Isolation and identification of natural products and biosynthetic pathways from *Photorhabdus* and *Xenorhabdus*

Dissertation

zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturwissenschaftlichen-Technischen Fakultät III

Chemie, Pharmazie, Bio- und Werkstoffwissenschaften

der Universität des Saarlandes

von

Alexander Oliver Brachmann

Saarbrücken

2009

Tag des Kolloquiums:	18. Dezember 2009
Dekan:	Prof. Dr.-Ing. S. Diebels
Vorsitzender:	Prof. Dr. J. Walter
Berichterstatter:	Prof. Dr. H.B. Bode
	Prof. Dr. M.J. Schmitt
Akad. Mitarbeiterin:	Dr. T. Binz

„[…]. Das Rad verschwindet aus dem Straßenbild. Die Verkehrsmittel der Zukunft werden keine Räder haben, sondern nur gleiten oder schweben. Der Straßenverkehr wird in einiger Zeit seinen Höhepunkt erreicht haben und dann abflauen. […]. Wenn man nicht fliegt oder fährt, dann rollt man. Aber das Fliegen wird der neue Mensch vorziehen. Jeder wird sein leicht zu bedienendes Flugzeug haben, das Flugzeug des kleinen Mannes wird der Massenartikel der Industrie sein.

So wird die Technik Triumphe feiern, und über Raum und Zeit fliegen. Zwei Ausklänge kann diese Entwicklung haben: entweder einen weltverheerenden Krieg oder die Weltgemeinschaft: keine Grenzen mehr, da die Sterne erreichbar sind. Die Welt ist eine einzige Familie.**“**

Zeitungsartikel „Im Jahre 2000“ aus dem Saarlouiser Journal vom 16.November 1928, eines unbekannten Autors, der die Zukunft aus der Vorhersage eines Fachmanns beschreibt.

Vorveröffentlichung von Teilen der Dissertation:

Teile der hier vorliegenden Arbeit wurden vorab mit Genehmigung des Fachbereichs, vertreten durch den Mentor, in folgenden Beiträgen vorab veröffentlicht:

Publikationen:

Brachmann, **A.O.**; **Forst**, **S.**; **Furgani**, **G.M.**; **Fodor**, **A. and Bode**, **H.B.** (2006) Xenofuranones A and B: phenylpyruvate dimers from *Xenorhabdus szentirmaii*. *J. Nat. Prod.*, **69**, 1830-1832.

Brachmann, **A.O.**; **Joyce**, **S.A.**; **Jenke-Kodama**, **H.**; **Schwär**, **G.**; **Clarke**, **D.J. and Bode**, **H.B.** (2007) A type II polyketide synthase is responsible for anthraquinone biosynthesis in *Photorhabdus luminescens*. *ChemBioChem*, **8**, 1721-1728.

Joyce, **S.A.**; **Brachmann**, **A.O.**; **Glazer**, **I.**; **Lango**, **L.**; **Schwär**, **G.**; **Clarke**, **D.J. and Bode**, **H.B.** (2008) Bacterial biosynthesis of a multipotent stilbene. *Angew. Chem. Int. Ed.*, **47**, 1942-1945.

Brachmann, **A.O.**; **Schwär**, **G.**; **Bode**, **H.B.** (2008) *Photorhabdus* and *Xenorhabdus*: potent secondary metabolite producers. IOBC wprs Bulletin, **31**, 151-156

Reimer, **D.**; **Luxenburger**, **E.**; **Brachmann**, **A.O.**; **Bode**, **H.B.** (2009) A new type of pyrrolidine biosynthesis is involved in the late steps of xenocoumacin production in *Xenorhabdus nematophila*. *ChemBioChem*, **10**, 1997-2001

Tagungsbeiträge:

Brachmann, **A.O.**; **Joyce**, **S.A.**; **Clarke**, **D.J.**; **Bode**, **H.B.**; Biosynthesis Of Anthraquinones And Stilbenes In *Photorhabdus luminescens* (Poster), VAAM - Biology of Bacteria Producing Natural Products, Tübingen, 20-22 Oktober, 2006

Brachmann, **A.O.**; **Joyce**, **S.A.**; **Clarke**, **D.J.**; **Bode**, **H.B.**; Stilbene und Anthrachinone Naturstoffe aus *Photorhabdus* (Vortrag), 28. Tübinger-Göttinger Gespräche zur Chemie von Mikroorganismen, Zellingen-Retzbach, September, 2007

Brachmann, **A.O.**; **Joyce**, **S.A.**; **Clarke**, **D.J.**; **Schwär**, **G.**; **Bode**, **H.B.**; Unusual biosynthesis of multifunctional stilbenes in *Photorhabdus luminescens* (Vortrag), VAAM - Biology of Bacteria Producing Natural Products, Nonnweiler-Otzenhausen, 4-6 Oktober, 2007

Brachmann, **A.O.**; **Bode**, **H.B.**; A Different Way: Biosynthesis Of Multifunctional Stilbenes in *Photorhabdus* (Poster), VAAM - GBM Jahrestagung, 9-11 März, Frankfurt am Main, 2008

Brachmann, **A.O.**; **Bode**, **H.B.**; A Different Way: Biosynthesis Of Multifunctional Stilbenes in *Photorhabdus* (Poster), International Conference on New Directions in Molecular Genetics and Genomics - Applications in Natural Product Producing Organisms, 10-11 April, Freiburg, 2008

Brachmann, **A.O.**; **Bode**, **H.B.**; The blue pigment indigoidine – a silent gene cluster in *Photorhabdus* heterologously expressed in *E. coli* (Poster), GDCh – Chemie für die Life Sciences, 2-5 September, Frankfurt am Main 2009.

Table of contents

Vorveröffentlichungen IV
Table of contents VI
Abstract VII
Zusammenfassung VIII
Introduction 1
Natural products and entomopathogenic bacteria 2
A brief historical reflection on the taxonomy of entomopathogenic nematodes and bacteria 3
The life cycle of *Heterorhabditis* and *Steinernema* 4
The genus *Photorhabdus* 6
The genus *Xenorhabdus* 8
Secondary metabolites of *Photorhabdus* and *Xenorhabdus* 10
Polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) 14
Outline of the work 17
Chapter 1 19
Bacterial biosynthesis of a multipotent stilbene
Chapter 2 43
A type II polyketide synthase is responsible for anthraquinone biosynthesis in *Photorhabdus luminescens*
Chapter 3 62
Xenofuranone A and B: Novel phenylpyruvate dimers from *Xenorhabdus szentirmaii*
Chapter 4 73
Heterologous expression of a silent indigoidine synthetase from *Photorhabdus luminescens* TT01 and identification of two putative genes involved in indigoidine production
Statement about the author´s efforts in chapters 1-4 88
Discussion 89
Stilbenes 91
Anthraquinones 101
Xenofuranones 109
Reference List 111
Danksagung 125

Abstract

The entomopathogenic bacteria of the genera *Photorhabdus* and *Xenorhabdus* display perfect model organisms to gain insights into the sophisticated interplay between symbiosis and pathogenicity. Moreover, numerous publications in the last years have demonstrated that these bacteria represent a rich source of secondary metabolites, which is exemplified in this work with the description of the novel xenofuranone compounds.

The recently available genome sequence of *Photorhabdus luminescens* TT01 pointed out that many biosynthetic gene clusters remain silent as the corresponding product cannot be detected. The heterologous expression of a nonribosomal peptide synthetase, which resulted in the successful production of indigoidine, depicts one way to gain access on these cryptic gene clusters. In addition the genome sequence also enabled the identification of biosynthesis genes of the already known compound families of stilbenes and anthraquinones. Thereby a type II polyketide synthase cluster was identified, which is responsible for anthraquinone biosynthesis, representing only the second known type II PKS derived compound from a Gram-negative bacterium. Furthermore the identification of genes involved in stilbene biosynthesis led to the discovery of a unique and novel pathway, strongly differing from plant derived stilbenes.

Zusammenfassung

Entomopathogene Bakterien der Gattungen *Photorhabdus* und *Xenorhabdus* eignen sich hervorragend als Modelorganismen um Einblicke in das komplizierte Wechselspiel zwischen Symbiose und Pathogenität zu erhalten. Darüber hinaus haben zahlreiche Publikationen der letzten Jahre gezeigt, dass diese Bakterien eine reiche Quelle an Sekundärstoffen darstellen. In der vorliegenden Arbeit wird dies anhand der neu beschriebenen Xenofuranone verdeutlicht.

Die veröffentlichte Genomsequenz von *Photorhabdus luminescens* TT01 offenbarte, dass die meisten Biosynthese Gencluster „verwaist“ sind, das heißt es ließ sich bisher kein dazugehöriges Produkt detektieren. Die erfolgreiche heterologe Expression einer nichtribosomalen Peptidsynthetase und der damit verbundenen Produktion von Indigoidin, zeigte eine Möglichkeit auf um Zugang zu solchen „verwaisten“ Genclustern zu erhalten. Des Weiteren erlaubte die Genomsequenz nach Biosynthesegenen zu suchen, deren Produkte wie zum Beispiel die Stilbene oder die Anthrachinone bereits bekannt waren. Auf diese Weise konnte ein Typ II Polyketidsynthasecluster der für die Biosynthese der Anthrachinone verantwortlich ist identifiziert werden. Die Anthrachinone sind damit erst das zweite bekannte Beispiel eines Typ II PKS erzeugten Produktes aus einem Gram-negativen Bakterium. Zusätzlich gelang es die Gene, die an der Stilbenbiosynthese beteiligt sind zu identifizieren und damit einen neuen und einzigartigen Stoffwechselweg, welcher stark abweichend zur pflanzlichen Biosynthese funktioniert zu beschreiben.

Introduction

Natural products and entomopathogenic bacteria

Today no one can think of a life without drugs, as they have become indispensable agents helping to overcome and cure from numerous diseases and pathological dysfunctions. A pivotal role hereby refers to natural products as they have evolved in a biological context including structural complexity and stereochemistry.[13] Although synthetic compounds are obtained in greater numbers and less time, they do not reach the relevance of natural products in the pharmaceutical area. Namely, 60% of the drugs that have reached the market over the past 20 years have their origins in nature.[108] Among them are well known drugs of cancer treatment like taxol and doxorubicin, but also anti-infectives like tetracycline and vancomycin. The significance of natural products is underlined by the fact that 68.3% of anti-infective and even 79.8% of cancer therapy drugs are natural products or inspired by natural products.[33] Additionally to their medical relevance they also play an important role in agriculture where they are used for treating crop pests.[31]

The discovery of penicillin by Alexander Fleming was a milestone and the initiation of natural product research. Since then more than 50.000 natural products with a molecular weight less than 2500 have been characterized from microorganisms.[37] More than half of them are produced by bacteria of the genus *Streptomyces*. Many of these bacterial natural products also referred to as secondary metabolites play an essential role in pharmaceutical applications as antibiotics, cytostatics or immunosuppressive agents.[37,38,134] Unfortunately, the misuse of antibiotics led to an increase of multidrug-resistant bacteria with the effect that former controllable or almost vanished diseases become a menace again.[200] But also threats of new diseases brought about searching for unexplored sources of new natural products.[56,146]

A sustainable way to discover new natural products is to look at the known and cultivable strains. However, a problem to cultivable microorganisms is that many biosynthetic genes remain dormant under standard laboratory conditions. The optimization of culture conditions or heterologous expression of these so called silent or cryptic pathways is one approach to obtain new metabolites.[75] But even the co-cultivation of other bacteria or mimic of the ecological environment can help to successfully induce silent pathways.[162] Another way to novel natural products is delivered by the metagenome technology, in which DNA is extracted from environmental samples and cloned into large insert libraries.[35] It is estimated that only less than 1% of all microorganisms have been cultivated, due to cultivation difficulties.[180] The metagenome technology opens the access to make the genetic and

functional diversity of these uncultured microorganisms available and provides a rich resource for novel natural chemistry.[71,156] With this technology it is also possible to gain access to the potential resource of bacterial symbionts, for example in the microflora of insects or marine sponges.[51,193] Symbiotic bacteria can be found in many associations with eukaryotes and have been proved to be a rich source of secondary metabolites.[148] However, most of them are unculturable with traditional cultivation methods and only available through metagenome approaches.

One exception represent the auspicious and barely investigated entomopathogenic bacteria *Photorhabdus* and *Xenorhabdus* in the order of Enterobacteriaceae.[198] They unite the advantage of being easily cultivable with or without host and display appropriate model organisms to study the change of symbiotic and pathogenic relationships.[28,55] Together with their symbiotic nematode partner they form a deadly and effective alliance against many soil living insects. Their ecological niche is adapted to eliminate saprophytic competitors and therefore they represent a potential source of antibacterial, antifungal, insecticidal and nematicidal compounds, which might become promising pharmaceutical antibiotics or biocides.

Moreover, they have been established as an alternative biological control agent for plant protection in place of synthetic insecticides, which is also indicated by the onset of commercial mass production.[47] The increasing danger of insecticide resistance and bioaccumulation of synthetic pesticides in food are growing problems of the human society.[5,121,136] The application of entomopathogenic nematode/bacteria associations which act specific against certain insects and leaving beneficial animals unaffected add further to the interest in these organisms.

A brief historical reflection on the taxonomy of entomopathogenic nematodes and bacteria

The genera *Photorhabdus* and *Xenorhabdus* are entomopathogenic bacteria living in symbiotic mutualism with **e**ntomo**p**athogenic **n**ematodes (EPN) and are exclusively associated with the genera *Heterorhabditis* and *Steinernema*. Taxonomically these bacteria are classified into the γ-subclass of Proteobacteria in the family of Enterobacteriaceae.

Nematode parasites of insects have been known since the 17th century, but in 1929 it was Glaser and Fox who first isolated a nematode from a beetle grub of *Popillia japonica*, which was described in the same year by Steiner as *Neoaplectana* and which should later become the genus *Steinernema*.[172] But it took until 1965 before Poinar and Thomas were able

to isolate a symbiotic living bacterium from the intestinal lumen of a nematode, which was identified as a new species and assigned into the genus *Achromobacter* named *Achromobacter nematophilus*.[186] As the 1926 introduced genus *Achromobacter* combined a number of different species including Gram positive and negative bacteria, the generic name was rejected in 1974 as not well defined and a *nomen dubium*.[72]

In the meantime several new bacterial strains were obtained from the nematode genus *Neoaplectana*, but also from a nematode belonging to a different and new entomopathogenic genus assigned *Heterorhabditis*. In 1979 as a causality of the generic rejection and in order to accommodate all these entomopathogenic and nematophilic bacteria Thomas and Poinar erected the new bacterial genus *Xenorhabdus*. The name is composed of the Greek noun xenos meaning “enemy stranger” and the Greek/Latin noun rhabdos/rhabdus meaning “rod” referring to the life habitat and phenotype of these bacteria.[185] The new isolated heterorhabditidae associated bacterium was named *Xenorhabdus luminescens* due to its ability to produce bioluminescence. But very soon it became obvious that this new strain showed many differences compared to the rest of the genus *Xenorhabdus*, apart from its different nematode host and capability of bioluminescence. Therefore, in 1993 Boemare introduced the new genus *Photorhabdus* to cope with the outstanding characteristics of this bacterium.[14] This fact led to a pleonasm in the species name *Photorhabdus luminescens.*

The genera *Photorhabdus* and *Xenorhabdus* endure to date and encompass all isolated bacteria from entomopathogenic nematodes of *Heterorhabditis* and *Steinernema*, respectively.

The life cycle of Heterorhabditis and Steinernema

The life cycle of Heterorhabditidae and Steinernematidae is very similar in most features. Both form a so called **i**nfective **j**uvenile (IJ), also called **d**auer **j**uvenile (DJ), a nonfeeding (mouth and anus are closed), long-term survival, soil-dwelling and developmental arrested stage of the nematode, representing the only stage of the nematode outside of the insect. In this stage the IJ outlasts in the soil and waits for a suitable insect prey.

One can distinguish between two different foraging behaviours, namely a cruiser and an ambusher strategy.[24] The ambusher or sit-and-wait strategy is followed by *Steinernema carpocapsae*, which means the nematode waits passively near the soil surface until a suitable insect larva passes and uses the chance to enter the insect through natural openings like mouth, anus and spiracles. *Heterorhabditis bacteriophora* practices a cruiser strategy,

meaning the nematode seeks actively for prey in the soil attracted due to volatile signal molecules.[140,150] Unlike *Steinernema* species it also possesses a dorsal "tooth"- like structure which even enables it to penetrate through the intersegmental sections of the insect integument.[94]

Before entering into the insect the nematode strips off its outer cuticula to uncover mouth and anus. Once inside the prey the bacteria are released into the haemocoel. In case of *Heterorhabditis* it could been shown that after a lag phase of 30 minutes the IJ regurgitates the bacteria, which are located in the intestine of the nematode, in a pulsatile and staggered manner at an average rate of one cell every two minutes.[26] In Steinernematidae the bacteria are kept in special vesicles in the intestine and the bacteria are released by defecation through the anus when the nematode begins to ingest haemolymph.[10,169]

Both nematode and bacteria overcome immune response by a variety of different mechanisms which kill the insect due to septicaemia within 48 hours. Many of these mechanisms remain to be characterized but some of them are disclosed in the following sections. Inside the host the bacteria multiply very fast and help to exploit and decompose the organic matrix of the insect, thereby supporting the reproduction of the nematodes. In addition, the bacteria themselves are also a nutrient source for the nematodes. Moreover, they help to protect the insect carcass from infection with opportunistic competitors by secreting metabolites into their environment.

