Myxococcus xanthus - a myxobacterial model strain as multiproducer of secondary metabolites

Dissertation zur Erlangung des Grades des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät III

(Chemie, Pharmazie, Bio- und Werkstoffwissenschaften)

der Universität des Saarlandes

von

Peter Meiser

Saarbrücken, 2008

Tag des Kolloquiums: 14.08.2008

Dekan:	Prof. Dr. Uli Müller
Berichterstatter:	Prof. Dr. Rolf Müller Prof. Dr. Claus Jacob
Vorsitzender der Prüfungskommission:	Prof. Dr. Uli Kazmaier

Danksagung

Mein besonderer Dank gilt Herrn Prof. Dr. Rolf Müller für die freundliche Aufnahme in den Arbeitskreis sowie für die Überlassung des sehr interessanten Themas. Ausdrücklich bedanken möchte ich mich für sein Engagement und die jederzeit vorhandene Bereitschaft zu Diskussionen und die wertvollen Anregungen.

Herzlich bedanken möchte ich mich bei Herrn Prof. Dr. Claus Jacob für die Übernahme des Korreferates, sowie bei Herrn Prof. Dr. Uli Kazmaier für die Übernahme des Prüfungsvorsitzes.

Dr. Helge B. Bode gilt mein Dank für die permanente Hilfsbereitschaft, vor allem zu Anfang meiner Doktorandenzeit. Effiziente Vorgehensweise in einem vielschichtigen Arbeitsfeld wurde mir von ihm stets nahe gelegt und vorgelebt.

Dr. Axel Sandmann sei gedankt für seine stete Diskussionsbereitschaft und die Anleitung in molekularbiologischen Techniken.

Thorsten Klefisch danke ich für die sehr produktive Zusammenarbeit während seiner Diplomarbeit und der anschließenden gemeinsamen Zeit.

Dr. Yasser Elnakady und Dr. Jan Hegermann sei gedankt für die Zusammenarbeit im Bereich der Proteomanalytik und der Elektronenmikroskopie.

Frau Birgitta Lelarge danke ich für das regelmäßige Korrekturlesen von Manuskripten und die Hilfe bei bürokratischen Problemstellungen jeglicher Art.

Ich möchte mich bei allen jetzigen und ehemaligen Arbeitskollegen bedanken für die gute Zusammenarbeit und freundliche Atmosphäre, ich wünsche allen dass es auch nach oder gerade wegen dem Umzug in den Neubau so gut weiter läuft wie bisher.

Bei meinen studentischen Wahlpflichtpraktikanten Marc Harenberg, Nico Reinhold und Helena Wortmann möchte ich mich für die produktive und kurzweilige Zusammenarbeit bedanken.

Schließlich möchte ich mich ausdrücklich bei meiner Familie bedanken, allen voran bei meinen Eltern die mich immer unterstützt haben.

Bei meinem Sohn Julian, der es sehr gut verstand mir immer wieder klar zu machen, dass es neben einzelligen Organismen mit sozialem Verhalten noch viel wichtigere höhere Organismen gibt, die Anspruch auf Aufmerksamkeit haben, und der mich dabei auch in der scheinbar schon erforschten Welt immer wieder auf Besonderheiten hinwies.

Meiner Frau Henny, die mich selbstlos unterstützt und mir großes Verständnis entgegen bringt, gilt der größte Dank.

Vorveröffentlichungen der Dissertation

Teilergebnisse dieser Arbeit wurden mit Genehmigung des Fachbereichs, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikationen:

Meiser, P., H. B. Bode, and R. Müller. 2006. DKxanthenes: Novel secondary metabolites from the myxobacterium *Myxococcus xanthus* essential for sporulation. Proc. Natl. Acad. Sci. USA **103**:19128-19133

Simunovic, V., J. Zapp, S. Rachid, D. Krug, P. Meiser, and R. Müller. 2006. Myxovirescin biosynthesis is directed by hybrid polyketide synthases/ nonribosomal peptide synthetase, 3-hydroxy-3-methylglutaryl CoA synthases and trans-acting acyltransferases. ChemBioChem **7**:1206-1220

Wenzel, S. C., P. Meiser, T. Binz, T. Mahmud, and R. Müller. 2006. Nonribosomal peptide biosynthesis: Point mutations and module skipping lead to chemical diversity. Angew. Chem. Int. Ed. **45**:2296-2301

Bode, H. B., P. Meiser, T. Klefisch, N. Socorro D.J.Cortina, D. Krug, A. Göhring, G. Schwär, T. Mahmud, Y. A. Elnakady, and R. Müller. 2007. Mutasynthesis-derived myxalamids and origin of the isobutyryl-CoA starter unit of myxalamid B. ChemBioChem **8**:2139-2144.

Meiser, P., and R. Müller. 2008. Two functionally redundant Sfp-type 4'-phosphopantetheinyl transferases differentially activate biosynthetic pathways in *Myxococcus xanthus*, ChemBioChem. **9**:1549-53

Meiser, P., K. J. Weissman, H. B. Bode, D. Krug, J. S. Dickschat, A. Sandmann, and R. Müller. 2008. DKxanthene Biosynthesis – Understanding the Basis for Diversity-Oriented Synthesis in Myxobacterial Secondary Metabolism, Chemistry and Biology **15**:771-781

Tagungsbeiträge:

H. B. Bode, P. Meiser and R. Müller. New Secondary Metabolites From The Myxobacterium *Myxococcus Xanthus* (Poster P40), Biology of Bacteria Producing Natural Products, Jena, 26.-28.09.2004

Meiser, P., H. B. Bode, and R. Müller. New secondary metabolites from the myxobacterium Myxococcus xanthus: a combined genomic and analytical approach (Poster 13.9) BioPerspectives, Wiesbaden 10.-12.05.2005

Meiser, P., H. B. Bode, and R. Müller. A secondary metabolite essential for sporulation? (Vortrag) 1th European Myxo Meeting, Marburg 18.19.02.2007

Meiser, P., H. B. Bode, and R. Müller. A secondary metabolite as gatekeeper for developmental sporulation in *Myxococcus xanthus* (Poster PE 001) Annual conference for General and Applied Microbiology, Osnabrück 01.-04.04. 2007

Meiser, P., H. B. Bode, and R. Müller. The DK-xanthenes**:** Novel Secondary Metabolites from *Myxococcus Xanthus* Essential for Developmental Sporulation (Vortrag), 34th International Conference on the Biology of the Myxobacteria, Granada/Spanien 14.-18.07.2007

Table of contents

Abstract

Within the last three decades, myxobacteria have been established as proficient producers of secondary metabolites that exhibit various biological activities. The recently finished genome sequencing project of *Myxococcus xanthus* DK1622 unveilled the – at least hypothetical – potential of this myxobacterial model strain to be a multiproducer of secondary metabolites as well. Applying a combined genetic and analytical approach, indeed several natural products and the corresponding biosynthetic gene clusters were identified and analyzed in our institute. Among them, four families of metabolites – myxochelins, myxochromids, myxalamids and myxovirescins – were already known from other myxobacterial species. The DKxanthene secondary metabolite family, however, resembles a novel class of compounds that appears to be unique to myxobacteria, and they were shown to be present in all *Myxococcus xanthus* strains investigated to date as well as in the closely related species *Stigmatella aurantiaca*. Studies aiming at the investigation of the biological function of these compounds strongly suggest that they are required for a proper progress of the developmental program that culminates in the formation of fruiting bodies and mature myxospores. DKxanthenes are synthesized by a hybrid polyketide synthase - nonribosomal peptide synthetase machinery, and the detailed analysis of the biosynthetic gene cluster proposes the presence of at least one iteratively acting polyketide synthase module that is in large part responsible for the diversity of metabolites from this family.

Zusammenfassung

Myxobakterien wurden in den letzten drei Jahrzehnten als außergewöhnliche Quelle sogenannter Sekundärmetabolite mit vielseitigen biologischen Wirkungen etabliert. Duch die vollständige Sequenzierung des Genoms von *Myxococcus xanthus* wurde das – zumindest theoretische – Potenzial dieses myxobakteriellen Modellstammes als Multiproduzent von Sekundärmetaboliten aufgezeigt. Im Rahmen einer kombiniert genetisch-analytischen Vorgehensweise konnten im Rahmen dieser Arbeit tatsächlich einige Naturstoffe und die dazugehörigen Biosynthesegene identifiziert werden. Darunter sind vier Familien von Sekundärstoffen - die Myxocheline, Myxochromide, Myxalamide und die Myxovirescine - die bereits von anderen Myxobakterien bekannt waren. Des Weiteren konnte eine neue Familie von Naturstoffen - die DKxanthene - charakterisiert werden. Diese scheinen spezifisch für Myxobakterien zu sein, da sie in allen bisher analysierten *M. xanthus* Stämmen und auch in der nahe verwandten Spezies *Stigmatella aurantiaca* identifiziert werden konnten. DKxanthene spielen eine wichtige Funktion für den Lebenszyklus des Produzenten, da sie für die geregelte Fruchtkörper- und Myxosporenbildung notwendig sind. Die DKxanthen-Biosynthese wird durch einen kombinierten Polyketidsynthase-nichtribosomale Peptidsynthetase Biosyntheseapparat gesteuert. Die detaillierte Analyse des Biosynthese-Genclusters weist hierbei auf die Mehrfachnutzung von mindestens einem Polyketidsynthase-Modul hin; eines von mehreren Besonderheiten, die zu der chemischen Diversität dieser Naturstoffe führt.

Introduction

1. Myxobacteria

Myxobacteria are obligate aerobic, chemotrophic Gram-negative δ proteobacteria that most commonly inhabit the soil. The recent discovery of novel myxobacterial species from marine as well as moderate halophilic environments (53,54) proves the ability of myxobacteria to adapt towards differing environmental conditions. The vegetative cells of myxobacteria are typically rod-shaped, 3-12 µm in length and 0.7-1.2 µm in width (110). When grown in nutrient-rich liquid media, myxobacteria usually grow as independent cells. However, on solid surfaces such as an agar plate, they interact with each other and swarm on the agar surface in order to gain access to nutrients that are not in immediate proximity to the bacteria. Many myxobacterial species secrete lytic enzymes which allow them to prey on other bacteria and yeasts (65,107) and to digest the released proteins, lipids and nucleic acids. In absence of any nutrient, myxobacteria stop the swarming behaviour and instead start to build up aggregates, a process that finally culminates in the formation of fruiting bodies. This complex life cycle that includes the formation of myxospores within the mature fruiting body is one of the outstanding features of myxobacteria.

Myxobacteria constitute the order *Myxococcales*, which can be further differentiated into the suborders *Cystobacterineae*, *Nannocystineae and Sorangiineae (109)*. *Sorangium cellulosum* from the latter suborder exhibits the unique capability among myxobacteria to degrade cellulose and to use it even as the sole carbon-source, a property that supports the isolation of *Sorangium* species from environmental samples (110).

Eight genera are known from the suborder *Cystobacterineae*, which is furthermore split into two families: The *Myxococcaceae* constituting of the genera *Myxococcus*, *Corallococcus* and *Pyxidicoccus* while *Archangium*, *Cystobacter*, *Hyalangium, Mellitangium* and *Stigmatella* belong to the *Cystobacteraceae (109)*.

Among them, the genus *Myxococcus* and especially the species *Myxococcus xanthus represents* the best studied organism among the myxobacteria since its life-cycle can be investigated very reproducible under laboratory conditions.

Another remarkable feature of myxobacteria - which might at least in part be caused by the complex life cycle when compared to other bacteria - is the overall large size of myxobacterial genomes. The recently sequenced genome of *Sorangium cellulosum* So ce56 resembles the so far largest known bacterial genome with a size

of 13.0338 Mb (118). The genome size of *Stigmatella aurantiaca* was estimated as 9.5 Mb (123), and the *Myxococcus xanthus* DK1622 genome was shown to be 9.14 Mb in size (44,66). The extraordinary large size as well as the high number of genes in the *Myxococcus* genome in comparison to all other so far sequenced non-myxobacterial δ proteobacteria was supposed to be at least in part based on extensive gene duplications. These gene duplications do not evolve by coincidence but rather include genes which are important for the myxobacterial life cycle, such as genes encoding proteins necessary for intercellular signalling (44).

1.1 Cooperative behaviour and life cycle of *Myxococcus xanthus*

Myxobacteria are assumed to be one of the earliest models in nature that explored the possible advantages of progressing from a single cell state to a social multicellular life style (30,122). However, myxobacteria retained their capability to grow as single cells rather than to further differentiate into obligate multicellular organisms. Multicellular behaviour requires several prerequisites like the ability to distinguish between sibling cells and cells from foreign species, spatial morphogenesis as well as the capability to exchange information from cell to cell, a process which is described as intercellular signalling (66,124). Several classes of mutants unable to form fruiting bodies or spores were identified and shown to arrest at specific time points during development, inferring the blocking of important signals. These mutants were grouped according to the pattern of synergism that lead to complementation of the developmental defect of one group when mixed with another group (47). Currently, five groups (A-E) of rescuable mutants are recognized, which have defects in the *asg, bsg, csg, dsg* or *esg* genes, respectively (27,47).

Multicellular social behaviour is induced when cells are starved on solid surfaces and when the bacteria “sense” that the overall cell number is high enough to enter the developmental process which will favour the survival of the population.

M. xanthus moves by gliding in the direction of its long axis by using two motility systems. Social (S-) motility is dependent on the presence of type IV pili that attach to nearby cells and subsequently are retracted, thus pulling the cells together (63,64). Adventurous (A-) motility enables the cells to glide as individuals. The mechanism underlying A-motility is unclear until now- the extrusion of slime from the cell poles through nozzle-like structures as the force for the propulsion was proposed (158),

and recently, a novel model describing focal adhesion complexes was introduced. Here, the attachment of intracellular motor complexes to membrane-spanning adhesion complexes and to the cytoskeleton were supposed to power motility by pushing against the substratum and thus moving the cell forward (93).

Starvation induces a shift from (adventurous) swarming behaviour towards the migration to aggregation centers which can be seen after about 4-6 hours. Within the first 24 hours of development, these loose aggregates become hemispherical mounds that each contain up to 1.000.000 cells. Approximately 10-20 % of the cells within the mounds differentiate into non-motile, heat- and sonication-resistant myxospores. The remaining cells undergo autolysis, most presumably in order to provide the essential nutrients for the surviving cells to undergo the differentiation into myxospores (157). Cells left outside the fruiting body remain rod shaped, but have a different protein expression pattern when compared with vegetative cells and myxospores. The role of these "peripheral rods" was proposed to be a backup of cells that take advantage of low nutrient levels that would not support germination and outgrowth of the developmental spores (102-104). Alternatively, peripheral rods might play a role in defending the dormant fruiting body from consumption and colonization by other microbes (124) (**Figure 1** pictures the time series of fruiting body formation of a *M. xanthus* submerged culture).

Besides the described differentiation into specialized cell types during fruiting body formation, *M. xanthus* cells growing under vegetative conditions undergo a phase variation between two alternate phases which are depicted by their pigmentation phenotype - the predominant colony type exhibiting the characteristic yellow colour that is the name giver of the species *M. xanthus* (yellow phenotype), meanwhile cells from the other phase are almost unpigmented and accordingly, this state is named the tan phenotype (18,19). Pure populations of mutants locked into the tan phase were shown to be unable to form fruiting bodies. Despite starting the differentiation process, cells did not form true spores after developmental induction, but rather developed phase-dark, round forms that were unable to complete the maturation to heat- and sonication-resistant, refractile spores (81,82). This defect in development could be restored by the addition of phase-variation proficient cells from predominant yellow cultures. In such mixtures, the tan-phase locked mutants were preferentially represented among the viable spores, and specific roles for each cell type were proposed: The tan cells within a population were suggested to be the progenitors of

spores, whereas the yellow cells might direct fruiting body formation and spore maturation through intercellular signalling (82,124). Similar observations were made in further experiments: Extracellular complementation of sporulation deficient group C mutants with group B strains showed only slight, if any, synergy in developmental complementation when yellow group C populations were used. Inclusion of a few percent of tan group C cells strongly stimulated the developmental synergism (62). Experiments investigating the glucosamin-induced cell lysis and subsequent sporulation of *M. xanthus* cells revealed that tan cells are more resistant to glucosamin-induced lysis, but yet were required for glucosamin-induced sporulation (97). Even though these observations strongly suggest an involvement of phase variation in the developmental life cycle of *M. xanthus*, still little is known about the mechanism of phase variation.

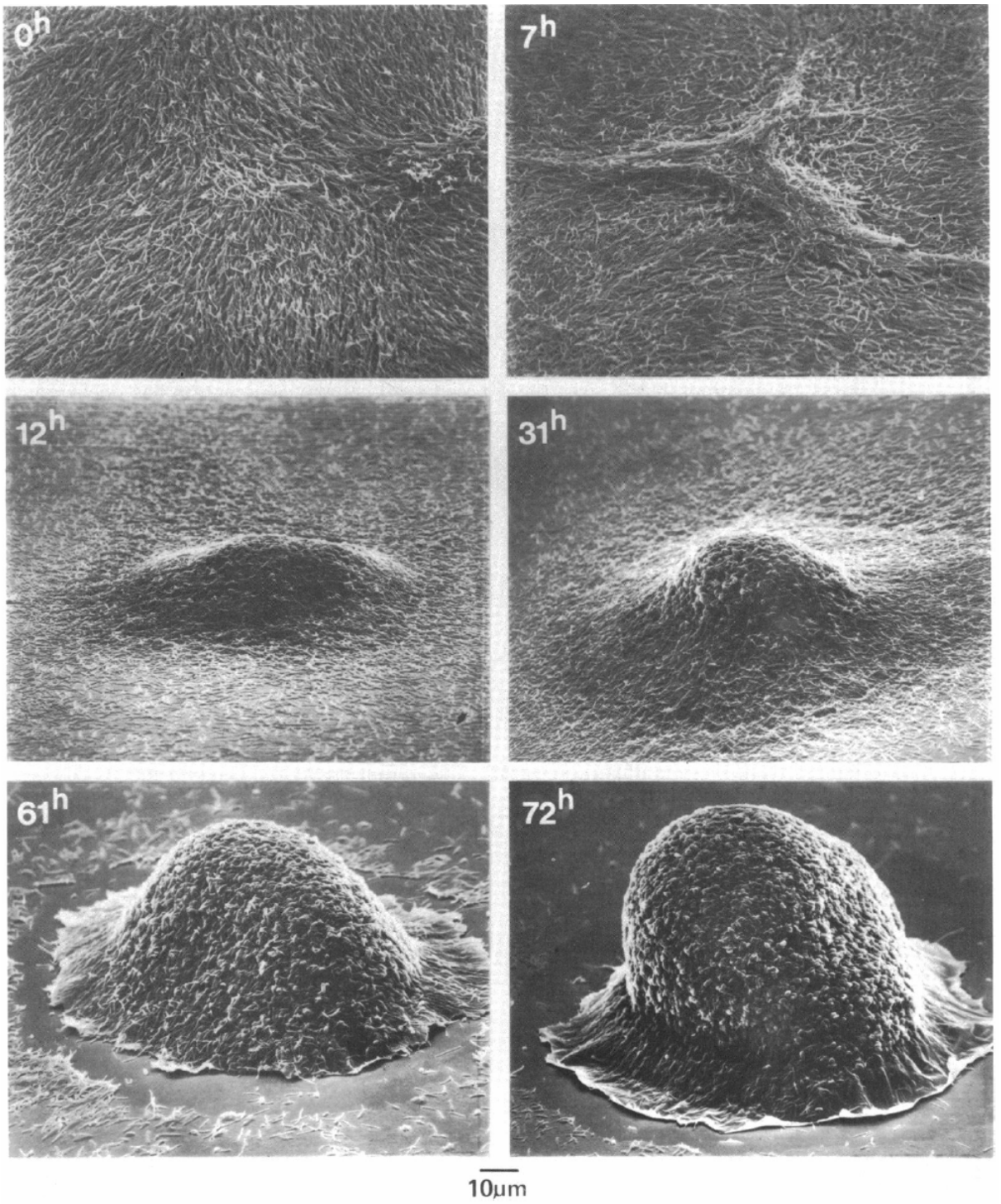


Figure 1. Fruiting body formation of *Myxococcus xanthus* in submerged culture (adopted from Kuner and Kaiser, 1982 (76))

2. Natural products

2.1 Natural products and their relevance in clinical therapy

Secondary metabolites from plants, fungi as well as bacteria constitute a daily growing library of compounds of natural origin that not only exhibit a vast diversity of chemical structures. More importantly, natural products and compounds which were developed from natural product lead structures, proved to be useful in the treatment of various diseases and in agrochemical applications such as crop-fungicides. Besides the use of complex therapeutic mixtures (such as extracts of herbs with boiled water) which was applied already thousands of years ago and which is still in use even in occidental medicine, the discovery and purification of single compounds with biological activities resembles a milestone of modern medical treatment. Among these, the discovery of penicillin - which is still in clinical use for the treatment of bacterial infections - by Alexander Fleming in 1928 from the fungus *Penicillium notatum* (83) and the discovery of morphine as the pain-relieving principle of the poppy *Papaver somniferum* L. more than two hundred years ago by Friedrich Sertürner (101) picture early hallmarks of this new era of natural product research. Since then, natural products have been established as useful sources for novel, non-synthetic drugs and drug-leads. Seventythree percent of the drugs used in clinical treatment of cancer are not derived from synthetic chemistry; 47% represent the natural products themselves or are directly derived thereof (100). Examples for potent antitumor drugs from natural producers are paclitaxel and doxorubicin. Further important fields of therapeutics besides cytostatic agents include various antibiotics-such as the already mentioned β-lactam antibiotics, but also erythromycin, tetracycline, streptomycin or vancomycin-, in addition immunosuppressive drugs like cyclosporine and rapamycin as well as the cholesterol-lowering agent lovastatin (selected structures are shown in **Figure 2**).

Besides the broad application of natural products in medicine, **Figure 2** exemplifies a further characteristic feature of secondary metabolites: the unique structural diversity found in nature. The recruitment of various different building blocks as well as the activity of diverse modifying enzymes leads to the construction of very often highly complex chemical structures exhibiting various stereocenters. On average, natural products differ from most synthetic structures by the incorporation of a higher number

of oxygen atoms and a higher steric complexity, whereas synthetic drugs usually comprise more nitrogen-, sulphur- and halogen-containing groups. Natural products can be regarded as privileged structures since they were selected for by evolutionary pressure, thus enabling the specific interaction with diverse biological targets (72).

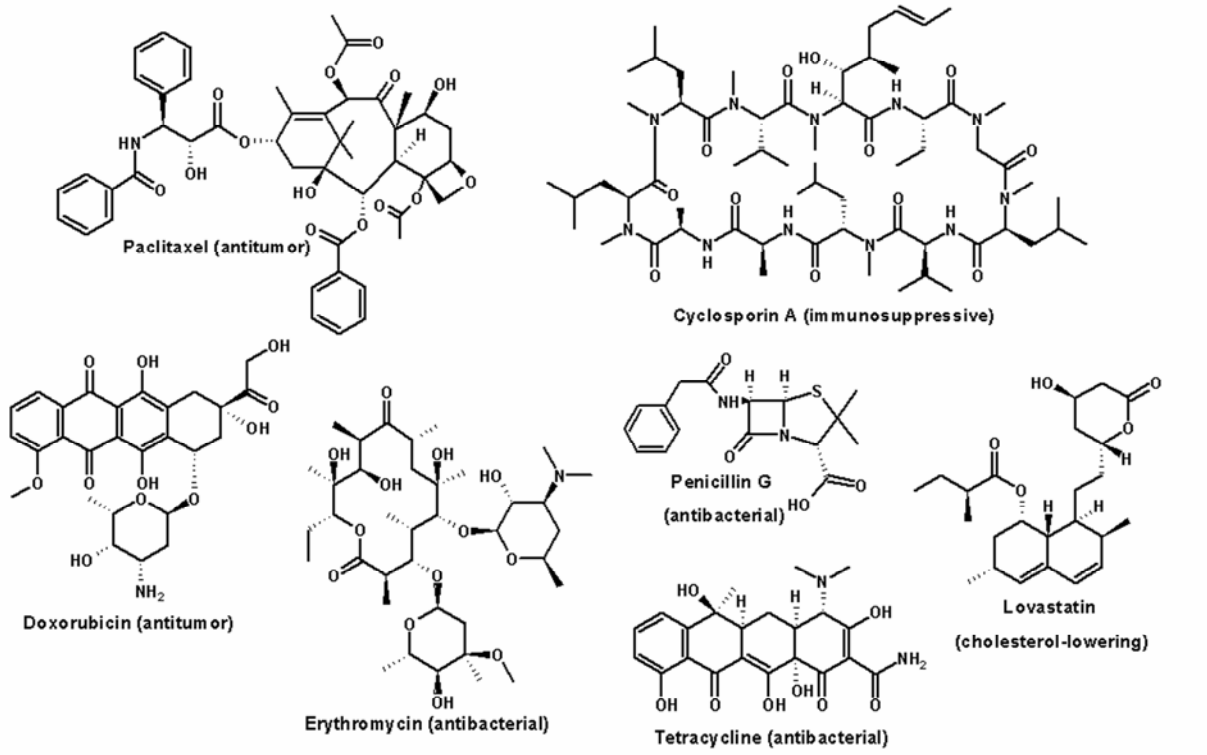


Figure 2. Selected chemical structures of natural products with diverse modes of action

In order to produce these diverse structures, microorganisms often employ large multifunctional enzyme complexes. Three frequently used systems are polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs) as well as hydrid-systems thereof which use activated carboxylic acids or amino acids, respectively, as simple building blocks that are incorporated into the growing precursor molecule (34,134,145,146). The genes encoding the biosynthetic enzymes are frequently found to be clustered on a small segment of the respective bacterial chromosome.

