unknown region of the FK506 gene cluster from Streptomyces tsukubaensis containing genes which encode the provision of unusual building blocks for FK506 biosynthesis as well as regulatory genes. Among others, we identified a group of genes encoding biosynthesis of the extender unit which forms the allyl group at carbon 21 of FK506. Based on our results, we propose a biosynthetic pathway for the provision of an unusual five-carbon extender unit which is carried out by a novel diketide synthase complex, small but rather complex biosynthetic machinery. We have also identified three regulatory genes, two of which have a profound influence on the biosynthesis of FK506. Genes involved in regulation and provision of substrate supply and their role in the biosynthesis and yield of FK506 during the biosynthetic process will be presented.

In addition, based on the recently discovered biosynthetic machinery we have designed a novel chemobiosynthetic process for production of FK506. Thus, novel aspects and application of biosynthetic/metabolic engineering approaches of this new biosynthetic machinery, as well as opportunities for the purpose of process development/improvement and drug discovery will be presented.

Abstract received: 30/06/2011 20:24:47 *********

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Therapeutic production in Escherichia coli using retrosynthetic design of metabolic pathways
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Progress in DNA technology and computational tools is leading to new developments in synthetic biology and metabolic engineering, enabling thus new ways to produce molecules of therapeutic interest. We are focusing on antimicrobial production in Escherichia coli with the aim to develop a therapeutic circuit for an in situ delivery to treat infectious diseases. The biosynthetic pathway of the antimicrobial is designed by the bioretrosynthesis method, which consists of the iterative application of reversed biotransformations (i.e. reversed enzymes-catalyzed reactions) starting from the target product to reach endogenous substrates. The retrosynthesis method uses the coding of the product, the reactants and the reactions into molecular signatures. A molecular signature is defined by its atomic signatures, which are canonical representations of the subgraph surrounding the atoms up to a certain distance that is defined by the signature height (named h). Using a reaction specificity search based on molecular signatures, reactions producing the intermediates or the target compound are also predicted. Candidate pathways are then ranked to select the pathways to implement in E. coli. The ranking function is based on several criteria such as inhibitory effects, host compatibility (codon usage, homology) and cytotoxicity of intermediates. Compound toxicity parameters were predicted by a structure-activity relationship model developed from our own experimental library of IC50 (half maximal inhibitory concentration) values. The IC50 is the concentration of chemical that inhibits 50% of the bacterial growth, and is used to determine the susceptibility of bacteria to the chemical. Furthermore, we use predictive tools (the MolSig package) developed by our group in order to estimate structure-activity relationships for enzyme activity and reaction efficiency at each step of the identified pathways. Pathways of interest are implemented in E. coli. Current tests in the lab involve synthesis of compounds with antimicrobial activity. In order to select the targeted compounds, we use our toxicity predictor to estimate the antimicrobial activity of such compounds since IC50 values provide a measure of the effectiveness of a compound in inhibiting bacterial growth. Thus, this work introduces a novel way of producing drugs that is applied to therapeutics, and in general to any compound of industrial interest.

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Investigation of the regio- and stereoselective intermolecular oxidative phenol coupling in Streptomycetes

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The oxidative phenol-coupling is a widespread biosynthetic procedure in the secondary metabolism of plants, fungi and bacteria. The oxidative phenol-coupling is an essential element for the configuration of the final structure of a wide range of antibiotics and other bioactive compounds. Mainly it is used to assemble biaryl structures by buildung up new C C-bonds between phenolic moieties. Compared to conventional methods of organic synthesis, this intermolecular biosynthetic step proceeds in almost all cases in a selective way. Particularly Streptomycetes, which are known for their enormous spectrum of secondary metabolites, produce a lot of substances with a biaryl-axis as structure characteristic. Even though lots of compounds and their biosynthesis are extensively investigated, it is still not known which enzymes are involved in the catalysis of this essential and selective biosynthetic process.
Here we present first results towards the elucidation of the biocatalytic system which is responsible for the regioselective intermolecular oxidative phenol-coupling in Streptomycetes.
The two compounds Julichrome Q3-3 from Streptomyces afghaniensis and Setomimycin from Streptomyces aurantiacus where chosen as model substances. The subunits of either compound derive from the same polyketide precursor and differ only marginally from each other. (1) The significant difference between the two compounds is that in Julichrome Q3-3 the subunits are connected at the C7-position while in Setomimycin the subunits are connected at the C10-position. (2,3)
For this purpose both substances were isolated and characterized, reconfirming structure already known. Furthermore the genomes of both strains are currently analyzed. By gen deletion experiments potential polyketide clusters were indentified. Subsequently, tailoring genes likely to be responsible for oxidative phenol-coupling, can be disrupted and the resulting mutant strains analyzed for production of the natural product.