Within this environment the nematode undergoes a very complex life cycle. After entering the host it exits the dauer stage triggered by a food signal which is produced by the bacteria.[179] This change from nonfeeding to feeding stage is also called "recovery". When recovery takes place the nematode develops into a fourth stage juvenile J4 before becoming a male or egg laying female adult. The insect domiciliated nematodes reproduce for 2-4 generations within the insect running from the egg through different juvenile stages J1, J2, J3, J4 and adult (Figure 1). On the other hand, nematodes of the genus *Heterorhabditis* are able to use another additional and extraordinary way to progeny in which IJs turn into self-fertile egg laying hermaphrodites. Thereafter the offspring can also develop into hermaphrodites, females and males, but the development of IJs is mainly restricted to intrauterine hatching and matricide in hermaphrodites, also known as *endotokia matricida* (Figure 1).[24,27,91]

Finally, when the nutrients are depleted the nematodes develop into the IJ form. The bacterial symbiont of *Heterorhabditis* is transmitted maternally, as the IJs consume the bacteria enriched cavity of the mother nematode. Only one or two *Photorhabdus* cells colonize per IJ and reproduce within the nematode to a mature population of 50 to 150 cell

forming units per IJ.[69] The source of IJ colonizing bacteria in *Steinernema* has not been investigated so far, but the colonization process is analogue to the *Heterorhabditis-Photorhabdus* event.[122] Thus, dependent on the size of the insect larva up to several hundred thousand IJs emerge from the disembowelled insect cadaver.[187]

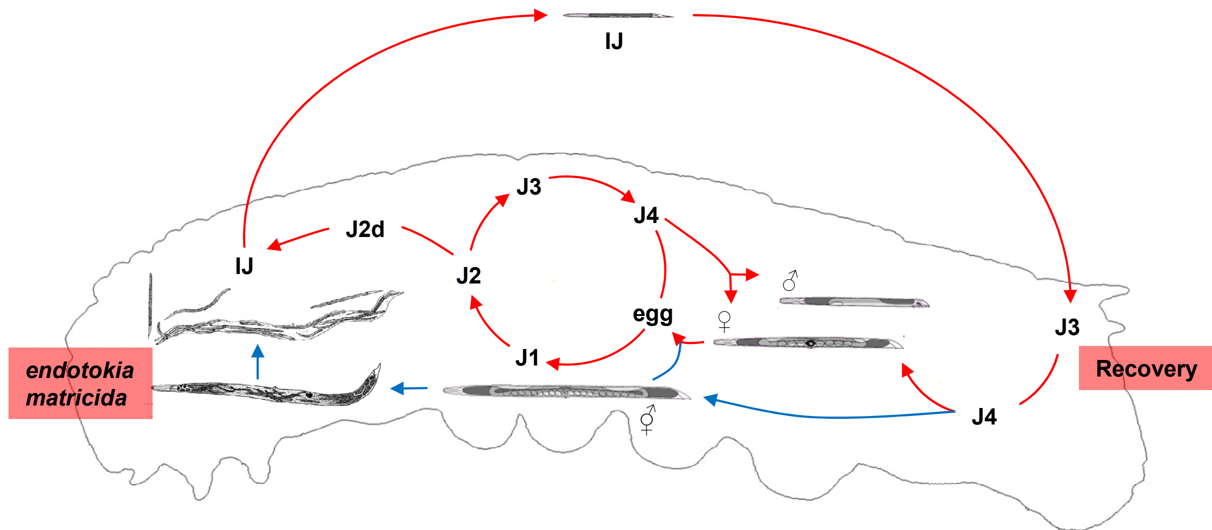


Figure 1. Life cycle of *Steinernema* and *Heterorhabditis*. The entomopathogenic phase is induced by the recovery of the IJ. The nematodes undergo different developmental stages before developing into IJs again (highlighted by red arrows). *Heterorhabditis* is able to undergo an alternative way to progeny, in which hermaphrodites occur (highlighted by blue arrows). Here IJs are generated by intrauterine hatching and matricide, a process called *endotokia matricida*.

The genus Photorhabdus

The genus *Photorhabdus* comprises the three species *Photorhabdus luminescens*, *Photorhabdus temperata* and *Photorhabdus asymbiotica*, which all include several further subspecies and are always, associated with nematodes of the genus *Heterorhabditis*. *Photorhabdus* forms Gram-negative, asporogenous, rod-shaped (2-6 × 1-1.4 µm) peritrichious cells. All strains are mesophilic bacteria with an optimal growth temperature at 28-30 °C, only some strains exhibiting an even broader temperature range between 16-38 °C.[53,145] The fact that *Photorhabdus* cells cannot reduce nitrate but can produce iso-branched fatty acids is very uncommon to Enterobacteriaceae and keeps them detached from other genera in this family.[89,182]

Another uncommon and remarkable feature is their capability to produce bioluminescence. The bioluminescence is obtained by a set of *lux* genes, which share high syntheny to lux genes from the phylogenetically distant genera *Vibrio* and *Photobacterium*, suggesting a horizontal gene transfer.[63] Nevertheless, *Photorhabdus* is currently the only

known non-marine luminous bacterium, but the biological function of the produced bioluminescence is still elusive.

Photorhabdus is also marked by another laboratory relevant trait. One can observe a variation in colony forms after prolonged subculturing, a primary and a secondary form which are also referred to as phase 1 and phase 2 forms. The primary form is displayed by pigmentation, the adsorption of dyes like bromothymol blue or neutral red, the ability to constitute inclusion bodies and to bioluminesce, but also to produce lipases, phospholipases and proteases. The secondary form has lost these characteristics and can be further discriminated by a decreased support of nematode growth and antibiotic production. It has been shown that isolates from nematodes are always in primary form and the conversion is unidirectional from primary to secondary form.[66,192]

In principle all *Photorhabdus* isolates are obtained from infected insects or their associated nematode host and no free living strains have been detected yet. However, some isolates of human wounds draw attention as clinical relevant strains causing invasive soft tissue and disseminated bacteremic infections. It was the first time that the bacterium was not recognized to be associated with a nematode and according to this the strain was given the name *P. asymbiotica*. But the epithet turned out to be a misnomer, as in 2006 Gerrard *et al*. succeeded in identifying the nematode symbiont.[64] For all that, the reports of *Photorhabdus asymbiotica* isolates from human wounds revealed that this strain represents an opportunistic human pathogen, in which the nematode part during infection is still unclear.[50,65]

A very important part in the pathogenicity of *Photorhabdus* is provided by the production of insecticidal toxins, whereof one family are the toxin complex (Tc) proteins, which consist of high molecular weight proteins. These toxins mediate an oral activity against many insects and represent an alternative to the entomopathogenic *Bacillus thuringiensis* toxins, as one protein was already expressed in transgenic plants conferring insect resistance.[194,195] Another effective protein is the ***m**akes **c**aterpillar **f**loppy* (Mcf) toxin, which derived its name from the phenotype, which is induced by Mcf treatment that leads to a rapid loss of body turgor making the caterpillar floppy. Mcf is a potential toxin inducing apoptosis on the insect haemocytes and midgut epithelium. The extraordinary potency of Mcf was evinced by the conclusion that non-entomopathogenic *E. coli* harbouring the *mcf* gene were able to persist within the insect and furthermore were even able to kill the insect.[34,42]

However, small molecules play also an important part in pathogenicity and symbiosis. Watson *et al*. succeeded in showing that an *exbD* homologue, a component of the TonB complex, is needed for the uptake of small iron scavenging siderophore molecules in

Photorhabdus temperata. A mutation in this gene generated a less virulent phenotype that was unable to support nematode development.[197] Further evidence of the involvement of small molecules in symbiosis was gained from a mutation in the gene *ngrA*. This gene encodes a phosphopantetheinyltransferase that is required to activate polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), which are responsible for the production of secondary metabolites.[25] The mutation resulted in a phenotype unable to support IJ recovery and let presume that small molecules also act as signalling molecules. A detailed overview on secondary metabolites of *Photorhabdus* is given in the section of secondary metabolites.

The life cycle of *Photorhabdus* implicates a switch between mutualism in the nematode and pathogenicity in the insect as well. This change is subject to regulatory networks and some regulatory proteins have been described. A mutation in the gene *pgbE1* that is part of the seven gene *pgbPE* operon rendered *Photorhabdus* to an attenuated virulence. It could also be shown that pgbE1 is required for a proper colonisation of the IJ.[9] The already alluded phase variation is also discussed as an adaptation to the respective virulent or symbiotic phenotype. A mutation in the *hexA* gene, a transcriptional regulator, was sufficient to restore most of primary-specific characteristics in a secondary form, whereas overexpression of *hexA* in the primary form triggered a conditional phenotypic variation towards secondary form.[92,93] But phase variation is also influenced by the AstR-AstS two-component signal transduction system (**a**daptation to **st**ationary-phase **r**egulator and **s**ensor), which positively regulates the universal stress proteins UspA, UspB and UspC. These stress proteins help to respond and protect on changes in the environment. Mutants in the *astR* gene were shown to undergo phase variation much earlier than the wildtype.[40]

The genus Xenorhabdus

The genus *Xenorhabdus* comprises of twenty highly diverse species *X. nematophila* [186], *X. bovienii*, *X. poinarii*, *X. beddingii*,[1] *X. japonica* [137], *X. budapestensis*, *X. ehlersii*, *X. innexi*, *X. szentirmaii*,[111] *X. cabanillasii*, *X. doucetiae*, *X. griffiniae*, *X. hominickii*, *X. koppenhoeferi*, *X. kozodoii*, *X. mauleonii*, *X. miraniensis*, *X. romanii*, *X. stockiae* and *X. indica*.[175,184] All *Xenorhabdus* strains have been found in a mutualistic association with nematodes of the genus *Steinernema*. The cells are rod shaped, mesophilic, Gram-negative, asporogenous and peritrichous flagellated. Another striking characteristic is the occurrence of proteinaceous crystalline inclusion bodies in the stationary phase. One of these crystal proteins in *Xenorhabdus nematophila* is encoded in the gene *pixA*, but unlike the crystal proteins of

Bacillus thuringiensis they show no insecticidal activity and therefore it is hypothesized that they might help to support the growth of the nematode host.[74]

The genus *Xenorhabdus* is highly isolated in the family of Enterobacteriacea. There is only a 4% DNA/DNA relatedness to the type species of the type genus of Enterobacteriaceae. In addition they are unable to reduce nitrate and lack the enzyme catalase, which both are positive characteristics of other genera in this family.[49] Nevertheless, the affiliation to the family of Enterobacteriaceae is confirmed by phylogenetic analyses based on 16S rDNA and the existence of the enterobacterial common antigen.[18,149]

In *Xenorhabdus* as well as in *Photorhabdus*, insecticidal toxins contribute to the pathogenicity against insects. The cytotoxin *Xenorhabdus* α-xenorhabdolysin was purified from *X. nematophila* and showed an apoptotic and haemolytic activity. This cytotoxin is encoded by two genes *xaxA* and *xaxB* and homologues thereof can also be found in other entomopathogenic bacteria like *Photorhabdus* and *Pseudomonas entomophila*.[189]

The life cycle of *Xenorhabdus* comprises a switch between mutualism and pathogenesis. Some of the responsible elicitors could be described in the last years. For example, the CpxRA two-component system, representing a histidine kinase dependent signal transduction system was revealed to be a regulator between mutualism and pathogenicity in *Xenorhabdus nematophila*. Mutants in the CpxRA system were shown to be less virulent towards insects and exhibited a reduced expression level of the gene *mrxA*, which encodes a fimbrial subunit that is needed for the efficient colonization of the nematode host gut.[23,73] Another global regulator is Lrp, the leucine-responsive regulatory protein. This regulator was shown to have a great impact on the expression of more than one hundred proteins, leading to an attenuated virulence against insects and the inability to colonize and grow within the nematode host. Moreover, Lrp seems to affect the phase shift of *Xenorhabdus nematophila*.[32] The phenotypic variation of *Xenorhabdus* is a congruent trait to *Photorhabdus*, but unlike in *Photorhabdus* the phase switch is to some extent reversible in *Xenorhabdus*.[67]

Recent findings denoted that motility and regulation of motility are connected with the virulence of *Xenorhabdus nematophila* towards insects. For this it could be shown that mutants of *lrhA* encoding a transcriptional regulator exhibited a less virulent phenotype and a reduced swimming motility.[154] This connection is also confirmed by the identification of FliZ as an indirect activator of flagellin production and consequently responsible for motility, but moreover as direct regulator of the cytotoxin encoding *xaxAB* operon.[106]

Insight into the mechanism how the bacteria overcome the insect immune response was delivered by the observation that a bacterial compound, presumably benzylideneacetone

(see also next section) inhibits the insect phospholipase A_2.[103] This phospholipase plays an important role in the insect immune system as it turns on the eicosanoid biosynthesis pathway by hydrolyzing arachidonic acids from cellular phospholipids, which then mediate phagocytosis and nodulation.[142,143] This points out those secondary metabolites are conducive in the interchange between symbiosis and pathogenicity.

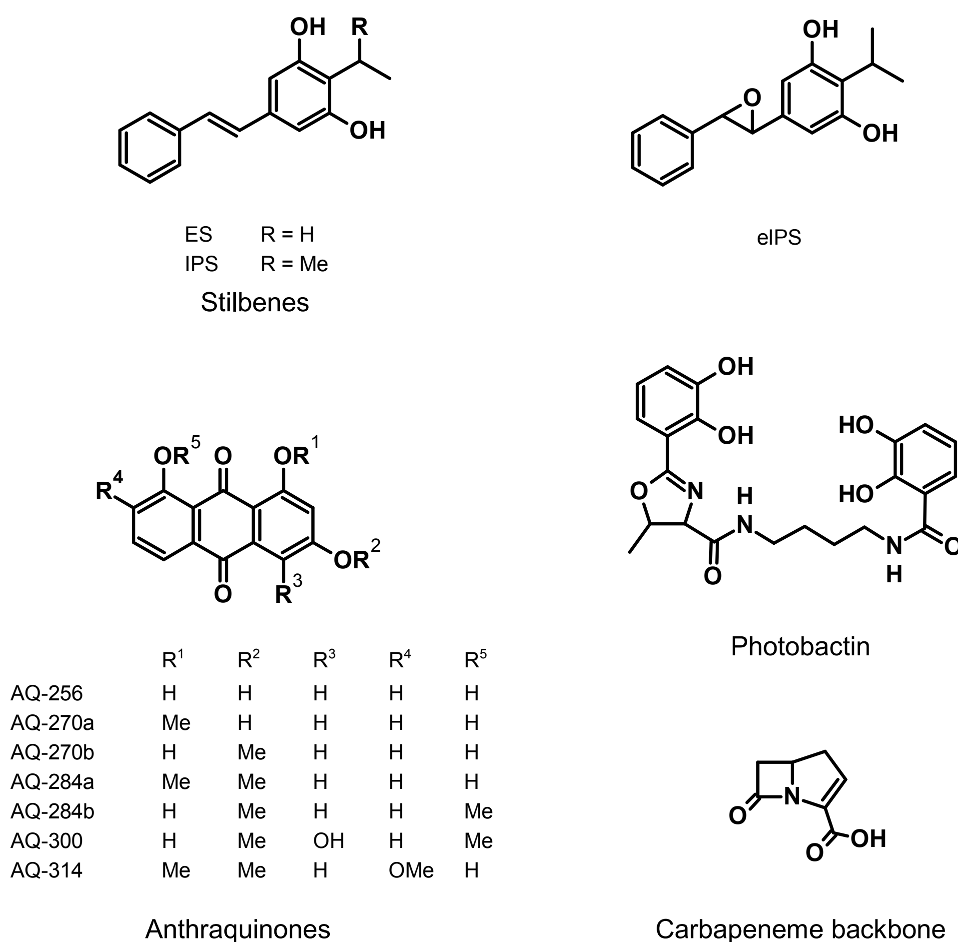
Secondary metabolites of Photorhabdus and Xenorhabdus

More than fifteen compound classes have been isolated from *Photorhabdus* and *Xenorhabdus* in the last three decades denoting a high structural biodiversity and specificity among these compounds.[17]

Four classes of compounds are specifically described in the genera *Photorhabdus*. Derzelle *et al*. were able to identify a gene cluster in *Photorhabdus* responsible for the biosynthesis of carbapenems, a class of *β*-lactam antibiotics that have a carbon in place of sulphur in the 5-membered ring system. This antibiotic class showed activity against a few Gram-negative bacteria.[39] The first isolated secondary metabolites from *Photorhabdus* were stilbenes namely 2-isopropyl-5-[(*E*)-2-phenylethenyl]benzene-1,3-diol (IPS) and 2-ethyl-5-[(*E*)-2-phenylethenyl]benzene-1,3-diol (ES),[144] a class of multipotent compounds which are part of this work and which will be described in detail in chapter 1 and the discussion. An epoxidized form of IPS 2-isopropyl-5-(3-phenyl-oxiranyl)benzene-1,3-diol (eIPS) was isolated recently from infected insect larvae and showed activity against many bacteria and even against a drug-resistant clinical strain of *Staphylococcus aureus*. Additionally, cytotoxicity against some human cancer cell lines was also observed.[84]

Yellowish to reddish pigments conferring the characteristic colour to most *Photorhabdus* strains are originated from a type II derived polyketide synthase (PKS), which forms polyaromatic anthraquinones.[16,155] These pigments show a weak antibacterial activity,[117,183] but the main mode of function is not yet unravelled. The biosynthesis of anthraquinones is described in chapter 2 and is also part of the discussion.