PKS systems can be further classified based on the chemical structure of the released product and on their mode of biosynthesis. For instance, fungal type I PKSs are characterized by the repetitive use of a single modular enzymatic system (a process described as iterative usage) (35,87). In contrast, the bacterial modular type I PKS is the most propagated one among bacteria. Here, the genes encoding the modular organized assembly lines are mostly clustered within the microbial genome,

and biosynthesis usually follows a colinear principle in which the backbone structure of the natural product can be correlated with the organization of the biosynthetic modules and catalytic domains (134). Typical products of modular type I PKSs are polyenes or macrolides such as erythromycin (149).

Bacterial type II PKSs consist of one set of discrete polypeptides that form a catalytic complex and which are used iteratively, usually leading to phenolic aromatic compounds such as actinorhodin (33,49). Finally, type III PKS systems belonging to the chalcon synthase and stilben synthase family are typical for the secondary metabolism of plants, but were recently also discovered in bacteria and fungi (5,7,45,121). Yet, more and more examples of PKSs that drop out of the borders of classified PKS systems emerge, and a new classification or the surrender of any classification system seems to be necessary (96,98).

The following description will focus on bacterial type I PKS systems and on nonribosomal peptide synthetases (NRPSs), since these are the most abundant in myxobacterial biosynthetic machineries and the relevant ones for this work. NRPSs have a similar structure as type I PKS systems (which will be called PKS in the following section for simplification).

2.2 Polyketide and nonribosomal peptide biochemistry

In both, polyketide and nonribosomal peptide biosynthesis, the growing chemical scaffold is build up by a series of condensing elongation steps with short carboxylic acid units or proteinogenic as well as nonproteinogenic amino acids, respectively. PKSs and NRPSs have a modular structure and follow the so-called “multiple carrier thio-template mechanism” (120,135). According to this model, repetitive catalytic units termed modules are responsible for the incorporation of one residue into the growing polyketide or nonribosomal peptide chain. During the assembly, the intermediates of the growing natural product remain tethered to the carrier protein of the respective module through a thioester-linkage. The carrier proteins themselves have to be activated through the activity of a phosphopantetheinyl transferase (Ppant transferase) that transfers a 4’-phosphopantetheine cofactor onto a conserved serine residue of the *apo*-carrier protein (**Figure 3**). The Ppant moiety serves two main functions during the natural product assembly: To activate the growing intermediate in a covalent and energy-rich linkage with the enzyme and furthermore to serve as a

flexible arm that facilitates the transport of the intermediates to the distinct catalytic centers - which are called domains - of the multifunctional enzyme (80,95,148).

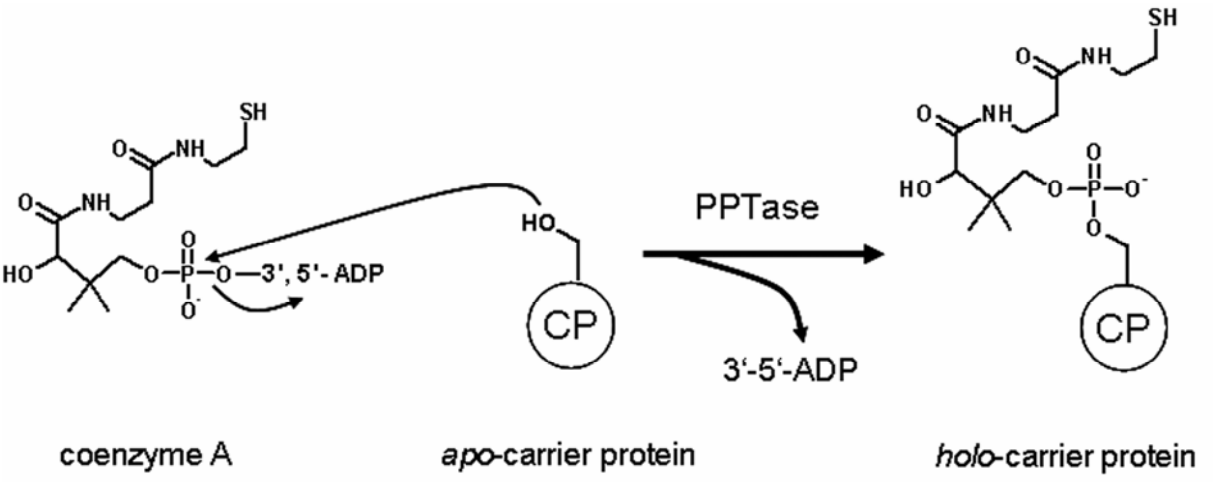


Figure 3. Activation of the *apo*-carrier protein (ACP or PCP) through the activity of a phosphopantetheinyl-transferase (PPTase)

Before attachment to the carrier protein (CP), the monomeric elongation unit has to be selected for by either acyltransferase (AT) or adenylation (A) domains in PKSs and NRPSs, respectively. After transfer to the carrier protein, a third catalytic domain accomplishes the chain extension resembling a thio-claisen condensation for PKSs and an amide bond formation in the case of NRPSs. The completely processed acyl-chain is finally released from its covalent thioester linkage through the activity of a termination domain such as a thioesterase (TE) or a reductive (R) domain.

Figure 4 shows the general processing of intermediates in PKS biochemistry. In the first step (**Figure 4**, **Step 1**) the acyl transferase (AT) domain selects for the respective CoA-activated starter or extender unit and transfers it onto the acyl carrier protein (ACP) of the corresponding biosynthetic module. Whereas the extender units are usually derived from malonyl-CoA and methylmalonyl-CoA, the starter units have been shown to be recruited from diverse sources of CoA-esters of short chain aliphatic as well as aromatic carboxylic acids. Subsequently, the ACP-bound intermediate of the upstream module (which is the loading module in the case of **Figure 4**) is transferred to a conserved cystein residue of the β-ketoacyl synthase (KS) domain of the downstream module and nucleophilically attacked by the α-carbon of the ACP-bound elongation unit (**Figure 4**, **Step 2**). After decarboxylative condensation of starter and elongation unit, the biosynthetic intermediate that now is extended by one additional C_2 unit remains attached to the ACP of the elongation module (**Figure 4**, **Step 3**). From here, the growing chain can be passed on to the

KS domain of the downstream module and thereby undergo another condensation reaction. Alternatively, in the presence of several additional catalytic domains, the intermediate might be channelled through several reductive steps before being passed on to the next module. A complete "reductive loop" (134) consists of a β-ketoacyl reductase (KR), a dehydratase (DH) and an enoyl reductase (ER) domain. The catalytic order of these domains is different from their order on the protein. KR domains catalyze the stereospecific reduction of a β-keto-function to the alcohol which can be further dehydrated and subsequently reduced to the α,β-methylene derivative through the activity of a DH and an ER domain, respectively (**Figure 4**, **Steps 4-6**).

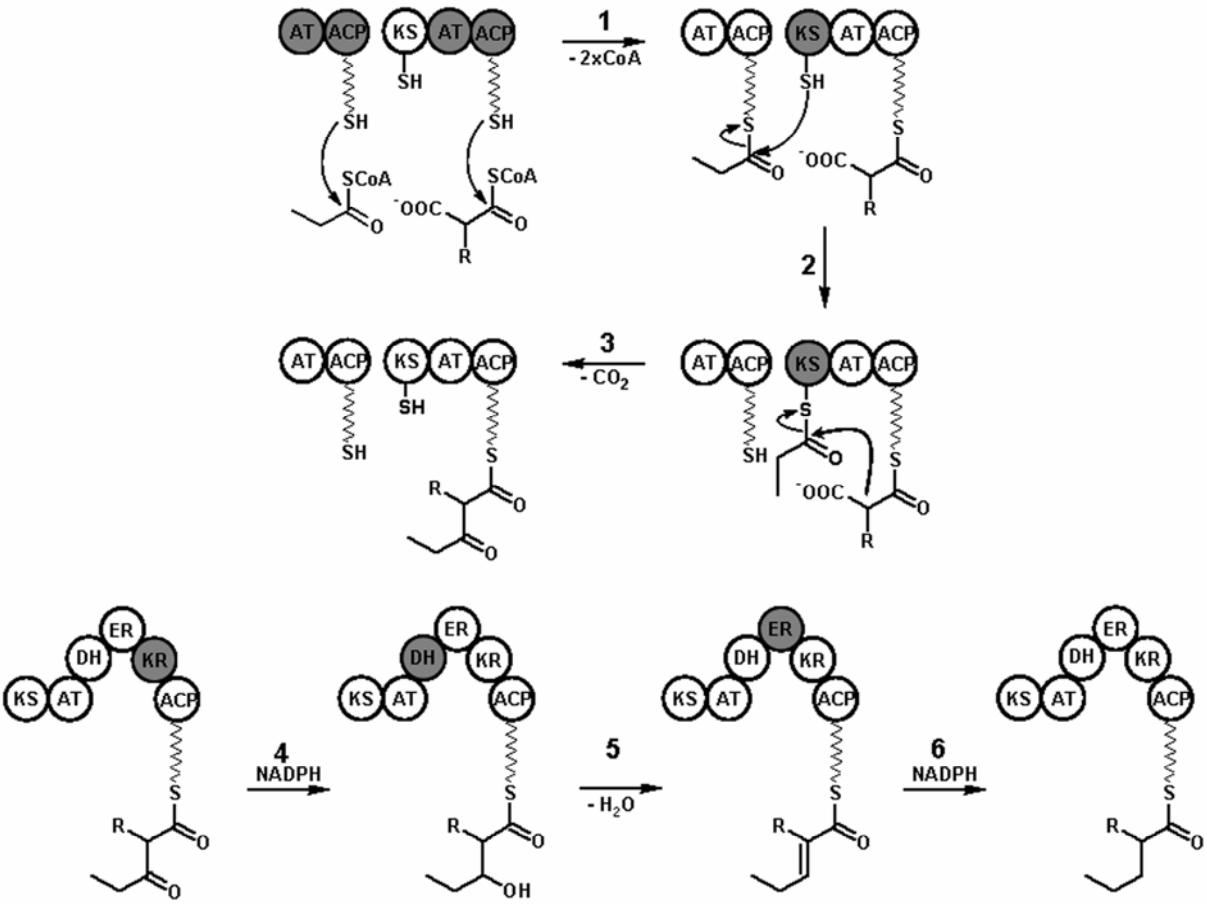


Figure 4. Schematic overview of polyketide synthase (PKS) biochemistry. The short chain carboxylic acid starter and extender units are selected for by acyl transferase (AT) domains and loaded onto the respective acyl carrier protein (ACP) (1). The thioester-linked intermediates subsequently undergo a decarboxylative condensation catalyzed by a β-ketoacyl synthase (KS) domain (2 and 3). Further optional domains might introduce additional structural modifications (4-6). Domains involved in catalysis of the respective reaction step are indicated by grey shading.

NRPS biochemistry follows a very similar procedure when compared to polyketide biosynthesis, as shown in **Figure 5**. Here, an adenylation (A) domain first selects for and furthermore activates the respective amino acid in an ATP-dependent reaction giving rise to its aminoacyl-adenylate derivative, which is subsequently transferred to the peptidyl carrier protein (PCP) (**Figure 5**, **Steps 1** and **2**). The condensing reaction - which in the NRPS case leads to an amide-bond formation - is catalyzed by the condensation (C) domain of the elongation (downstream) module (**Figure 5**, **Step 3**). Again, several modifying enzymes accomplish a higher diversity in natural product biosynthesis, a few examples are epimerization, (N)-methylation as well as heterocyclization (**Figure 5**, **Steps 4-6**). The latter is performed by specialized C domains (HC = heterocyclization domains) that catalyze the peptide bond formation and the subsequent cyclization and dehydration reaction, usually using cysteine, serine or threonine as the substrates. The resulting oxazoline (derived from serine and threonine) or thiazoline (derived from cysteine) ring system might be further oxidized to an oxazole or thiazole moiety (**Figure 5**, **Step 7**).

After the last elongation step, the enzyme-bound nonribosomal peptide, polyketide or NRPS/PKS-hydrid intermediate is released from its biosynthetic template, usually through hydrolytic cleavage catalyzed by a thioesterase (TE) domain that releases the free acid or, by intramolecular cyclization, a macrolacton- or macrolactam-structure.

Very common in nature are further chemical modifications that occur after the release of the natural product from the thio-template, such as glycosylations or additional oxygenation steps, many of which have proven to be essential for the bioactivity of the natural product (100).

A very high degree of conservation has been found for the delineated types of enzymes and domains, and several critical sequence motifs were identified that are necessary for the recognition (159) and activation (22,73,144) of the substrate, the attachment of the intermediate (156) as well as the catalytic activities such as condensing (73,156) and reductive reactions (112,156).

With the growing knowledge about polyketide and nonribosomal peptide biosynthesis, based on sequence as well as functional analysis of newly characterized biosynthetic gene clusters, the way is paved for the emerging field of combinatorial biosynthesis (21,28,90). The theoretically unlimited combination of PKS and NRPS systems and modifying enzymes opens up the opportunity for the

generation of even larger chemical libraries as well as structure-activity guided approaches in order to improve e.g. the selectivity of a natural product derived drug.

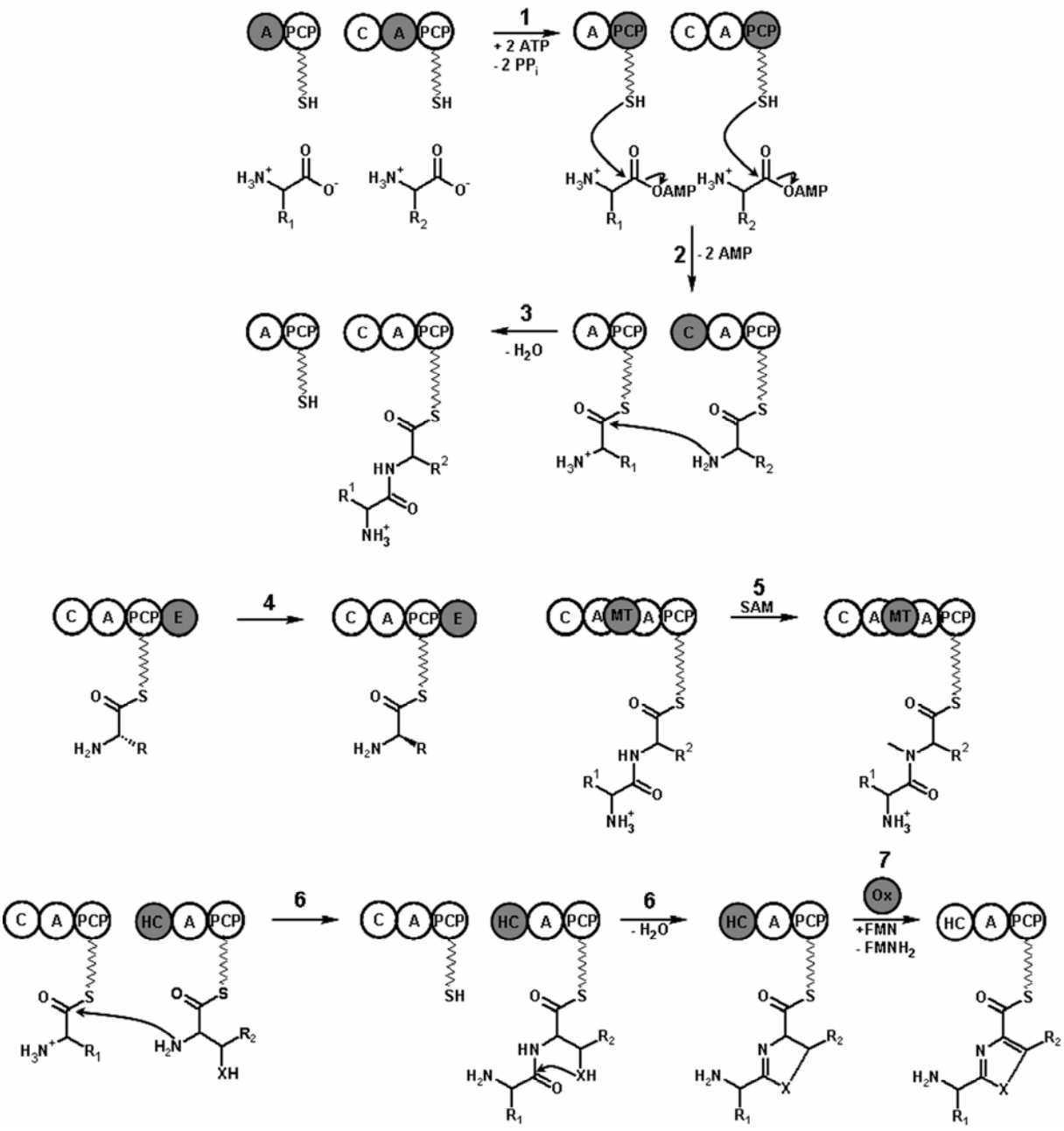


Figure 5. Schematic overview of nonribosomal peptide synthetase (NRPS) biochemistry. Amino acid starter and elongation units were selected and activated by adenylation (A) domains, attached to the peptidyl carrier protein (PCP) (1 and 2) and finally condensed by condensation (C) domains (3). Again, several optional domains (4-7) might lead to modifications in the chemical scaffold. Epimerization domains (4) generate an equilibrium of L- and the respective D-amino acids, and the C domain of the downstream module selects for one specific enantiomer. Domains involved in catalysis of the respective reaction step are indicated by grey shading (X = O, S).

3. Myxobacteria as multi-producers of secondary metabolites

Besides the readily established microbial secondary metabolite producers actinomycetes and fungi, the research on myxobacteria as novel sources for natural products only gained its legitimate attention within the last three decades. In a screening program performed at the former GBF (Gesellschaft für Biotechnologische Forschung, now Helmholtz Zentrum für Infektionsforschung, HZI), more than 100 new chemical core structures and additionally approximately 500 derivatives were isolated from altogether more than 7,500 different myxobacterial strains (for selected examples, see **Figure 6**) (13,43,108). Moreover, many of these natural products could be assigned diverse biological activities with unusual, rarely found mode of actions. Myxovirescin (141) and sorangicin (20,56) are examples for potent antibiotics from myxobacterial origin. In particular the latter, sorangicin, which targets the bacterial RNA polymerase beta subunit in a similar manner as the clinically used tuberculosis-antibiotic rifampicin has the potential to be a new drug-lead since sorangicin proved to be not as sensitive to mutations in the enzyme`s binding pocket which is shared between the two compounds (20). Myxothiazol (41,138,140), melithiazol (15,115), stigmatellin (78,137) and soraphen (8,16,39) are bioactive against several fungi, with soraphen being a potent inhibitor of the novel antifungal target-enzyme acetyl-CoA-carboxylase (8,16,39,106,111). Of special interest are compounds that interact with the eukaryotic cytoskeleton. Among these, the microtubuli-stabilizer epothilone (2,51,52) has served as a very promising drug-lead, with several natural and non-natural derivatives being investigated. Ixabepilone has recently finished the phase III clinical trials and was approved for the treatment of breast cancer by the FDA (24). Epothilone binds to tubulin and thus stabilizes the microtubuli in a similar fashion as the established anticancer-drug paclitaxel, but at least in part through a nonoverlapping mode of action (2,24). Avoidance of cross-resistances might be also achieved by targeting the cytoskeleton through a different mode of action exhibited by other bioactive secondary metabolites. This holds true for tubulysin (68,70,116) and disorazol (32,57,59), since these compounds do not stabilize but rather destabilize the microtubuli.

Furthermore, myxobacteria as well as other bacteria produce compounds that act as iron-chelators, so called siderophores, in order to sequester the essential metal from their environment (3). Myxochelin (77) was identified as the most often used iron-

chelating principle in myxobacteria. Additionally, it shows weak antibacterial activity against Gram positive bacteria and recently an inhibitory effect on the invasion of murine colon 26-L5 carcinoma cells was described *in vitro* (94).

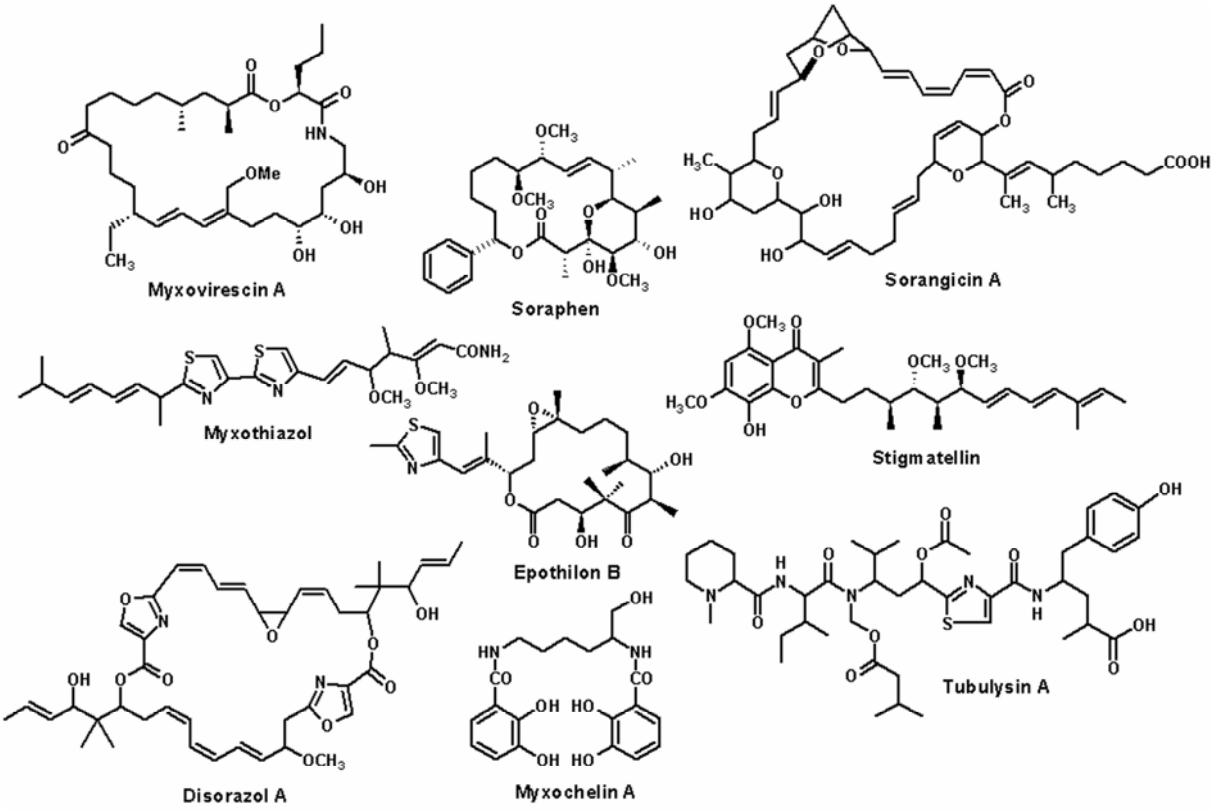


Figure 6. Selected myxobacterial natural products. Information about the respective biological activity or function is given in section 3

4. Outline of the present work

4.1 The potential of *Myxococcus xanthus* DK1622 as a multi-producer of secondary metabolites

Despite detailed investigations as the model strain for myxobacterial development, nothing was known about secondary metabolism in *M. xanthus* DK1622 until recently (13). The *Myxococcus xanthus* genome sequencing project (44) and the subsequent *in silico*-analysis revealed the presence of at least 18 biosynthetic gene clusters from the NRPS- or hybrid PKS/NRPS-type (**Figure 7**, red bars in layer 4), indicating the – at least hypothetical - potential of this strain to be a multi-producer of natural products. Remarkably, a large part of these 18 identified biosynthetic gene clusters

were shown to be located in close proximity to each other on the chromosome, with most of the biosynthetic genes being clustered between 1.5 and 3.5 Mb as well as between 4.4 and 5.8 Mb on the 9.14 Mb chromosome (44).