Abstract received: 30/06/2011 18:49:57 *********

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Secondary metabolite production in Aspergillus niger at near zero specific growth rates
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With the aim to study secondary metabolite production in the filamentous fungus Aspergillus niger approaching a specific growth rate of zero, we revisited and improved the retentostat cultivation method for A. niger. Retentostat cultures are continuous cultures with cell retention which, instead of maintaining a fixed specific growth rate, forces μ into a transient decline to approach zero. A new retention device was designed allowing reliable and near complete cell retention even at high flow rates. The carbon and energy-limited retentostat cultures were highly reproducible. The specific growth rate approached zero (<0.005 h-1) and the growth yield stabilized at a minimum (0.20 gdw g-1 maltose). The severe limitation led to asexual differentiation and the supplied substrate was used for spore formation (asexual differentiation) and secondary metabolite production. Genome-wide expression studies were performed at distinct phases during retentostat cultivation and revealed the up-regulation of many secondary metabolism genes. At least 14 putative secondary metabolite clusters were identified that were not expressed during vegetative growth but were then up-regulated during retentostat cultivation. Analysis of the secondary metabolite profile of A. niger growing at near zero specific growth rates revealed at least eight secondary metabolites of which six are known to be polyketide-derived. The appearance of the metabolites in the culture medium occurred in different phases either before, associate with and after the onset of conidiation. Finally, we show that aerial conidiation mutants are also affected in submerged conidiation and that the conidiation mutants have changed profiles of secondary metabolite production. We propose that the retentostat method developed for A. niger can be used in fundamental studies of differentiation and secondary metabolite production and is applicable to filamentous fungi in general.

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NRPSpredictor2: an established method for the prediction of NRPS adenylation domain specificity – update and examples of applications
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The products of non-ribosomal peptide synthetases (NRPS) are of high interest in academia and industry as they comprise molecules with antibiotic, antitumoral, anti-inflammatory, immunosuppressing, metal chelating and other interesting properties. While NRPS are composed of several enzymatic domains, its adenylation (A) domains determine which amino acids will be integrated in the synthesized peptide.
NRPSpredictor is a program that predicts which amino acid is likely to be activated by an NRPS A domain given its protein sequence. These predictions provide insights on the (putative) composition of the peptide synthesized by a novel (unknown) NRPS, a crucial step towards the still unreached automated prediction of the entire secondary metabolite based on sequence information.
The program relies on a machine learning approach to learn from information inherent in a dataset of currently 576 A domain sequences of NRPS proteins with known specificity. First, the residues of A domains that align with the active site residues of gramicidin synthetase A crystal structure (8 Å around the bound phenylalanine) are extracted and encoded into a numeric fingerprint according to their physico-chemical properties. Training and predictions are then made based on the fingerprints of each A domain.
The current version, NRPSpredictor2 predicts A domain specificity on four hierarchical levels, ranging from gross physicochemical properties of an A-domain's substrate (hydrophobic-aliphatic, hydrophobic-aromatic, hydrophilic) down to single amino acid substrates. The prediction accuracy has been assessed with crossvalidation. The service and references are available at http://nrps.informatik.uni-tuebingen.de.
Since its publication in 2005, NRPSpredictor has become one of the major computational methods for A domain specificity prediction. It has been cited >120 times by publications in the field of microbial genomes and NRPS/PKS biosynthesis clusters.
Here, we will provide an update of the algorithm as well as an overview of success cases in the community.