As mentioned before *Photorhabdus* also produces a catecholate siderophore named photobactin which contributes to the antibiosis in the insect cadaver.[25]



	R1	R2	R3	R4	R5
AQ-256	H	H	H	H	H
AQ-270a	Me	H	H	H	H
AQ-270b	H	Me	H	H	H
AQ-284a	Me	Me	H	H	H
AQ-284b	H	Me	H	H	Me
AQ-300	H	Me	OH	H	Me
AQ-314	Me	Me	H	OMe	H

Figure 2. Secondary metabolites of *Photorhabdus*. 2-isopropyl-5-[(*E*)-2-phenylethenyl]benzene-1,3-diol (IPS), 2-ethyl-5-[(*E*)-2-phenylethenyl]benzene-1,3-diol (ES) and 2-isopropyl-5-(3-phenyl-oxiranyl)benzene-1,3-diol (eIPS). As the carbapeneme derivative has not been isolated yet, only the basic structure is displayed.

Most of the isolated and elucidated secondary metabolites are originated from the genus *Xenorhabdus* in particular from *X. nematophila* and *X. bovenii*. Benzylideneacetone a small and heat stable molecule was identified from *X. nematophila*. Although this compound has been known for a long time before and even is used as food and cosmetic flavouring additive, it was never related to antibacterial activity. In this context it was the first report as an antibiotic with activity against several Gram-negative plant-pathogenic bacteria.[90] More pregnant was the evidence that benzylideneacetone acts as phospholipase A_2 inhibitor which mediates immunosuppression in the insect and consequentially might enhance the virulence of the bacteria and nematode complex.[103]

The xenocoumacins 1 and 2 are the major antibiotics in *X. nematophila.* They exhibit a broad biological spectrum as they show antibacterial activity against many Gram positive bacteria and bear a strong antiulcer activity. In addition xenocoumacin 1 also mediates an antifungal activity.[126] Very recently the biosynthesis gene cluster of xenocoumacins was

identified and revealed several independent transcriptional units. This seems consistent with the detection that xenocoumacin 2 is derived biosynthetically from xenocoumacin 1 in a much later phase of growth.[12,151,152]

The xenorhabdins are a class of natural products which belong to the class of pyrrothines that endow an unusual dithiol moiety. Pyrrothines with different moieties are also known from other bacteria, like thiomarinol from the marine *Alteromonas rava*.[81] Xenorhabdins were isolated from *X. nematophila* and *X. bovienii*, whereas the oxidized xenorxide derivatives are only known from the latter one. Several xenorhabdin derivatives have been described to confer antibacterial, antifungal and insecticidal activity.[114,125]

Other secondary metabolites that have been isolated are the indol derived compounds from *X. bovienii* and nematophin from *X. nematophila*.[112,113,115,144] All of them display good antibacterial activity particularly against the clinical relevant strain *Staphylococcus aureus*. Moreover, some of them are active against fungi of medical and agricultural importance.[113,116] The phenylacetamides are also another class of compounds which were isolated from *X. nematophila*.[141] These compounds own a significant cytotoxicity against several human cancer cell lines, which is mediated by apoptosis through activation of caspase, a cysteine protease.[88]
Most of the discussed compounds are small molecules and have a low molecular weight but recently the first peptides xenortides and xenematide could be isolated from *X. nematophila* showing a moderate insecticidal activity.[105] At the same time Reimer *et al.* succeeded in identifying the corresponding biosynthetic gene cluster, which consists of nonribosomal peptide synthetase (NRPS) enzymes (Reimer *et al.*, unpublished). In the same way a family of new linear peptides named rhabdopeptides were identified (Reimer *et al*., unpublished).

Two other natural products from the genus *Xenorhabdus* were made accessible from the recently described *X. szentirmaii*. Colonies of *X. szentirmaii* have a striking phenotype by their purple metallic colour. This colour is originated from the already known phenazine pigment iodinin which was first isolated from *Chromobacterium* strains.[29,30] However, the most abundant compounds in *X. szentirmaii* are represented by the xenofuranones whose biological function is completely unknown.[15]

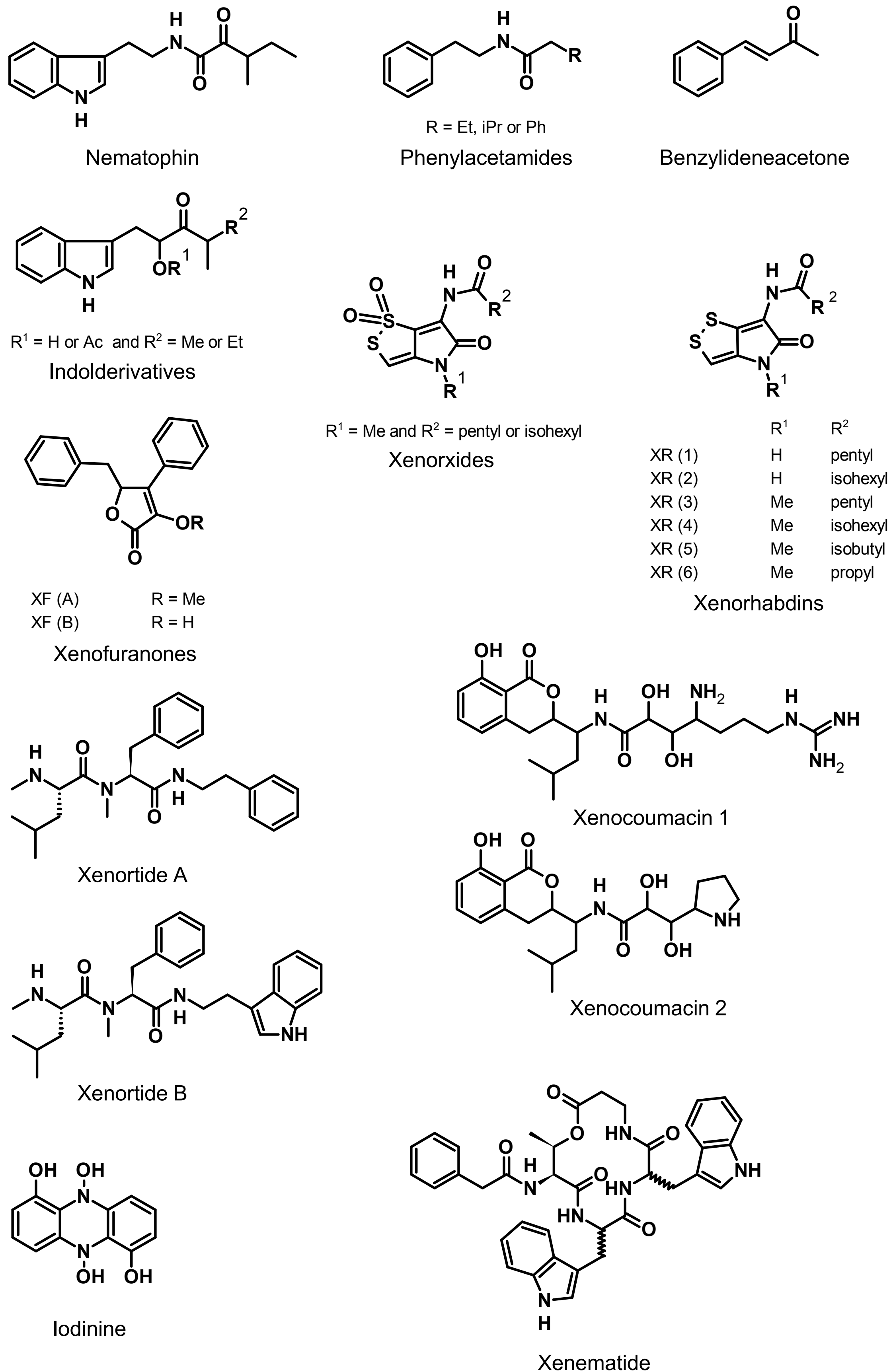


Figure 3. Secondary metabolites of *Xenorhabdus*

Polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS)

Many secondary metabolites are synthesized by special enzymes for example anthraquinones through a **p**oly**k**etide **s**ynthase (PKS), xenematides through a **n**on**r**ibosomal **p**eptide **s**ynthetase (NRPS) and xenocoumacins through a NRPS/PKS hybrid. Both, PKS and NRPS have in common that they work like an assembly line in which extender units are modified and connected through the action of special enzyme activities.[20]

The biosynthesis of polyketides is similar to the biosynthesis of fatty acids through the **f**atty **a**cid **s**ynthase (FAS). In contrast to FAS, PKS show a variable reduction of the resulting β-ketoacyl derivative and have access to a larger pool of extension and starter units.[173]

Today PKSs are separated into three types based on their functional organization. Type I PKS are large proteins which carry different enzymatic activities, also referred to as domains. A set of domains which is needed for the incorporation of one extender unit is termed module. A polypeptide can consist of several modules; thereby the nascent polyketide product stays in colinearity to the protein assembly line.[82] Type II PKS are discrete dissociable proteins which only own one enzymatic activity or domain, respectively. This type of PKS resembles the bacterial type II FAS. The discrete proteins work synergistically and can be used iteratively, thus the final product cannot be predicted.[77] Type III PKS also known as chalcone synthase (CHS) and stilbene synthase (STS), represent an enzyme family which produces many plant pigments and other common plant metabolites. Originally, their occurrence was only restricted to the plant kingdom, but since then a growing number of type III PKS have also been found in bacteria. Substantial differences to the PKS types explained before consist in the utilization of coenzyme A (CoA) esters instead of acyl-carrier protein (ACP) bound derivatives. Secondary, the catalytic activity of a single active site takes control of the complete biosynthesis including decarboxylation, condensation, cyclisation and aromatisation reactions.[4,165]

The following section will focus on the machinery of a type II PKS, as this type is most relevant for this work. Polyphenolic ring systems are typical type II PKS products, such as in actinorhodin or tetracycline. This type of PKS consists of several invidiual enzymes. A set of three enzymes, a ketosynthase α (KS_{α}), a ketosynthase β (KS_{β}) and an acyl carrier protein (ACP) were shown to be sufficient to produce the polyketide backbone when used iteratively. An instance which termed these set of enzymes minimal PKS.[77] Thereby the ACP has to be transformed from an inactive *apo*-form into an enzymatically active *holo*-form. This

activation step is catalyzed by a phosphopantetheinyltransferase (PPTase) which transfers a 4′-phosphopantetheinyl moiety of coenzyme A onto the β-hydroxygroup of a highly conserved serine residue of the ACP.[104,131] It is generally accepted that both ketosynthases form a heterodimer that moulds an interior pocket to protect the nascent polyketide from spontaneous condensations. With only few exceptions the chain extension initiates with acetate.[132] The origin of the starter unit is still a matter of debate as type II PKS lacks a starter unit loading acyltransferase (AT) like in type I PKS assembly lines. One possible mechanism is the decarboxylation of malonyl-ACP to acetyl-ACP by the KS_β domain which lacks the active cysteine site of the KS_α.[11] The acetyl group is then passed to prime the active KS_α (Figure 4). Afterwards the ACP is reloaded with a malonate extender unit again which subsequently acts as an electrophile that is transiently docked to the ACP until the KS_α driven carbon-carbon condensation is catalyzed. The β-ketoester is then transferred back to the KS_α and another cycle can begin (Figure 4).[77,178]

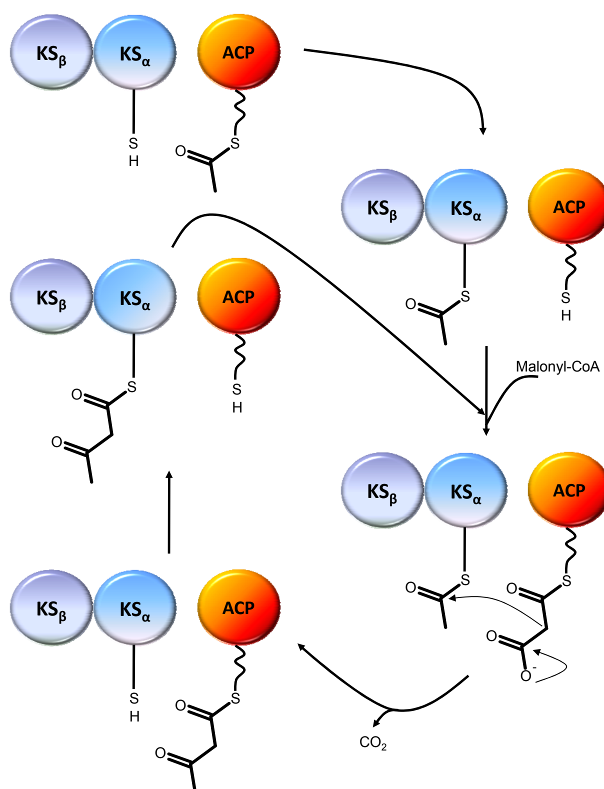


Figure 4. Polyketide synthesis by the type II minimal PKS. Priming of KS_α is presumably achieved by decarboxylation of malonyl-ACP to Acetyl-ACP by the KS_β domain. The acetyl group is then passed to the active site of the KS_α domain and the ACP is loaded with a malonate extender unit. The formation of an ACP bound β-ketoester takes place in a KS_α catalysed condensation reaction. The β-ketoester is then transferred to the KS_α domain again and with the reloading of malonate onto the ACP another extension cycle can begin.

The chain release factor for the final polyketide is still unknown as a thioesterase (TE) found in type I PKS is lacking.[77] Here, the TE facilitates hydrolysis of the final polyketide to the free acid or catalyses the intramolecular cyclisation to a macrolacton or macrolactam ring.[101] In type II PKS at least, it was shown that KS_{β} plays a role in determining the polyketide chain length and is therefore also called chain length factor (CLF) in literature.[19] Type II PKS gene clusters often comprise additional genes encoding for ketoreductases, cyclases or aromatases, which are crucial to define the polyketide folding pattern.[76] Furthermore the gene cluster can contain so called tailoring enzymes like oxygenases, glycosylases or methyltransferases, which are involved in post-PKS modifications. These modifications not only originate in a greater structural diversity, they also contribute to a proper bioactivity of the natural product.[127]

Nonribosomal peptides are synthesized by NRPS enzymes which are organized into domains and modules similar to PKS type I systems.[101] The loading of the starter and extender amino acid is adopted by the ATP dependent adenylation domain (A), which shows specificity for the activation of a certain amino acid.[177] The activated aminoacyl-AMP is then translocated to an ACP-analogue peptidyl-carrier protein (PCP), which also requires activation through a PPTase as described before. The peptide-bond formation is mediated by a condensation domain (C), which catalyses the nucleophilic attack of the amino group of the downstream activated amino acid onto the upstream activated aminoacylthioester. Successive amino acid incorporation into the growing peptide chain is accomplished by running through the remaining downstream located modules. The release of the final peptide chain is obtained by a TE leading to linear or cyclic peptides.[22,52,167]

Additional optional NRPS domains, which are located within single modules, increase the structural diversity of nonribosomal peptides. Examples for such additional domains are epimerization domains (E) which convert L- to D-amino acids, cyclisation domains (Cy) which facilitate the formation of heterocyclic elements like oxazoline and thiazoline rings from serine, threonine and cysteine and oxidation domains (Ox).[95,128,153,190] The utilization of more than 100 nonproteingenous amino acids through NRPS enzymes and post NRPS modifications allow the synthesis of an unimaginable number of different natural products.[87,158,170]

This structural diversity is expanded by the occurrence of NRPS/PKS hybrids, biosynthetic pathways in which NRPS and PKS modules directly interact with each other.[43,44]

Outline

The aim of this work was to obtain new insights into the secondary metabolism of *Photorhabdus* and *Xenorhabdus*. Therefore one part of this work was dedicated on the screening for new natural products of different *Photorhabdus* and *Xenorhabdus* strains. The other part was uniquely dedicated to the strain *Photorhabdus luminescens* and the identification of its major secondary biosynthetic pathways.

Since the pioneer work in the 1980s and early 1990s on natural products of *Photorhabdus* and *Xenorhabdus*, secondary metabolism became a stagnant research field in the last few years. Although both bacterial species are used as biological control agents in agriculture and thereby represent a potential source of bioactive molecules, no effort was put in the isolation of new metabolites and the elucidation of biosynthetic pathways of already known natural products.