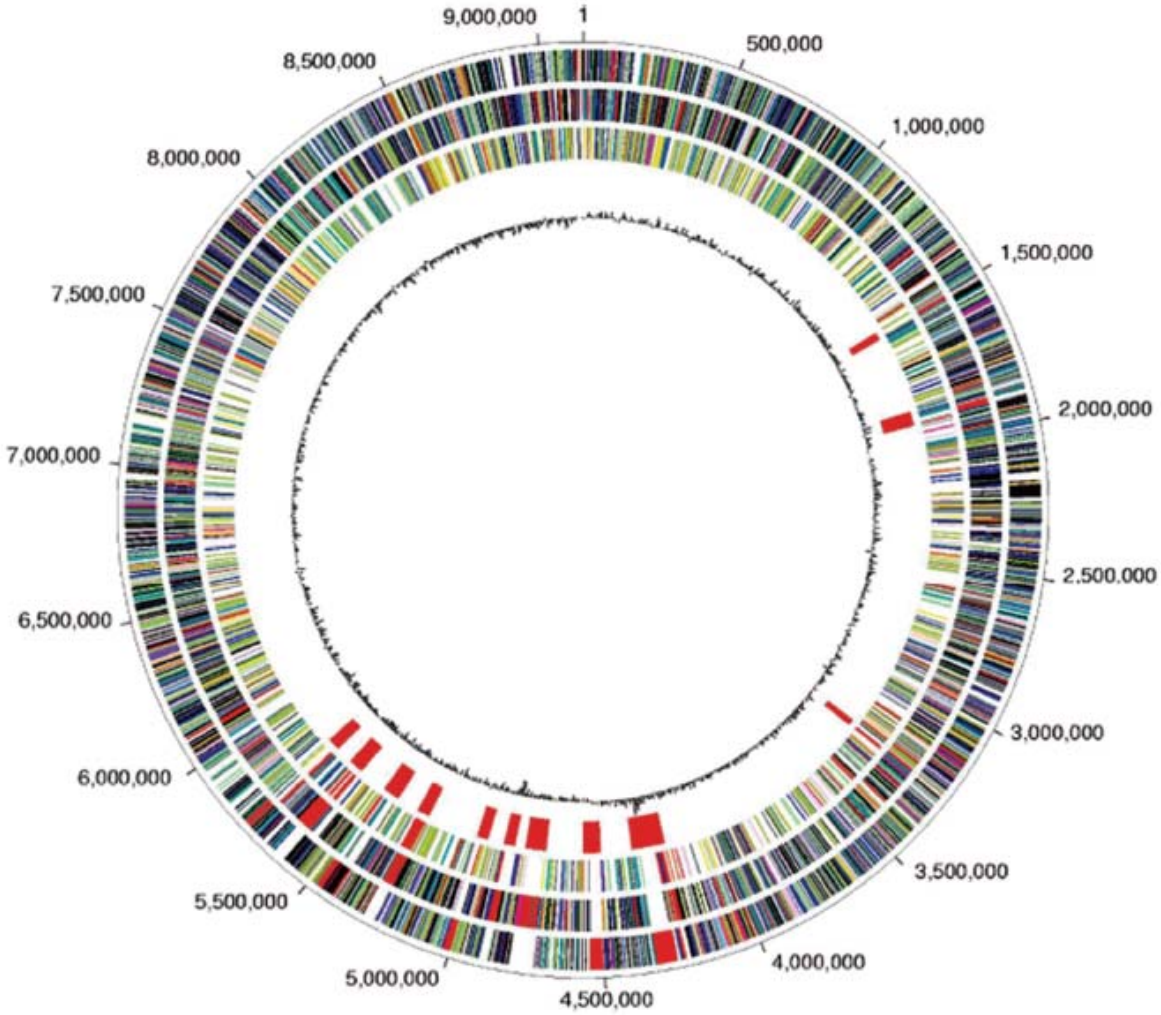


Figure 7. Genome map of *Myxococcus xanthus* DK1622. The red bars in layer 4 indicate the position of genes dedicated to secondary metabolism. Layer 1: Genes expressed in clockwise direction, layer 2: genes expressed in counterclockwise direction, layer 3: lineage-specific duplications, layer 5: GC nucleotide skew (adopted from Goldman et al. 2006 (44))

Altogether 8.6 % of the *M. xanthus* genome is dedicated to secondary metabolism, a percentage higher than in various other established secondary metabolite producers (9,105). Comparison of the newly identified biosynthetic gene clusters in *M. xanthus* DK1622 with gene clusters that were already known from other myxobacteria led to the identification of the biosynthetic gene clusters of myxalamid (cp. *Stigmatella aurantiaca* Sg a15), myxovirescin (cp. *Myxococcus virescens*), myxochromide (cp. *Stigmatella aurantiaca* DW4/3-1) and myxochelin (cp. *Stimatella aurantiaca* Sg a15)

(42,60,77,126-128,139,141,152,153) in *M. xanthus* (for chemical structures, see **Figure 8**). The mentioned metabolites - including in part novel derivatives - could be identified in *M. xanthus* DK1622 in the scope of this work. Structure elucidation was performed after applying large-scale fermentation and the subsequent isolation and one- and two-dimensional NMR analysis as well as HPLC-MS and MS/MS analyses in combination with feeding experiments (11,128,153) (myxochelin unpublished). Each family of metabolites will be described in more detail in the discussion section.

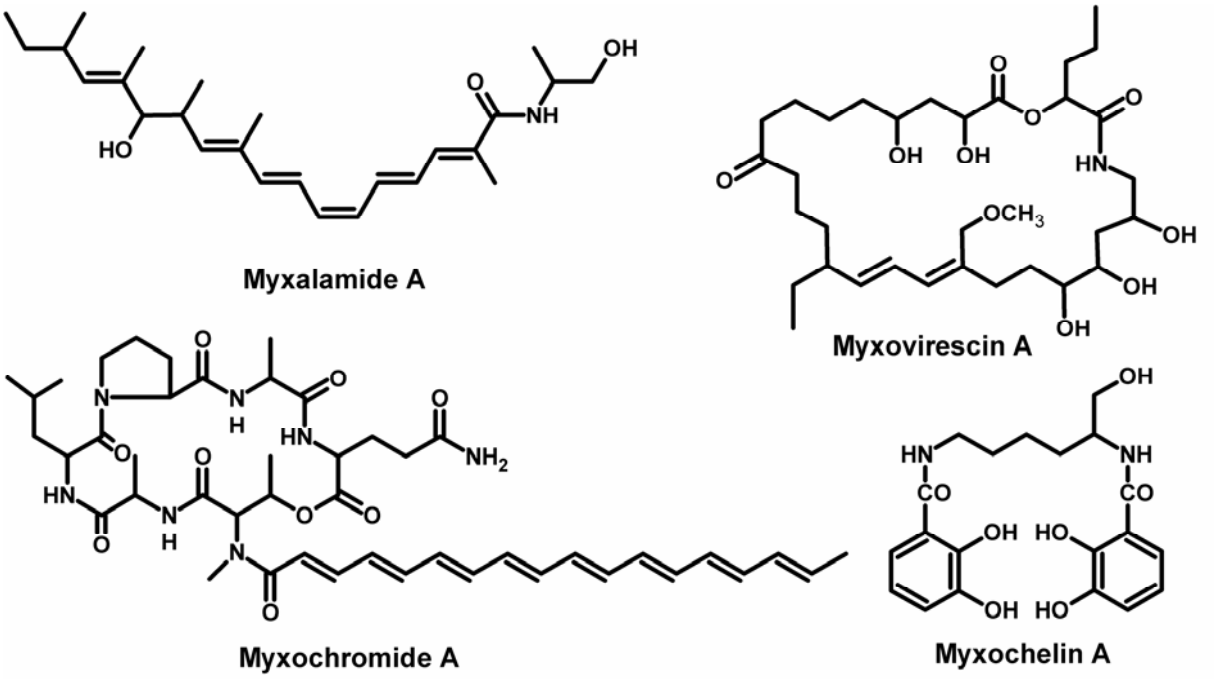


Figure 8. Natural products identified in *Myxococcus xanthus* extracts that were already described from other myxobacterial secondary metabolite producers

The structures and biological functions of further (novel) secondary metabolites from *M. xanthus* DK1622 were unknown at the starting point of this study. The isolation and structural characterization of natural products from *M. xanthus* DK1622 and, where possible, the determination of their physiological roles were hence a key issue of the presented thesis. The identification and optimization of production conditions for the respective metabolite represent a decisive point for a successful performance (10). Many biosynthetic gene clusters might be “switched off” under laboratory conditions (so called silent gene clusters), and it was one goal of this study to induce the production of these putatively novel compounds. In order to do so, several differing cultivation conditions, including the variation of media, temperature and

extraction procedures as well as induction of fruiting body formation and sporulation were applied, and the secondary metabolite profile was determinded by HPLC-MS.
Furthermore, the biosynthetic gene cluster responsible for the production of the pigments that give *M. xanthus* its characteristic yellow appearance was identified through transposon mutagenesis in the beginning of this work (performed by Dr. Helge B. Bode). Besides the purification and chemical characterization of the yellow pigments, the analysis of the biosynthetic machinery that drives their production as well as investigations into the biological function of these metabolites was a major goal of this work.

Chapter 1

The following article has been published in the
ChemBioChem journal, Vol.8-No.17, November 2007,
Pages 2139-44

Mutasynthesis-derived myxalamids and origin of the isobutyryl-CoA starter unit of myxalamid B

Helge B. Bode,*[a] Peter Meiser,[a] Thorsten Klefisch,[a] Niña Socorro d. J. Cortina,[a] Daniel Krug,[a] Anke Göhring,[a] Gertrud Schwär,[a] Taifo Mahmud,[b] Yasser A. Elnakady,[a] and Rolf Müller*[a]

Myxalamid biosynthesis

Mutasynthesis-derived myxalamids and origin of the isobutyryl-CoA starter unit of myxalamid B

Helge B. Bode,*[a] Peter Meiser,[a] Thorsten Klefisch,[a] Niña Socorro d. J. Cortina,[a] Daniel Krug,[a] Anke Göhring,[a] Gertrud Schwär,[a] Taifo Mahmud,[b] Yasser A. Elnakady,[a] and Rolf Müller*[a]

This article is available online at:

http://www3.interscience.wiley.com/journal/116833315/abstract?CRETRY=1&SRETRY=0

[a] *Dr. H.B. Bode, Apotheker P. Meiser, Apotheker T. Klefisch, M. Sc. N.S. d. J. Cortina, Dipl. Chem. D. Krug, Dipl. Chem. A. Göhring, G. Schwär, Dr. Y.A. Elnakady, Prof. Dr. R. Müller*
Institut für Pharmazeutische Biotechnologie
Universität des Saarlandes
Postfach 151150, 66041 Saarbrücken (Germany)
Fax: (+) 49-681-3025473
E-mail: h.bode@mx.uni-saarland.de, rom@mx.uni-saarland.de

[b] *Prof. Dr. T. Mahmud*
College of Pharmacy, Oregon State University
Corvallis, OR 97331-3507 (USA)

Supporting information for this article is available on the WWW under http://www.chembiochem.org/ or from the author.

Supporting Information for

Mutasynthesis-derived myxalamids and origin of the isobutyryl-CoA starter unit of myxalamid B

Helge B. Bode,*[a] Peter Meiser,[a] Thorsten Klefisch,[a] Niña Socorro d. J. Cortina,[a] Daniel Krug,[a] Anke Göhring,[a] Gertrud Schwär,[a] Taifo Mahmud,[b] Yasser A. Elnakady,[a] and Rolf Müller*[a]

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/contents/jc_2268/2007/f700401_s.pdf or from the author.

Chapter 2

The following article has been published in the
ChemBioChem journal, Vol.7-No.8, August 2006,
Pages 1206-1220

Myxovirescin A Biosynthesis is Directed by Hybrid Polyketide Synthases/Nonribosomal Peptide Synthetase, 3-Hydroxy-3-Methylglutaryl–CoA Synthases and trans-Acting Acyltransferases

M.S.Vesna Simunovic, Dr. Josef Zapp, Dr. Shwan Rachid, Dipl-Chem. Daniel Krug,
Apotheker Peter Meiser and Prof. Dr. Rolf Müller

Myxovirescin biosynthesis

Myxovirescin A Biosynthesis is Directed by Hybrid Polyketide Synthases/Nonribosomal Peptide Synthetase, 3-Hydroxy-3-Methylglutaryl–CoA Synthases and trans-Acting Acyltransferases

M.S.Vesna Simunovic, Dr. Josef Zapp, Dr. Shwan Rachid, Dipl-Chem. Daniel Krug, Apotheker Peter Meiser and Prof. Dr. Rolf Müller

This article is available online at:

http://www3.interscience.wiley.com/journal/112696128/abstract

Supporting Information

For

Myxovirescin A Biosynthesis is Directed by Hybrid Polyketide Synthases/Nonribosomal Peptide Synthetase, 3-Hydroxy-3-Methylglutaryl CoA Synthases and *trans*-Acting Acyltransferases

V. Simunovic, J. Zapp, S. Rachid, D. Krug, P. Meiser and Rolf Müller*

DOI: 10.1002/cbic.200600075

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/contents/jc_2268/2006/f600075_s.pdf or from the author.

Chapter 3

The following article has been published in the
Angew Chem Int Ed Engl journal, Vol.45-No.14, March 2006,
Pages 2296-301

Nonribosomal Peptide Biosynthesis: Point Mutations and Module Skipping Lead to Chemical Diversity

Silke C. Wenzel, Peter Meiser, Tina M. Binz, Taifo Mahmud and Rolf Müller*

Nonribosomal Peptide Biosynthesis: Point Mutations and Module Skipping Lead to Chemical Diversity**

Silke C. Wenzel, Peter Meiser, Tina M. Binz, Taifo Mahmud and Rolf Müller*

[*] Dipl.-Chem. S. C. Wenzel, Apotheker P. Meiser, Dipl.-Biol. T. M. Binz, Prof. Dr. R. Müller
Pharmaceutical Biotechnology, Saarland University
P.O. Box 151150, 66041 Saarbrücken (Germany)
Fax: (+49)681-302-5473
E-mail: rom@mx.uni-saarland.de
Prof. Dr. T. Mahmud
College of Pharmacy, Oregon State University
Corvallis, OR 97331-3507 (USA)

This article is available online at:

http://www3.interscience.wiley.com/journal/112467776/abstract

[**] The authors would like to thank B. Arbogast and L. Barofsky (the Mass Spectrometry Core of the Environmental Health Sciences Center at Oregon State University, NIH grant P30 ES00210) for the ESI/ICR and MALDI/TOF analysis and B. Hinkelmann and H. Schüler for performing a large scale fermentation. We are also grateful to Prof. Dr. T. M. Zabriskie, Dr. H. B. Bode and B. Lelarge for the critical review of this manuscript. This work was supported by grants from the BMB+F and the DFG. S.C.W. and R.M. would like to acknowledge a Kekulé fellowship of the Fonds der Chemischen Industrie and a DAAD fellowship.

Supporting Information for:

Nonribosomal Peptide Biosynthesis: Point Mutations and Module Skipping Lead to Chemical Diversity

Silke C. Wenzel, Peter Meiser, Tina M. Binz, Taifo Mahmud and Rolf Müller*

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/contents/jc_2002/2006/z503737_s.pdf or from the author.

Chapter 4

Identification and structure-elucidation of 3''-O-methylmyxochelin A (unpublished)

Summary

A novel, methylated derivative of myxochelin A was discovered in the course of an attempt to investigate the chemical principle underlying a strong cytotoxic activity found in *Myxococcus xanthus* extracts. For fractionation, an activity-guided approach was chosen. *M. xanthus* DK1050 was cultured in rich medium, extracted and fractionated applying several subsequent chromatographic separation steps, and fractions were tested for bioactivity. Since the main activity was finally observed in a fraction containing solely the known myxochelins A and B as well as an unknown compound, the separation-strategy was changed in order to purify the novel compound. Purification of a methylated myxochelin A and the testing for cytotoxicity in comparison to the known myxochelins did not unveil any difference in activity among the derivatives. Rather, the discovery of a methylated myxochelin A derivative suggests a novel mechanism of iron release.

Introduction

Siderophores are small molecules synthesized by bacteria, fungi and even some plants that exhibit a very high affinity towards Fe(III). In bacteria, they are usually build up from nonribosomal peptide synthetases and after their biosynthesis, they are secreted into the bacterial environment in order to sequester the essential trace element. From the exterior, the Fe(III)-siderophore complex is specifically recognized by outer membrane receptors and internalized, in Gram-negative bacteria usually mediated by the TonB-ExbB-ExbD protein complex (6). The intracellular iron release is a process less well understood. For enterobactin, a hydrolytic mechanism is described. In other systems, enzymes with ferric reductase activity are thought to be involved in iron release, since Fe(II)-siderophore complexes exhibit weaker affinities than the respective Fe(III) complexes (5). However, so far there is no experimental evidence for these hypotheses. In an activity-guided approach aiming at the isolation of a cytotoxic compound from *M. xanthus*, an O-methylated myxochelin derivative was isolated here. The discovery of the modified siderophore might provide useful information about the mechanism of iron release in myxobacteria.

Results:

Isolation and structure elucidation of 3''-O-methylmyxochelin A. Two liters of a *M. xanthus* culture was grown in CTT medium in presence of 1% of the adsorber resin Amberlite XAD-16, harvested and extracted with ethylacetate/methanol (1:4) and fractionated by gel permeation and isocratic RP-high performance liquid chromatography. The obtained fractions were tested for cytotoxicity applying the MTT test (12) on L929 mouse cell lines, and chemically analyzed using thin layer chromatography and HPLC-MS (-MS/MS). As the activity narrowed down to a fraction containing the known myxochelins A and B ($(M+H]^+$ m/z 404 and m/z 405) (2,9) and apparently only one further compound (m/z 419), the strategy was changed from the activity-guided to a classical analytical approach in order to purify the unknown compound by sequential RP-HPLC chromatography. The final yield of the purified compound was 2 mg. By ^{13}C NMR analysis (Table 1) the total number of carbons could be determined as 21, with 12 signals found between 114 and 150 ppm indicating the presence of several double bonds and two signals assignable to ester or amide bonds (δ_C169 and 170, respectively). Through ^1H NMR analysis six protons (δ_H 6.67-7.31) could be determined as part of an aromatic moiety, and heteronuclear single quantum correlation (HSQC) spectroscopy allowed their assignment to six carbons between 116 and 120 ppm. HSQC and heteronuclear multiple bond correlation (HMBC) as well as ^1H-^1H-correlated spectroscopy (^1H-^1H-COSY) experiments elucidating the chemical neighbourhood of these protons led to the identification of two separated dihydroxy-benzoyl moieties (COSY- and HMBC-correlations are shown in Figure 1). A singlet at 3.85 ppm indicated the presence of a methoxy-moiety. Weak ^1H-^1H-COSY correlation of the methyl protons of this methoxy-moiety to H-4'' as well as HMBC correlation to C-3'' indicates that OH-3'' of the respective dihydroxy-benzoyl moiety is methylated in the isolated metabolite. Each dihydroxy-benzoyl unit is attached to one amino-group (α- or ε-, respectively) of a reduced lysine as could be assigned through HMBC correlation of H-2 and H-6 of the lysinol moiety to C-7' and C-7'', respectively. The structure of the lysinol could be elucidated through ^1H-^1H-COSY, HSQC and HMBC experiments, leading to the final structure determination of 3''-O-methyl-myxochelin A (Figure 1 and Table 1).

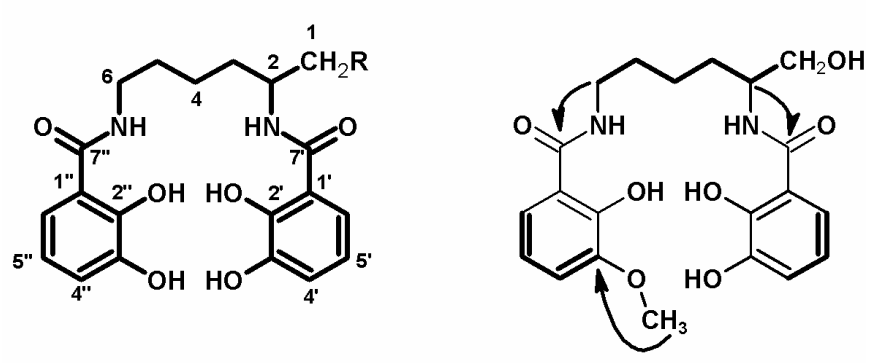


Figure 1. Numbering of myxochelins (A: R = OH, B: R = NH_2; left structure), and selected ^{1}H-^{1}H-COSY (bold lines) and HMBC (arrows) correlations of 3''-O-methylmyxochelin A (right structure)

Table 1. ^{13}C and ^{1}H spectral data of 3''-O-methylmyxochelin A in [D_6]DMSO at 500 MHz (^{1}H) and 125.7 MHz (^{13}C)

Number	^{13}C	^{1}H	*J*, Hz
1	64.8	3.61 dd	5.2/7.9
2	52.5	4.14 m	
3	31.4	1.63 m	
4	31.2	1.75 m	
5	29.9	1.68 m	
6	40.0	3.39 m	
1'	115.6		
2'	148.6		
3'	148.8		
4'	119.0	6.90 d	7.6
5'	119.2	6.67 t	7.9
6'	118.0	7.26 d	7.0
7'	170.0		
1''	114.5		
2''	149.1		
3''	148.4		
4''	116.0	7.05 d	8.2
5''	119.4	6.80 t	8.0
6''	120.5	7.31 d	8.2
7''	169.0		
3''-OMe	56.7	3.85 s	

s, singlet; d, doublet; m, multiplet; t, triplet; dd, doublet of doublet

Discussion:

Myxochelins are iron-chelating siderophores known from several myxobacteria (9,14) as well as non-myxobacterial species (11). As observed for many other siderophores, the iron-uptake is facilitated by the chelation of Fe(III) with –in this case two- 2,3-dihydroxy-benzoyl (catecholate) subunits. Enterobactin resembles a further siderophore from this class and its "iron-metabolism" including the mechanism of iron-release was investigated in detail (3). It is produced by several Gram-negative enteric bacteria and exhibits three such catecholate subunits which are connected with each other by a triserine-lactone backbone. Iron taken up from the environment as the Fe-enterobactin catecholate complex is primarily released intracellular by the esterase Fes (10). Yet, an alternative mechanism for iron release from enterobactin was proposed since synthetic analogues of enterobactin that were not susceptible to hydrolysis retained the ability to supply the cells with iron sufficiently. Here, iron release through reduction of Fe(III) from its salicylate complex enabled by protonation of the meta-hydroxy function of the catecholate structure was proposed (1).

At the onset of this work, nothing was known about the mechanism of iron-release from the myxochelins. A cleavage of one or both amide bonds would - in conformity with the iron-release from enterobactin - destroy the siderophore and would at least in part enable the reuse of the myxochelin building blocks. Besides this theoretical mechanism, the hereby reported discovery of an O-methylated myxochelin A derivative hints at an alternative mechanism. Driven by methylation, the Fe(III)-myxochelin complex is very likely shifted from the catecholate to the salicylate form, from which reduction of Fe(III) to Fe(II) and the subsequent iron release is significantly favoured. Through feeding experiments with L-methionine-*methyl*-d_3, it was demonstrated that the methylation is catalyzed within the cells (7). Furthermore, the presence of a methylated myxochelin B derivative is strongly proposed since a metabolite with the expected mass, fragmentation pattern as well as the expected mass shift in the described feeding experiment was observed. Yet, structure elucidation of this metabolite failed so far, mainly due to productivity reasons (7).

Since inactivation of seven preselected (O-)methyltransferase/SAM-dependent protein encoding genes from *M. xanthus* did not lead to a loss of the methylated derivative (7), the most promising methyltransferase (MXAN_5681; chosen according to its location in close proximity of genes encoding proteins with hypothetical

functions in iron metabolism) was chosen for heterologous expression in *E. coli* in order to pave the way for subsequent *in vitro* experiments (T. Klefisch, unpublished). Here, purified or chemically synthesized myxochelin will serve as the substrate that is supposed to be methylated in meta-position by the active methyltransferase. Apart from this necessary proof of principle, information about the regioselectivity of the methyltransferase can be gained from these experiments since at least in theory three additional phenolic groups might be methylated besides the observed 3''-OH function. In parallel to the expression experiment, myxochelin A as well as its methylated derivative is going to be synthesized in order to determine the coordination chemistry and reduction potential of both metabolites with respect to Fe(III) (in collaboration with M. Bartholomä, K. Hegetschweiler, Arbeitskreis für Komplexchemie, Saarland University). It is expected that the methylated myxochelin derivative forms a salicylate rather than the catecholate complex (as postulated for the unmethylated myxochelins) with ferric iron, and consequently, that the reductive release is highly favoured from the methylated form.

Taken all of these considerations together, an unprecedented mechanism of iron release is proposed based on the methylation-dependent distortion of the siderophore-iron complex: Ferric iron (Fe(III)) is sequestered from the environment by the siderophore myxochelin and taken up into the cell, and the iron-siderophore complex prevents the cell from direct toxic effects of Fe(III). The iron release from this complex might be triggered through methylation of the siderophore. As the most likely candidate protein for methylation, the methyltransferase MXAN_5681 is currently under investigation. This methyltransferase is part of an operon together with several other genes presumably involved in iron homeostasis. The operon is presumably under the transcriptional control of FUR (ferric uptake regulator protein), since a typical DNA-FUR binding box is located upstream of the first gene of the operon. FUR usually binds ferrous iron and subsequently binds to promoter regions as a homodimer, acting as a positive repressor. Without this repression – at low ferrous iron level - MXAN_5681 (or an alternative methyltransferase) might be expressed, leading to the (meta-OH)-methylation of the myxochelin-Fe(III) complex, the described shift in coordination, the reduction of Fe(III) to Fe(II) and thus eventually to the release of iron. This refined system for a crucial process of the bacterial cell would be another example among many decribing how *M. xanthus* is capable to perform a very complex and adjustable life cycle.