Abstract received: 29/06/2011 17:10:32 *********

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Functional analysis of silent polyketide synthase genes of Penicillium chrysogenum

A major challenge in the battle against infection diseases is the resistance of pathogens against the commonly used antibiotics. The discovery of new antimicrobial compounds based on new chemical structures may solve this problem and yield a new generation of antibiotics. The functional analysis of cryptic secondary metabolite gene clusters is a promising tool for novel bioactive compounds discovery as many of these gene clusters are not expressed under laboratory conditions.
Polyketides represent a diverse group of bioactive compounds that are widely used as therapeutics due to their antibiotic and cytostatic properties. Polyketides are produced by multifunctional enzymes – polyketide synthases – that are encoded by associated (PKS) genes. Here we report on the functional analysis of (silent) PKS genes in the filamentous fungi Penicillium chrysogenum. Sequencing data showed the presence of 20 putative PKS genes in the genome [1], none of which have been characterized before. The expression of most is silent under laboratory conditions. To activate their expression two strategies are used: i) promoter replacement and ii) activation of the complete biosynthetic pathway through local regulator deletion or overexpression. Strong promoters were chosen from the P.chrysogenum genome and used for cloning in the appropriate expression plasmids. Growth media of strains with expression of silent PKS genes were obtained and analyzed using HPLC and mass spectrometry to reveal the products of these PKS clusters. We will report on the identification of pigments produced by PKS genes.

Abstract received: 30/06/2011 18:32:46 *********

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Activation of silent nonribosomal peptide synthetase gene clusters in Penicillium chrysogenum
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The filamentous fungus Penicilium chrysogenum is a well-known for its penicillin production. Penicillin and other secondary metabolites are produced by nonribosomal peptide synthetases (NRPS) or polyketide synthetases (PKS). Analysis of P. chrysogenum genome reveals the presence of six silent or low-expressed NRPS gene clusters, including three potentially siderophore biosynthetic genes and two hybrid synthases (PKS-NRPS). Replacement of the native promoter by the strong pcbC promoter led to the over-expression of one of the NRPS genes and associated product formation whose structure is currently analysed. Expression of secondary metabolites genes is also strongly related to chromatin structure and posttranslational modifications of histones. Overexpression of LaeA, a global regulator, with methyltransferases activity changed the transcript level of several secondary metabolites genes in P. chrysogenum. Also the significant upregulation of NRPS and PKS genes was observed in deletion of histone de-acetylase, suggesting epigenetic, as a key tool for silent gene activation. This approach is now used to identify the products of the silent NRPS and PKS genes.

Abstract received: 27/06/2011 15:02:22 *********

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Evolutionary and functional conservation of PAS-LuxR transcriptional regulators among polyene gene clusters

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Mainly produced by Streptomyces, polyene macrolides constitute a major-class of antifungal agents with hardly replaceable applications in medicine and food industry. Most of the currently available knowledge about the regulatory mechanisms that govern the biosynthesis of these compounds has been determined from the study of pimaricin production in Streptomyces natalensis.
Two positive transcriptional regulators are present in the pimaricin gene cluster of S. natalensis: pimR and pimM. Unlike PimR, highly conserved PimM homologous proteins are encoded in every known polyene gene cluster, suggesting a conserved functional role for these regulators in the modulation of the biosynthesis of such antifungal agents. The architecture of PimM is composed essentially by a PAS sensor domain in its N-terminal third, and a LuxR-type DNA-binding domain in the C-terminal one. Recently, we have determined the multiple gene targets for PimM in the pimaricin gene cluster, and the DNA-binding sequences in their promoter regions, and a transcriptional activation model mediated by this regulator has been proposed.
Here we show that PimM binding sites are conserved in promoter regions belonging to different polyene biosynthetic gene clusters in other Streptomyces species (like the nystatin, amphotericin or filipin ones), being type I polyketide synthases, glycosilation genes and ABC transporters the general targets for these PAS-LuxR transcriptional regulators. These new targets, characterized by EMSA and footprinting analysis, have been employed to refine the consensus binding sequence determined for PimM in S. natalensis.
The expression of pimM enhances filipin production in S. avermitilis and amphotericin production in S. nodosus, suggesting a general bottleneck in polyene biosynthesis. Additionally, we demonstrate that PAS-LuxR homologous regulators are fully exchangeable: nysRIV, amphRIV or pteF expression in a DeltaPimM S. natalensis mutant restores pimaricin production (which is undetectable in the mutant culture broths). That means not only that orthologous regulators are able to bind every target promoter in the pimaricin cluster of S. natalensis, but also that these proteins are able to activate transcription from all of them. Taken together, these results demonstrate that the function of PAS-LuxR homologous transcriptional regulators is fully conserved among polyene gene clusters.