The published genome of *P. luminescens laumondii* TT01 in 2003 confirmed once again the potential of *Photorhabdus* as a multiproducer of secondary metabolites. Thus, the genome annotation revealed that 7.5 % of the genome encode 22 different secondary metabolites associated gene clusters.[17,45]

The now available genome data of TT01 offers different approaches to gain more and new information of biosynthetic pathways and their secondary metabolites. Exploiting this fact, one major scope of this work was to identify the biosynthetic pathway of the bacterial stilbenes and anthraquinone pigments. Special interest was drawn on stilbene synthesis as *Photorhabdus* represents the only currently known non plant stilbene producer. However, stilbenes are very common plant metabolites which are originated through a type III PKS stilbene synthase, but a corresponding enzyme is absent in *Photorhabdus*. Therefore it was challenging to identify the required enzymes. First evidence of enzymes required for the production of stilbenes was given by a phenylalanine ammonium lyase (PAL) mutant generated by the group of David Clarke, which was also the starting point for the supplied work.[199]

Another focus was weighed on the red anthraquinone pigments of *Photorhabdus*. These polyaromatic polyketides are typical type II PKS derived compounds. The genome data revealed a single type II cluster, which is very rare in Gram-negative bacteria and so far only the second example known.[16,159] Accordingly, the main interest was to verify the biosynthetic origin of the anthraquinones, but also to gain a deeper insight into the mechanisms of this still not well understood type of PKS.

Attention was also paid to biosynthetic gene clusters for which no product could be assigned. As already pinpointed the genome analysis revealed 22 biosynthetic gene clusters, but only four compound classes were known at the beginning of this work. This instance indicates that many secondary products are still unidentified, because either they are produced in minor amounts and are difficult to isolate and detect by conventional analytic methods or they are not produced at all. Therefore it was also an effort to express one of these so called silent or cryptic gene clusters with the help of a heterologous host and to identify the corresponding product.

Chapter 1

The following article has been published in the
Angew Chem Int Ed Engl., Vol.47, No.10, 2008, pp 1942-1945

Bacterial biosynthesis of a multipotent stilbene

Susan A. Joyce, Alexander O. Brachmann, Itamar Glazer, Lea Lango, Gertrud Schwär, David J. Clarke*, and Helge B. Bode*

Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA
The definitive version is available at www3.interscience.wiley.com
DOI: 10.1002/anie.200705148

Chapter 2

The following article has been published in the
Chembiochem., Vol.8, No.14, 2007, pp 1721-1728

A type II polyketide synthase is responsible for anthraquinone biosynthesis in *Photorhabdus luminescens*

Alexander O. Brachmann,[a] Susan A. Joyce,[b] Holger Jenke-Kodama, [c] Gertrud Schwär,[a] David J. Clarke,[b] and Helge B. Bode*[a]

Copyright 2007 Wiley-VCH Verlag GmbH & Co. KGaA
The definitive version is available at www3.interscience.wiley.com
DOI: 10.1002/cbic.200700300

Chapter 3

The following article has been published in
J.Nat.Prod., Vol.69, No.12, 2006, pp 1830-1832

Xenofuranone A and B: Novel phenylpyruvate dimers
from *Xenorhabdus szentirmaii*

Alexander O. Brachmann,[†] Steven Forst,[‡] Ghazala M. Furgani,[§]
Andras Fodor,[⊥] and Helge B. Bode[†]*

Copyright 2006 Amercian Chemical Society
The definitive version is available http://pubs.acs.org
DOI: 10.1021/np060409n

Chapter 4

Heterologous expression of a silent indigoidine synthetase

from *Photorhabdus luminescens* TT01

and identification of two putative genes involved

in regulation of indigoidine production

(unpublished)

Summary

The entomopathogenic bacterium *Photorhabdus luminescens* TT01 is a potential producer of secondary metabolites exemplified by stilbenes and anthraquinones. Nevertheless, no indigoidine production was ever reported in *Photorhabdus* although genes similar to the *Erwinia chrysanthemi* indigoidine pathway can be also found in the genome of *P. luminescens* TT01. Heterologous expression of *indC* (*plu2185*) in *E. coli* led to the production of indigoidine and confirmed its function. Moreover, it could be demonstrated that the genes *indA* (*plu2187*) and *indB* (*plu2182*), which are thought to be involved in indigoidine biosynthesis in *E. chrysanthemi*, are not essential for indigoidine production in *Photorhabdus*. By contrast, the coexpression of several upstream of *indC* located genes revealed two putative genes *plu2183* and *plu2185* that might play a role in the regulation of indigoidine production.

Introduction

The blue diazadiphenoquinone pigment indigoidine is produced by a large group of different bacteria like *Corynebacterium insidiosum*, *Pseudomonas indigofera*, *Arthrobacter polychromogenes* and the phytopathogenic *Erwinia chrysanthemi*.[6,12] Blue pigmented cultures have been described as early as 1890, but the slow partial loss of the exocyclic nitrogen hindered the elucidation of the indigoidine structure.[4] Finally, synthetically produced indigoidine and its hydrolysis products provided evidence for its 5,5´-diamino-4,4´-dihydroxy-3,3´-diazadiphenoquinone-(2,2´) structure.[4,5] The characterization and identification of a nonribosomal peptide synthetase (NRPS) of *Erwinia chrysanthemi* involved in indigoidine production shed light on the biosynthetic origin.[9] NRPSs are widely distributed among bacteria and are associated with the biosynthesis of many peptide antibiotics. These enzymes are large proteins which consist of multiple catalytical domains for the incorporation and processing of amino acids. The indigoidine (ind) synthetase contains an adenylation domain (A), an oxidation domain (Ox), a peptidyl-carrier domain (PCP) and a thioesterase domain (TE). Recent *in vitro* experiments demonstrated that the adenylation domain of the indigoidine synthetase preferentially uses L-glutamine as substrate and only precursor for indigoidine production.[13] Based on this fact, the final indigoidine formation is presumably achieved through condensation of two intramolecular cyclised glutamines which are further oxidised (Figure 1).

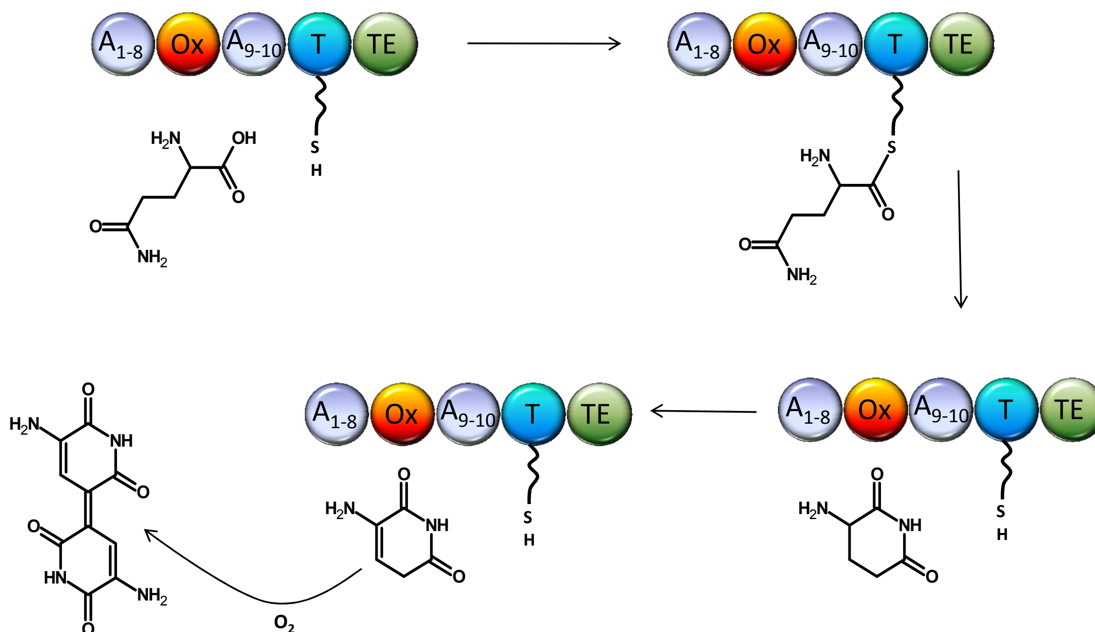


Figure 1. The proposed biosynthesis of indigoidine. Glutamine is activated by the indigoidine synthetase IndC, cyclised by an intramolecular amide bond formation and then oxidized to give 4-aminocyclohex-4-ene-1,3-dione. A condensation of two molecules forms the final indigoidine. The indigoidine synthetase is a single module nonribosomal peptide synthetase consisting of an adenylation domain (A); an oxidation domain (Ox), which is embedded between the conserved motifs 1-8 and 9-10 of (A); a peptide carrier protein (T) and a thioesterase (TE).

Genome analysis of *Photorhabdus luminescens* TT01 showed that homologues of *Erwinia chrysanthemi* indigoidine synthetase IndC and two other proteins IndA and IndB, both dedicated to the indigoidine biosynthetic pathway, are also existent in *Photorhabdus* (Figure 2). In *Erwinia* the genes *indA* and *indB* probably constitute an operon, but no specific function could be assigned to IndA whereas IndB shows similarity to phosphatases that are involved in the antibiotic synthesis of naphthomycin, ansatrienin and mitomycin C of some *Streptomyces* strains.[9] However, the absence of indigoidine in extracts of *Photorhabdus* implied that indigoidine production could be subject to a strong regulation mechanism or that *indC* is not functional at all. In order to elucidate these possibilities the corresponding genes *indC* and *indA* were heterologously expressed in *E. coli*. Furthermore a set of six genes including *indB*, which is located upstream of *indC* was also heterologously coexpressed.

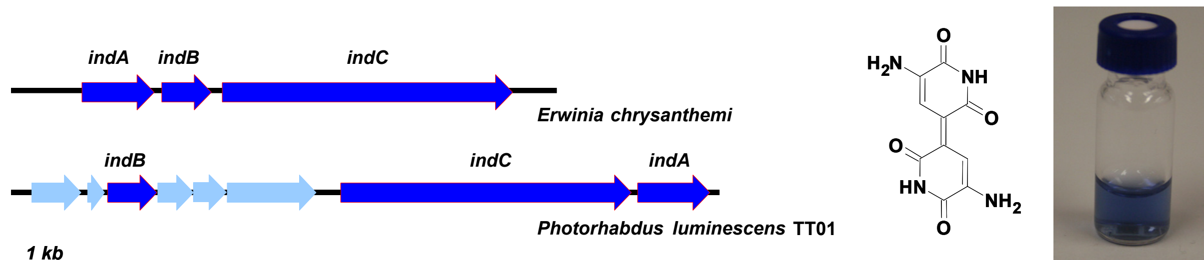


Figure 2. Organisation of the indigoidine biosynthesis gene cluster in *Photorhabdus luminescens* TT01 and *Erwinia chrysanthemi*, furthermore an indigoidine enriched extract from *E. coli*.

Results

Heterologous expression of *indC* and *indA* in *E. coli* EC100D *pir*-116. A 5150 bp fragment containing *indC* and *indA* was amplified from genomic DNA of *Photorhabdus luminescens* TT01 and cloned via restriction sites *Sph*I and *Sac*I into pUC18 plasmid. The amplification product also contained the original *indC* promotor. The obtained construct pUC18indCA was transformed by electroporation into *E. coli* EC100D *pir*-116 cells. For the activation of the PCP domain of IndC, the gene *mtaA* encoding a phosphopantetheinyltransferase was coexpressed on plasmid pSUmtaA containing a compatible selection marker and origin of replication.[2] Liquid cultures expressing both plasmids changed their colour into dark blue in less than 24 hours. Liquid cultures only expressing the pUC18indCA construct without coexpression of *mtaA* stayed unchanged (Figure 3). In order to prove if *indA* is essential for the biosynthesis the complete gene was deleted from plasmid pUC18indCA resulting in the new plasmid pUC18indC. Liquid cultures expressing pUC18indC and pSUmtaA were also able to produce the blue pigment without significant difference to the expression of pUC18indCA with pSUmtaA.

Figure 3. Heterologous expression of pUC18indCA in *E. coli* EC100D *pir*-116 coexpressed with pSUmtaA (left tube) and without pSUmtaA (right tube).

Identifiaction of indigoidine. After 24 h a 250 ml liquid culture of blue pigment producing *E. coli* EC100D *pir*-116 expressing pUC18ind and pSUmtaA was lyophilized. The dried extract was washed once with water and lyophilized again. For HRESIMS measurement the dried extract was dissolved in DMSO/Methanol (50:50) containing 1% formic acid and analysed via direct injection. Mass spectrum analysis yielded a peak at 248.05412 *m*/*z* corresponding to the molecular formula of indigoidine ($C_{10}H_8N_4O_4$) which was also consistent with the results of Takahashi *et al*.[13]

Heterologous coexpression of a set of six genes including *indB*. A cluster of six genes including *indB* is located upstream of *indC*. In order to test if *indB* or another gene of this cluster is involved in the production or regulation of the blue pigment those were coexpressed with pUC18indCA. For this, a fragment of 3898 bp containing the open reading frames (ORFs) *plu2180*, *plu2181*, *plu2182* (*indB*), *plu2183*, *plu2184* and *plu2185* was amplified and cloned via *Xba*I and *Sph*I into the *mtaA* free backbone of pSUmtaA resulting in plasmid pABcind. The coexpression of all three plasmids necessitated the modification of pSUmtaA to have three different selection markers and compatible origins of replication in hand. This was achieved through the exchange of the chloramphenicol resistance against a kanamycin cassette and exchange of the p15A ori against a *pir* dependent R6K ori resulting in plasmid pABmtaA02. A significant difference was observed in the production of the blue pigment by coexpression of pUC18indCA, pABcind and pABmtaA02 in comparison to pUC18indCA and pABmtaA02 without pABcind. The coexpression of pABcind led to a faint greenish colour of the liquid culture (Figure 4 and Table1). To exclude that this effect was only limited through the additional selection marker chloramphenicol of plasmid pABcind, the plasmid was replaced in a control experiment by the empty chloramphenicol conferring vector pSU19. However, coexpression of pSU19 displayed the similar phenotype to pUC18indCA and pABmta02, proving that the effect is linked to the genes of pABcind and not to the selection marker. Subsequently, single in frame deletions of each gene were generated in pABcind to determine which gene or genes were responsible for the observable change of indigoidine production. Each deletion construct was then coexpressed in triplicates with pUC18indCA and pABmtaA02. For deletion constructs pABcindΔ2180, pABcindΔ2181, pABcindΔindB and pABcindΔ2184 no significant differences were detected in comparison to pABcind cultures. Instead, pABcindΔ2183 and pABcindΔ2185 constructs exhibited similar phenotypes to the dark blue pUC18indCA and pABmtaA02 liquid cultures. The use of pUC18indC instead of pUC18indCA provided the same results in all experiments (Table 1).

Table 2: Plasmids used in this work

Plasmid	Genotype	Phenotype	Source / Reference
pUC18indCA	pUC ori, Ap^r, *plu2186*, *plu2187*	deep blue*	This work
pUC18indC	pUC ori, Ap^r, *plu2186*	deep blue*	This work
pABcind	p15A ori, Cm^r, *plu2180-2185*	greenish**	This work
pABcindΔ2185	pABcind Δ*2185*	blue**	This work
pABcindΔ2184	pABcind Δ*2184*	greenish**	This work
pABcindΔ2183	pABcind Δ*2183*	deep blue**	This work
pABcindΔindB	pABcind Δ*2182*	greenish**	This work
pABcind Δ2181	pABcind Δ*2181*	greenish**	This work
pABcind Δ2180	pABcind Δ*2180*	greenish**	This work
pUC18indC::*kan*	pUC ori, Ap^r, Km^r,*plu2186*::*kan*	no colour*	This work
pSUmtaA	p15A ori, Cm^r, *mtaA*	--	Gaitatzis *et al.* (2001)
pABmtaA01	R6Kγ ori, Cm^r, *mtaA*	--	This work
pABmtaA02	R6Kγ ori, Km^r, *mtaA*	--	This work
pMR06kan	pUC ori, Km^r	--	M. Ring
pSU19	p15A ori, Cm^r	--	Bartolomé *et al.* (1991)
pDS132	R6Kγ ori, Cm^r	--	Nadège *et al.* (2004)

* coexpressed with a compatible *mtaA* containing plasmid

** coexpressed with a compatible *mtaA* containing plasmid and pUC18indCA or pUC18indC

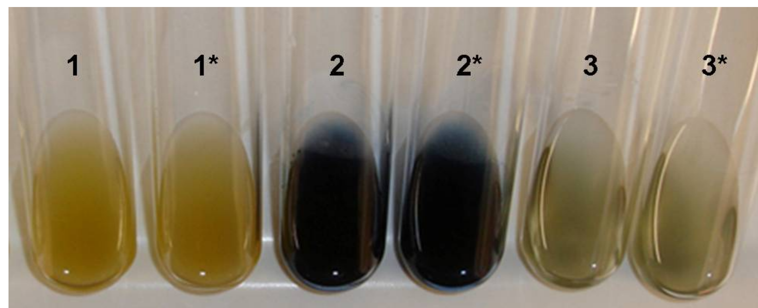


Figure 4. Heterologous coexpression of pABcind with pUC18indCA and pABmta02 (3). Expression of pUC18indCA with pABmta02 (2) and without pABmta02 (1). Cultures marked with an asterisk (*) carry pUC18indC instead of pUC18indCA.