Materials and methods:

Isolation of the compound. *M. xanthus* DK1050 (13), a stable yellow derivative of strain *M. xanthus* FB (4) was cultivated at 30°C in two liters of CTT medium (8) in presence of 1% Amberlite XAD-16. Cells and XAD were harvested after three days of growth and extracted repeatedly with overall 500 ml of ethylacetate/methanol (1:4). Subsequently, the crude extract was first separated by gel permeation chromatography (Sephadex LH20 in methanol) into six fractions. Fractions exhibiting strong activity in the MTT-test were pooled and further separated applying several sequential isocratic RP-HPLC purification steps, yielding 2 mg of purified 3''-O-methylmyxochelin A (Jasco HPLC (PU-2087, UV-2075), Nucleosil 250/21 RP18, Macherey-Nagel, MeOH/H_2O 70:30, detection at 254 nm).

Structure elucidation. The structure of 3''-O-methylmyxochelin A was elucidated using 1D (^1H, ^{13}C) and 2D NMR (^1H,^1H-Cosy, HSQC, HMBC) analysis on Bruker DRX 500 or Bruker Avance 500 spectrometers using [D_6]DMSO. HPLC-MS and -MS/MS-experiments were performed using an acetonitrile:water gradient (5-95 % acetonitrile with 0.1% formic acid, HPLC: Agilent 1100 series, MS: Bruker HCT Plus ion trap).

Reference List

1. **Abergel, R. J., J. A. Warner, D. K. Shuh, and K. N. Raymond**. 2006. Enterobactin protonation and iron release: Structural characterization of the salicylate coordination shift in ferric enterobactin. J. Am. Chem. Soc. **128**:8920-8931.

2. **Ambrosi, H. D., V. Hartmann, D. Pistorius, R. Reissbrodt, and W. Trowitzsch-Kienast**. 1998. Myxochelins B, C, D, E and F: A new structural principle for powerful siderophores imitating nature. Eur. J. Org. Chem. 541-551.

3. **Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones**. 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27**:215-237.

4. **Dworkin, M.** 1962. Nutritional requirements for vegetative growth of *Myxococcus xanthus*. J. Bacteriol. **84**:250-257.

5. **Fontecave, M., J. Coves, and J. L. Pierre**. 1994. Ferric reductases or flavin reductases. Biometals **7**:3-8.

6. **Higgs, P. I., P. S. Myers, and K. Postle**. 1998. Interactions in the TonB-dependent energy transduction complex: ExbB and ExbD form homomultimers. J. Bacteriol. **180**:6031-6038.

7. **Klefisch, T.** 2007. Diploma thesis: Untersuchungen zur Bio- und Mutasynthese von Myxalamiden und Myxochelinen aus *Myxococcus xanthus*.Saarland University.

8. **Kroos, L. and D. Kaiser**. 1984. Construction of Tn5lac, a transposon that fuses lacZ expression to exogenous promoters, and its introduction into Myxococcus xanthus. P. Natl. Acad. Sci. USA **81**:5816-5820.

9. **Kunze, B., N. Bedorf, W. Kohl, G. Höfle, and H. Reichenbach**. 1989. Myxochelin A, a new iron-chelating compound from *Angiococcus disciformis* (Myxobacterales). Production, isolation, physico-chemical and biological properties. J. Antibiot. **42**:14-17.

10. **Lin, H., M. A. Fischbach, D. R. Liu, and C. T. Walsh**. 2005. In vitro characterization of salmochelin and enterobactin trilactone hydrolases IroD, IroE, and Fes. J. Am. Chem. Soc. **127**:11075-11084.

11. **Miyanaga, S., T. Obata, H. Onaka, T. Fujita, N. Saito, H. Sakurai, I. Saiki, T. Furumai, and Y. Igarashi**. 2006. Absolute configuration and antitumor activity of myxochelin A produced by *Nonomuraea pusilla* TP-A0861. J. Antibiot. **59**:698-703.

12. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods **65**:55-63.

13. **Ruiz-Vazquez, R. and F. J. Murillo**. 1984. Abnormal motility and fruiting behavior of Myxococcus xanthus bacteriophage-resistant strains induced by a clear-plaque mutant of bacteriophage Mx8. J. Bacteriol. **160**:818-821.

14. **Silakowski, B., B. Kunze, G. Nordsiek, H. Blöcker, G. Höfle, and R. Müller**. 2000. The myxochelin iron transport regulon of the myxobacterium *Stigmatella aurantiaca* Sg a15. Eur. J. Biochem. **267**:6476-6485.

Chapter 5.1

The following article has been published in the
PNAS journal, Vol.103-No.50, December 2006,
Pages 19128-33

DKxanthenes: Unique secondary metabolites from the myxobacterium **Myxococcus xanthus** *required for developmental sporulation*

Peter Meiser*, Helge B. Bode* and Rolf Müller*†

Biological sciences: Microbiology

DKxanthenes: Unique secondary metabolites from the myxobacterium *Myxococcus xanthus* required for developmental sporulation

Peter Meiser*, Helge B. Bode* and Rolf Müller*†

*Pharmaceutical Biotechnology, Saarland University, PO Box 151150, 66041 Saarbrücken, Germany; Phone: ++49 681 3025474, Fax: ++49 681 3025473, email: rom@mx.uni-saarland.de

This article is available online at:

http://www.pnas.org/content/103/50/19128

Supporting information for:

DKxanthenes: Unique secondary metabolites from the myxobacterium *Myxococcus xanthus* required for developmental sporulation

Peter Meiser*, Helge B. Bode* and Rolf Müller*†

Supporting information for this article is available on the WWW under http://www.pnas.org/content/103/50/19128/suppl/DC1#F6 or from the author.

Chapter 5.2

The following article has been published in the
Chemistry and Biology journal, Vol 15, August 2008, Pages 771-781

DKxanthene Biosynthesis – Understanding the Basis for Diversity-Oriented Synthesis in Myxobacterial Secondary Metabolism

Peter Meiser,[1] Kira J. Weissman,[1] Helge B. Bode, [1] Daniel Krug, [1] Jeroen S. Dickschat,[1,2] Axel Sandmann,[1] and Rolf Müller[1,*]

DKxanthene Biosynthesis – Understanding the Basis for Diversity-Oriented Synthesis in Myxobacterial Secondary Metabolism

Peter Meiser,[1] Kira J. Weissman,[1] Helge B. Bode, [1] Daniel Krug, [1] Jeroen S. Dickschat,[1,2] Axel Sandmann,[1] and Rolf Müller[1,*]

[1]Institut für Pharmazeutische Biotechnologie, Saarland University, P.O. Box 151150, 66041 Saarbrücken, Germany

[2]Present address: Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, United Kingdom

[*]Correspondence: rom@mx.uni-saarland.de

This article is available online at:

http://www.chembiol.com/content/article/abstract?uid=PIIS1074552108002391

SUPPLEMENTAL DATA

DKxanthene Biosynthesis – Understanding the Basis for Diversity-Oriented Synthesis in Myxobacterial Secondary Metabolism

Peter Meiser,[1] Kira J. Weissman,[1] Jeroen S. Dickschat,[1,2] Axel Sandmann,[1] Helge B. Bode,[1] Rolf Müller[1,*]

[1]Institut für Pharmazeutische Biotechnologie, Saarland University, P.O. Box 151150, 66041 Saarbrücken, Germany

[2]Present address: Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, United Kingdom

[*]Correspondence: rom@mx.uni-saarland.de

Supporting information for this article is available on the WWW under http://www.chembiol.com/cgi/content/full/15/8/771/DC1/ or from the author.

Chapter 6

The following article has been published in the
ChemBioChem journal, Vol.9-No.10, July 2008,
Pages 1549-1553

Two functionally redundant Sfp-type 4'-phosphopantetheinyl transferases differentially activate biosynthetic pathways in Myxococcus xanthus

Peter Meiser[a] and Rolf Müller[a]*

Communications:

Two functionally redundant Sfp-type 4'-phosphopantetheinyl transferases differentially activate biosynthetic pathways in *Myxococcus xanthus*

Peter Meiser[a] and Rolf Müller[a]*

This article is available online at:

http://www3.interscience.wiley.com/journal/119427677/abstract

[a] P. Meiser, Prof. Dr. R. Müller*
Institut für Pharmazeutische Biotechnologie, Universität des Saarlandes
P.O. Box 151150, 66041 Saarbrücken (Germany)
Phone: (+49) 681 3025474. Fax: (+49) 681 3025473
E-mail: rom@mx.uni-saarland.de

Supporting Material for:

Two functionally redundant Sfp-type 4'-phosphopantetheinyl transferases differentially activate biosynthetic pathways in *Myxococcus xanthus*

Peter Meiser[1] and Rolf Müller[1*]

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/contents/jc_2268/2008/z800077_s.pdf or from the author.

Statement about the author's effort in Chapters 1-6

Chapter 1: The author performed in part the chemical analysis of the described feeding experiments. Isolation of [2-^{13}C]-acetate enriched myxalamid B from several *M. xanthus* and *S. aurantiaca* strains as well as isolation of the two novel mutasynthesis derived myxalamid derivatives was performed by the author or in close collaboration with the author. The author participated in the subsequent structure elucidation by NMR.

Chapter 2: Isolation of myxovirescins was performed in collaboration with the author.

Chapter 3: Myxochromid A_3 was isolated and the structure was elucidated by the author.The structures of myxochromids A_2 and A_4 could be clarified by feeding experiments and subsequent LC-MS (MS/MS) analysis. The absolute configuration of the amino acids from mxyochromid A_3 was determined by the author.

Chapter 4: The described work was performed by the author if not indicated differently in the text.

Chapter 5.1: The major part of the work was performed by the author (80%). Transposon mutagenesis was performed by Helge B. Bode. Structure elucidation was performed by Helge B. Bode and the author.

Chapter 5.2: The major part of the work was performed by the author (80%). Detailed docking domain analysis was performed by Kira J. Weissman. Inactivation of *dkxG* in *S. aurantiaca* and synthesis of Pyrrolyl-2-carboxyl-SNAC were performed by Jeroen S. Dickschat.

Chapter 6: The work was performed by the author in large part. Anke Göhring supported the HPLC-MS analysis and Michael W. Ring performed fatty acid analyses.

Discussion

Discussion:

During the ongoing process and finally with the finishing of the genome sequencing project in 2006 (44), it became increasingly apparent that *Myxococcus xanthus* might be not only a suitable model organism for myxobacterial development, but also a proficient producer of secondary metabolites (12,13). At least 18 biosynthetic gene clusters could be identified, among them six nonribosomal peptide synthetases and 12 hybrid polyketide synthase/ nonribosomal peptide synthetase systems. Four of these biosynthetic gene clusters were readily assigned to secondary metabolites already known from other myxobacterial strains, namely the myxalamids, the myxovirescins, the myxochelins and the myxochromides. A fifth family of natural products was identified in the course of this work (**Figure 9**). Disruption of this gene cluster by transposon mutagenesis as well as targeted plasmid integration led to a constant tan phenotype. Purification of the corresponding compounds and subsequent structure elucidation clarified the chemical structure of the yellow pigments which were named Dkxanthenes in honour of Dale Kaiser (91).

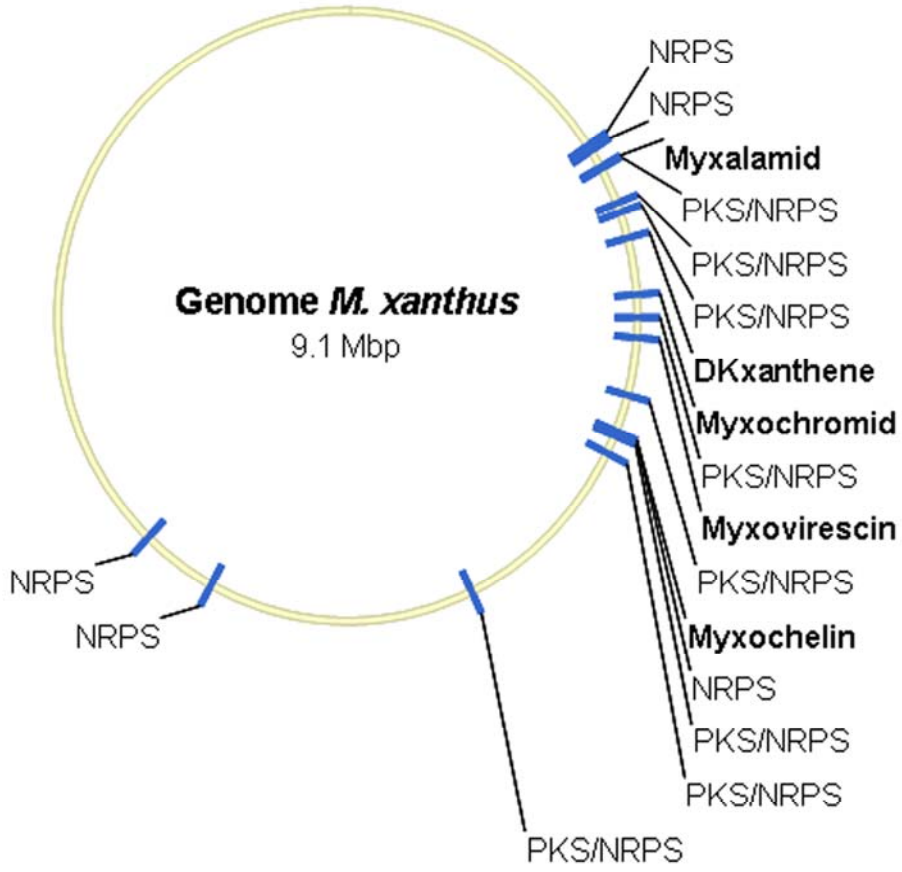


Figure 9. Genomic map of *M. xanthus* indicating the number and type as well as the location of secondary metabolite gene clusters on the chromosome. Gene clusters that could be correlated to the produced secondary metabolite are indicated.

1. Myxalamid

Myxalamids resemble a class of hybrid PKS/NRPS natural products that were first isolated from *Myxococcus xanthus* Mx x12 and which exhibit antibiotic activity against yeast, Gram-positive bacteria and eukaryotic cell lines (11,42,61). The biosynthetic gene cluster was first characterized from *Stigmatella aurantiaca* Sg a15 as one of very few combined PKS/NRPS systems reported at the time (127). Furthermore, a novel type of chain release through reduction of the terminal PCP-bound alanine moiety to 2-amino-propanol was described (127). Sequence comparison revealed the presence of a very similar gene cluster in *M. xanthus* DK1622 as well (on average over 80 % sequence identity on the DNA-level), with slight differences when compared to the biosynthetic gene cluster of *S. aurantiaca*. For instance, the last polyketide synthase elongation module is split into two separated proteins in *S. aurantiaca* but is encoded on one gene in *M. xanthus* (11). From the biosynthetic point of view, the most apparent difference can be observed in the starter unit utilization: *S. aurantiaca* Sg a15 produces myxalamid B as the major metabolite but also myxalamids C and D in easily detectable amounts, while myxalamid A is only produced in trace amounts. *M. xanthus* DK1622 in contrast produces myxalamid A by far as the major myxalamid metabolite besides smaller amounts of myxalamid B and C (**Figure 10**, A).

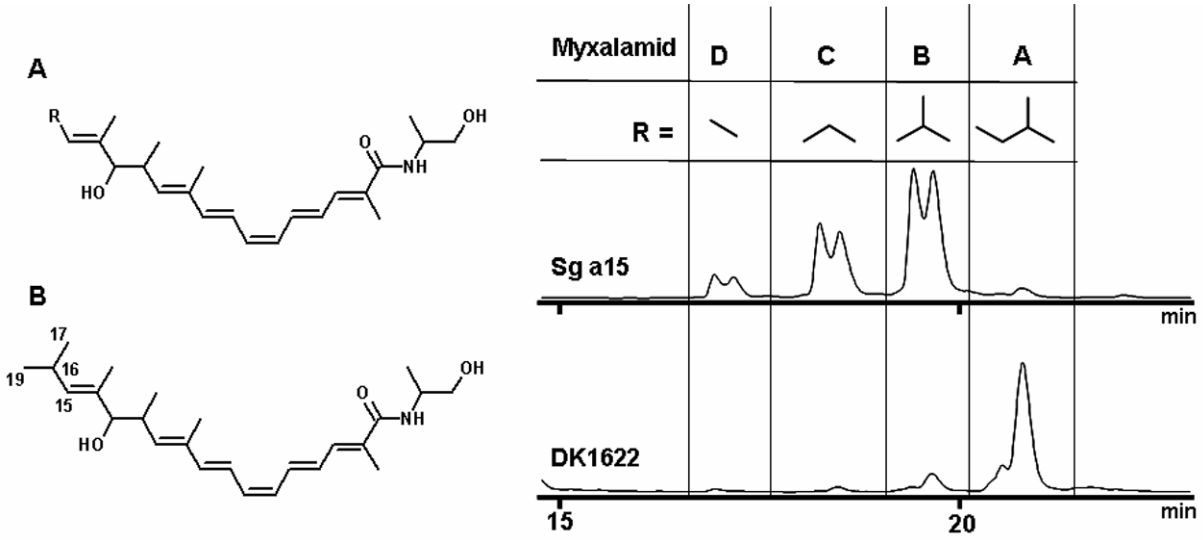


Figure 10. (A) Structural myxalamid derivatives and HPLC-UV chromatograms of *S. aurantiaca* and *M. xanthus*; (B) Numbering of the myxalamid B carbon atoms of the starter unit.

This might be explained by a more promiscuous loading module AT domain of *M. xanthus* for larger starter units such as 2-methyl-butyryl-CoA (which is used in myxalamid A biosynthesis), or by an improved precursor supply of the respective starter unit to the myxalamid biosynthetic pathway of the respective strains. Analysis of the *S. aurantiaca* and *M. xanthus* AT loading domains reveal a very high homology but also a noteworthy difference in the specificity conferring residues (**Figure 11**). Sequence alignment analyses reveal that residues 198-201 typically exhibit an HAFH motif for malonate- and a YASH motif for methylmalonate extender unit selection (26,159). According to the selection of starter units that differ from the most widely used elongation units methylmalonate- and malonate-CoA, the loading domains of both myxalamid biosynthetic pathways differ in these motifs. Hence, they also show a different motif when compared to each other, with a very unusual proline residue at position 199 of the *M. xanthus* loading AT domain (**Figure 11**).

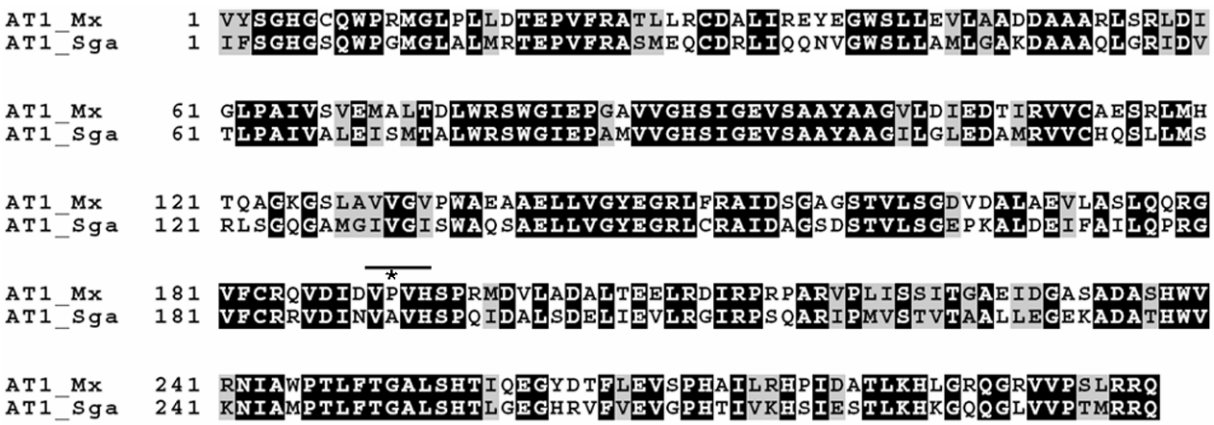


Figure 11. Sequence alignment (black shading: identical amino acid residues; grey shading: similar residues) of loading module AT domains of *Myxococcus xanthus* (Mx) and *Stigmatella aurantiaca* Sg a15 (Sga). Substrate specificity conferring residues 198-201 are indicated by a black bar and the unusual proline residue is displayed by an asterisk.

Remarkably, this uncommon residue is also found in the AT domain of the loading module in avermectin biosynthesis (55) that as well selects 2-methyl-butyryl-CoA as the starter unit. Thus, residue 199 might be a promising candidate for point mutagenesis approaches in order to shift the substrate specificity of the AT loading domain of *S. aurantiaca* towards an increased incorporation of 2-methyl-butyryl-CoA. However, P199 is also found in module 4 of both biosynthetic pathways and such might solely resemble another unusual feature of myxobacterial biochemistry that differs from published model systems (**Table 1**) (155).

Table 1. AT domain specificity motifs of proteins/ modules [B-F/ (L, 1-7)] for myxalamid biosynthetic pathways of *Stigmatella aurantiaca* (Mxa_S) and *Myxococcus xanthus* (Mxa_M)

$MxaF_S$ (L)	VAVH	$MxaF_M$ (L)	VPVH
MxaFs (1)	VASH	$MxaF_M$ (1)	VASH
MxaEs (2)	VASH	$MxaE_M$ (2)	VASH
MxaDs (3)	VASH	$MxaD_M$(3)	VASH
MxaCs (4)	VPSH	$MxaC_M$ (4)	VPSH
MxaCs (5)	YAFH	$MxaC_M$ (5)	YAFH
MxaCs (6)	HAFH	$MxaC_M$ (6)	HAFH
MxaBs (7)	VASH	$MxaB_M$ (7)	VASH

Despite differences in substrate specificity and quantities of substrate incorporation, the enzymatic machineries of both biosynthetic pathways apparently tolerate a broad range of short chain carboxylic acid CoA esters as starter units of the respective loading module. For this reason, a mutasynthetic approach (150) was applied in order to test for the acceptance of various carboxylic acid starter units that are naturally not available and thus not build into the myxalamid scaffold. In order to increase the production yield and to simplify the purification of potentially novel myxalamid derivatives, these starter units were fed to *M. xanthus* and *S. aurantiaca* mutants that had a defect in the *bkd* locus (88,89). Such mutants lack the activity of the branched-chain keto acid dehydrogenase that is required for the degradation of the branched-chain amino acids (iso-)leucine and valine to the respective branched-chain carboxylic acids (92). Among them, 2-methylbutyryl-CoA and isobutyryl-CoA are used as natural precursors for myxalamid A and B biosynthesis, respectively, and a decreased quantitiy of these starter units would consequently lead to the depletion of the particular metabolites. This approach was readily performed in the generation of novel avermectin derivatives (29,55).

Unexpectedly, both *M. xanthus* and *S. aurantiaca bkd* mutants retained the ability to produce myxalamid B (with isobutyryl-CoA as the starter unit), although at a reduced production level when compared to the wild type. In *M. xanthus bkd* mutants, a second form of Bkd activity is responsible for the biosynthesis of isobutyryl-CoA derived from valine as could be shown by feeding experiments with [D_8]-valine. *S. aurantiaca bkd* mutants instead use this pathway to a lesser extend when compared to the wild type but rather employ an alternative biosynthetic pathway branching from the mevalonic acid isoprenoid biosynthesis (11,88,89). This pathway has already been described for the biosynthesis of iso fatty acids in *S. aurantiaca bkd* mutants

(89), and in myxalamid biosynthesis the isobutyryl-CoA starter unit is supplied by α- and β-oxidation of iso-odd fatty acids as was shown by feeding experiments.

The incorporation pattern of [2-^{13}C]-acetate was determined by NMR analysis after feeding of the precursor and purification of myxalamid B from the respective strains. Only the *S. aurantiaca bkd* mutant shows a significant labelling of carbons C-15, C-17 and C-19 (for numbering, see **Figure 10**, B), as it is expected for HMG-CoA derived isobutyryl-starter units (**Figure 12**, myxalamid B labelling pattern from *M. xanthus* strains correlates with the one obtained for *S. aurantiaca* wild type).