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Gliotoxin pathway reconstruction as a prerequisite for pathway engineering
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Gliotoxin (GT) is the prototype of the infamous family of ETP (epi-dithiodiketopiperazine) toxins that are produced by several microorganisms. A hallmark of ETPs is the cyclopeptide scaffold equipped with a transannular disulfide bridge. Two individual routes rationalize gliotoxin-mediated toxicity. First, through redox cycling, reactive oxygen species (ROS) may be formed, which can severely damage the host cell. Second, gliotoxin can target proteins that are essential for vital functions. Notably, invasive aspergillosis caused by this fungus is the leading cause for death in immunocompromized patients. Since knowledge of the molecular basis and enzymology of ETP biosynthesis could greatly assist in developing diagnostics and antifungal therapy, this area has been the focus of intense research over the past years. Because its role in virulence and high cytotoxicity makes gliotoxin an interesting metabolite, it would be very beneficial to obtain the potential to manipulate the gliotoxin pathway to produce derivatives that are potential drugs. Therefore it is necessary to reveal all steps of the pathway that finally lead to the production of GT in Aspergillus fumigatus. We analyzed the biosynthetic route of GT with different methods from analytical chemistry to molecular biology. We could identify some of the key steps of the pathway and started to characterize the corresponding enzymes in detail. Some of them namely GliT and GliG are the funding members of completely new classes of enzymes and would certainly play a role in the construction of new natural product pathways as they perform unique reactions. Taken together the reconstruction of the gliotoxin pathway is a prerequisite for the selective manipulation of the pathway and give exciting insights into natural product biosynthesis.

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Synthetic Biology at BASF SE
Hartwig Schroeder GVF/D BASF SE

Industrial biotechnology research and production at BASF SE covers different products from vitamins such as vitamin B2, amino acids, organic acids such as lactic acid and succinic acid, optically active compounds such as pharmaceutical relevant intermediates or enzymes for industrial purposes or nutrition. My work has been contributing to all these fields in the last years. New approaches have been made to combine enzymes and pathways from different organisms to produce compounds which were not accessible previously by chemical synthesis such as diaminopentane. This product has been produced in multi 100kg amounts and has been used to produce bio-based polyamides with superior product perfomance. Lately natural product production employing new molecular methods have been applied at BASF.

Abstract received: 09/06/2011 10:30:17 *********

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Development of synthetic biobrick parts in actinomycetes.
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Actinomycetes are Gram-positive bacteria with a high GC-content. Especially members of the genus Streptomyces produce many pharmaceutically relevant secondary metabolites, including antibiotics and anticancer drugs. However, there is a limited number of biotechnological applications available as opposed to genetic model organisms like Bacillus subtilis or Escherichia coli.
To tackle the need for basic biobrick parts in streptomycetes we developed a synthetic promoter library. Synthetic promoters are less likely to be regulated and thereby their activity should be less dependent on impacts like nutrition supply or growth phase. The need for a library of promoters with a certain range of promoter strengths arises from different research approaches. Strong promoters can be used for the overproduction of a gene of interest, whereas genes responsible for the production of substances which are toxic to the cell in high concentrations require only sparse expression.
We chose the constitutive, well-known, and widely-used ermEp1 promoter (Bibb et al., 1985) and based our synthetic promoter derivatives on its -10 and -35 consensus sequences. The sequences located upstream, in between and downstream of these consensus sequences were completely randomized using degenerated primers. The library of synthetic promoters was cloned in front of the highly sensitive gusA reporter gene, encoding the enzyme beta-D-glucuronidase. After integration of the plasmid into the chromosome of Streptomyces lividans TK24 the gusA reporter gene system enabled us to pre-screen promoter strengths by sight with the substrate X-Gluc, which will show beta-D-glucuronidase activity via blue color on the plate. Exact and fast quantification of desired promoter strengths was then accomplished spectrophotometrically with p-nitrophenyl-beta-D-glucuronide as a substrate. Using this system we created promoters whose strengths ranged from 8 % up to 741 % when compared to ermEp1. Thus, covering a wide variety of promoter strengths we supply a helpful tool for the transcription of genes to a desired extent.
In addition, the plasmid designed for this assay can be easily adapted to compare the activities of known promoters, investigate them under different conditions as well as measure terminator efficiency.