Indigoidine production as new blue-white screening system. To ascertain if plasmid pUC18indC could represent an alternative blue/white screening system, a kanamycin cassette was inserted into *indC*. For this pUC18indC was digested with *Bcu*I and *Hind*III cutting out a 221 bp fragment within the adenylation domain of *indC*. Hereafter the kanamycin cassette was inserted via *Bcu*I and *Hind*III restriction sites and ligated into the digested pUC18indC vector. The resulting plasmid pUC18indC::*kan* was then coexpressed with pSUmtaA. Neither agar colonies nor liquid cultures exhibited a blue phenoytpe in comparison to the control strain expressing the intact pUC18indC and pSUmtaA plasmids (Figure 5). Strikingly, indigoidine producing strains exhibited smaller colonies and slower growth in comparison to the non producing control strain.

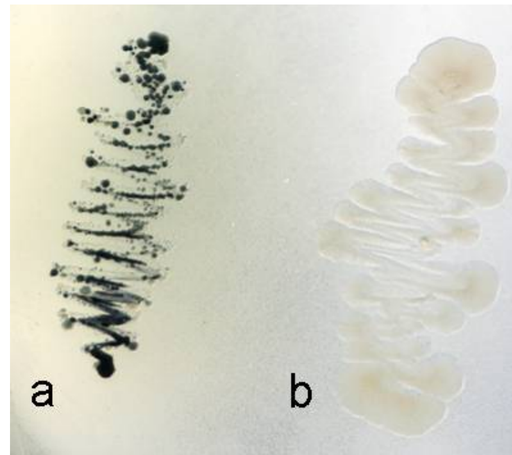


Figure 5. Blue/white screening with *E. coli* EC100D *pir*-116 expressing pABmta02 with pUC18indC (a) and pUC18indC::*kan* (b).

Discussion

The production of the blue pigment indigoidine was reported from many bacteria, but the identification was restricted to the analysis of their phenotype. The recent identification of the responsible biosynthesis enzymes also allowed analysing different bacterial genomes for the presence of indigoidine biosynthesis genes.[9] Surprisingly, indigoidine synthetases were also found in bacteria which are not known to produce indigoidine or to exhibit a blue phenotype. *Photorhabdus luminescens* TT01 belongs to the latter one. Several reasons can be accountable for the absence of indigoidine in *Photorhabdus*. Firstly, the responsible enzymes are not functional. Secondly, indigoidine is produced in very low amounts and analytically unverifiable. Thirdly, the expression of the responsible enzymes is strictly controlled by

regulation elements (silent genes) and fourthly indigoidine is only produced under certain physiological conditions which are not known.

In this work it could at least be shown that the indigoidine synthetase IndC of *Photorhabdus* is functional and that two candidate genes *plu2183* and *plu2185* might influence the regulation of indigoidine production. Furthermore it could be shown that *indA* and *indB*, both clustered with *indC* and dedicated to indigoidine biosynthsis in *Erwinia chrysanthemi* are present in *Photorhabdus*, but are not essential for the formation of indigoidine. Indeed, crystal structure elucidation of an IndA like protein revealed a new protein fold and a possible ligand analog,[7] but its function in indigoidine biosynthesis is still elusive. In case of IndB similarities with two phosphatases, which are involved in the production of antibiotics are found, but it does not seemed to be required for indigoidine production in *Photorhabdus*.[9] For example in *Vogesella indigofera* another indigoidine producing bacterium harboring the *indC* indigoidine synthetase non homologues of *indA* and *indB* are present,[9] which corroborates the hypothesis that exclusivley IndC is sufficient to produce indigoidine and that IndA and IndB might only have regulatory functions at most.

The obvious fact that a single NRPS gene *indC* is responsible for the blue pigment indigoidine offers the possibility to use indigoidine production as an alternative blue/white screening system. The insertion of a kanamycin casette into the adenylation domain of *indC* led clearly to an indigoidine deficient phenotype and therefore displays a well defined selection system. For a more convenient application the necessary PPTase can also be integrated into the genome of the host strain. In comparison to the conventional blue/white screening such a system would be independent from the addition of X-Gal. Moreover, the decreased growth rate of indigoidine producing colonies in comparison to non producing colonies (which have an insert) contribute to the promotion and better selection of the desired positive clones. Therefore indigoidine blue/white screening is a promising alternative cloning system to already established systems.

The blue pigment indigoidine is produced by a vast number of taxonomically different bacteria. The wide distribution leads to the assumption that the biosynthetic genes for indigoidine production originate from an evolutionary old ancestor and have been conserved in many different bacteria since then. Furthermore it suggests that indigoidine confers an important physiological advantage to bacteria as it is produced by bacteria from diverse ecological niches. Nevertheless, to date there is only little information about the biological role of indigoidine available. Only one possible function is described by Reverchon *et al.* who

found indications that indigoidine of *Erwinia chrysanthemi*, a plant pathogenic bacterium, might protect the bacteria against the reactive oxygen species generated during the plant defence response.[9] A similar function in the ecological niche of *Photorhabdus* is conceivable as one immune response of arthropods is the oxidative burst, an enzyme induced generation of reactive oxygen intermediates (ROIs).[8] Therefore indigoidine might operate as an oxide or radical scavenger to overcome an insecticidal oxidative burst and therefore also benefit the symbiotic nematode partner. Dietrich *et al.* described another interesting role for redox-active phenazine molecules, in which pyocyanin for example acts as an intercellular signal molecule dependent on the stress response regulator SoxR, which is widely distributed in enteric bacteria. Hence, they were able to show that pyocyanin mediates bacterial colony size and structure of a bacterial population.[1] A process which might be helpful to adapt and endure different environmental changes. Astonishingly indigoidine was never detected in the wild type strain *Photorhabdus luminescens* TT01, neither in solely *Photorhabdus* cultures nor under tripartite conditions together with nematode and insect.

Hence it seems obvious that indigoidine production in *P. luminescens* TT01 is dependent on a strong and so far unknown regulation, as functionality of indigoidine synthetase *indC* could be shown in this work. A known indigoidine regulator is protein PecS, a regulatory protein which negatively modulates the expression of many virulence genes in *Erwinia chrysanthemi* and which represses *indA* and *indC* expression by binding to the promoter regions.[9,10] However, neither homologues of PecS nor PecM, which moderates the efflux of indigoidine were identified in the genome of strain TT01.[10] Instead, two possible candidates *plu2183* and *plu2185* were identified in this work, which might play a major role in the regulation of indigoidine production in *Photorhabdus*. Both proteins can be also found in other bacteria, but until now no possible function was disclosed as no conserved domains within the proteins have been detected. However, an assumable function as DNA binding proteins seems inconsistent when even typical helix-turn-helix motifs are absent. Therefore both proteins might rather interact directly with the indigoidine synthetase. Deletions mutants of *plu2183* and *plu2185* in *Photorhabdus* will give more information on their involvement in indigoidine biosynthesis.

Definitely remarkable is the existence of *plu2180*-*plu2185* homologues which are also clustered together with *indC* in *Serratia proteamaculans* (Figure 6) (http://genome.jgi-psf.org/finished_microbes/serpr/serpr.info.html). In comparison, all proteins show a high similarity to *P. luminescens* proteins and only a little disarrangement of the genes upstream of *indC* is present (Figure 6 and Table 2). The successful isolation of *Serratia proteamaculans*

from entomopathogenic nematodes (personal communication N.Waterfield) supports the hypothesis of a horizontal gene transfer between both genera.

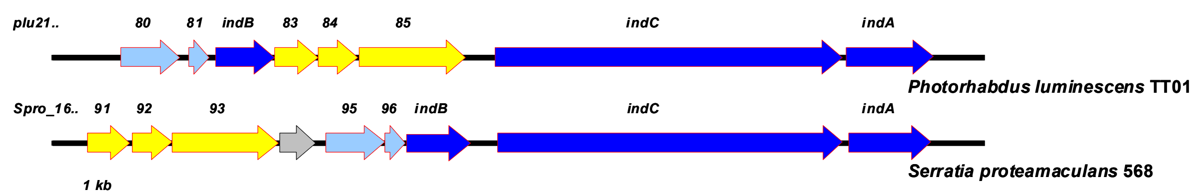


Figure 6. Comparison of the indigoidine biosynthesis gene clusters from *P. luminescens* and *S. proteamaculans*. The different colours serve to recognize the homologues easier.

Table 1. Comparison of homologous proteins in *P. luminescens* and *S. proteamaculans* 568. *S. proteamaculans* 568 display the closest homologues to *P. luminescens*, except for Plu2181 (*Agrobacterium radiobacter* K84, 47/70 % identities/positives) and for Plu2182 (*Streptomyces albus* J1074, 51/62 % identities/positives).

Photorhabdus luminescens TT01				*Serratia proteamaculans* 568	
Protein	Size (aa)	Deduced function	Identities/Positives (%)	Protein	Size (aa)
Plu2180	218	phosphoribosyl transferase	60/76	Spro_1695	216
Plu2181	74	4-Oxalocrotonate tautomerase	42/63	Spro_1696	71
IndA (Plu2182)	222	phosphatase	51/65	Spro_1697	231
Plu2183	158	unknown function	50/66	Spro_1691	152
Plu2184	145	unknown function	50/70	Spro_1692	147
Plu2185	392	unknown function	56/73	Spro_1693	393
IndC (Plu2186)	1284	indigoidine synthetase	50/66	Spro_1698	1278
IndB (Plu2187)	318	unknown function	62/76	Spro_1699	308

In view of Plu2185 *Streptomyces clavuligerus,* another indigoidine synthetase harbouring organism possesses a homologue which shows 44% identities and 61% positives on the amino acid level. This finding is interesting in several points because *S. clavuligerus* belongs to the group of Gram-positive bacteria and it lacks the aforementioned genes *plu2180-plu2184*. On the other hand, gene *plu2185* is not far away located from *indC* and indicates that even in this distantly related organism *plu2185* might contribute to indigoidine regulation. However, one has to pinpoint that neither *plu2185* nor *plu2183* homologues have been identified from the numerous other indigoidine producing bacteria. Hence even if the

regulatory function of both proteins can be proven, a generalized mechanism for indigoidne production cannot be concluded.

Materials and Methods

Fermentation. All expression experiments were carried out in *E. coli* EC100D *pir*-116 cells (*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG pir-116(DHFR)*) from Epicentre Biotechnologies. EC100D strains were cultivated at 30°C and 180 rpm on rotary shaker in 50 ml Erlenmeyer flasks containing 20 ml Luria-Bertani (LB) broth (pH 7.0) or in test tubes containing 5 ml LB. Appropriate antibiotics were added to LB liquid and agar cultures when necessary at following concentrations: ampicillin 100 µg/ml, kanamycin 50 µg/ml and chloramphenicol 34 µg/ml. An indigoidine production culture was cultivated in a 1 l Erlenmeyer flask containing 250 ml LB and inoculated with a 24h preculture of the same medium (0.1 % v/v).

Recombinant DNA techniques. Restriction digestions, ligations, DNA electrophoresis, transformation and electroporation of DNA were performed using standard molecular biology techniques.[11] Plasmid and DNA isolation were performed with GeneJet™ plasmid miniprep and genomic DNA purification kits (Fermentas). Subcloning was performed with CloneJET™ PCR cloning kit (Fermentas) when necessary. Information on genes and sequence comparison was extracted from the NCBI blast databank. All primers that were used in this work are listed in Table 3.

High resolution MS analysis. The extract was dissolved in DMSO/Methanol (50:50) containing 1% formic acid and analysed via direct injection on a Thermo LTQ Orbitrap Hybrid FT mass spectrometer.

Table 3. Oligonucleotides used in this work

Oligonucleotide	(5´-3´) Sequence
indBpUCSacI	ACCTACGAGCTCAAAAATACTCCAGTATTACCGTGG
indBpUCPaeI	GCCTGAGCATGCCCCTTTTCATTGGCTCTTAAT
indCfw	ACCTTGCAGATTATATTTCTAT
indCrv	GTTAATGTCATGATAATAATGG
CindfwXbaI	ATCTTTTCTAGAATCGATATGTGATGATCTAAA
CindrvPaeI	TTATTCGCATGCTAAATATTCCAATATTATTCAAT
pABCindFw	CCAGAGCGATGAAAACGTT
pABCindRv	AGTGAATACCACGACGATTT
Fw2185	CCCTACCCTTATTTTAACTCAG
Rv2185	ACTCCTTTGCTTCCTCCTTCT
Fw2184	AAGCTCAGCATTAGTTACTTCCCC
Rv2184	TGGAGTACTGTTTGTGTTAATTGG
Fw2183	TCATTTAAGTATATTTTCCTCAATA
Rv2183	TTTTCTTTGTTCTATTTGGCAGTA
Fw2182	ATCAAACAATGTACCATCTAAATCG
Rv2182	TGTTTTGATGACGTACTTCGTATT
Fw2181	CATCTTTCAAGTTGCCATAGGG
Rv2181	ATGATTTTTTAAAGGTATTTT
Fw2180	CAGCCGTACTTGGTCTCAAATA
Rv2180	ATCTATACCCTATGGCAACTTGAA
KanHindIIIfw	TAAAGCTTGATAGCTAGACTGGGCGGTT
KanBcuIrv	TAAAGCTTATGTGTGCGCGTCTCAGA
pSUmtaAfw	ACTGCCTTAAAAAAATTACG
pSUmtaArv	CAGTGAGCGCAACGCAATT
R6KoriFw	CCATGTCAGCCGTTAAGT
R6KoriRv	GAGGATCTGAAGATCAGCAG
mtaR6Kfw	AGTCACGACGTTGTAAAACGAC
mtaR6Krv	CTCGAGAAGGCAGTGAGGATCTGAAGATCA
Kanfw	GTGAAAACCTCTGACACATG
Kanrv	ATGCCGATATCCTATTGG

Construction of pUC18indCA, pUC18indC and pABCind. A 5150 bp fragment containing *indC* and *indB* was amplified from genomic DNA of *Photorhabdus luminescens* TT01 with primers indBpUCSacI and indBpUCPaeI and cloned via restriction sites *Sph*I and *Sac*I into the pUC18 plasmid giving pUC18indCA. To yield pUC18indC a 6350 bp fragment excluding *indB* was amplified from pUC18indCA with primers indCfw and indCrv. PCR products were 5´-phosphorylated, ligated and transformed into *E. coli* EC100D pir-116. Plasmid accuracy was verified by digestion with *Sph*I and *Sac*I resulting in a single fragment of 6350 bp. For pABCind a 3898 bp fragment containing *plu2180*, *plu2181*, *plu2182* (*indB*), *plu2183*, *plu2184* and *plu2185* was amplified from genomic DNA of *P. luminescens* TT01 with primers CindfwXbaI and CindrvPaeI and cloned via *Sph*I and *Xba*I into pSU19.

Construction of in frame deletions of pABcind. In frame deletions were constructed referring to a modified method described previously.[3] For each deletion two primer pairs were

designed. One primer pair consisted of a forward primer (pABCindFw) covering one half of the p15A ori sequence and the second primer (Rv2180-2185) was designed to cover upstream of the designated DNA sequence to be excluded. The second primer pair consisted of a reverse primer (pABCindRv) covering the other part of the p15A ori sequence and a primer (Fw2180-2185) which covered the end of the designated DNA sequence to be excluded. The different fragments were amplified from pABcind by PCR with use of Phusion Polymerase (Finnzymes). Purified PCR products were 5′-phosphorylated with use of T4 polynucleotide kinase (Fermentas) applying the manufacturers protocol and purified again. Ligation of the corresponding PCR fragments in a ratio of 1:1 resulted in the plasmid deletion constructs pABcindΔ2180, pABcindΔ2181, pABcindΔindB, pABcindΔ2184, pABcindΔ2183 and pABcindΔ2185. Then the plasmids were transformed into *E. coli* EC100D *pir*-116 cells via electroporation. Only cells with complemented and intact p15A ori were able to grow on chloramphenicol agar plates. In frame deletion integrity was verified by DNA sequencing.

Construction of pABmtaA02. For construction of a compatible *mtaA* containing plasmid pSUmtaA was used as a template. In a first step a fragment of 3260 bp excluding the p15A ori was amplified from pSUmtaA with primers pSUmtaAfw and pSUmtaArv. The PCR product was fused with a fragment of 396 bp amplified from pDS132 with primers R6KoriFw and R6KoriRv containing the *pir* dependent R6Kγ ori. Both PCR products were 5′-phosphorylated and then ligated. The resulting plasmid pABmtaA01 was transformed by electroporation into *E. coli* EC100D *pir*-116. Only cells with intact plasmid containing R6Kγ ori and chloramphenicol resistance were able to grow on selection agar plates. In a second step a fragment of 1595 bp excluding *cat* was amplified from pABmtaA01 with primers mtaR6Kfw and mtaR6Krv. The PCR product was fused with a fragment of 877 bp amplified from pMR06 with primers Kanfw and Kanrv containing the kanamycin resistance cassette and afterwards treated similar to the first step. The resulting plasmid pABmtaA02 was transformed by electroporation into *E. coli* EC100D *pir*-116 and only cells with pABmtaA02 containing R6Kγ ori and kanamycin resistance were able to grow on selection agar plates.