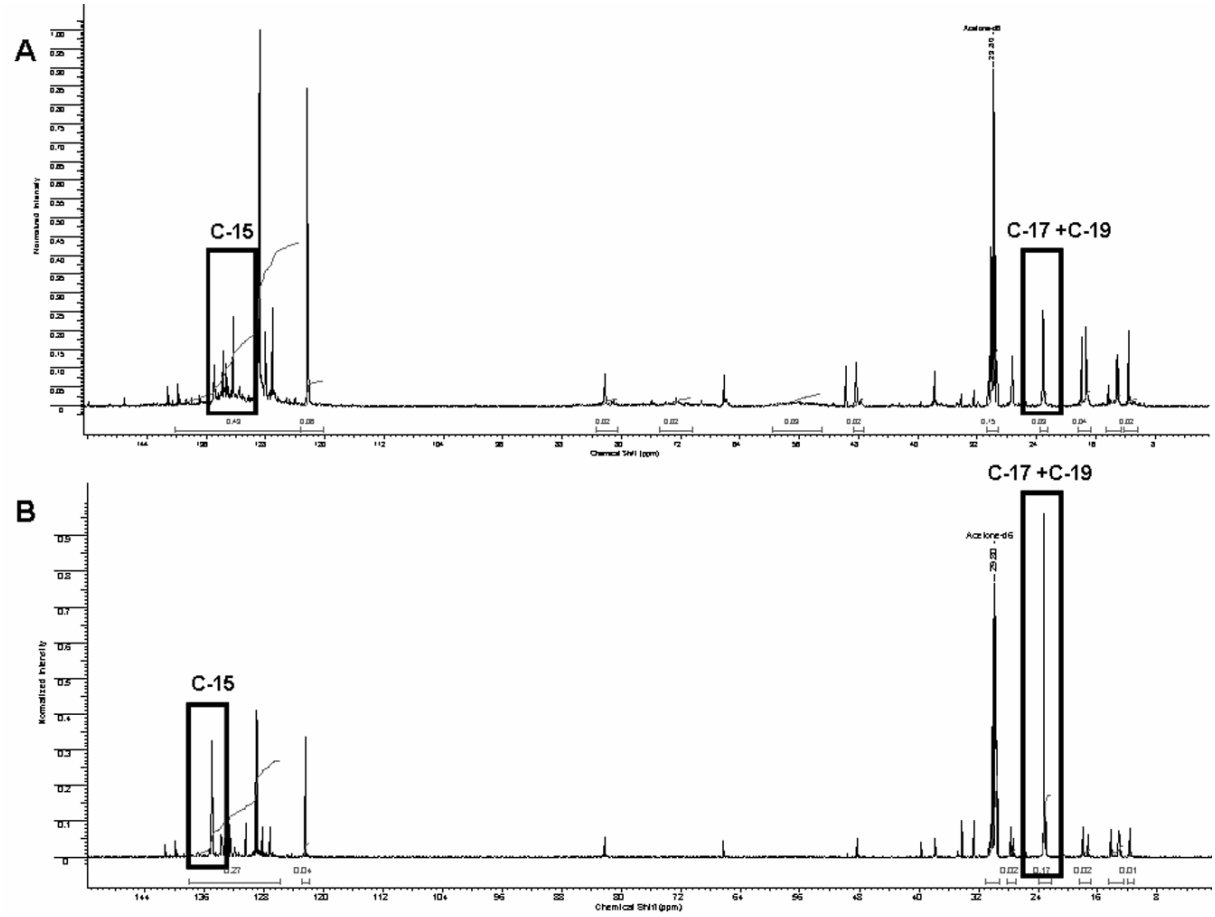


Figure 12. ^{13}C NMR analysis of myxalamid B from (A) *S. aurantiaca* wild type and (B) *S. aurantiaca bkd* mutant after feeding of [2-^{13}C]-acetate. Increased signals for C-15, C-17 and C-19 in the *bkd* mutant are indicated.

For mutasynthesis, altogether 19 carboxylic acids that were naturally not used as starter units for myxalamid biosynthesis were fed to cultures of *M. xanthus* and *S. aurantiaca bkd* mutants. Indeed, nine novel myxalamid derivatives (which were named MS-1 to MS-9) were produced and accounted for up to 60% of the total myxalamids produced from the strain (11). As a proof of principle, two of these novel non-natural secondary metabolites (carrying a cyclopropyl- or cyclopentyl-starter unit, respectively) were chosen for isolation from the *M. xanthus bkd* strain and

subsequently their structures were confirmed by one- and two-dimensional NMR analysis (71).

Meanwhile *M. xanthus* produces all novel derivatives MS-1 to MS-9 after feeding of the respective precursor, *S. aurantiaca* produces only compounds MS-6 to MS-8. One explanation might be a higher flexibility of the intrinsic acyl-CoA ligase of *M. xanthus* when compared to the CoA ligase of *S. aurantiaca*. Alternatively, or in addition, this finding indicates again that the *M. xanthus* loading AT domain might be more flexible especially towards larger starter units, a hypothesis which is supported by the higher yield of myxalamid A relative to all other myxalamids produced in this strain. Myxalamids thus seem to be a feasible system for further investigations aiming at alterations of starter unit selection, be it by point mutagenesis, domain or whole module swap experiments.

2. Myxovirescin

Myxovirescins are a family of compounds with antibiotic activity, primarily against Gram-negative bacteria. They were first isolated from *Myxococcus virescens* Mx v48 and have to date been found exclusively in the genus *Myxococcus* (40,141,142). Simunovic et al. could show that at least two members from this family of compounds were produced from *M. xanthus* DK1622 as well, myxovirescin A and C (**Figure 13**) (131).

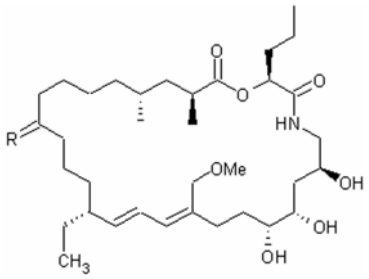


Figure 13. Myxovirescin A: R = O, myxovirescin C: R = H, H

Myxovirescin biosynthesis gives another interesting example for myxobacterial biosynthetic machineries that differ from textbook logic. Among these is the discovery of an AT-less (23) multimodular PKS system that is fed through the trans-acting AT domain TaV as well as the incorporation of rarely used β-alkyl moieties into the myxovirescin scaffold by an enzyme complex employing an HMG-CoA-like synthase. Detailed information about myxovirescin biosynthesis can be found in (128-131).

3. Myxochromid

Myxochromids (**Figure 14**) are a family of compounds that were originally isolated from *Myxococcus virescens* Mx v48 (139), and no biological function could be assigned to these metabolites so far. Besides myxochromids from the subfamily A which are produced from several *Myxococcus* species, myxochromids S that exhibit small but conspicuous structural differences to the A family were recently isolated from *S. aurantiaca* DW4/3-1 (**Figure 14**, A) (152). This paved the way for a detailed analysis of commonalities and in particular of the differences of both biosynthetic pathways (153).

In the scope of this work, myxochromid A_3 was isolated from *M. xanthus* DK1050 and its structure was elucidated by one- and two-dimensional NMR analysis. The original published structure (139) of these lipohexapeptides could be revised such that the α-carboxyl group of a glutamine rather than the γ-carboxyl group of a glutamate residue forms the ester linkage with the hydroxy group of the N-methylated threonine moiety (**Figure 14**, B and C). The structures of myxochromids A_2 and A_4 could be assigned by HPLC-MS and MS/MS experiments in combination with feeding experiments employing [$^{13}C_4$,$^{15}N_1$]-L-threonine that lead to a specifically labelled threonine-polyketide side chain MS/MS fragment (153).

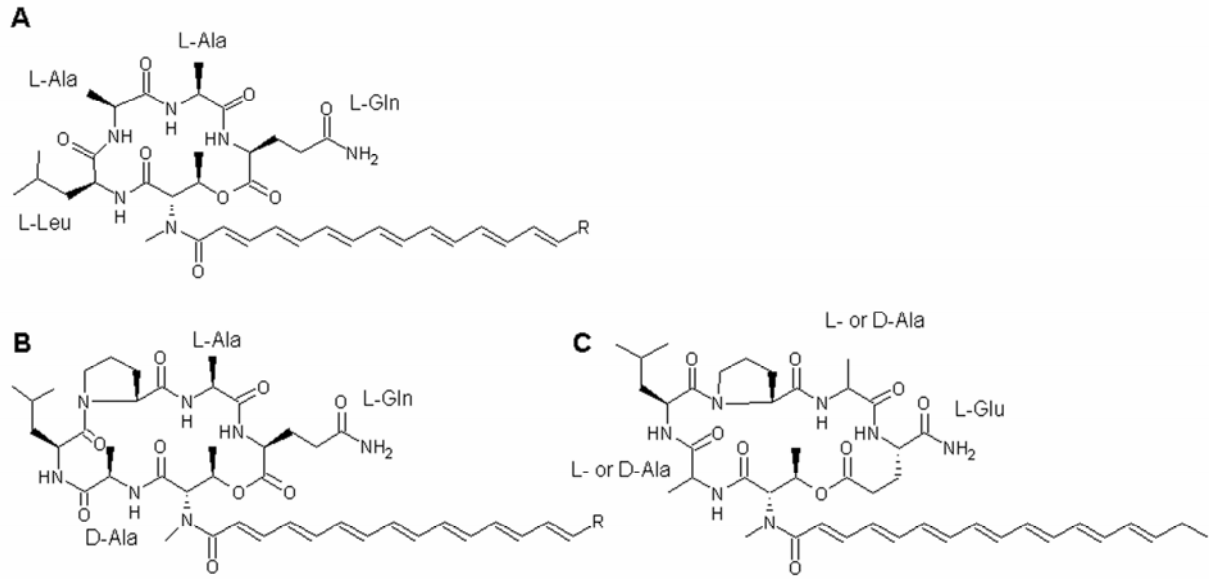


Figure 14. (A) Myxochromid S; S_1: R = Me, S_2: R = Et, S_3: R = CH=CH-Me
(B) Myxochromid A; A_2: R = Et, A_3: R = CH=CH-Me, A_4: R = CH=CH-Et
(C) Structure of the originally published myxochromid A

The myxochromid A and S megasynthetases are composed of seven biosynthetic modules. The first module resembles the only PKS present in both assembly lines, and heterologous expression experiments unambiguously demonstrated that this PKS acts iteratively with the intrinsic capacity to produce polyketide chains of varying lengths (151,152). Surprisingly, the length of the primarily processed polyketide chains in *M. xanthus* and *S. aurantiaca* differs as myxochromids A carry a 17-19 carbon atom side chain wherease myxochromids S harbour a 16-18 fatty acid extension (**Figure 14**, A and B), implying a differing intrinsic control of the number of iterations applied in both systems and/ or a differing preference of both biosynthetic pathways in starter unit selection (acetate-CoA vs. propionate-CoA).

Determination of the absolute configurations of the amino acids incorporated into myxochromid A revealed that all amino acids except alanine exhibit the L configuration. For alanine, both L and D configured amino acids were assigned in equal amounts which correlates well with the results for myxochromid A from *Myxococcus virescens* Mx v48 (139). Analysis of the biosynthetic gene cluster makes the structure shown in **Figure 14** (B) the most likely candidate since module 2 (incorporating alanine in myxochromid A biosynthesis) comprises an epimerization domain that catalyzes the conversion from L- to D-configured amino acids (84,153). In contrast, all amino acids of myxochromid S were assigned as L-configured. Furthermore, the peptide core of myxochromids S consists only of five amino acids instead of six as observed for myxochromids A, with the proline moiety missing and the order of alanine and leucine switched in myxochromid S relative to myxochromid A.

Wenzel et al. demonstrated that point mutations of the respective A domains led to the shift towards selection of differing amino acids rather than by genetic exchange of the particular A domain or the complete modules. Intriguingly, the C domains of the respective modules appear to be flexible to a certain degree and fail to perform their gatekeeper role in accepting only one specific incoming intermediate. On the other hand, further (point) mutations might have led to changes in C domain discrimination abilities of the respective module allowing the assembly using an alternativel amino acid residue.

More strikingly, myxochromid S biosynthesis constitutes the first example for the skipping of a complete NRPS module as could be shown by *in vitro* experiments. Expression of the carrier protein domains from modules 4 of the myxochromid A and

S biosynthetic pathways, respectively, and coexpression of the 4'-phosphopantetheinyl transferase MtaA activated the myxochromid A carrier protein but not the myxochromid S carrier protein. The flexibility in substrate activation and recognition as well as the obvious tolerance towards point mutations that alter domain specificities or even abolish their function makes the myxochromid biosynthetic pathway a promising system for further investigations aiming at site-directed mutagenesis and domain or module swaps of both biosynthetic pathways (119).

4. Myxochelin

Myxochelins (**Figure 15**) are siderophores of the catecholate-type that were first characterized from the myxobacterium *Angiococcus disciformis* An d30 (77). It did not take a long time until they became apparent to be generally used small molecules for iron-uptake among the myxobacteria (besides the hydroxamate-type siderophore nannochelin from *Nannocystis exedens* Na e483 (79)). Recently, myxochelin A was isolated from the actinomycete *Nonomuraea pusilla* TP-A0861 (94) indicating that myxochelins might be widely used as siderophores in the bacterial world. The gene cluster for myxochelin biosynthesis was described from *Stigmatella aurantiaca* Sg a15 (126). Mutants defective in myxochelin biosynthesis had to be supplemented with Fe(III) in order to grow, emphasizing the essential role of the myxochelins in iron-homeostasis. Furthermore, inactivation of the myxochelin biosynthetic gene cluster in *M. xanthus* was not possible when standard inactivation conditions were applied (Socorro D.J.Cortina, N., not published). Here, supplementation with Fe(III) will most likely support a more successful approach. Myxochelin biosynthesis employs an unusual two-fold usage of a condensation domain that attaches two 2,3-dihydroxy benzoic acid moieties to the α- and the ε-amino group of L-lysine, respectively (37). The readily processed natural product is finally released from the thio-template by the rarely found reductive release mechanism as was subsequently described for myxalamid biosynthesis as well. Myxochelin B is produced from myxochelin A most presumably via an aldehyde intermediate (126). Strikingly, myxochelin biosynthesis could be reconstituted *in vitro* by expression of the respective biosynthetic enzymes in presence of the necessary precursors and co-factors (37). Besides a weak antibacterial activity against several Gram-positive bacteria described in the original report of Kunze et. al (77), Miyanaga et al. discovered a weak cytotoxic activity and, more promising, a potent *in vitro* inhibition of tumor cell invasion at concentrations that did not show any cytotoxic acitivity (94).
The hereby described discovery of a novel 3''-O-methylated myxochelin A derivative from *M. xanthus* within an acitivity-guided approach aiming at the isolation of the cytotoxic principle of *M. xanthus* cell/ XAD-extracts might possibly have been guided by this novel reported cytotoxicity of the myxochelins. Yet, it remains to be questioned whether myxochelins resemble the actual cytotoxic principle of the extracts, considering the stronger cytotoxic activity of myxalamides (11) as well as

further putative compounds that might be active at concentrations in which they are not even detectable with standard analytical chemistry methods.

The drastic effects of the inactivation of the myxochelin biosynthetic pathway in *S. aurantiaca* and *M. xanthus* (which was not possible, see above) rather suggest that these metabolites truly are the iron-chelating natural products of these strains. Proteins known to be responsible for the uptake of Fe(III)-siderophore complexes such as TonB-dependent receptors (50) are encoded in the genome of *M. xanthus* (data not shown) and make it likely that siderophore secretion and reuptake takes place in a similar manner as reported for well-investigated siderophore systems like enterobactin in *E. coli*. Concerning iron-release from the Fe(III)-siderophore complex, nothing is known for myxobacteria so far. The actively driven methylation (71) of one meta-hydroxy group of the myxochelins most presumably destroys the catecholate Fe(III)-siderophore complex, and, if at all, only the weaker salicylate complex can be formed. For the enterobactin system, such an (artificially) induced shift through protonation towards the salicylate coordination (1) was shown to make the reductive release of Fe(II) from the complex much more favourable. Methylation in comparison to protonation of the meta-hydroxy group leads to a non-pH dependent shift that is not reversible and which appears to be better to control by induction of a methyltransferase than by the reversible transfer of a proton. Thus, the discovery of 3''-O-methylmyxochelin A might have opened the door for further insights into siderophore-mediated iron homeostasis in (myxo-)bacteria. Enzymes involved in iron-uptake and –release that are only found in bacteria certainly are interesting targets for future antibiotic research.

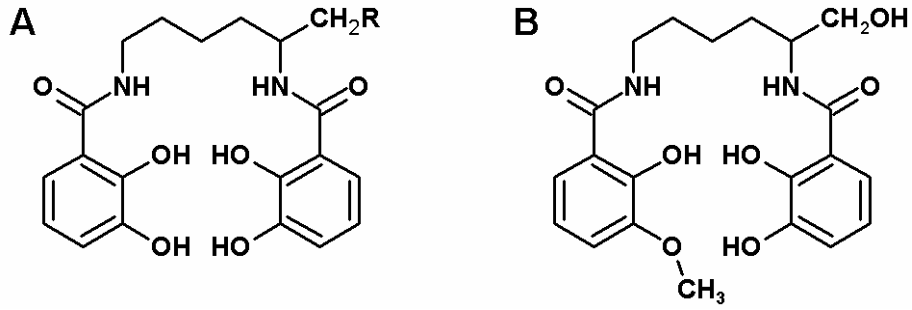
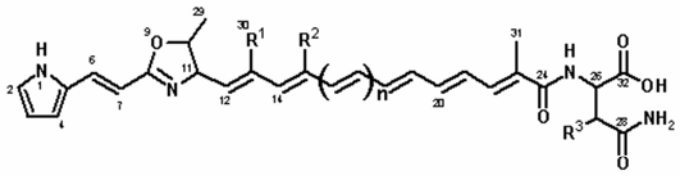


Figure 15. (A) Structure of myxochelin A and B (A: R = OH, B: R = NH_2) and (B) the newly identified 3''-O-methylated myxochelin A derivative

5. DKxanthene

The DKxanthenes (**Figure 16**) constitute the first and so far sole family of compounds that was primarily reported from *M. xanthus* DK1622 (91). They appear to be characteristic for *M. xanthus*, since strains from various differing locations worldwide invariably produce these metabolites (74). Yet, as could be demonstrated during this work, they are produced from other myxobacterial species (at least *S. aurantiaca*) as well and thus are not suitable as chemotaxonomic marker for *M. xanthus*. DKxanthenes are prominently represented within the cells and are not secreted to the medium, as was observed during the course of isolation of these metabolites (91). Isolation was severely hampered due to unfavourable chromatographic properties, the presence of several derivatives that had to be separated from each other and the tendency of these metabolites to appear as double-peaks, which even occurred during the ongoing separation process. The latter is most likely caused by *cis-trans* isomerization of one (or multiple) double bond(s) of the polyketide chain. Consequently, the purification required several sequential separation steps and was performed in the dark whenever possible to limit isomerization (and even degradation) during the workup (91). Finally, sufficient amounts of DKxanthene-534 and DKxanthene-560 were obtained for detailed one- and two-dimensional NMR analysis. Further metabolites were identified in *M. xanthus* extracts and their structures were determined by mass spectrometric analysis (MS and MS^n) in combination with feeding experiments employing [$^{13}C_4$, $^{15}N_1$]-threonine and [D_6]-propionate (91) (**Figure 16**).



M. xanthus		n	R^1	R^2	R^3	*S. aurantiaca*
✓	DKxanthene-492	0	CH_3	H	H	
✓	DKxanthene-504	1	H	H	H	✓
✓	DKxanthene-508	0	CH_3	H	OH	
✓	DKxanthene-518	1	CH_3	H	H	✓
✓	DKxanthene-520	1	H	H	OH	
✓	DKxanthene-530	2	H	H	H	✓
✓	DKxanthene-534	1	CH_3	H	OH	
✓	DKxanthene-544	2	CH_3	H	H	✓
✓	DKxanthene-548	1	CH_3	CH_3	OH	
✓	DKxanthene-556	3	H	H	H	✓
✓	DKxanthene-560	2	CH_3	H	OH	
✓	DKxanthene-574	2	CH_3	CH_3	OH	
✓	DKxanthene-586	3	CH_3	H	OH	

Figure 16. DKxanthene structures determined from *M. xanthus* and *S. aurantiaca*. Presence of the respective derivative in each strain is indicated by checkmarks. Numbering of atoms is shown at the structure.

As observed for other polyenes, NMR structure elucidation of the conjugated double bond system was complicated due to signal overlap, and no clear assignment of coupling partners and coupling constants was possible. Here, changing the deuterated NMR-solvent system from DMSO to methanol and methanol/ benzene mixtures of differing composition allowed a more clear assignment, and in combination with the obtained UV-data and calculation of theoretical chemical shifts an *all-trans* configuration was proposed for the polyene chain (91) (**Figure 17**).

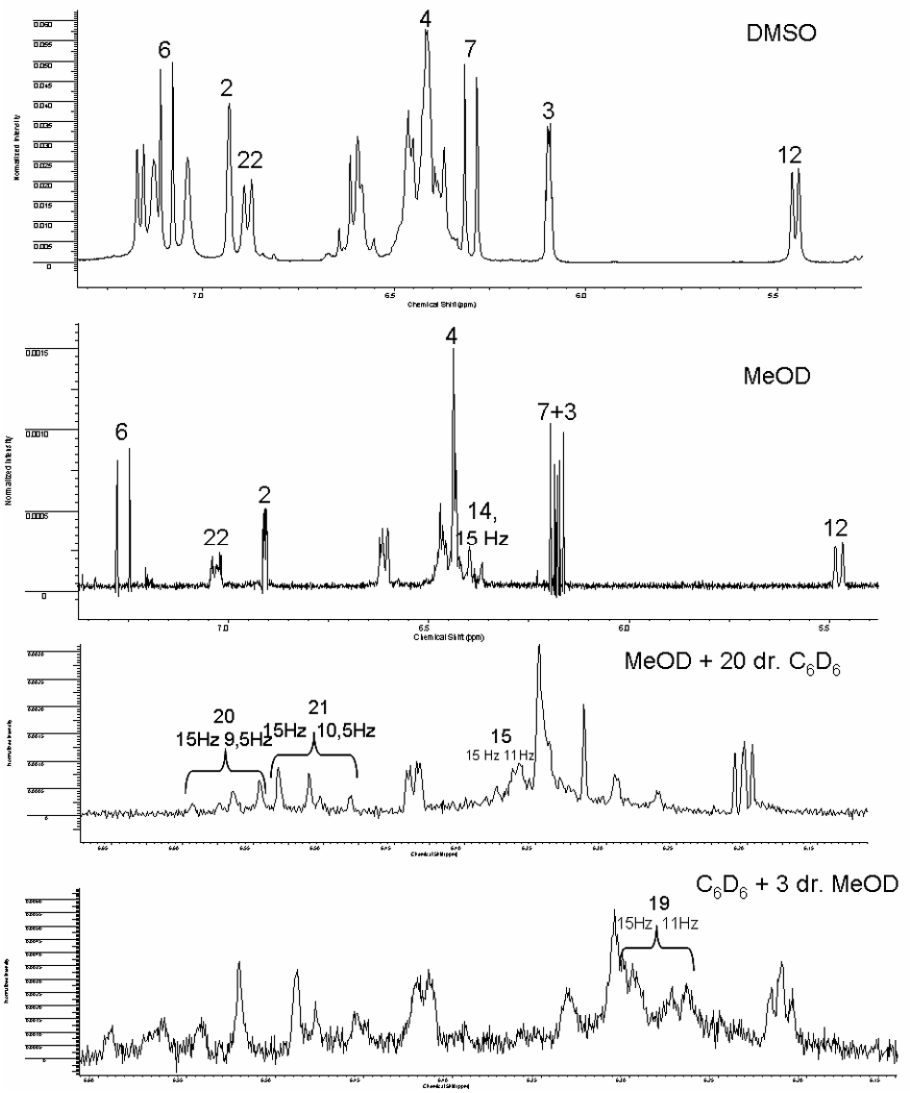


Figure 17. Selected ^{1}H-NMR spectra of DKxanthene-534 applying different deuterated solvent systems (given in the upper right corners). Numbers above the signals indicate the corresponding proton. Signals were shifted under different solvent conditions and hidden coupling constants were released as indicated.

The DKxanthene biosynthetic gene cluster was first discovered in *M. xanthus* DK1622, and shortly after that in *S. aurantiaca* DW4/3-1 as well, enabling the informative comparison of both biosynthetic pathways. As can be readily proposed from the structure of the DKxanthenes, the biosynthetic gene cluster encodes a hybrid PKS/NRPS megasynthetase. The production of a broad natural diversity which is restricted to specific parts of the molecule seems to be a characteristic feature for DKxanthene biosynthesis. Meanwhile the structures of the starter unit, the oxazoline ring and the presence of a terminal asparagine moiety are unvaried in all derivatives known so far, the architecture of the polyene chain is more flexible with respect to its length and methylation pattern. Furthermore, the asparagine moiety is

hydroxylated in the main but not all derivatives in *M. xanthus*, and none hydroxylated metabolites were identified in *S. aurantiaca* (**Figure 16**).

The latter is most likely due to the absence of *dkxD* in the biosynthetic gene cluster of *S. aurantiaca* (dkx_{DW}), which encodes a FAD-dependent monooxygenase in the *M. xanthus* cluster (dkx_{DK}) (**Figure 18**, A).