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Biosynthesis and Mutasynthesis of Trans-AT Polyketides: Mupirocin and Thiomarinol
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Thiomarinol A is the principal metabolite produced by the marine bacterium Alteromonas rava and is a broad spectrum antibiotic displaying particularly potent activity against methicillin-resistant Staphylococcus aureus (MRSA). It is highly unusual in being a hybrid antibiotic; effectively two separate classes of antibiotic fused together. It is composed of a polyketide portion, closely analogous to mupirocin (a mixture of pseudomonic acids, e.g. PA-A), a clinically important antibiotics active against MRSA produced by Pseudomonas fluorescens, to which is appended a non-ribosomal peptide derived pyrrothine moiety. Pyrrothine analogues such as holomycin are already known to act as antibiotics. This hybridisation of two antibiotic classes renders thiomarinol a more potent antibiotic than either of its constituent parts, and even more interestingly, it displays activity against mupirocin resistant strains of MRSA.

The plasmid-borne gene cluster responsible for thiomarinol A production has been sequenced. In common with mupirocin it represents a member of the growing family of antibiotics produced via the trans-AT class of modular polyketide synthases. Targeted mutations of both the mupirocin (mup)1 and thiomarinol (tml) clusters have provided information on the biosynthesis and have allowed mutasynthesis experiments leading to a variety of new pyrrothine and mupirocin based metabolites. These and other studies on mupirocin and thiomarinol biosynthesis will be described.

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Rule-based simulation of synthetic integron construction.
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Integron structures allow organisms to react quickly to environmental stress by shuffling of gene cassettes through excision and incorporation by site-specific recombination. This process is best illustrated by rapid acquisition of multi-drug antibiotic resistance. Various groups are currently investigating how to harness this mechanism for applications in synthetic biology, by directed evolution experiments. Our work aims to simulate such experiments via a rule-based description of gene cassettes, taking into account such factors as excision probability, incorporation bias and compatibility in plasmid transfer. We predict the combinatorial outcomes, which will then be calibrated by experiment. With the objective of control and optimisation of directed evolution, as well as deeper understanding of the integron mechanism and ultimately to serve as a basis for computational metabolic optimisation. We present initial methodology and results for a simple assembly of gene cassettes, which could represent for example the carotenoid biosynthetic pathway.

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Modelling of homologous recombination in modular polyketide synthases combined with synthetic biology as a strategy for producing biologically active lead compounds,
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Modular polyketide synthases (PKS) are an attractive target for synthetic biology approaches with the possibility of reusing synthesized components in many constructs. A major stumbling block in the reprogramming of PKS has been the low product yield after most manipulations. Homologous recombination probably plays a major role in the natural evolution of modular PKS and would occur in regions of high sequence similarity, which may well favour better product yield compared to junctions typically selected for in vitro manipulation. However, it is not clear whether recombination between existing clusters can cover a comparable chemical space compared to a fully combinatorial approach.
We decided to model homologous recombination between pairs of PKS clusters to determine the potential to generate novel chemical entities. The recombination model assumed a core of sequence identity in a region of high sequence similarity. The ClustScan annotation program developed a data format for modular PKSs that links DNA sequence to module architecture and the chemical structure of the polyketide backbone. This format was used to develop an algorithm to determine whether a recombinant would produce a polyketide product and predict the chemical structure. For ease of use, this was implemented as a program (CompGen) with a graphical user interface. Recombination between 47 well-characterised clusters was modeled. This yielded 20,187 recombinants that were predicted to produce a product and 11,796 unique chemical entities. The details of the recombinants were entered into a database (http://bioserv.pbf.hr/cms/). The chemical structures can be exported in a standard SMILES format. These were used to calculate the molecular weights and degree of reduction of the predicted products. These data showed that they covered a significant region of chemical space. The large number of recombinants and their chemical diversity indicate that this is an attractive target for synthetic biology to produce novel lead compounds.