Construction of blue/white screening reference plasmid pUC18indC::*kan*. For pUC18indC::*kan* plasmid pUC18indC was digested with *Bcu*I and *Hind*III. A kanamycin resistance cassette was amplified with primers KanHindIIIfw and KanBcuIrv from plasmid pMR06 and digested in the same way. Both products were purified and ligated to give plasmid pUC18indC::*kan* and then transformed by electroporation into *E. coli* EC100D *pir*-

116. Only cells with a kanamycin cassette insertion in pUC18indC confered kanamycin and ampicllin resistance.

Reference List

1. **Dietrich, L. E., T. K. Teal, A. Price-Whelan, and D. K. Newman**. 2008. Redox-active antibiotics control gene expression and community behavior in divergent bacteria. Science **321**:1203-1206.

2. **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.

3. **Jin, C. J., X. Cai, H. Ma, Y. Xue, J. H. Yao, and X. B. Yao**. 2007. An efficient site-directed mutagenesis method for Co1E1-type ori plasmid. Analytical Biochemistry **363**:151-153.

4. **Knackmuss, H. J.** 1973. Chemistry and biochemistry of azaquinones. Angewandte Chemie-International Edition in English **12**:139-145.

5. **Kuhn, R., H. Bauer, and H. J. Knackmuss**. 1965. Struktur und Synthesen des Bakterienfarbstoffs Indigoidin. Chem. Ber. **98**:2139-2154.

6. **Kuhn, R., M. P. Starr, D. Kuhn, H. Bauer, and H. J. Knackmuss**. 1965. Indigoidine and other bacterial pigments related to 3,3'-bipyridyl. Arch. Mikrobiol. **51**:71-84.

7. **Levin, I., M. D. Miller, R. Schwarzenbacher, D. McMullan, P. Abdubek, E. Ambing, T. Biorac, J. Cambell, J. M. Canaves, H. J. Chiu, A. M. Deacon, M. DiDonato, M. A. Elsliger, A. Godzik, C. Grittini, S. K. Grzechnik, J. Hale, E. Hampton, G. W. Han, J. Haugen, M. Hornsby, L. Jaroszewski, C. Karlak, H. E. Klock, E. Koesema, A. Kreusch, P. Kuhn, S. A. Lesley, A. Morse, K. Moy, E. Nigoghossian, J. Ouyang, R. Page, K. Quijano, R. Reyes, A. Robb, E. Sims, G. Spraggon, R. C. Stevens, B. H. van den, J. Velasquez, J. Vincent, X. Wang, B. West, G. Wolf, Q. Xu, O. Zagnitko, K. O. Hodgson, J. Wooley, and I. A. Wilson**. 2005. Crystal structure of an indigoidine synthase A (IndA)-like protein (TM1464) from *Thermotoga maritima* at 1.90 A resolution reveals a new fold. Proteins **59**:864-868.

8. **Neumann**. 2008. Das Immunsystem der Invertebraten, p. 245-269. Immunbiologie. Springer Berlin Heidelberg.

9. **Reverchon, S., C. Rouanet, D. Expert, and W. Nasser**. 2002. Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi* and role of this blue pigment in pathogenicity. J. Bacteriol. **184**:654-665.

10. **Rouanet, C. and W. Nasser**. 2001. The PecM protein of the phytopathogenic bacterium *Erwinia chrysanthemi*, membrane topology and possible involvement in the efflux of the blue pigment indigoidine. J Mol Microbiol Biotechnol **3**:309-318.

11. **Sambrook, J., E. F. Fritsch, and T. Maniatis**. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

12. **Starr, M. P., G. Cosens, and H. J. Knackmuss**. 1966. Formation of the Blue Pigment Indigoidine by Phytopathogenic *Erwinia*. Appl. Microbiol **14**:870-872.

13. **Takahashi, H., T. Kumagai, K. Kitani, M. Mori, Y. Matoba, and M. Sugiyama**. 2007. Cloning and characterization of a *Streptomyces* single module type non-ribosomal peptide synthetase catalyzing a blue pigment synthesis. J. Biol. Chem. **282**:9073-9081.

Statement about the author´s efforts in Chapters 1-4

Chapter 1: The author performed all feeding experiments and chemical analyses including fatty acid profiles. The isolation of the ^{13}C enriched stilbenes was also performed by the author. The generation of mutants were carried out in cooperation with the group of David Clarke. Nematode assays were performed by I. Glazer and L. Lango.

Chapter 2: The author performed all feeding experiments and chemical analyses. The isolation of the ^{13}C enriched anthraquinone and mutactin was also performed by the author. The deletion mutant was generated by the group of David Clarke, other mutants were generated in collaboration with G. Schwär. Structure elucidation of mutactin was perfomed by Helge B. Bode. The erection of a phylogenetic tree was performed by H. Jenke-Kodama.

Chapter 3: The work in whole was performed by the author, except structure elucidation which was performed together with Helge B. Bode.

Chapter 4: The work in whole was performed by the author.

Discussion

Discussion

During this work it came apparent that bacteria of the genera *Photorhabdus* and *Xenorhabdus* represent rich sources of secondary metabolites as we succeeded in identifying and isolating new derivatives of known compound classes like 5-[(*E*)-2-phenylethenyl]-2-(propan-2-yl)benzene-1,3-diol (cis-isopropylstilbene, cisIPS, see chapter 5.1) and 5-(2-phenylethyl)-2-(propan-2-yl)benzene-1,3-diol (IPB, see chapter 5.1), but also of novel secondary metabolites like xenofuranones and 4-hydroxy-6-isobutyl-3-(5-methylhexyl)-2*H*-pyran-2-one (unpublished data). This richness of putative natural compounds is also underlined by the identification of 22 biosynthetic gene clusters in the genome of only a single species *Photorhabdus luminescens* TT01, representing notable 7.5% of the overall genome sequence (Figure 1).[12] However, many of the respective products are still unknown as they are not produced at all under laboratory conditions. The heterologous expression of such a "silent gene" cluster in this work and identification of indigoidine demonstrated a prosperous way to gain access to so far unknown secondary metabolites (see chapter 4 for discussion).

In the following the natural compound families of stilbenes, anthraquinones and xenofuranones, which were the main focus of this work are discussed in detail in their possible biological function and their biosynthetic origin.

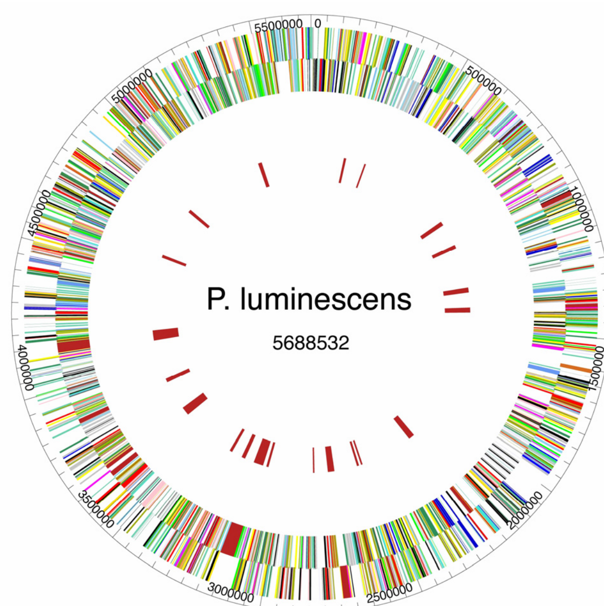


Figure 1. Genome map of *Photorhabdus luminescens* TT01. The red bars in the inner circle represent the gene clusters involved in secondary metabolism (Genome map provided by H.B. Bode)

Stilbenes

Stilbenes have been exclusively known as a class of natural compounds from many plants. Over 30 differently substituted stilbenes and stilbene glycosides are described, among them the extensively investigated polyphenol resveratrol (more than 2700 www.pubmed.gov citations). In general, they act as phytoalexins which are produced in response to an injury of the plant. Their purpose is to protect the plant against bacterial and fungal infections, but also from ozone and harmful ultraviolet radiation.[97,174] Moreover, stilbenes and in particular resveratrol displayed interesting and useful pharmacological properties. It is known that resveratrol is an activator of the protein sirtuin1 (SIRT1), a longevity gene that modulates the response of an organism to aging and nutritional status.[129] SIRT1 mimics a calorie restriction thus helps to delay the onset and reduces the incidence of age-related diseases.[130] Beside this anti-ageing effect, resveratrol exhibits properties as an antioxidant, anti-inflammatory and anti-proliferative molecule.[57] In the meantime it was shown that not only plants can produce stilbenes, but also the bacterium *Photorhabdus luminescens*. The broad spectrum of stilbene activities makes *Photorhabdus luminescens* an interesting bacterial stilbene producer which can genetically be exploited to increase and produce different stilbene derivatives. The abundant stilbenes in *P. luminescens* isopropylstilbene (IPS, Figure 1B) and ethylstilbene (ES, Figure 1B) can be regarded as 2-alkylated derivatives of pinosylvin (Figure 1A), which was shown to exhibit an antibacterial and antifungal activity as well.[107] However, up to date there is no data available on the pharmacological properties of the bacterial stilbenes, only for ES it was shown that RNA synthesis was inhibited by accumulating the intracellular regulatory compound guanosin-3´,5´-bis-pyrophosphate (ppGpp) leading to an inhibition of bacterial growth.[181] The multifunctional properties of this class of compounds point out that beside the known biological functions of bacterial stilbenes and the functions found in this work, further effects might still wait to be discovered in appropriate bioassays.

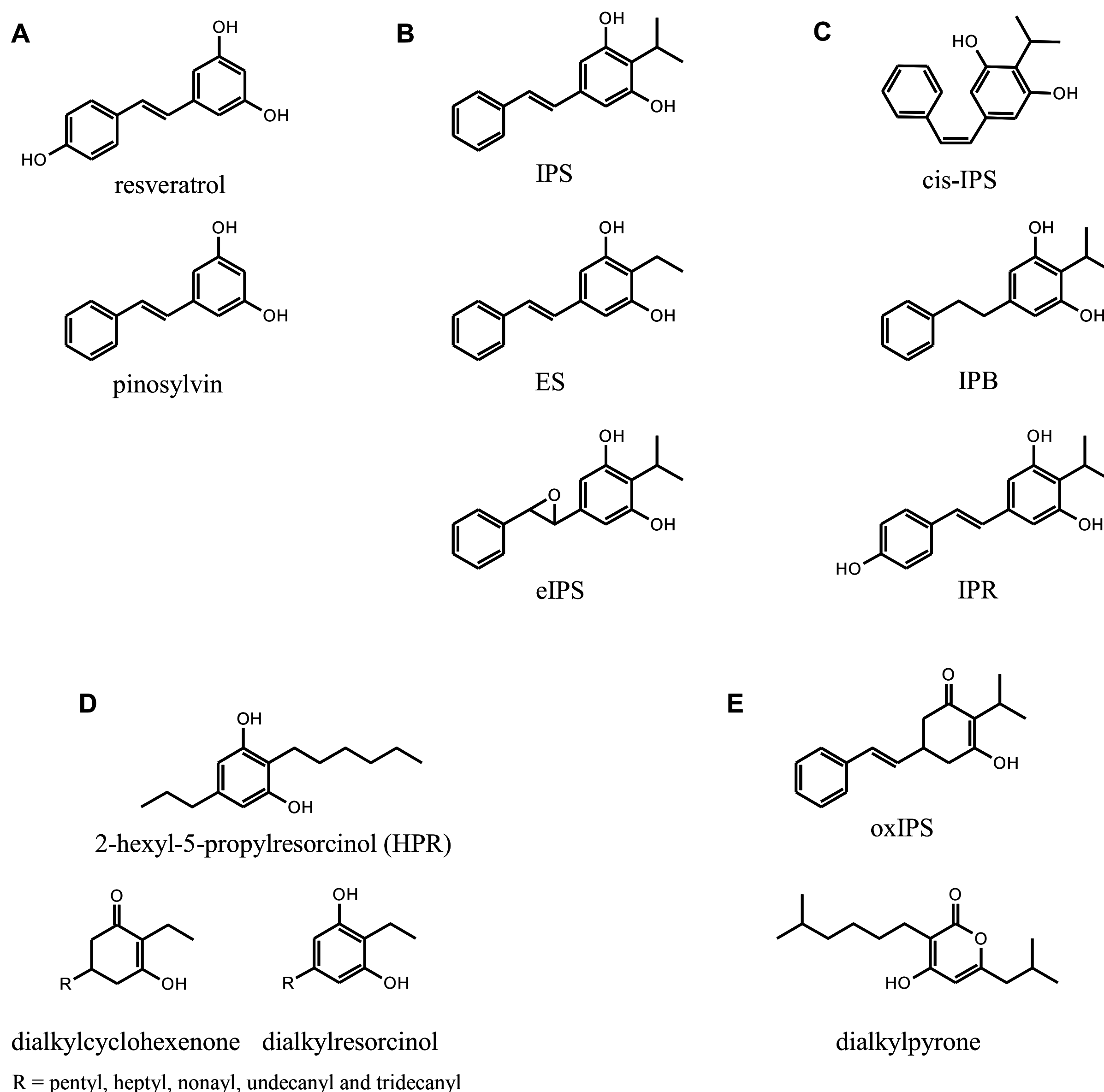


Figure 1. (A) Type III PKS derived plant stilbenes resveratrol and pinosylvin. (B) Isolated stilbenes from *Photorhabdus luminescens* 2-isopropyl-5-[(*E*)-2-phenylethenyl]benzene-1,3-diol (IPS), 2-ethyl-5-[(*E*)-2-phenylethenyl]benzene-1,3-diol (ES) and 2-isopropyl-5-(3-phenyl-oxiranyl)benzene-1,3-diol (eIPS). (C) A new and minor stilbene identified in *Photorhabdus luminescens* 2-isopropyl-5-[(*Z*)-2-phenylethenyl]benzene-1,3-diol (cis-IPS) and two new derivatives which were derived by feeding of phenylpropionic acid and coumaric acid to a PAL (StlA) deficient strain yielding 2-isopropyl-5-[2-phenylethyl]benzene-1,3-diol (IPB) and 2-isopropyl-5-[(*E*)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol (IPR). (D) Heterologous expression of the *stlC*, *stlD* and *stlE* homologues of *Pseudomonas aurantiaca* led to the production of the dialkylresorcinol 2-hexyl-5-propylbenzene-1,3-diol (HPR). The heterologous expression of the *Photorhabdus* derived genes led to the production of different dialkylcyclohexenones instead of the expected dialkylresorcinols. (E) Novel compounds isolated from the strain *Photorhabdus luminescens* ssp. *thracensis* DSM 15199, 2-isopropyl-3-hydroxy-5-[(*E*)-2-phenylethenyl]cyclohex-2-en-1-one (oxIPS) and the dialkylpyrone 4-hydroxy-3-(5-methylhexyl)-6-(2-methylpropyl)-2*H*-pyran-2-one.

Prior to this work it was considered that stilbene biosynthesis is only achieved through the action of a stilbene synthase. This enzyme is a representative of the type III PKS family that yields stilbenes from the incorporation of a cinnamoyl-CoA derivative and sequential elongation with three malonyl-CoA units. Due to the fact that bacterial stilbenes are substituted at the 2-carbon either with an isopropyl or ethyl moiety it would necessitate the incorporation of different extender units for a stilbene synthase. Indeed, it was recently shown that type III PKS are also able to utilize ethyl- and methylmalonyl-CoA and not only malonyl-CoA extender units.[176] Furthermore, a type III PKS was reported that used different CoA extender units in a strictly controlled order.[60] For this reason a bacterial type III PKS utilizing malonyl-CoA and isopropylmalonyl-CoA or ethylmalonyl-CoA extender units could not be ruled out. However, genome analysis revealed that no similar genes of bacterial or plant type III PKS were existent in *P. luminescens* TT01. Only the identification of a stilbene biosynthesis involved gene operon harbouring the genes *stlC*, *stlD* and *stlE* established an unprecedented way of biosynthesising stilbenes. Nevertheless, homologues of these genes were also found in *Pseudomonas aurantiaca* were those encode proteins responsible for the production of 2-hexyl-5-propylbenzene-1,3-diol (HPR) a 2,5-dialkylresorcinol (Figure 1D).[138] Additionally, Nowak-Thompson *et al.* provided evidence that dialkylresorcinol is formed by a head-to-head condensation of two β-keto acyl precursors. A similar mechanism can be also assumed for the formation of stilbenes (Figure 2).