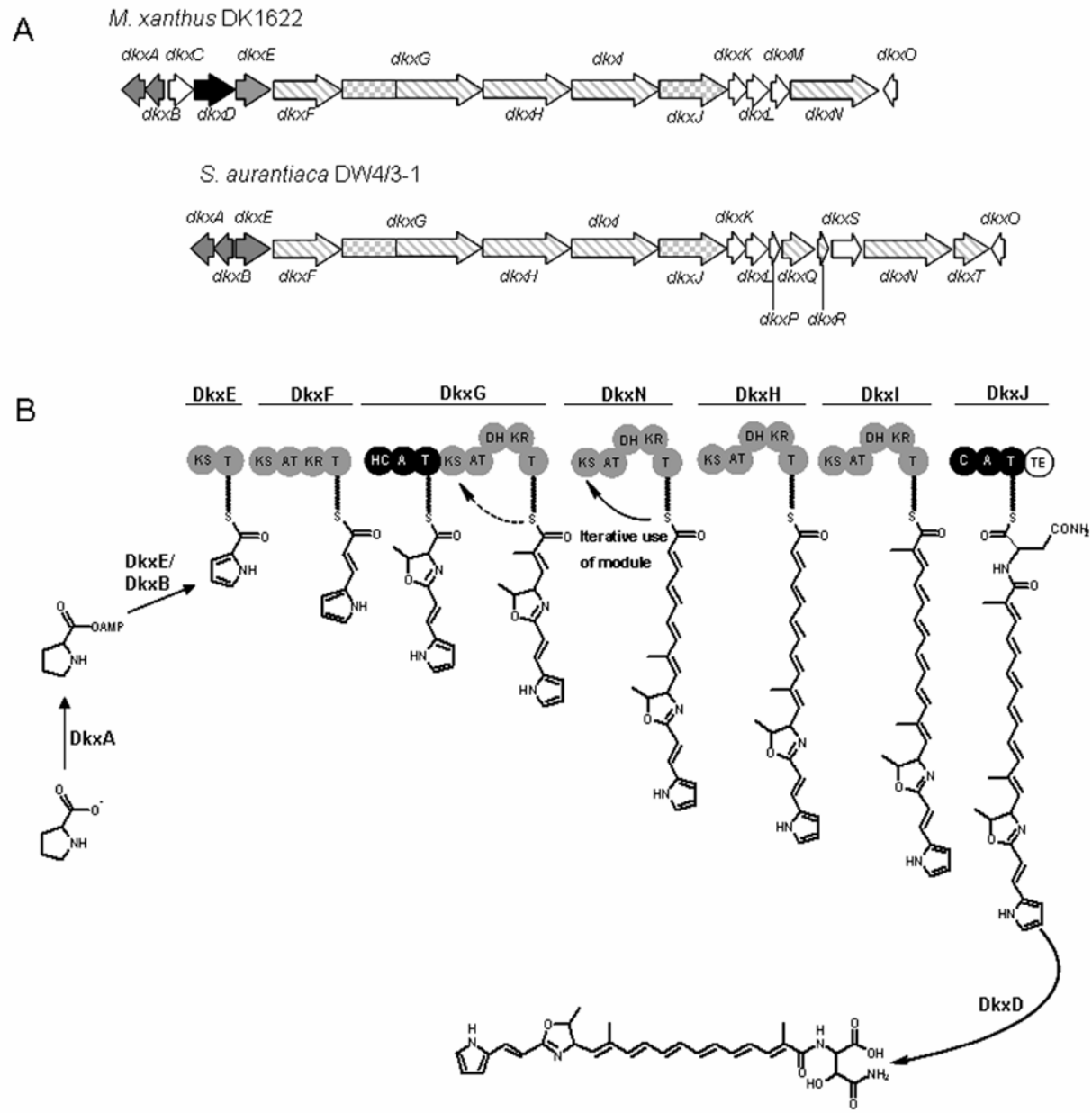


Figure 18. (A) DKxanthene biosynthetic gene clusters of *M. xanthus* and *S. aurantiaca*. Genes encoding enzymes involved in starter unit biosynthesis (dark grey), hydroxylation (black), PKS formation (hatched) and NRPS (chequered) biochemistry are indicated. (B) Model for DKxanthene biosynthesis. The pyrrole carboxylic acid starter unit is derived from proline and processed as described recently (147). Four PKS modules perform altogether at least six rounds of chain extensions (and seven in the case of DKxanthene-534 as shown). DkxN appears to be the best candidate for programmed iteration, and DkxG might iterate in a randomly occurring

process. Notice the different order of enzymes when compared to the order of genes in the DKxanthene gene cluster.

One common characteristic of type I polyketide biochemistry is the colinearity of biosynthetic enzymes with the chemical structures generated from the multimodular machinery. This colinearity rule is not absolute, however, and examples exist in which single modules within type I PKS systems were used more than once. So far, one single module was always shown or postulated to be responsible for this programmed or aberrant iteration (4,38,48). Iteration is proposed for DKxanthene biosynthesis, since altogether four PKS modules assemble the polyketide chain that requires at least one and up to four repetitive uses of at least one module. Alternatively, extra PKS modules might be located elsewhere in the genome, an option that can not be ruled out by the chosen approach (here, successful heterologous expression of the DKxanthene cluster is required for unambiguous proof). Yet this option appears to be unlikely as there is no precedent for such a case, and second, docking domain analysis (113) hints to the well assorted arrangement of DKxanthene modules DkxG, DkxN, DkxH, DkxI and DkxJ (**Figure 18**, B). This order might as well reason the observed pattern of DKxanthene derivatives since the docking domain interaction of DkxG and DkxN appears to be not as optimized as the others, possibly providing a basis for the postulated programmed iterative usage of DkxN and the possible aberrant iterative use of DkxG.

The DKxanthenes were tested for their therapeutic potential in antibacterial (Gram-positive and –negative), antifungal and cytotoxicity assays. However, in none of the tests applied a mentionable activity was observed (data not shown). Rather, as these metabolites remain within the cells and since earlier reports (18,82,124) indicated the involvement of the yellow pigments in developmental processes of *M. xanthus*, we set out to determine a potential role of the DKxanthenes for the producing organism itself. First, mutants defective in DKxanthene biosynthesis were tested for fruiting body formation in comparison to their parent wild type strain *M. xanthus* DK1050. The build-up of fruiting bodies was observed for all mutants tested, but an apparent delay in aggregation and completion of fruiting body formation indicated that the developmental process is hampered in absence of DKxanthenes. The formation of refractile spores was confirmed after 72 hours of development (**Figure 19**).

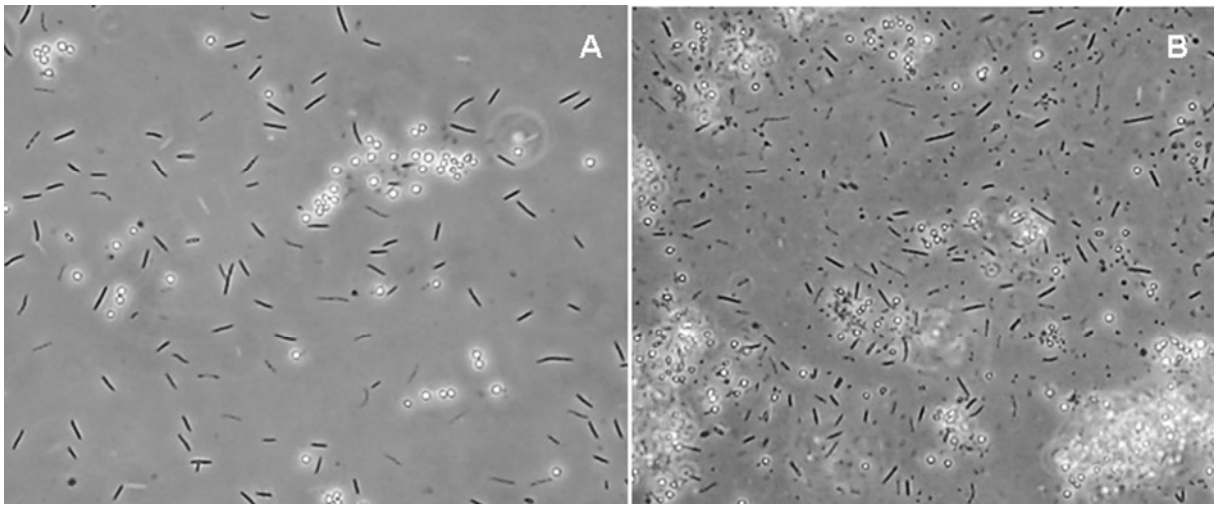


Figure 19. (A) *M. xanthus* DK1050 wild type and (B) DKxanthene-negative mutant derived thereof after 72h of development, after resuspension. Remaining vegetative cells/peripheral rods are rod-shaped, and spores are round and refractile.

Unexpectedly, when spores of wild type versus mutant were tested for viability after 3 days of development (after ultrasound and heat treatment), no viable spores could be recovered from the mutants. Testing of ongoing time-points during development revealed that this defect persisted until day seven, and altogether a maximum recovery rate of 25% of viable spores was observed when compared to the wild type. However, this defect could be complemented at least in part by codevelopment of the mutant strains with wild type, and, moreover, by the addition of purified DKxanthene-534 to the developing cells (91). Similar results were obtained in preliminary studies that dealt with the phenomenon of phase variation: *M. xanthus* cells are able to alternate between two colony types. The first and usually predominant phase is characterized by the yellow pigmentation and rough, swarming colonies (yellow phase). In the alternate phase, the cells are tan and mucoid with smooth edges (tan phase). Both phases are interconvertible. Mutants that are locked in the tan phase were very inefficient in fruiting body formation and unable to produce heat- and sonication-resistant spores. However, when mixed with predominantly yellow cultures, the spores produced from these mixtures were derived predominantly from the phase-locked tan mutants (82).

DKxanthenes resemble in large part polyenes, a class of metabolites that very often exhibit protective properties against various damaging agents including free radicals, and since an UV-protective effect of the yellow pigments as well as the UV-induced conversion of tan cells to yellow cells was described before (18), the antioxidative

potential of DKxanthene-534 was determined in two test systems. The strung-out structure and a large lipophilic part with a hydrophilic “C-terminal” hydroxy-asparagine moiety make it likely that the DKxanthenes are located within the membrane, where they could play a similar role in protecting fatty acids from oxidation and furthermore in rigidifying the membrane to maintain the required membrane fluidity as was described before for carotenoids, members of a family with similar polyene structure (46). Indeed, an antioxidative activity could be assigned to the DKxanthenes, supporting this hypothesis. Yet, it remains questionable whether this activity is a sufficient explanation for all of the effects observed during development, and it appears rather likely that pleiotropic effects lead to the overall phenotype.

Thus, the phenotype of DKxanthene-negative mutants was further investigated by electron microscopy (performed by Dr. Jan Hegermann) and proteomic studies (performed by Dr. Yasser Elnakady). In short, wild type and mutant cells were developed in submerged culture or on nutrient-deficient agar, harvested at selected time points and prepared for the subsequent analysis.

Electron micrographs allowed insights into the defect of DKxanthene-deficient myxospores. Meanwhile, no apparent difference was observed for vegetative cells, developmental spores of the mutants differ in size and morphology from the wild type. Spores from DKxanthene-negative mutants are slightly but unambiguously larger than wild type spores, a difference that is more significant after 72 hours of development but that is still existent after 5 days. Furthermore, lipid droplets within the spores are smaller in size but larger in number in the mutant, and retain longer within the myxospores during development. Finally, apparent differences exist with respect to spore cortex and coat that both consist of several layers (136). DKxanthene-negative mutants produce spores with less condensed and less defined spore cortex and coat (**Figure 20**).

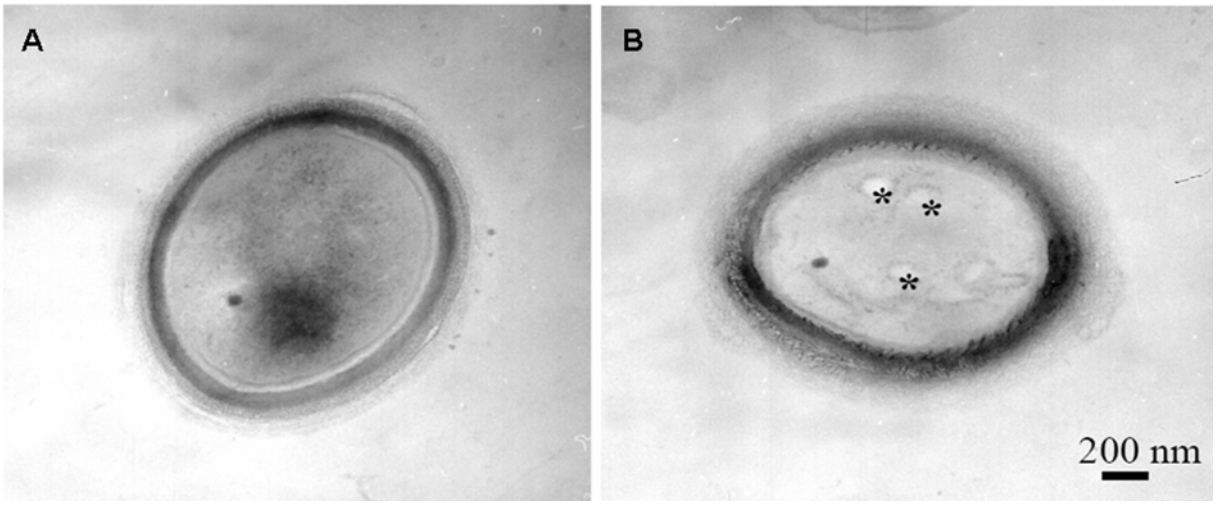


Figure 20. Electron micrographs of (A) wild type and (B) DKxanthene-negative mutant spores after 72 hours of development. The presence of lipid droplets is indicated by asterisks.

A very similar effect was observed in spores that were induced by the addition of glycerol. Glycerol-induced sporulation is independent from fruiting body formation and appears already after 2 hours time of induction (31) (**Figure 21**).

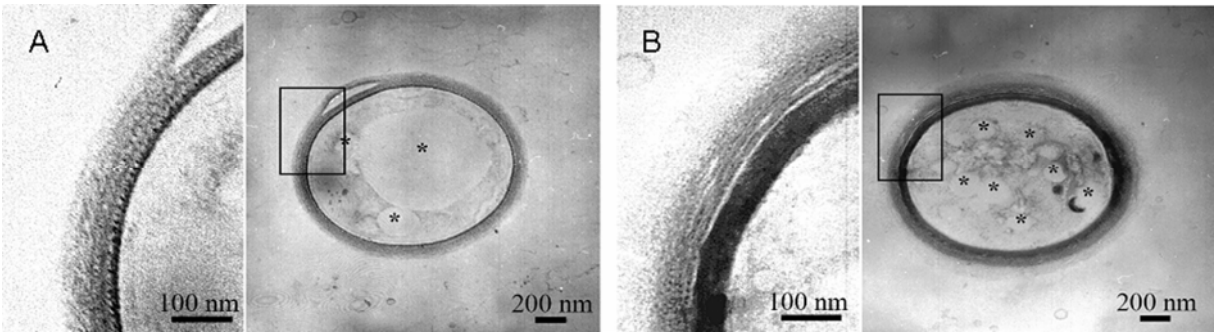


Figure 21. Electron micrographs of (A) wild type and (B) DKxanthene- negative mutant spores derived from glycerol-induction.

This indicates that maturation of the mutant spores is at least delayed, and furthermore a direct or indirect involvement of the DKxanthenes in lipid metabolism seems likely, e.g. by emulsifying lipids from the lipid droplets that are further processed during the reorganization that takes place during spore's maturation (114). For proteomic analysis, a two-dimensional fluorescence difference gel electrophoresis (2D DIGE) approach with subsequent MALDI-TOF peptide identification was chosen. Here, two samples can be compared to each other within one gel, and different labelling of the respective proteins allows the differential in gel detection as well as comparison of both samples without further treatment of the gel.

Four parallel samples of wild type versus mutant were analyzed that way under vegetative as well as developmental conditions (Meiser and Elnakady, unpublished). For vegetative cells (= time-point 0h of development), altogether nine proteins were detected that appeared to be differentially expressed (taken a ratio of a more than 1.5-fold increase resp. decrease in protein expression in all four samples as cutoff) (**Table 2**). Eight of them were up- and one was down-regulated in the DKxanthene-negative mutant. Unfortunately, identification succeeded only for five of the up-regulated proteins. These include a dehydrogenase (glucose/ sorbosone family), the glycine cleavage system T protein, a FG-GAP repeat protein, FibA and ProV. Among the proteins identified, especially the three latter appear to be interesting findings. FibA represents the most abundant (known) extracellular matrix-bound protein that shows homologies to M4 zinc metalloproteases, was proven to be important for stimulation of cells by the chemoattractant phosphatidylethanolamine (PE) and for formation of discrete aggregation foci (17,69). Yet, although FibA resembles a protein known to be involved in developmental processes and is thus a promising candidate for the interpretation of the effects caused by the absence of DKxanthenes, its quantification by proteome analysis was observed to be not perfectly reliable (in former experiments employing *M. xanthus* wild type and different mutant strains) and thus makes the interpretation of this finding difficult (Y. Elnakady, M.O. Altmeyer, personal communication). However, another protein that typically interacts with extracellular matrix proteins is up-regulated in the mutant as well: FG-GAP repeat proteins constitute a family of proteins that are predicted to fold into a ß-propeller domain. Such domains were reported to be part of phosphatidylinositol phospholipase D as well as integrins and contain FG (phenylalanyl-glycyl) and GAP (glycyl-alanyl-prolyl) consensus sequence repeats (132). Integrins are cell adhesion molecules found in eukaryotes, and cell adhesive events are mediated by transmembrane receptors that regulate adhesion, embryonic development by controlling cell migration, growth and differentiation (85). Integrins bind to diverse ligands from the extracellular matrix and cell surface receptors. One might therefore speculate that one such ligand might be FibA. The binding of invasins from enteropathogenic bacteria to integrins allows the internalization into the eukaryotic host cell (58). Binding to ligands is dependent of divalent cations and acidic residues were identified as key components of integrin-binding ligands (85). Only one protein in the databases shows high homologies to MXAN_1005, a FG-GAP repeat protein

from *S. aurantiaca* DW4/3-1 (58% identity/ 72% similarity). Much less significant homologies are found for the described eukaryotic integrin family of proteins.

ProV represents a homologue of OpuAA, the ATP-binding/hydrolyzing subunit of an osmoregulated ABC transporter system (OpuA) (143). It was shown for *Lactococcus lactis* that OpuA can act both as osmosensor and osmoregulator. Experiments employing artificial phospholipid bilayers suggested that OpuA is activated by sensing changes in the physical status of the lipid bilayer via lipid/protein interactions (143). The upregulation of ProV might therefore be another indicator for the affected integrity of the cell membrane of DKxanthene-negative mutants as well as for the membrane-associated localization of the DKxanthenes.

MXAN_2249 (ProV) and MXAN_1005 (FG-GAP repeat protein) were inactivated by plasmid insertion mutagenesis and tested for fruiting body formation. Both mutants retained the ability to produce fruiting bodies (data not shown). Closer inspection of both strains is currently under investigation in our laboratory.

Table 2. Differentially expressed proteins identified in vegetative cells of wild type and DKxanthene-negative mutants (Up- or down-regulation of proteins refers to the mutant in comparison to the wild type)

Gene name/ MXAN	Gene product	Regulation
MXAN_6745	Dehydrogenase, glucose/ sorbosone family	2.60 up
gcvT/ MXAN_3040	Glycine cleavage system T protein	1.74 up
MXAN_1005	FG-GAP repeat protein	2,63 up
fibA/ MXAN_6106	Matrix-associated zinc metalloprotease FibA	1.76 up
proV/ MXAN_2249	Glycine betaine/ L-proline ABC transporter, ATP-binding protein	1.96 up

Differences in proteome pattern strongly increased after 24 hours of development. Altogether at least 28 proteins were differentially regulated, and 19 proteins were down- and nine proteins up-regulated. Among them, 12 could be identified (Table 3).

Table 3. Differentially expressed proteins of wild type and DKxanthene-negative mutants identified after 24 hours of development (Up- or down-regulation of proteins refers to the mutant in comparison to the wild type)

Gene name/ MXAN	Gene product	Regulation
MXAN_7110	Peptidyl-prolyl *cis-trans* isomerase, FKBP-type	1.58 up
MXAN_5430	Development-specific protein S (Spore coat protein S)	1.66 up
MXAN_6451	Hypothetical protein	1.73 up
MXAN_6035	2-oxoglutarate dehydrogenase, E1 component	1.77 down
MXAN_6911	TonB-dependent receptor	1.93 down
MXAN_2408	Translation elongation factor G	1.50 down
pckGl MXAN_1264	Phosphoenolpyruvate carboxykinase	1.50 down
MXAN_5856	Acetate-CoA ligase	1.63 down
MXAN_5266	Hypothetical protein	1.50 up
MXAN_3068	Translation elongation factor Tu	1.53 up
MXAN_3434	Hypothetical protein	2.12 up
MXAN_4863	Adventurous gliding motility protein AgmK	2.33 down

One peptidyl-prolyl *cis-trans* isomerase is among the identified proteins, which is upregulated in the mutant strain (MXAN_7110) (a second peptidyl-prolyl *cis-trans* isomerase (MXAN_6153) that is down-regulated (1.86 down) in the mutant was identified as well but needs to be reconfirmed). Besides the role of these enzymes in prolyl *cis-trans* isomerization during protein folding, recent studies have uncovered that prolyl-isomerization can also function as a molecular timer in a number of biological and pathological processes, including cell signalling, gene expression and infection (86). Consequently, it appears within the realms of possibility that the identified enzymes could play a role in triggering cell differentiation at specific time points during development. The differential regulation of two enzymes of this class at contrastive levels might be due to the delayed developmental progession of the DKxanthene-negative mutants. Mutants defective in these enzymes might possibly be unable to develop or might arrest at specific time points during development. Unfortunately, disruption of both genes by plasmid insertion did not succeed, indicating that they are essential for cell viability.

The most significantly down-regulated protein AgmK represents another remarkable finding. It is part of the adventurous gliding motility apparatus, one of two known motility systems (A and S) in *M. xanthus* (64,93) that are separated from each other but that are used as coordinated motility engines. Even though no significant differences for A- and S-motility were observed for DKxanthene-negative mutants in comparison to the wild type (91), the decreased production of A-motility proteins might be one reason for the reported differing colony morphology of tan phase variants.

Three enzymes involved in primary metabolism (MXAN_6035, MXAN_1264 and MXAN_5856) are identified as down-regulated in the mutants at comparable level. The formation of myxospores involves the remodelling of the peptidoglycan layer, with the amount of peptidoglycan increasing relative to the surface area during the metamorphosis from rod-shaped vegetative cells towards the spherically shaped spores. Furthermore, novel spore envelope polysaccharides are synthesized, and trehalose production strongly increases during sporulation (122). Thus, gluconeogenesis is extended during the sporulation process, giving further evidence for a delayed or hampered mature spore formation in the DKxanthene-negative mutants. It is worth mentioning that a functionally similar protein to MXAN_1264 (a phosphoenolpyruvate carboxykinase, a key enzyme for gluconeogenesis), PykA from *S. aurantiaca* (a pyruvate kinase, involved in glycolysis) was identified as “indole-binding protein” essential for fruiting body formation (133).

Protein S, the most abundant and development-specific protein of *M. xanthus*, however, is upregulated in the mutant strain. This appears to contrast with the phenomena described so far, but two possible explanations might be adduced. First, the overproduction of protein S might be a counter-reaction of the defective spores to compensate for the unusual architecture of the spore coat. The function of protein S during sporulation is not yet known, but some data suggest that protein S might function as a cement that holds the spores together within the fruiting body (67). Second, the higher quantities of protein S in the mutant protein extract are due to an intrinsic property of protein S as well as the analytic method used. Protein S synthesis starts early in development and the protein first accumulates in the soluble fraction of the cytoplasm. Later in development (15 to18 h), it can be found in the periplasm as well, from where it is assembled into the spore coat starting at 24 to 30 hours of development. It was speculated that the production of protein S and its

retention in the cytoplasm before transport across the cytoplasmic membrane must occur since cells were unable to produce the massive amount of protein S immediately before spore formation (transport might be accomplished by a developmentally regulated transport protein, or alternatively, by major changes in the cell membrane that allow protein S to transverse the membrane) (99). As the applied 2D DIGE approach is very suitable for soluble proteins but in contrast less applicable for the reliable detection of membrane proteins, the observed results might again mirror the retarded maturation of the DKxanthene-negative spores. Meanwhile a certain proportion of protein S is assembled into the spore coat of *M. xanthus* wild type after 24 hours – and thus is detracted from detection - the major part of this protein is still accumulated in the cytoplasm in the case of the mutants, giving rise to a seemingly higher yield of protein S in the DKxanthene-negative mutants.

In summary, our data suggest that DKxanthene-negative mutants form fruiting bodies and spores, but the progession of this process is at least delayed, if not impaired in general. The underlying mechanism appears to be complicated, which is not surprising considering the complex biochemical changes that must occur within the developing cells. Moreover, the scenario should be even more complicated for tan phase variants as the tan phenotype should be induced by a regulatory network that controls the behaviour of a single cell within millions of congeners that pass through the developmental program.