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Identification of a highly flexible glycosyltransferase from Saccharothrix espanaensis
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Therapeutically important antibiotics, such as erythromycin and vancomycin, contain sugars attached to the aglycon. The interaction of these sugars with the cellular target is usually important or even essential for the biological activity of such antibiotics. In addition to the pharmacodynamic properties glycosylation has been found to alter also the kinetic performance of a drug including solubility and bioavailability. To benefit from this, molecular biological engineering of glycoside moieties was developed. This approach has been shown to be a promising tool to modify the antimicrobial potency of small molecules.
Glycosyltransferases (GTs) catalyze the reaction of an activated nucleotide sugar with an aglycon to form glycosidic bonds. This chemoenzymatic attachment is both site-specific and stereoselective, thus providing a significant advantage to chemical synthesis. However GTs usually possess a high substrate specificity which hinders their application in altering the glycosylation pattern of a vast variety of small molecules. This leads to two possibilities: either using many different GTs to glycosylate different compounds or to find a few highly flexible GTs.
Here we report the discovery of the GT Ses60310 from Saccharothrix espanaensis. This enzyme is shown to glycosylate several different polycyclic aromatic compounds such as alizarin, quercetin and novobiocinic acid. Additionally the high flexibility of Ses60310 is emphasized by the acceptance of -OH as well as -NH2 nucleophiles and even different sugar-donors. Furthermore Ses60310 is active in vitro and in vivo. Consequently it is possible to perform isolated in vitro reactions as well as to exploit the ability of full cell systems to provide activated sugars as precursor very cheaply. In other words with Ses60310 we found a promising GT with broad substrate specificity which may be successfully used as a tool to generate a variety of different drug candidates.

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Optimization Of Terpenoid Precursors in Thermofibida fusca Using in silico Modeling

Natural products play an important role in manufacturing of several active pharmaceutical ingredients (APIs). APIs or precursors of APIs can be produced in living organisms with the major challenge of designing and optimizing metabolic pathways to obtain the compounds of interest. In this capacity, living organisms can act as renewable catalysts with high product specificity to produce APIs with potential cost savings over purely synthetic chemistry synthesis routes. Thus, efforts are being made to understand and design industrially usable microorganisms that can act as a host system for the purpose of production of these compounds. Cellulolytic organisms are being heavily studied for the production of biofuels, given that lignocellulosic biomass would be a cheap, abundant, and renewable starting material for chemical production. A challenge with cellulolytic microorganisms is that they are typically poorly characterized and often difficult to genetically manipulate. Here we propose that the aerobic, cellulolytic actinobacterium, Thermobifida fusca, may be a good candidate for cellulolytic production of APIs. Our group has recently developed a genetic engineering technique for T. fusca and has constructed a genome-scale metabolic model to study its metabolic capabilities. In the current study, in silico analysis of the non-mevalonate pathway (or DXP pathway) will be performed using a genome-scale metabolic model of T.fusca. The DXP pathway leads to the production of terpenoid precursors that have applications in nutraceutics and pharmaceutics. Modeling analysis illustrates the presence of the genes required to form: 1) Gerenyl pyrophosphate GPP, which is a substrate for producing monoterpenoids, 2) Farnesyl pyrophosphate or FPP, which is sesquiterpenoids precursor, and 3) Geranyl geranyl pyrophosphate GGPP, which is a diterpenoids precursor. The constructed metabolic model provides a basis for conducting in silico strain design predictions to optimize the production of terpenoid precursors through metabolic flux analysis to provide a rational basis for determining the distinct possible target gene sets for increased product synthesis. In parallel with these computational predictions, experimental work is being conducted to verify specific biochemical pathways and to implement novel pathways in T. fusca. Overall, this work will provide a computational basis for terpenoid production from cellulose and experimental testing and implementation of genetically modified strain designs.

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The structural basis of beta-methyl branch formation in trans-AT polyketide biosynthesis
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Type I modular Polyketide synthases (PKSs) are giant multi-functional enzymes involved in the production of secondary metabolites in a wide range of microorganisms. The bacillaene synthase (pksX) from B. subtilis 168 is a member of the trans-AT PKS family and is responsible for the biosynthesis of the antibiotic compound bacillaene. Bacillaene is a potent broad-spectrum bacteriostatic agent, which coupled with its minimal toxicity in eukaryotic systems suggests significant potential for use as an antibiotic in the treatment of animals and man. As in other trans-AT synthases, the bacillaene system employs a five enzyme “HCS cassette” to introduce a distinctive β-methyl branch into the functional product. Provision of a detailed structural and functional description of the HCS cassette enzymes will not only provide significant insight into branch incorporation, but may also reveal new routes towards the exploitation of this unique biosynthetic activity for the derivatisation of natural product based pharmacological agents. Here I present high resolution crystal structures of selected enzymes from the bacillaene HCS cassette. These structures provide significant mechanistic insight into this highly unusual and exploitable PKS branching pathway, and offers a framework for the manipulation of HCS cassette enzymes, towards the derivitsation of antibiotic natural products.