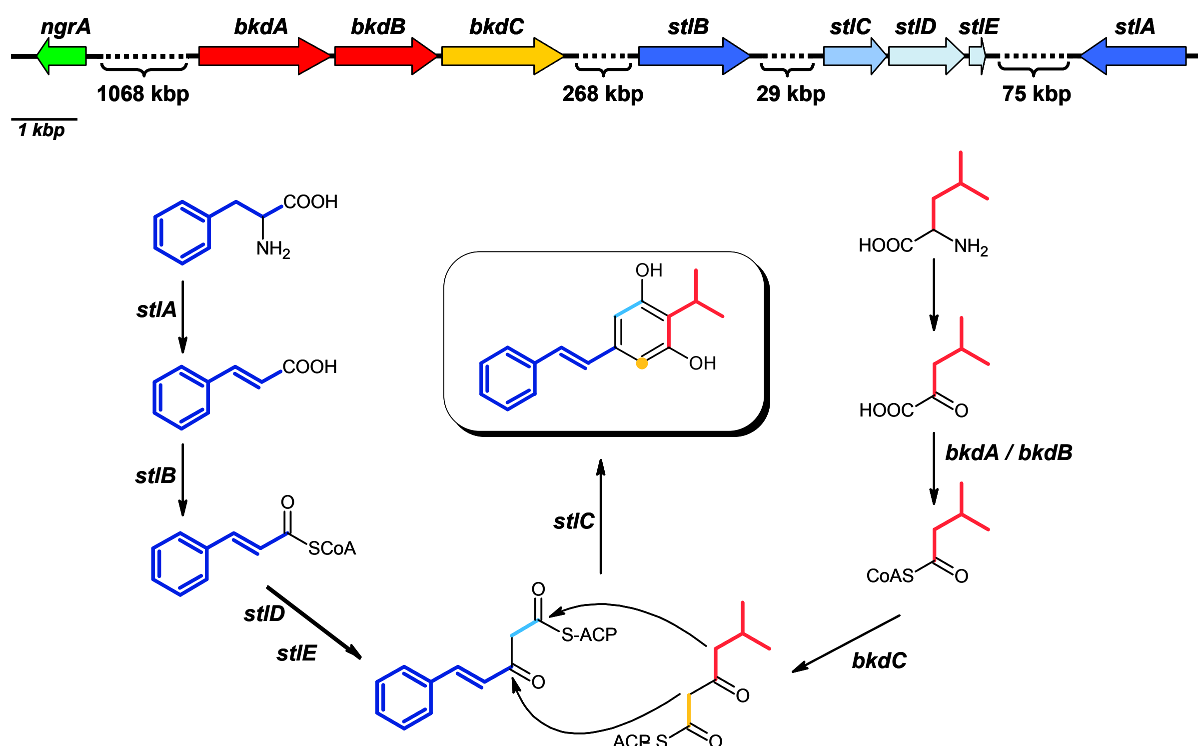


Figure 2. Genes involved in IPS biosynthesis and proposed IPS biosynthesis. Brackets display the genomic gaps between the pictured genes. Gene *ngrA* encodes for a phosphopantetheinyltransferase (PPtase) required for activation of acyl carrier proteins (ACPs); *bkdA*, *bkdB*, *bkdC* encode for the branched chain keto acid dehydrogenase complex (Bkd) involved in the formation of iso-branched precursors in iso-fatty acid and IPS biosynthesis; *stlB* encodes for the cinnamoyl-CoA ligase; *stlC* encodes for a "condensing enzyme"; *stlD* encodes for a ketosynthase; *stlE* encodes for an acyl carrier protein (ACP); *stlA* encodes for a phenylalanine ammonium lyase (PAL). The proposed biosynthetic IPS pathway shows the chemical structures of all intermediates and probably involved enzymes leading to two β-keto-acyl precursors which undergo a head-to-head condensation to form the final IPS product.

To verify if the three gene operon found in *Photorhabdus* has the same function as the homologue in *Pseudomonas aurantiaca*, the operon was expressed heterologously in *E. coli*. Unexpectedly the heterologous expression in *E. coli* of the *Photorhabdus* derived three gene operon (unpublished data) resulted in the production of dialkylcyclohexenones (Figure 1D) instead of dialkylresorcinols (Figure 1D), indicating that a subsequent aromatisation of the six membered carbon ring does not take place. This observation implicated that another enzyme catalyzed step is necessary to oxidize the cyclohexenone to an aromatic ring. But more astonishingly neither dialkylcyclohexenones nor dialkylresorcinols were detected in a phenylalanine ammonium lyase inactivation mutant *stlA*. Although one would expect at least the formation of non- or iso-branched dialkylresorcinols. One possible explanation was delivered from *Photorhabdus luminescens* ssp. *thracensis* who produces the new compounds 4-hydroxy-3-(5-methylhexyl)-6-(2-metyhlpropyl)-2*H*-pyran-2-one, a dialkylpyrone (Figure 1E) and 2-isopropyl-3-hydroxy-5-[(*E*)-2-phenylethenyl]cyclohex-2-en-1-one (oxIPS), a

second ring oxidized IPS derivative beside the known stilbenes (unpublished data, Figure 1E). This new oxIPS displays a cyclohexenone ring as found in the dialkylcyclohexenones. Thus it appears that oxIPS might represent an intermediate product of stilbene biosynthesis, which is further processed by a so far unknown aromatase-like enzyme. In *Photorhabdus luminescens* ssp. *thracensis* the enzyme appears to be ineffective in the total conversion of oxIPS into IPS. A reasonable explanation might be originated from an altered enzyme recognition site leading to a hampered conversion of oxIPS or in case of the dialkylcyclohexenones to a total substrate refusal. But as long as a putative enzyme is not identified the question of aromatisation keeps unsolved. Further experiments in *E. coli* showed that *stlD* encoding a ketosynthase is essential for the biosynthesis of dialkylcyclohexenones. On the contrary, the loss of the *stlE* encoding acyl carrier protein (ACP) seems to be complemented at least in parts by the *acpP* encoded fatty acid synthase ACP of *E. coli*. However, the production of dialkylcyclohexenones is reduced especially for long chain derivatives (Figure 3, unpublished data).

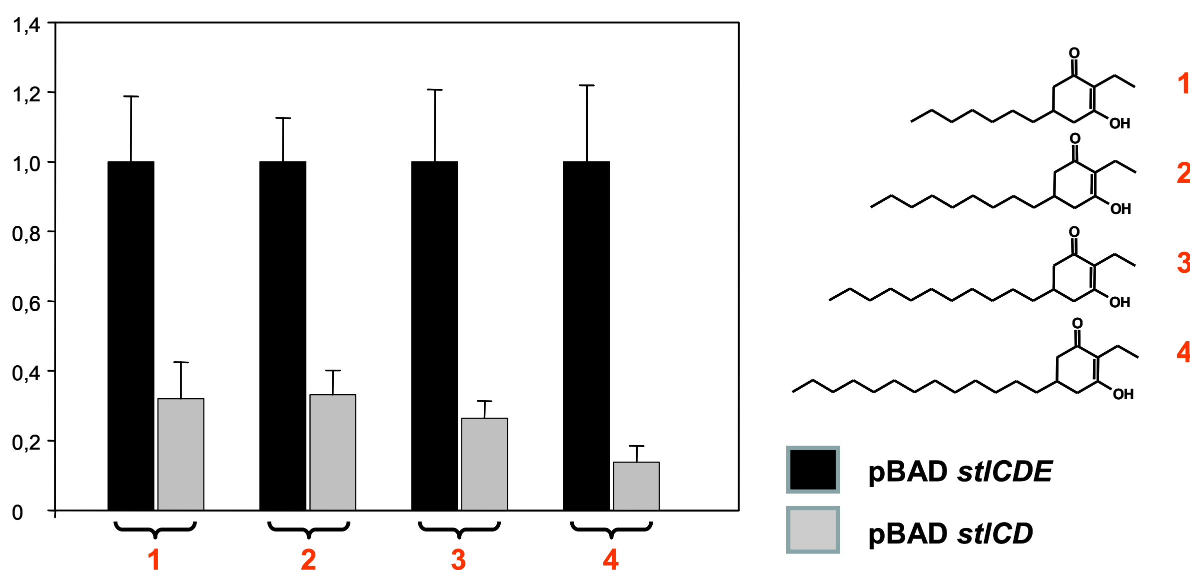


Figure 3. Quantification (triplicates) of arabinose inducible plasmids in *E. coli* DH10B. Black highlighted bars display the construct containing *stlC*, *stlD* and *stlE* (a gift from N.Waterfield). Grey highlighted bars display the construct containing *stlC* and *stlD*. A construct only containing *stlC* was not capable of dialkylcyclohexenone production (the deletion constructs were generated similar to pABcind plasmid deletions constructs of chapter 4). Numbers 1-4 represent the major dialklycyclohexenones identified in the extracts. The data indicates that the loss of ACP encoding *stlE* is compensable with the *E. coli* own ACP, but reduced in the total production of dialkylcyclohexenones and the tendency to use long acyl chain precursors. The relative abundance of each dialkylcyclohexenone was denoted with =1 and standard deviations are displayed.

In this work it was proven that the branched chain keto acid dehydrogenase (bkd) complex is needed for the formation of the iso-branched precursor of stilbene synthesis.

Deletion mutants of each *bkd* gene were shown to be deficient in iso-branched fatty acid and IPS production. In chapter 1 IPS was identified as a factor required for nematode recovery, but not sufficient for full recovery. In this context it is worth mentioning that iso-branched fatty acids, especially iso15:0 and iso17:0 were shown to be essential for growth and development in the closely related nematode *Caenorhabditis elegans*.[99,100] Iso15:0 and iso17:0 belong to the dominantly produced fatty acids of all investigated *Photorhabdus* strains so far and might play a similar role. Therefore, the Bkd complex is a special gateway for the production of primary and secondary metabolites (Figure 4), with both probably contributing to the sophisticated interaction of bacteria and nematodes. The heterologous expression of the *bkd* locus in *E. coli*, which normally exhibits no iso-fatty acids at all, succeeded in the production of iso-fatty acids like iso15:0, iso16:0 and iso17:0 (Figure 5).

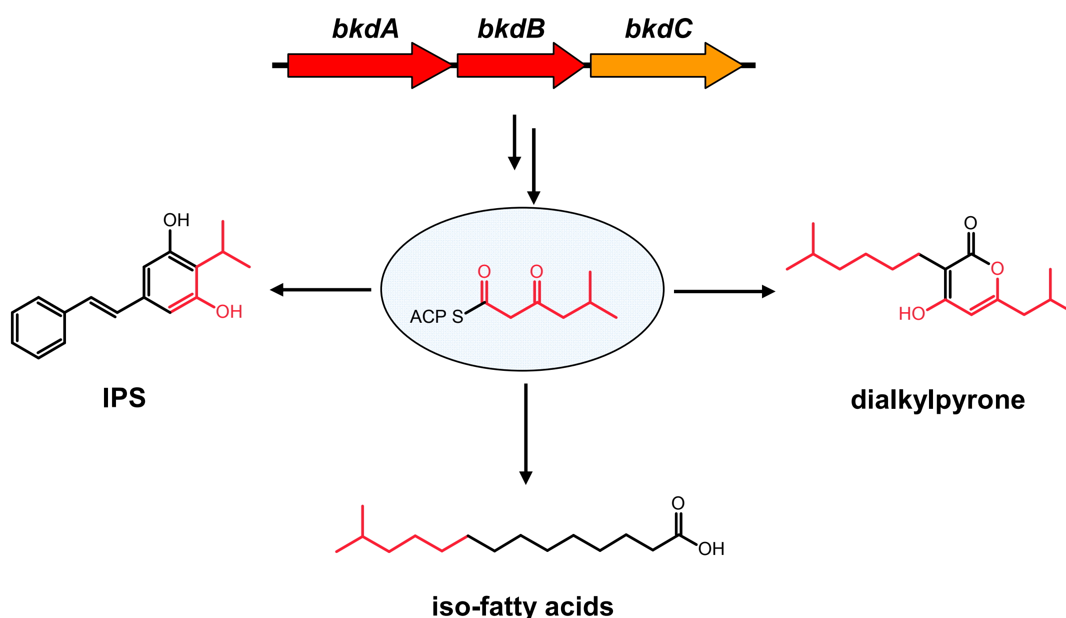


Figure 4. The (bkd) branched chain keto acid dehydrogenase (Bkd) complex of *Photorhabdus luminescens* consisting of *bkdA*, *bkdB* and *bkdC* takes a key role in the production of different metabolites in the way of providing precursors for primary and secondary metabolism. It was shown that incorporation of Bkd derived precursors are not only found in iso-fatty acids of the primary metabolism but also in secondary metabolites as IPS and the novel isolated dialkylpyrone 4-hydroxy-3-(5-methylhexyl)-6-(2-metyhlpropyl)-2*H*-pyran-2-one (unpublished data).

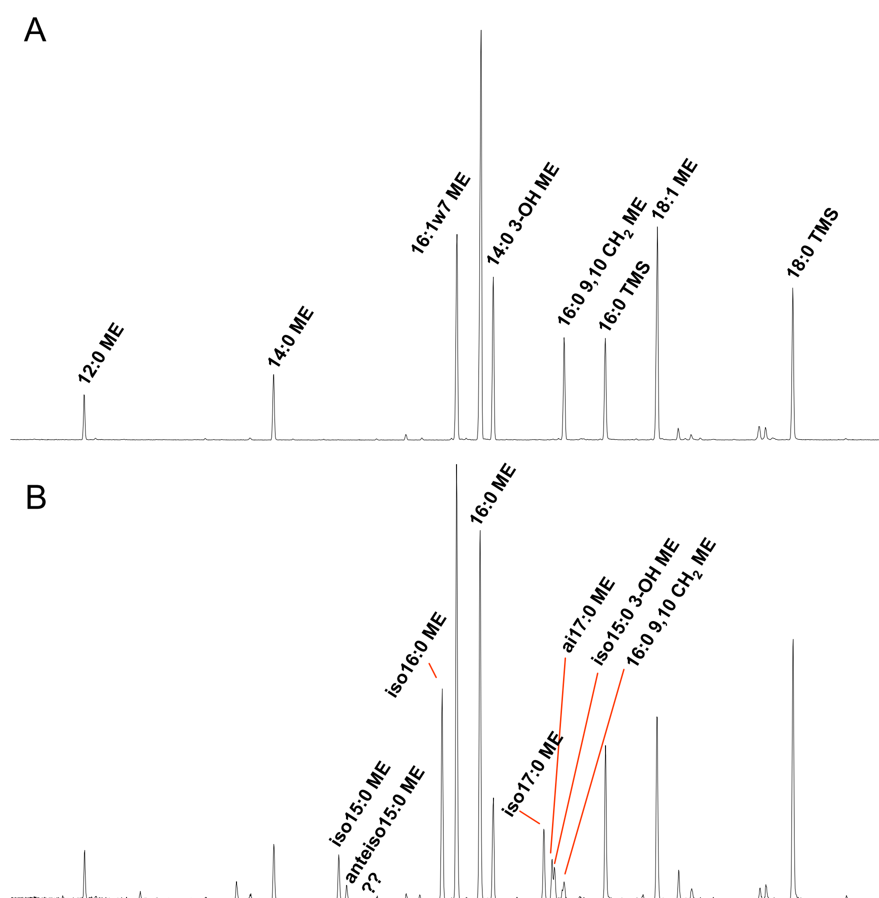


Figure 5. (A) Fatty acid profile of *E. coli*. (B) Fatty acid profile of *E. coli* with heterologous expression of the *bkd* complex and the phosphopantetheinyltranserase encoding gene *mtaA*.

Feeding experiments of different phenylpropanoids and cinnamic acid derivatives to an anthraquinone and stilbene deficient (ASD) mutant provided information on the CoA ligase encoded by *stlB* (Figure 6). Here it was shown that StlB only exhibits restricted substrate flexibility, as only cinnamic acid and coumaric acid were accepted for the biosynthesis of stilbenes (unpublished data). The incorporation of coumaric acid resulted in very small traces of two isomers, probably *cis*- and *trans*- forms of 5-[(*E*)-2-(4-hydroxyphenyl)ethenyl]-2-(propan-2-yl)benzene-1,3-diol (isopropylresveratrol, IPR; Figure 1C and 6). Even more surprising was the incorporation of phenylpropionic acid to yield IPS and 5-(2-phenylethyl)-2-(propan-2-yl)benzene-1,3-diol (IPB; Figure 1C and 6). The production of IPS implicates that phenylpropionate is to some extent oxidized. However, in *E. coli* biodegradation of phenylpropionic and cinnamic acid is initiated by HcaECFD, a dioxygenase introducing molecular oxygen into the phenyl ring, which was recently also reported for *P. luminescens*.[21,41] Hence, it seems obvious that a second oxidation mechanism is available. Therefore *Photorhabdus* might posses a dehydrogenase which can accept phenylpropionate as an incidental substrate. Another possibility is the direct involvement of the CoA ligase StlB in the oxidation of phenylpropionate to cinnamic acid. But also a later

enzyme catalysed oxidation of IPB is conceivable. Within these analyses it was also possible to identify a *cis*-IPS (Figure 1C) as a new and minor stilbene derivative of the wildtype strain *P. luminescens* TT01.