6. Other (putative) natural products from **M. xanthus** *and activation of carrier proteins from secondary metabolite biosynthetic pathways*

As described, the genome sequence of *M. xanthus* DK1622 indicates the potential of this strain to produce at least 18 different secondary metabolites. Five of these could be characterized at least in part within this work. It appears unlikely that the remaining biosynthetic gene clusters do not serve any function for the organism since maintaining the genetic information requires energy and the loss of non-functional gene clusters would be the expected consequence. However, no additional secondary metabolite was discovered in *M. xanthus* extracts so far except for one compound with a molecular mass of 582 that was identified in *M. xanthus* extracts and which is only generated and recovered reproducibly if cultivation is performed in presence of XAD-2 adsorber resin (unpublished observation). Several explanations might be adduced: First, most simple but as well very likely, the metabolites might not be produced under laboratory conditions which are quite remote from the conditions of the natural habitat of the bacteria. The induction of specific biosynthetic pathways might require environmental “signals” such as various nutrients, shifts in temperature, oxygen supply, pH or moistness, and even the competition with other microbes in the soil. In contrast, laboratory conditions are set up in order to be highly reproducible, including constant temperatures, pH and, of course, sterile conditions that only allow the strain of interest to grow. Assuming that all natural products have a biological function for the producing organism by acting as signalling or regulatory molecules within cells or cell communities to maintain homeostatic conditions (25), it might be possible that they are superfluous in standard laboratory environment. Alternatively, at least some of the secondary metabolites are produced even under laboratory conditions, but at concentrations which do not allow any analytical detection or which make it difficult to differentiate between background noise and the signal of the small molecule. This hypothesis is supported by recent investigations into the proteome of *M. xanthus* applying a two-dimensional chromatographic separation of tryptic peptides from *M. xanthus* protein lysate with subsequent mass spectrometric analysis (117). This approach readily allows identification of high-molecular weight PKS and NRPS proteins, and among them, not only proteins from biosynthetic pathways associated with the production of known natural products were detected

but also proteins from six biosynthetic gene clusters with so far unknown products. The presence of all biosynthetic proteins of the respecitve gene cluster is of course the prerequisite for biosynthesis, but appears to be likely with respect to the above mentioned criterion of modest energy house keeping. Statistical approaches such as principle component analysis (PCA) (75) help to unveil the production of secondary metabolites that are produced only in trace amounts, e.g. by comparing the wild type strain with mutants defective in one particular biosynthetic pathway (74).

A further problem to overcome besides low production yields is featured by the complex media in which the bacteria are cultivated. They provide a strong background of signals that have to be discriminated against the “true” natural products, and even matching of the pure media extracts (background) against the bacterial culture grown in media from the same batch might be misleading since the non-natural product background changes during fermentation due to degradation of media components as well as cell lysis (**Figure 22**, A and B). The best comparison for background subtraction would thus be a bacterial culture grown in production medium that does not produce any secondary metabolites. Encouraged from results of inactivation experiments of *mtaA* in *S. aurantiaca* DW4/3-1 (36,125) leading to the complete abolishment of known secondary metabolite production in this strain, a similar approach was chosen for *M. xanthus*. MtaA from *S. aurantiaca* DW4/3-1 represents a 4’-phosphopantetheine transferase (PPTase) with broad substrate specificity from the Sfp-type (95) essential for activation of carrier proteins of secondary metabolite biosynthetic pathways. A PPTase (MXAN_3485, renamed to MxPpt1) with very high sequence homology to MtaA was identified in *M. xanthus* and inactivated. Unexpectedly, the inactivation did not lead to a complete loss of natural product formation but rather to significant changes in the metabolite pattern (**Figure 22**, C).

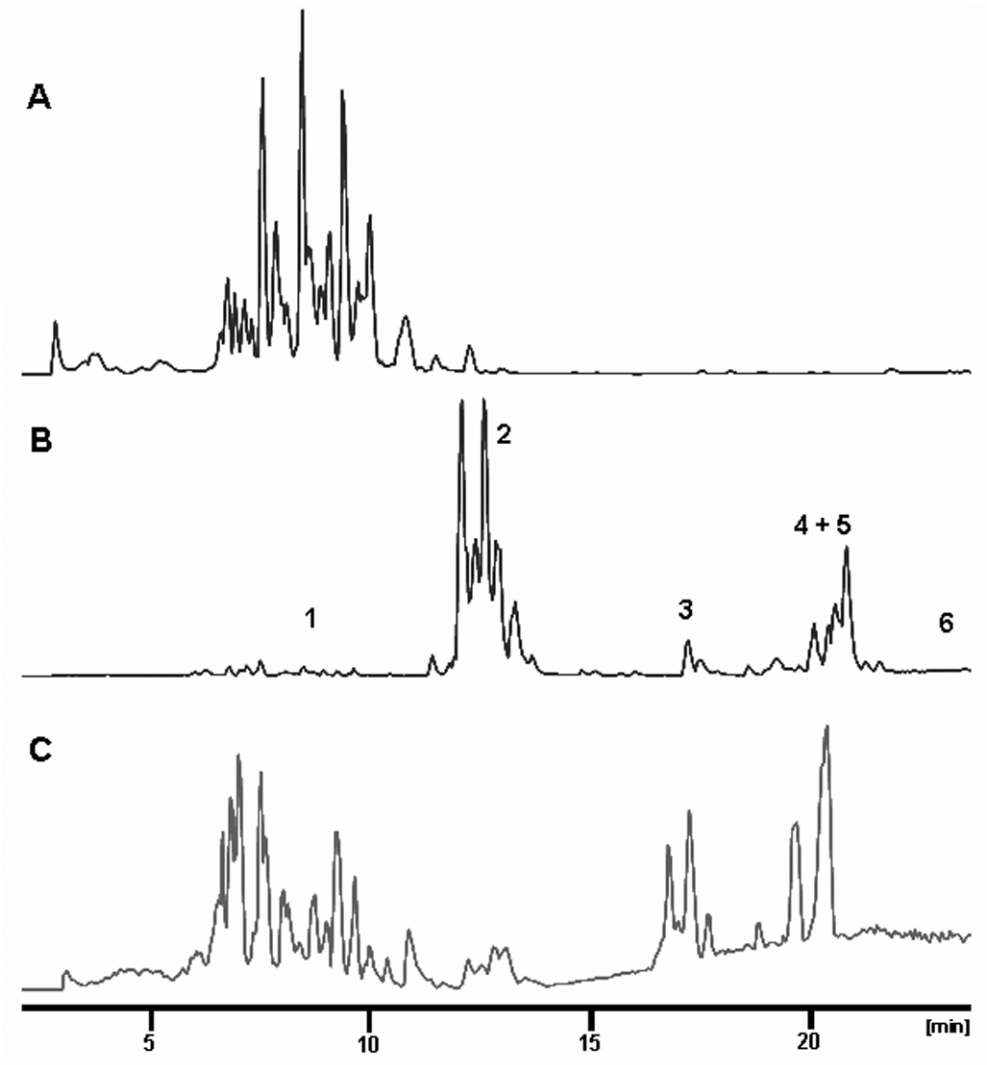


Figure 22. Base peak chromatograms (BPC) of (A) CTT medium (B) *M. xanthus* DK1050 grown in CTT and (C) the mutant in which MxPpt1 is inactive in CTT. Metabolites are indicated by numbers: 1 Myxochelin, 2 DKxanthene, 3 Lyso-PE (a species of lipids (6)), 4 Myxovirescin, 5 Myxalamid, 6 Myxochromid

Subsequent analysis of the *M. xanthus* genome revealed the presence of another Sfp-type PPTase (MXAN_4192, renamed to MxPpt2) and (as expected) one AcpS-like PPTase (MXAN_4350, renamed to MxAcpS). AcpS-like PPTases are thought to activate fatty acid synthases and type II polyketide synthases. Disruption of MxAcpS as well as the creation of a MxPpt1/ MxPpt2 double mutant did not succeed, but the inactivation of MxPpt2 and overexpression of MxPpt1 and MxPpt2 led to further insights into the roles of both enzymes in secondary metabolite production. In short, apparently both enzymes are necessary for the proper activation of all (known and analyzed) natural product biosynthetic pathways and are able to complement at least in part the loss of function of each other. Furthermore, both enzymes appear to be specific for certain biosynthetic pathways. This interpretation is exemplified in the

case of the *mxPpt1* inactivation mutant by the resulting strong decrease in DKxanthene and myxovirescin production and the increase in myxalamid production (the latter most presumably due to increased precursor supply caused by the decreased competition from other biosynthetic pathways). Remarkably, a change in fatty acid profile was observed in all PPTase mutants as well when compared to the wild type.

As a conclusion, the creation of a clean “natural product free background strain” failed, but the attempt to do so allowed further insights into the complex metabolic network used by *M. xanthus.* The suggested use of Sfp-type PPTases in addition to MxAcpS for the activation of fatty acid biosynthesis carrier proteins gives another example for the connection of primary and secondary metabolism in this strain, a feature that was described before, e.g. for the supply with branched chain carboxylic acid precursors for primary and secondary metabolite production (11,14,88).

One promising alternative towards the attempt to discriminate between secondary metabolites and the occurring background noise in the natural producer is given by heterologous expression of the respective biosynthetic pathways in phylogenetically distant host organisms. This approach, even though laborious, comprises several advantages: First, the identification of the metabolite should be easier since the mutant strain containing the gene cluster can be compared directly to its progenitor strain (wild type). Second, the biosynthetic pathway can be uncoupled from (unknown) required signals for biosynthesis by employing strong constitutive or inducible promoters that drive transcription of the gene cluster. Thus, gene clusters that are for some reason “silent” in the natural producer can be activated and the natural product can subsequently be identified and isolated (45). A similar effect would be expected from the introduction of strong promoters into the biosynthetic gene cluster in the natural producer itself. Third, gene clusters expressed in heterologous hosts are often easier to manipulate genetically when compared to the original host, as is the case for *E. coli* and pseudomonads when compared to myxobacteria. This opens the door for better manipulation of the biosynthetic pathway, e.g. mutations aiming at alteration of binding pocket substrate specificity or the repairing of aberrations identified in the sequence of conserved residues that might be responsible for non-production. Finally, as the tools and the knowledge for heterologous production become more and more sophisticated, the number of examples for secondary metabolites that are produced in higher quantities in the

heterologous host when compared to the natural producer will steadily increase (151,154). Beyond this, further insights into the chemical diversity of the *M. xanthus* secondary metabolome should pave the way for a better understanding of the role natural products play for this extraordinary organism.

Reference List

1. **Abergel, R. J., J. A. Warner, D. K. Shuh, and K. N. Raymond**. 2006. Enterobactin protonation and iron release: Structural characterization of the salicylate coordination shift in ferric enterobactin. J. Am. Chem. Soc. **128**:8920-8931.

2. **Altmann, K.-H., G. Höfle, R. Müller, and K. Prantz**. 2008. The Epothilones: An Outstanding Family of Anti-Tumour Agents: From Soil to the Clinic. Springer, Wien, New York.

3. **Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones**. 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27**:215-237.

4. **Arakawa, K., F. Sugino, K. Kodama, T. Ishii, and H. Kinashi**. 2005. Cyclization mechanism for the synthesis of macrocyclic antibiotic lankacidin in *Streptomyces rochei*. Chem. Biol. **12**:249-256.

5. **Austin, M. B. and J. P. Noel**. 2003. The chalcone synthase superfamily of type III polyketide synthases. Nat. Prod. Rep. **20**:79-110.

6. **Avadhani, M., R. Geyer, D. C. White, and L. J. Shimkets**. 2006. Lysophosphatidylethanolamine is a substrate for the short-chain alcohol dehydrogenase SocA from *Myxococcus xanthus*. J. Bacteriol. **188**:8543-8550.

7. **Bangera, M. G. and L. S. Thomashow**. 1999. Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. J. Bacteriol. **181**:3155-3163.

8. **Bedorf, N., D. Schomburg, K. Gerth, H. Reichenbach, and G. Höfle**. 1993. Isolation and structure elucidation of soraphen A1, a novel antifungal macrolide from *Sorangium cellulosum*. Liebigs Ann. Chem. 1017-1021.

9. **Bentley, S. D., K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood**. 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature **417**:141-147.

10. **Bode, H. B., B. Bethe, R. Hofs, and A. Zeeck**. 2002. Big effects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem **3**:619-627.

11. **Bode, H. B., P. Meiser, T. Klefisch, N. Socorro D.J.Cortina, D. Krug, A. Göhring, G. Schwär, T. Mahmud, Y. A. Elnakady, and R. Müller**. 2007. Mutasynthesis-derived myxalamids and origin of the isobutyryl-CoA starter unit of myxalamid B. ChemBioChem **8**:2139-2144.

12. **Bode, H. B. and R. Müller**. 2005. The impact of bacterial genomics on natural product research. Angew. Chem. Int. Ed. **44**:6828-6846.

13. **Bode, H. B. and R. Müller**. 2006. Analysis of myxobacterial secondary metabolism goes molecular. J. Ind. Microbiol. Biotechnol. **33**:577-588.

14. **Bode, H. B., M. W. Ring, G. Schwär, R. M. Kroppenstedt, D. Kaiser, and R. Müller**. 2006. 3-Hydroxy-3-methylglutaryl-coenzyme A (CoA) synthase is involved in the biosynthesis of isovaleryl-CoA in the myxobacterium *Myxococcus xanthus* during fruiting body formation. J. Bacteriol. **188**:6524-6528.

15. **Böhlendorf, B., M. Herrmann, H. J. Hecht, F. Sasse, E. Forche, B. Kunze, H. Reichenbach, and G. Höfle**. 1999. Antibiotics from gliding bacteria - Melithiazols A-N: New antifungal ß-methoxyacrylates from myxobacteria. Eur. J. Org. Chem. 2601-2608.

16. **Böhlendorf, B., G. Hofle, M. Kiffe, A. C. OSullivan, D. Schummer, and M. Sutter**. 1997. Chemistry and fungicidal activity of soraphen A (1 alpha) derivatives. Acs Sym Ser **658**:249-266.

17. **Bonner, P. J., W. P. Black, Z. Yang, and L. J. Shimkets**. 2006. FibA and PilA act cooperatively during fruiting body formation of *Myxococcus xanthus*. Mol. Microbiol. **61**:1283-1293.

18. **Burchard, R. P., A. C. Burchard, and J. H. Parish**. 1977. Pigmentation phenotype instability in *Myxococcus xanthus*. Can J Microbiol **23**:1657-1662.

19. **Burchard, R. P. and M. Dworkin**. 1966. A bacteriophage for *Myxococcus xanthus* - Isolation, characterization and relation of infectivity to host morphogenesis. J. Bacteriol. **91**:1305-&.

20. **Campbell, E. A., O. Pavlova, N. Zenkin, F. Leon, H. Irschik, R. Jansen, K. Severinov, and S. A. Darst**. 2005. Structural, functional, and genetic analysis of sorangicin inhibition of bacterial RNA polymerase. EMBO J. **24**:674-682.

21. **Cane, D. E., C. T. Walsh, and C. Khosla**. 1998. Harnessing the biosynthetic code: combinations, permutations, and mutations. Science **282**:63-68.

22. **Challis, G. L., J. Ravel, and C. A. Townsend**. 2000. Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chem. Biol. **7**:211-224.

23. **Cheng, Y. Q., G. L. Tang, and B. Shen**. 2003. Type I polyketide synthase requiring a discrete acyltransferase for polyketide biosynthesis. Proc. Natl. Acad. Sci. USA **100**:3149-3154.

24. **Cortes, J. and J. Baselga**. 2007. Targeting the microtubules in breast cancer beyond taxanes: The epothilones. Oncologist **12**:271-280.

25. **Davies, J.** 2007. Small molecules: the lexicon of biodiversity. J. Biotechnol. **129**:3-5.

26. **Del Vecchio, F., H. Petkovic, S. G. Kendrew, L. Low, B. Wilkinson, R. Lill, J. Cortes, B. A. Rudd, J. Staunton, and P. F. Leadlay**. 2003. Active-site residue,

domain and module swaps in modular polyketide synthases. J. Ind. Microbiol. Biotechnol. **30**:489-494.

27. **Downard, J., S. V. Ramaswamy, and K. S. Kil**. 1993. Identification of esg, a genetic locus involved in cell-cell signaling during *Myxococcus xanthus* development. J. Bacteriol. **175**:7762-7770.

28. **Du, L. H., C. Sanchez, and B. Shen**. 2001. Hybrid peptide-polyketide natural products: Biosynthesis and prospects toward engineering novel molecules. Metab. Eng. **3**:78-95.

29. **Dutton, C. J., S. P. Gibson, A. C. Goudie, K. S. Holdom, M. S. Pacey, J. C. Ruddock, J. D. Bu'Lock, and M. K. Richards**. 1991. Novel avermectins produced by mutational biosynthesis. J. Antibiot. **44**:357-365.

30. **Dworkin, M.** 1966. Biology of the myxobacteria. Annu. Rev. Microbiol. **20**:75-106.

31. **Dworkin, M. and S. M. Gibson**. 1964. A system for studying microbial morphogenesis: rapid formation of microcysts in *Myxococcus xanthus*. Science **146**:243-244.

32. **Elnakady, Y. A., F. Sasse, H. Lünsdorf, and H. Reichenbach**. 2004. Disorazol A1, a highly effective antimitotic agent acting on tubulin polymerization and inducing apoptosis in mammalian cells. Biochem. Pharmacol. **67**:927-935.

33. **Fernandez-Moreno, M. A., E. Martinez, L. Boto, D. A. Hopwood, and F. Malpartida**. 1992. Nucleotide sequence and deduced functions of a set of cotranscribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin. J. Biol. Chem. **267**:19278-19290.

34. **Finking, R. and M. A. Marahiel**. 2004. Biosynthesis of nonribosomal peptides. Annu. Rev. Microbiol. **58**:453-488.

35. **Fujii, I., N. Yoshida, S. Shimomaki, H. Oikawa, and Y. Ebizuka**. 2005. An iterative type I polyketide synthase PKSN catalyzes synthesis of the decaketide alternapyrone with regio-specific octa-methylation. Chem. Biol. **12**:1301-1309.

36. **Gaitatzis, N., A. Hans, R. Müller, and S. Beyer**. 2001. The *mtaA* gene of the myxothiazol biosynthetic gene cluster from *Stigmatella aurantiaca* DW4/3-1 encodes a phosphopantetheinyl transferase that activates polyketide synthases and polypeptide synthetases. J. Biochem. (Tokyo) **129**:119-124.

37. **Gaitatzis, N., B. Kunze, and R. Müller**. 2001. In vitro reconstitution of the myxochelin biosynthetic machinery of *Stigmatella aurantiaca* Sg a15: Biochemical characterization of a reductive release mechanism from nonribosomal peptide synthetases. Proc. Natl. Acad. Sci. USA **98**:11136-11141.

38. **Gaitatzis, N., B. Silakowski, B. Kunze, G. Nordsiek, H. Blöcker, G. Höfle, and R. Müller**. 2002. The biosynthesis of the aromatic myxobacterial electron transport inhibitor stigmatellin is directed by a novel type of modular polyketide synthase. J. Biol. Chem. **277**:13082-13090.

39. **Gerth, K., N. Bedorf, H. Irschik, G. Höfle, and H. Reichenbach**. 1994. The soraphens: a family of novel antifungal compounds from *Sorangium cellulosum* (Myxobacteria). I. Soraphen A1 alpha: fermentation, isolation, biological properties. J. Antibiot. **47**:23-31.

40. **Gerth, K., H. Irschik, H. Reichenbach, and G. Höfle**. 1982. The myxovirescins, a family of antibiotics from *Myxococcus virescens* (Myxobacterales). J. Antibiot. **35**:1454-1459.

41. **Gerth, K., H. Irschik, H. Reichenbach, and W. Trowitzsch**. 1980. Myxothiazol, an antibiotic from *Myxococcus fulvus* (myxobacterales). I. Cultivation, isolation, physico-chemical and biological properties. J. Antibiot. **33**:1474-1479.

42. **Gerth, K., R. Jansen, G. Reifenstahl, G. Höfle, H. Irschik, B. Kunze, H. Reichenbach, and G. Thierbach**. 1983. The myxalamids, new antibiotics from *Myxococcus xanthus* (Myxobacterales). I. Production, physico-chemical and biological properties, and mechanism of action. J. Antibiot. **36**:1150-1156.

43. **Gerth, K., S. Pradella, O. Perlova, S. Beyer, and R. Müller**. 2003. Myxobacteria: Proficient producers of novel natural products with various biological activities - past and future biotechnological aspects with the focus on the genus *Sorangium*. J. Biotechnol. **106**:233-253.

44. **Goldman, B. S., W. C. Nierman, D. Kaiser, S. C. Slater, A. S. Durkin, J. Eisen, C. M. Ronning, W. B. Barbazuk, M. Blanchard, C. Field, C. Halling, G. Hinkle, O. Iartchuk, H. S. Kim, C. Mackenzie, R. Madupu, N. Miller, A. Shvartsbeyn, S. A. Sullivan, M. Vaudin, R. Wiegand, and H. B. Kaplan**. 2006. Evolution of sensory complexity recorded in a myxobacterial genome. Proc. Natl. Acad. Sci. USA **103**:15200-15205.

45. **Gross, F., N. Luniak, O. Perlova, N. Gaitatzis, H. Jenke-Kodama, K. Gerth, D. Gottschalk, E. Dittmann, and R. Müller**. 2006. Bacterial type III polyketide synthases: Phylogenetic analysis and potential for the production of novel secondary metabolites by heterologous expession in pseudomonads. Arch. Microbiol. **185**:28-38.

46. **Gruszecki, W. I. and K. Strzalka**. 2005. Carotenoids as modulators of lipid membrane physical properties. Biochim. Biophys. Acta **1740**:108-115.

47. **Hagen, D. C., A. P. Bretscher, and D. Kaiser**. 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev Biol **64**:284-296.

48. **He, J. and C. Hertweck**. 2003. Iteration as programmed event during polyketide assembly; Molecular analysis of the aureothin biosynthesis gene cluster. Chem. Biol. **10**:1225-1232.

49. **Hertweck, C., A. Luzhetskyy, Y. Rebets, and A. Bechthold**. 2007. Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork. Nat. Prod. Rep. **24**:162-190.

50. **Higgs, P. I., P. S. Myers, and K. Postle**. 1998. Interactions in the TonB-dependent energy transduction complex: ExbB and ExbD form homomultimers. J. Bacteriol. **180**:6031-6038.

51. **Höfle, G.** 1999. Epothilone - a natural product on the road to becoming a medicine, p. 21-34. *In* G. f. B. F. mbH (ed.), Scientific Annual Report, Braunschweig.

52. **Höfle, G. and H. Reichenbach**. 2005. Epothilone, a myxobacterial metabolite with promising antitumor activity, p. 413-450. *In* G. M. Cragg, D. G. Kingston, and D. J. Newman (ed.), Anticancer agents from natural products. Taylor & Francis, Boca Raton.

53. **Iizuka, T., Y. Jojima, R. Fudou, M. Tokura, A. Hiraishi, and S. Yamanaka**. 2003. *Enhygromyxa salina* gen. nov., sp. nov., a slightly halophilic myxobacterium isolated from the coastal areas of Japan. Syst. Appl. Microbiol. **26**:189-196.

54. **Iizuka, T., Y. Jojima, R. Fudou, and S. Yamanaka**. 1998. Isolation of myxobacteria from the marine environment. FEMS Microbiol. Lett. **169**:317-322.

55. **Ikeda, H., T. Nonomiya, M. Usami, T. Ohta, and S. Omura**. 1999. Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. Proc. Natl. Acad. Sci. USA **96**:9509-9514.

56. **Irschik, H., R. Jansen, K. Gerth, G. Höfle, and H. Reichenbach**. 1987. The sorangicins, novel and powerful inhibitors of eubacterial RNA polymerase isolated from myxobacteria. J. Antibiot. **40**:7-13.

57. **Irschik, H., R. Jansen, K. Gerth, G. Höfle, and H. Reichenbach**. 1995. Disorazol A, an efficient inhibitor of eukaryotic organisms isolated from myxobacteria. J. Antibiot. **48**:31-35.

58. **Isberg, R. R. and P. Barnes**. 2001. Subversion of integrins by enteropathogenic *Yersinia*. J. Cell Sci. **114**:21-28.

59. **Jansen, R., H. Irschik, H. Reichenbach, V. Wray, and G. Höfle**. 1994. Disorazoles, highly cytotoxic metabolites from the Sorangicin-producing bacterium *Sorangium cellulosum,* strain So ce12. Liebigs Ann Chem 759-773.

60. **Jansen, R., G. Reifenstahl, K. Gerth, H. Reichenbach, and G. Höfle**. 1983. Antibiotika aus Gleitenden Bakterien, XV: Myxalamide A, B, C und D, eine Gruppe homologer Antibiotika aus *Myxococcus xanthus* Mx x12 (Myxobacterales). Liebigs Ann. Chem. **7**:1081-1095.