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Characterization of pyrrolamide biosynthetic pathways

Congocidine (or netropsin) is one of the two best known molecules of the pyrrolamide family. This molecule binds into the minor groove of the DNA double helix in a sequence specific manner (succession of four A/T bases). Recently, we characterized the congocidine biosynthetic pathway in Streptomyces ambofaciens (Juguet et al, 2009). Analysis of the congocidine cluster showed an original biosynthetic pathway involving an iterative Non Ribosomal Peptide Synthetase and new enzymes or pathways for the biosynthesis of the three precursors involving in the molecule assembly.

We have undertaken the isolation and characterization of the biosynthetic pathways of other members of the pyrrolamide family, distamycin, netropsin (other name of congocidine) and pyrronamycins produced by Streptomyces distallicus, Streptomyces netropsis, and an uncharacterized Streptomyces respectively. The objective of the study is first to improve our understanding of the biosynthetic mechanisms involved in pyrrolamide syntheses and regulation and secondly to manipulate the identified gene clusters to synthesize new pyrrolamide derivatives.

The characterization of the netropsin and pyrronamycin clusters is in progress. We also showed that Streptomyces distallicus produce both congocidine (netropsin) and distamycin. Preliminary results suggest the existence of two different clusters, each of them directing the biosynthesis of one pyrrolamide.

Once the whole clusters will be isolated, sequenced and analyzed, genetic manipulation could be used to combine biosynthetic genes of the different biosynthetic pathways identified to create new “unnatural” natural products. An example of such combinatorial biosynthesis could be the biosynthesis of congocidine derivatives able to bind DNA covalently. It had been suggested that pyrronamycin B binds DNA covalently (Asay et al, 2000) through its hydroxylated guanidinyl group. Once identified in the pyrronamycin cluster, the gene encoding the guanidinyl hydroxylating enzyme could be transferred to a congocidine producing strain. As congocidine contains also a guanidinyl group, hydroxylated derivatives of congocidine should be obtained and their DNA binding properties tested.

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Searching for Genes Cluster Encoding the Biosynthesis of Echinocandins in Aspergillus nidulans and Glarea lozoyensis fungi

Echinocandins are large lipopeptide molecules which inhibitor -(1,3)-glucan synthesis, so that fungal walls are damaged. They are strongly fungicidal against most Candida spp and fungistatic against Aspergillus spp. Glarea lozoyensis (G.lozoyensis) and Aspergillus nidulans (A. nidulans) NRRL 8112 fungi produce echinocandins as pneumocandin B (Cancidas) and echinocandin B (Anidulfungin) respectively. These commercial drugs are in clinical use for the treatment of systemic, life-threatening fungal infections in humans.

Pneumocandin B and echinocandin B were commercialized since 70 but the knowledge about the biosynthesis at genetic and biochemistry level still very poor. Echinocandins compounds are structurally characterized by a cyclic hexapeptide acylated with a long side chain; they are non ribosomical peptides (NRPS) and their interest is that they contain a number of unusual hydroxylated amino acids, including trans 3- and trans 4-hydroxy-L-proline (P3H and P4H respectively).

3-Hydroxyproline and 4-hydroxyproline as free amino acids are uncommon but they have been found in some peptide antibiotics such as etamycin, telomycin; ect… Moreover, the a-ketoglutarate-dependent conversion of free proline to cis-3-hydroxy-L-proline and trans-4-hydroxy-L-proline has been demonstrated in a number of organisms. Although proline hydroxylation is proved for fungi, none of the corresponding enzymes has ever been characterized. Our previous experiments suggest no conserved domain present in fungi from bacteria thus; we proceed to sequence G.lozoyensis and A. nidulans genome which will allow us to screen for NRPS clusters involved in echinocandins biosynthesis using bioinformatics tool as first approach.

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Genome mining of marine actinobacteria for secondary metabolite biosynthesis gene clusters: tools and applications.

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Marine actinobacteria represent a rich source of natural products with diverse biological activities. However, their full potential for production of bioactive compounds is never revealed via conventional screening. Poor growth in the laboratory conditions and our inability to mimic signals triggering expression of secondary metabolite gene clusters are the likely reasons for the latter. Genome sequencing and mining, followed by cloning and heterologous expression of the identified gene clusters might help to reveal the hidden biosynthetic potential of marine actinobacteria. New bioinformatic tool and vector/host systems that are being developed to address this imoprtant problem will be presented and discussed.

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