61. **Jansen, R., G. Reifenstahl, K. Gerth, H. Reichenberg, and G. Höfle**. 1983. Myxalamide A,B,C und D, eine Gruppe homologer Antibiotika aus *Myxococcus xanthus* Mx x12 (Myxobacterales). Liebigs Ann. Chem. 1081-1095.

62. **Janssen, G. R. and M. Dworkin**. 1985. Cell-cell interactions in developmental lysis of *Myxococcus xanthus*. Dev. Biol. **112**:194-202.

63. **Kaiser, D.** 1979. Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76**:5952-5956.

64. **Kaiser, D.** 2000. Bacterial motility: how do pili pull? Curr. Biol. **10**:R777-R780.

65. **Kaiser, D.** 2003. Coupling cell movement to multicellular development in myxobacteria. Nat. Rev. Microbiol. **1**:45-54.

66. **Kaiser, D.** 2004. Signaling in myxobacteria. Annu. Rev. Microbiol. **58**:75-98.

67. **Kaiser, D., C. Manoil, and M. Dworkin**. 1979. Myxobacteria: cell interactions, genetics, and development. Annu Rev Microbiol **33**:595-639.

68. **Kaur, G., M. Hollingshead, S. Holbeck, V. Schauer-Vukasinovic, R. F. Camalier, A. Domling, and S. Agarwal**. 2006. Biological evaluation of tubulysin A: a potential anticancer and antiangiogenic natural product. Biochem. J. **396**:235-242.

69. **Kearns, D. B., P. J. Bonner, D. R. Smith, and L. J. Shimkets**. 2002. An extracellular matrix-associated zinc metalloprotease is required for dilauroyl phosphatidylethanolamine chemotactic excitation in *Myxococcus xanthus*. J. Bacteriol. **184**:1678-1684.

70. **Khalil, M. W., F. Sasse, H. Lunsdorf, Y. A. Elnakady, and H. Reichenbach**. 2006. Mechanism of action of tubulysin, an antimitotic peptide from myxobacteria. ChemBioChem **7**:678-683.

71. **Klefisch, T.** 2007. Untersuchungen zur Bio- und Mutasynthese von Myxalamiden und Myxochelinen aus *Myxococcus xanthus*. Diploma thesis, Saarland University.

72. **Koehn, F. E. and G. T. Carter**. 2005. The evolving role of natural products in drug discovery. Nat. Rev. Drug Discovery **4**:206-220.

73. **Konz, D. and M. A. Marahiel**. 1999. How do peptide synthetases generate structural diversity? Chem. Biol. **6**:R39-R48.

74. **Krug, D., G. Zurek, O. Revermann, M. Vos, G. J. Velicer, and R. Müller**. 2008. Discovering the hidden secondary metabolome of *Myxococcus xanthus*: a study of intraspecific diversity. Appl. Environ. Microbiol. **published ahead of print**.

75. **Krug, D., G. Zurek, B. Schneider, C. Bässmann, and R. Müller**. 2007. Analysis of secondary metabolites from myxobacteria using ESI-TOF-MS and PCA. LC-GC Europe, The Applications Book **March 2007**:41-42.

76. **Kuner, J. M. and D. Kaiser**. 1982. Fruiting Body Morphogenesis in Submerged Cultures of *Myxococcus xanthus*. J. Bacteriol. **151**:458-461.

77. **Kunze, B., N. Bedorf, W. Kohl, G. Höfle, and H. Reichenbach**. 1989. Myxochelin A, a new iron-chelating compound from *Angiococcus disciformis* (Myxobacterales). Production, isolation, physico-chemical and biological properties. J. Antibiot. **42**:14-17.

78. **Kunze, B., T. Kemmer, G. Höfle, and H. Reichenbach**. 1984. Stigmatellin, a new antibiotic from *Stigmatella aurantiaca* (Myxobacterales). I. Production, physico-chemical and biological properties. J. Antibiot. **37**:454-461.

79. **Kunze, B., W. Trowitzsch-Kienast, G. Höfle, and H. Reichenbach**. 1992. Nannochelins A, B and C, new iron-chelating compounds from *Nannocystis exedens* (myxobacteria). Production, isolation, physico-chemical and biological properties. J. Antibiot. **45**:147-150.

80. **Lambalot, R. H., A. M. Gehring, R. S. Flugel, P. Zuber, M. LaCelle, M. A. Marahiel, R. Reid, C. Khosla, and C. T. Walsh**. 1996. A new enzyme superfamily - the phosphopantetheinyl transferases. Chem. Biol. **3**:923-936.

81. **Laue, B. E. and R. E. Gill**. 1994. Use of a phase variation-specific promoter of *Myxococcus xanthus* in a strategy for isolating a phase-locked mutant. J. Bacteriol. **176**:5341-5349.

82. **Laue, B. E. and R. E. Gill**. 1995. Using a phase-locked mutant of *Myxococcus xanthus* to study the role of phase variation in development. J. Bacteriol. **177**:4089-4096.

83. **Lerner, P. I.** 2004. Producing penicillin. N Engl J Med **351**:524.

84. **Linne, U., S. Doekel, and M. A. Marahiel**. 2001. Portability of epimerization domain and role of peptidyl carrier protein on epimerization activity in nonribosomal peptide synthetases. Biochemistry **40**:15824-15834.

85. **Loftus, J. C., J. W. Smith, and M. H. Ginsberg**. 1994. Integrin-mediated cell adhesion: The extracellular face. J. Biol. Chem. **269**:25235-25238.

86. **Lu, K. P., G. Finn, T. H. Lee, and L. K. Nicholson**. 2007. Prolyl cis-trans isomerization as a molecular timer. Nat. Chem. Biol. **3**:619-629.

87. **Ma, Y., L. H. Smith, R. J. Cox, P. Beltran-Alvarez, C. J. Arthur, and T. J. Simpson**. 2006. Catalytic relationships between type I and type II iterative polyketide synthases: The *Aspergillus parasiticus* norsolorinic acid synthase. ChemBioChem **7**:1951-1958.

88. **Mahmud, T., S. C. Wenzel, E. Wan, K. W. Wen, H. B. Bode, N. Gaitatzis, and R. Müller**. 2005. A novel biosynthetic pathway to isovaleryl-CoA in myxobacteria: The involvement of the mevalonate pathway. ChemBioChem **6**:322-330.

89. **Mahmud, T., H. B. Bode, B. Silakowski, R. M. Kroppenstedt, M. Xu, S. Nordhoff, G. Höfle, and R. Müller**. 2002. A novel biosynthetic pathway providing precursors for fatty acid biosynthesis and secondary metabolite formation in myxobacteria. J. Biol. Chem. **277**:23768-32774.

90. **McDaniel, R., A. Thamchaipenet, C. Gustafsson, H. Fu, M. Betlach, and G. Ashley**. 1999. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. Proc. Natl. Acad. Sci. USA **96**:1846-1851.

91. **Meiser, P., H. B. Bode, and R. Müller**. 2006. DKxanthenes: Novel secondary metabolites from the myxobacterium *Myxococcus xanthus* essential for sporulation. Proc. Natl. Acad. Sci. USA **103**:19128-19133.

92. **Michal, G.** 1999. Biochemical Pathways. Spektrum Akad. Verlag, Heidelberg.

93. **Mignot, T., J. W. Shaevitz, P. L. Hartzell, and D. R. Zusman**. 2007. Evidence that focal adhesion complexes power bacterial gliding motility. Science **315**:853-856.

94. **Miyanaga, S., T. Obata, H. Onaka, T. Fujita, N. Saito, H. Sakurai, I. Saiki, T. Furumai, and Y. Igarashi**. 2006. Absolute configuration and antitumor activity of myxochelin A produced by *Nonomuraea pusilla* TP-A0861. J. Antibiot. **59**:698-703.

95. **Mootz, H. D., R. Finking, and M. A. Marahiel**. 2001. 4 '-phosphopantetheine transfer in primary and secondary metabolism of *Bacillus subtilis*. J. Biol. Chem. **276**:37289-37298.

96. **Moss, S. J., C. J. Martin, and B. Wilkinson**. 2004. Loss of co-linearity by modular polyketide synthases: a mechanism for the evolution of chemical diversity. Nat. Prod. Rep. **21**:575-593.

97. **Mueller, C. and M. Dworkin**. 1991. Effects of glucosamine on lysis, glycerol formation, and sporulation in *Myxococcus xanthus*. J. Bacteriol. **173**:7164-7175.

98. **Müller, R.** 2004. Don't classify polyketide synthases. Chem. Biol. **11**:4-6.

99. **Nelson, D. R. and D. R. Zusman**. 1983. Transport and localization of protein S, a spore coat protein, during fruiting body formation by Myxococcus xanthus. J. Bacteriol. **154**:547-553.

100. **Newman, D. J. and G. M. Cragg**. 2007. Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. **70**:461-477.

101. **Norn, S., P. R. Kruse, and E. Kruse**. 2005. History of opium poppy and morphine. Dan. Medicinhist. Arbog. **33**:171-184.

102. **O'Connor, K. A. and D. R. Zusman**. 1991. Analysis of *Myxococcus xanthus* cell types by two-dimensional polyacrylamide gel electrophoresis. J. Bacteriol. **173**:3334-3341.

103. **O'Connor, K. A. and D. R. Zusman**. 1991. Behavior of peripheral rods and their role in the life cycle of Myxococcus xanthus. J. Bacteriol. **173**:3342-3355.

104. **O'Connor, K. A. and D. R. Zusman**. 1991. Development in *Myxococcus xanthus* involves differentiation into two cell types, peripheral rods and spores. J. Bacteriol. **173**:3318-3333.

105. **Omura, S., H. Ikeda, J. Ishikawa, A. Hanamoto, C. Takahashi, M. Shinose, Y. Takahashi, H. Horikawa, H. Nakazawa, T. Osonoe, H. Kikuchi, T. Shiba, Y. Sakaki, and M. Hattori**. 2001. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. Proc. Natl. Acad. Sci. USA **98**:12215-12220.

106. **Pridzun, L., F. Sasse, and H. Reichenbach**. 1995. Inhibition of fungal acetyl-CoA carboxylase: A novel target discovered with the myxobacterial compound soraphen, p. 99-109. *In* G. K. Dixon, C. L.G., and D. W. Hollomon (ed.), Antifungal agents. BIOS Scientific Publishers Ltd, Oxford, UK.

107. **Reichenbach, H.** 1984. Myxobacteria: a most peculiar group of social prokaryotes, *In Myxobacteria*, edited by E. Rosenberg. Springer-Verlag, Berlin

108. **Reichenbach, H.** 2001. Myxobacteria, producers of novel bioactive substances. J. Ind. Microbiol. Biotechnol. **27**:149-156.

109. **Reichenbach, H.** 2005. Myxococcales, p. 1059-1144. *In* D. J. Brenner, N. R. Krieg, and J. T. Staley (ed.), Bergey's manual of systematic bacteriology. Springer.

110. **Reichenbach, H. and M. Dworkin**. 1992. The Myxobacteria, p. 3416-3487. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The Procaryotes. Springer-Verlag, New York.

111. **Reichenbach, H. and G. Höfle**. 1994. Discovery of a new antifungal mechanism of action: soraphen - an almost-success story, p. 5-22. *In* Scientific Annual Report. Gesellschaft für Biotechnologische Forschung mbH, Braunschweig.

112. **Reid, R., M. Piagentini, E. Rodriguez, G. Ashley, N. Viswanathan, J. Carney, D. V. Santi, C. R. Hutchinson, and R. McDaniel**. 2003. A model of structure and catalysis for ketoreductase domains in modular polyketide synthases. Biochemistry **42**:72-79.

113. **Richter, C. D., D. Nietlispach, R. W. Broadhurst, and K. J. Weissman**. 2007. Multienzyme docking in hybrid megasynthetases. Nat. Chem. Biol. **4**:75-81.

114. **Ring, M. W., G. Schwar, V. Thiel, J. S. Dickschat, R. M. Kroppenstedt, S. Schulz, and H. B. Bode**. 2006. Novel iso-branched ether lipids as specific markers of developmental sporulation in the myxobacterium *Myxococcus xanthus*. J. Biol. Chem. **281**:36691-36700.

115. **Sasse, F., B. Böhlendorf, M. Herrmann, B. Kunze, E. Forche, H. Steinmetz, G. Höfle, H. Reichenbach, and M. Hermann**. 1999. Melithiazols, new beta-methoxyacrylate inhibitors of the respiratory chain isolated from myxobacteria. Production, isolation, physico-chemical and biological properties. J. Antibiot. **52**:721-729.

116. **Sasse, F., H. Steinmetz, J. Heil, G. Höfle, and H. Reichenbach**. 2000. Tubulysins, new cytostatic peptides from myxobacteria acting on microtubuli: Production, isolation, physico-chemical and biological properties. J. Antibiot. **53**:879-885.

117. **Schley, C., M. O. Altmeyer, R. Swart, R. Müller, and H. CG**. 2006. Proteome analysis of *Myxococcus xanthus* by off-line two-dimensional chromatographic separation using monolithic poly-(styrene-divinylbenzene) columns combined with ion-trap tandem mass spectrometry. J. Proteome Res. **5**:2760-2768.

118. **Schneiker, S., O. Perlova, O. Kaiser, K. Gerth, A. Alici, M. O. Altmeyer, D. Bartels, T. Bekel, S. Beyer, E. Bode, H. B. Bode, C. J. Bolten, J. V. Choudhuri, S. Doss, Y. A. Elnakady, B. Frank, L. Gaigalat, A. Goesmann, C. Groeger, F. Gross, L. Jelsbak, L. Jelsbak, J. Kalinowski, C. Kegler, T. Knauber, S. Konietzny, M. Kopp, L. Krause, D. Krug, B. Linke, T. Mahmud, R. Martinez-Arias, A. C. McHardy, M. Merai, F. Meyer, S. Mormann, J. Munoz-Dorado, J. Perez, S. Pradella, S. Rachid, G. Raddatz, F. Rosenau, C. Ruckert, F. Sasse, M. Scharfe, S. C. Schuster, G. Suen, A. Treuner-Lange, G. J. Velicer, F. J. Vorholter, K. J. Weissman, R. D. Welch, S. C. Wenzel, D. E. Whitworth, S. Wilhelm, C. Wittmann, H. Blöcker, A. Pühler, and R. Müller**. 2007. Complete genome

sequence of the myxobacterium *Sorangium cellulosum*. Nat. Biotechnol. **25**:1281-1289.

119. **Schott, J.** 2007. Modifikation und heterologe Expression der Myxochromid S Megasynthetase durch Red/Et Rekombination.Naturwissenschaftlich Technische Fakultät III, Universität des Saarlandes.

120. **Schwarzer, D. and M. A. Marahiel**. 2001. Multimodular biocatalysts for natural product assembly. Naturwissenschaften **88**:93-101.

121. **Seshime, Y., P. R. Juvvadi, I. Fujii, and K. Kitamoto**. 2005. Discovery of a novel superfamily of type III polyketide synthases in *Aspergillus oryzae*. Biochem. Biophys. Res. Commun. **331**:253-260.

122. **Shimkets, L. J.** 1990. Social and developmental biology of the myxobacteria. Microbiol. Rev. **54**:473-501.

123. **Shimkets, L. J.** 1993. The myxobacterial Genome, p. 85-108. *In* M. Dworkin and D. Kaiser (ed.), Myxobacteria II. American Society for Microbiology, Washington DC.

124. **Shimkets, L. J.** 1999. Intercellular signaling during fruiting-body development of *Myxococcus xanthus*. Annu Rev Microbiol **53**:525-549.

125. **Silakowski, B., B. Kunze, and R. Müller**. 2001. Multiple hybrid polyketide synthase/non-ribosomal peptide synthetase gene clusters in the myxobacterium *Stigmatella aurantiaca*. Gene **275**:233-240.

126. **Silakowski, B., B. Kunze, G. Nordsiek, H. Blöcker, G. Höfle, and R. Müller**. 2000. The myxochelin iron transport regulon of the myxobacterium *Stigmatella aurantiaca* Sg a15. Eur. J. Biochem. **267**:6476-6485.

127. **Silakowski, B., G. Nordsiek, B. Kunze, H. Blöcker, and R. Müller**. 2001. Novel features in a combined polyketide synthase/non-ribosomal peptide synthetase: The myxalamid biosynthetic gene cluster of the myxobacterium *Stigmatella aurantiaca* Sga15. Chem. Biol. **8**:59-69.

128. **Simunovic, V.** 2007. Biosynthesis and regulation of production of the antibiotic myxovirescin A *in Myxococcus xanthus* DK1622.Naturwissenschaftlich-Technische Fakultät III der Universität des Saarlandes.

129. **Simunovic, V. and R. Müller**. 2007. 3-Hydroxy-3-methylglutaryl-CoA-like synthases direct the formation of methyl and ethyl side groups in the biosynthesis of the antibiotic myxovirescin A. ChemBioChem **8**:497-500.

130. **Simunovic, V. and R. Müller**. 2007. Mutational analysis of the myxovirescin biosynthetic gene cluster reveals novel insights into the functional elaboration of polyketide backbones. ChemBioChem **8**:1273-1280.

131. **Simunovic, V., J. Zapp, S. Rachid, D. Krug, P. Meiser, and R. Müller**. 2006. Myxovirescin biosynthesis is directed by hybrid polyketide synthases/ nonribosomal peptide synthetase, 3-hydroxy-3-methylglutaryl CoA synthases and trans-acting acyltransferases. ChemBioChem **7**:1206-1220.

132. **Springer, T. A.** 1997. Folding of the N-terminal, ligand-binding region of integrin alpha-subunits into a beta-propeller domain. Proc. Natl. Acad. Sci. USA **94**:65-72.

133. **Stamm, I., F. Lottspeich, and W. Plaga**. 2005. The pyruvate kinase of *Stigmatella aurantiaca* is an indole binding protein and essential for development. Mol. Microbiol. **56**:1386-1395.

134. **Staunton, J. and K. J. Weissman**. 2001. Polyketide biosynthesis: a millennium review. Nat. Prod. Rep. **18**:380-416.

135. **Stein, T., J. Vater, V. Kruft, A. Otto, B. Wittmann-Liebold, P. Franke, M. Panico, R. McDowell, and H. R. Morris**. 1996. The multiple carrier model of nonribosomal peptide biosynthesis at modular multienzymatic templates. J. Biol. Chem. **271**:15428-15435.

136. **Tengra, F. K., J. L. Dahl, D. Dutton, N. B. Caberoy, L. Coyne, and A. G. Garza**. 2006. CbgA, a protein involved in cortex formation and stress resistance in *Myxococcus xanthus* spores. J. Bacteriol. **188**:8299-8302.

137. **Thierbach, G., B. Kunze, H. Reichenbach, and G. Höfle**. 1984. The mode of action of stigmatellin, a new inhibitor of the cytochorme b-c1 segment of the respiratory chain. Biochim. Biophys. Acta **765**:227-235.

138. **Thierbach, G. and H. Reichenbach**. 1981. Myxothiazol, a new antibiotic interfering with respiration. Antimicrob. Agents Chemother. **19**:504-507.

139. **Trowitzsch Kienast, W., K. Gerth, H. Reichenbach, and G. Höfle**. 1993. Myxochromid A: Ein hochungesättigtes Lipopeptidlacton aus *Myxococcus virescens*. Liebigs Ann. Chem. 1233-1237.

140. **Trowitzsch, W., G. Reifenstahl, V. Wray, and G. Höfle**. 1980. Myxothiazol, an antibiotic from *Myxococcus fulvus* (Myxobacteraoles) II. Structure elucidation. J. Antibiot. **33**:1480-1490.

141. **Trowitzsch, W., V. Wray, K. Gerth, and G. Höfle**. 1982. Structure of Myxovirescin A, a new macrocyclic antibiotic from gliding bacteria. J. Chem. Soc., Chem. Commun. 1340-1341.

142. **Trowitzsch-Kienast, W., K. Schober, V. Wray, K. Gerth, H. Reichenbach, and G. Höfle**. 1989. Zur Konstitution der Myxovirescine B-T und Biogenese des Myxovirescins A. Liebigs Ann. Chem. 345-355.

143. **van der Heide, T. and B. Poolman**. 2000. Osmoregulated ABC-transport system of *Lactococcus lactis* senses water stress via changes in the physical state of the membrane. Proc. Natl. Acad. Sci. USA **97**:7102-7106.

144. **von Döhren, H., R. Dieckmann, and M. Pavela-Vrancic**. 1999. The nonribosomal code. Chem. Biol. **6**:R273-R279.

145. **Walsh, C. T.** 2003. Polyketide antibiotic biosynthesis: assembly-line enzymology, p. 175-193. *In* Antibiotics: actions, origins, resistance. ASM Press, Washington, DC.

146. **Walsh, C. T.** 2008. The Chemical Versatility of Natural-Product Assembly Lines. Acc. Chem. Res. **41**(1):4-10

147. **Walsh, C. T., S. Garneau-Tsodikova, and A. R. Howard-Jones**. 2006. Biological formation of pyrroles: nature's logic and enzymatic machinery. Nat. Prod. Rep. **23**:517-531.

148. **Walsh, C. T., A. M. Gehring, P. H. Weinreb, L. E. Quadri, and R. S. Flugel**. 1997. Post-translational modification of polyketide and nonribosomal peptide synthases. Curr. Opin. Chem. Biol. **1**:309-315.

149. **Walsh, C. T.** 2004. Polyketide and nonribosomal peptide antibiotics: Modularity and versatility. Science **303**:1805-1810.

150. **Weissman, K. J.** 2007. Mutasynthesis - uniting chemistry and genetics for drug discovery. Trends Biotechnol. **25**:139-142.

151. **Wenzel, S. C., F. Gross, Y. Zhang, J. Fu, F. A. Stewart, and R. Müller**. 2005. Heterologous expression of a myxobacterial natural products assembly line in pseudomonads via Red/ET recombineering. Chem. Biol. **12**:349-356.

152. **Wenzel, S. C., B. Kunze, G. Höfle, B. Silakowski, M. Scharfe, H. Blöcker, and R. Müller**. 2005. Structure and biosynthesis of myxochromides S1-3 in *Stigmatella aurantiaca*: Evidence for an iterative bacterial type I polyketide synthase and for module skipping in nonribosomal peptide biosynthesis. ChemBioChem **6**:375-385.

153. **Wenzel, S. C., P. Meiser, T. Binz, T. Mahmud, and R. Müller**. 2006. Nonribosomal peptide biosynthesis: Point mutations and module skipping lead to chemical diversity. Angew. Chem. Int. Ed. **45**:2296-2301.

154. **Wenzel, S. C. and R. Müller**. 2005. Heterologe Expression von komplexen myxobakteriellen Naturstoff-Biosynthesewegen in Pseudomonaden. Biospektrum **11**:628-631.

155. **Wenzel, S. C. and R. Müller**. 2007. Myxobacterial natural product assembly lines: fascinating examples of curious biochemistry. Nat. Prod. Rep. **24**:1211-1224.

156. **White, S. W., J. Zheng, Y. M. Zhang, and Rock**. 2005. The structural biology of type II fatty acid biosynthesis. Annu. Rev. Biochem. **74**:791-831.

157. **Wireman, J. W. and M. Dworkin**. 1977. Developmentally induced autolysis during fruiting body formation by *Myxococcus xanthus*. J. Bacteriol. **129**:796-802.

158. **Wolgemuth, C., E. Hoiczyk, D. Kaiser, and G. Oster**. 2002. How myxobacteria glide. Curr. Biol. **12**:369-377.

159. **Yadav, G., R. S. Gokhale, and D. Mohanty**. 2003. Computational approach for prediction of domain organization and substrate specificity of modular polyketide synthases. J. Mol. Biol. **328**:335-363.

Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form in einem Verfahren zur Erlangung eines akademischen Grades vorgelegt.

Lebenslauf/ Curriculum vitae

Persönliche Daten

Name:	Peter Meiser
Familienstand:	verheiratet
Staatsangehörigkeit:	deutsch
Geburtsdaten:	09.02.1977 in Saarbrücken/Deutschland

Schulbildung

1983 – 1987:	Grundschule Hohe Wacht in Saarbrücken
1987 – 1996:	Gymnasium am Schloß in Saarbrücken, Abitur

Zivildienst

1996-1997:	Zivildienst im Zentrallabor des Saarbrücker Winterbergklinikums

Hochschulausbildung/ Approbation

1997- 2002:	Studium der Pharmazie an der Universität des Saarlandes
1998:	Famulatur in der Park-Apotheke, Merzig
2000:	Einjähriger Studienaufenthalt am Mary Washington College, Fredericksburg, Virginia/USA
2002/2003:	Praktisches Jahr der Apothekerausbildung: Adler-Apotheke in Berlin-Lichtenberg und Krankenhausapotheke der Uniklinik Homburg
2003:	Approbation zum Apotheker
2004-2008:	Dissertation unter der Leitung von Prof. Dr. Rolf Müller, Thema „ *Myxococcus xanthus*- a myxobacterial model strain as multiproducer of secondary metabolites"
2007:	Fachapotheker für Pharmazeutische Analytik
ab 04/2008:	Wissenschaftlicher Mitarbeiter in der medizinisch-wissenschaftlichen Abteilung der Ursapharm Arzneimittel GmbH & Co. KG