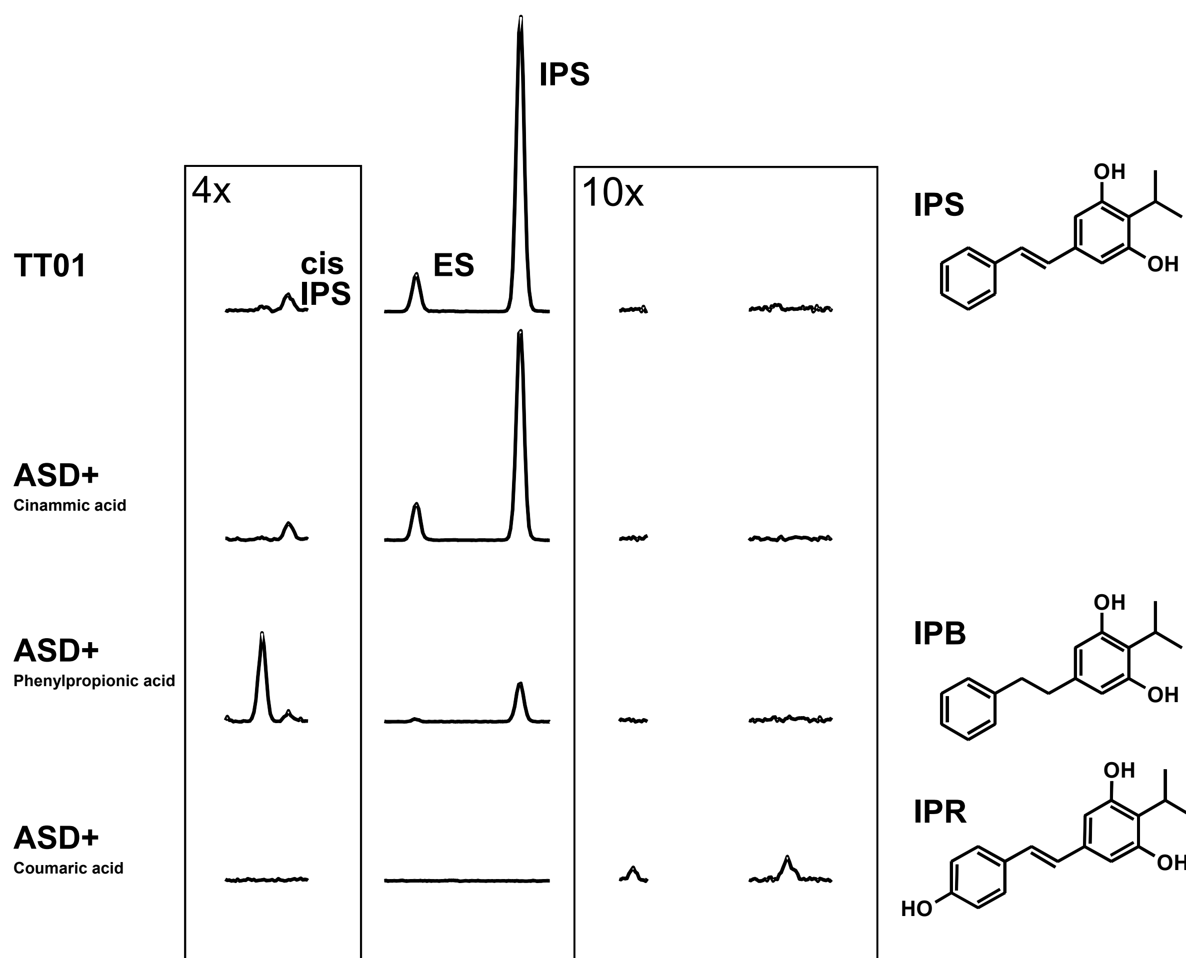


Figure 6. GC-MS analysis of differently fed anthraquinone stilbene deficient (ASD) cultures. The ASD mutant is anthraquinone and stilbene deficient and was generated by gene disruption of the phosphopantetheinyltransferase encoding *antB* in the phenylalanine ammonium lyase *stlA* disrupted mutant BMM901 (Williams *et al*. 2005). Analysis of the wildtype strain *Photorhabdus luminescens* TT01 established the identification of a *cis*-IPS as minor compound in the extract. Feeding of cinnamic acid to an ASD mutant led to the expected complementation of the wildtype stilbene profile. Feeding of phenylpropionic acid led to the unexpected production of IPS and IPB. Feeding of coumaric acid led to production of two IPR isomers. None new stilbene derivatives were detected after the feeding of ferulic, coffeic and other cinnamic acid derivatives. The pictured frames display GC chromatogram cutouts which are enlarged four and ten times respectively.

Epoxy-IPS (eIPS, Figure 1B) is another stilbene derivative isolated from *P. luminescens* which showed pronounced antimicrobial activity.[84] The production of eIPS can be very often observed after the formation of IPS and might be the first step in stilbene degradation. The possible involvement of a cytochrome P450 monooxygenase in introducing oxygen was investigated by adding ancymidol to liquid cultures of the good eIPS producer *P. luminescens* strain IthC13. Ancymidol is an inhibitor of some P450 monooxygenases,[163] but

no decrease or elimination of eIPS production was detected in strain IthC13 (unpublished data). Therefore, there is no data available to give further information on the formation of eIPS.

Further experiments indicated that bacterial stilbenes might play an important role as quorum sensing molecules. Swarming assays with a stilbene deficient *P. luminescens* TT01 mutant on LB agar plates supplied with resveratrol showed a significant inhibition of cell swarming. Similar phenotypes were reported from resveratrol treated *Proteus mirabilis* bacteria. Moreover, Wang *et al.* succeeded in identifying RsbA (regulator of swarming behaviour) a histidine phosphotransmitter of a bacterial two component signalling system as the mediating factor of resveratrol activity in *P. mirabilis*.[191] Two component pathways represent signalling systems to monitor and respond on environmental changes, thus allow bacteria to act simultaneously in order to enhance phenotypes.[86,196] In *P. mirabilis* resveratrol could inhibit swarming and virulence factor expression through an RsbA dependent pathway,[191] while RsbA might function as a sensor of environmental conditions required to initiate swarming migration.[8] A homologue of RsbA (48% identities and 69% positives on amino acid sequence level) can be also found in the genome of *P. luminescens* TT01. Indeed, Easom and Clarke could show that swarming of *Photorhabdus* is not required for either pathogenicity or mutualism, but they also observed that swarming contributes to the competitive fitness of *Photorhabdus* during infection of the insect.[46] Based on the mentioned similarities to *P. mirabilis* and the effect of resveratrol on *Photorhabdus* swarming behaviour, it might be possible that IPS or ES might also function as signalling molecules in interaction with RsbA homologue Plu3047.

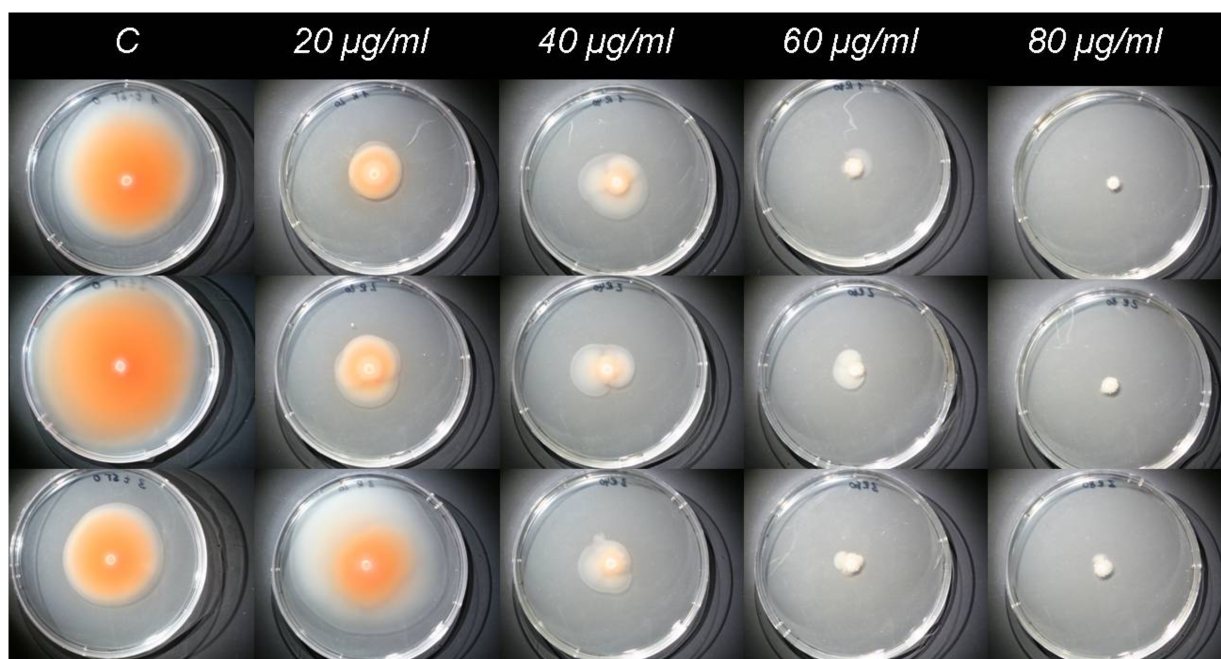


Figure 6. Swarm Assay triplicates of *Photorhabdus luminescens* TT01 on differently concentrated (µg/ml) resveratrol supplied swarming-Agar plates after 66 hours. The control (C) was plated on resveratrol free swarming-Agar. The minimal inhibition concentration (MIC) of resveratrol is > 100 µg/ml.

In summary, the data of this work revealed a new alternative way of stilbene biosynthesis via a head-to-head condensation of two β-keto acyl precursors. The crucial condensation step is thereby catalysed by the condensing enzyme StlC which is in combination with StlD also capable of dialkylcyclohexenone formation. The involved Bkd complex was not only shown to be required for iso-fatty acid but also for IPS and dialkylpyrone production, depicting an essential junction for primary and secondary metabolite pathways. The unexpected formation of a cyclohexenone ring instead of an aromatic ring and the resulting dialkylcyclohexenones suggest an involvement of a yet unknown enzyme, which is required for the aromatisation of the second ring in stilbene biosynthesis, but to date no potential candidate could be identified.

Anthraquinones

Anthraquinones are very widespread natural pigments which are produced by many different organisms. Among these organisms plants are the dominant sources. The most prominent plant produced anthraquinone, alizarin, was used as a red staining dye since the medieval times.[161] Another glycosylated anthraquinone dye of historical relevance was carminic acid, which occurs naturally in some scale insects.[161] But natural anthraquinones have been also found from fungal and bacterial sources, from the latter one especially in actinomycetes for example R1128 from *Streptomyces*.[83]

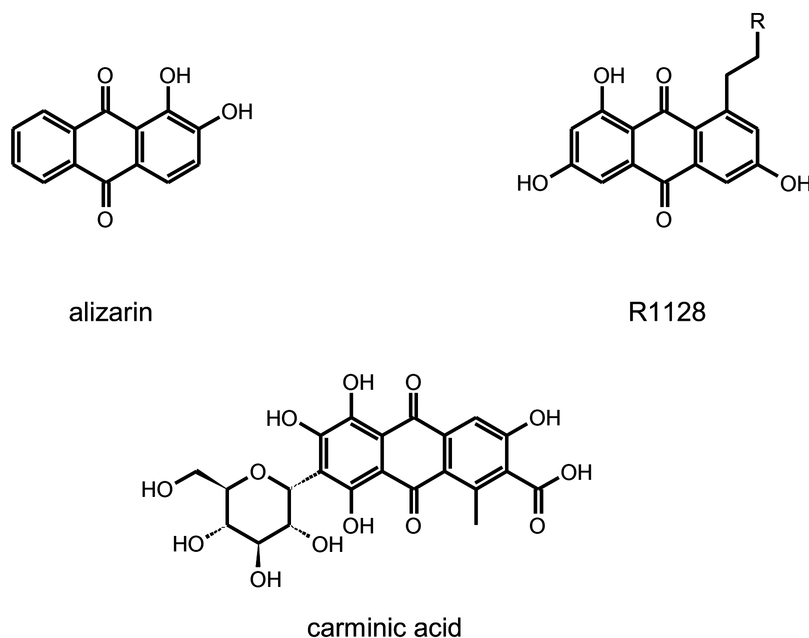


Figure 1. Natural anthraquinones. Alizarin is available as a glycosylated derivative from the plant *Rubia tinctorum* and was used as a staining dye since the Middle Ages. Another known staining dye is the gylcosylated carminic acid which can be isolated from scale insects where it is used as a deterring agent against predators. A bacterial type II PKS derived anthraquinone is R1128 from *Streptomyces* sp. No. 1128.

Photorhabdus represents the first Gram negative anthraquinone producer. Seven anthraquinone (AQ) derivatives have been described from *Photorhabdus* in the past and only a few of them were reported to exhibit a weak antibacterial activity. Although almost all *Photorhabdus* strains produce anthraquinones and hereby confer the characteristic red colour to colonies and liquid cultures, their real biological mode of function however is still elusive. Nevertheless, some possible biological roles can be deduced from other anthraquinones. For instance, it was shown that carminic acid as mentioned before displays an effective ant deterring agent.[48] In addition Bauer *et al*. could demonstrate with their experiments that foraging ants significantly avoid *Photorhabdus*/*Heterorhabditis* killed insect larvae as a prey, and were therefore suggesting the production of an unidentified deterring factor.[7] The

obtained findings strongly imply that *Photorhabdus* produced anthraquinones might be responsible for the observed behaviour. This conclusion is also underlined by bioassays showing that anthraquinone containing eggs of the beetle *Galeruca tanaceti* moderate a feeding deterrence against ants.[79] In this concern it is also worth mentioning that anthraquinones of the heartwood of teak plants contribute to the protection against termites.[157] Regarding these examples, one biological role of *Photorhabdus* produced anthraquinones might be the protection of the insect cadaver against scavengers in order to assure the complementation of the nematode life cycle. Moreover, anthraquinones might also function as bird repellents. Anthraquinones are commercially used to repel birds, for example at airports. The extremely bitter taste of anthraquinones urge them to suffocate and to prescind from the object.[31] *Heterorhabditis*/*Photorhabdus* associations can be very often found in bareley tree covered littoral zones,[135] which are easily observable and approachable for avian predators. Therefore an effective bird or animal repellent on infected insect larvae would benefit the protection against such scavengers. In this context it is also noteworthy that some anthraquinones have also been reported to bear antifungal activity and molluscicidal activity against some snails, which are further potential food competitors.[118,171] To gain more information of the biological role of *Photorhabdus* derived anthraquinones it would be necessary to setup suitable bioassays to examine if some of the discussed functions are also adapted by these compounds.

The biochemistry to anthraquinones can be distinguished according to two different biosynthetic pathways. One pathway follows the shikimate (chorismate)/*o*-succinylbenzoic acid pathway, whereas the other is a type II polyketide pathway.[98,110,188] In general, both pathways can be discriminated as chorismate/*o*-succinylbenzoic acid derived anthraquinones which are hydroxylated in only one ring and as polyketide derived products which are hydroxylated in both rings.[109] Anthraquinones from *Photorhabdus* represent typical polyketide derived compounds and the corresponding biosynthesis gene cluster is typical for type II PKS biosynthesis gene clusters with the classical minimal PKS consisting of an ACP and two ketosynthases, the catalytical active KS_{α} and the KS_{β} also referred as chain length factor CLF in literature. KS_{α} and KS_{β} form a heterodimer and are normally together with the ACP sufficient for the synthesis of the polyketide backbone.[76,77,178]

Some noticeable properties of the anthraquinone ketosythases have been already addressed in chapter two. Here it has to be highlighted that KS_{β} adopts a special rank in the row of other known type II KS_{β}, due to its additional C-terminal 120 amino acid end, which

shows no homology to other proteins or catalytic domains. In the same way, it is the first example of a KS_{β} harbouring aspartate instead of the widely conserved glutamine in the "active site". Bisang *et al.* succeeded in showing that KS_{β} with its conserved glutamine might be important for the initiation of polyketide synthesis by generating acetyl-KS through decarboxylase activity towards malonyl-ACP.[11] Indeed, anthraquinone biosynthesis also requires an acetate starter unit but the role of an aspartate mediated catalytic reaction has to be analyzed in future *in vitro* experiments. But even if KS_{β} does not fulfil a catalytic function it might have a structural function. This was recently confirmed by crystallographic and mass-spectroscopic analyses of the KS_{α}-KS_{β} heterodimer of actinorhodin. Here, both ketosynthases form an amphiphatic tunnel with polyketide synthesis taking place at the heterodimer interface and thereby influencing the polyketide chain length.[19,96] The "unnecessary" C-terminal end of KS_{β} AntE might contribute to a proper heterodimer interaction and polyketide chain length formation. Shortening of the C-terminal end will shed light on the biosynthetic function of the additional C-terminal end and KS_{β} at whole in anthraquinone biosynthesis.

Moreover it will be interesting to see if the *ant* minimal PKS cluster is able to form polyketides at all or even polyketides of different chain length. As mentioned before, normally it is assumed that the KS heterodimer and the ACP are sufficient to produce the polyketide product, but there is no general rule for this. It is becoming clear that it takes more than just the minimal PKS enzyme set to form the original polyketide chain for most type II PKS systems. Ectopic expression of the minimal spore pigment *whiE* from *Streptomyces coelicolor* for example generated a large array of more than 30 polyketides and pointed out that it does not independently control polyketide chain length.[168] For enterocin, it was the first report of a ketoreductase EncD that adopts a catalytical function in generating the polyketide chain hence representing the first example of an essential KR for polyketide production.[78] Additionally, disruption of an aromatase/cyclase from the oxytetracycline gene cluster resulted in the production of novel polyketides with shorter chain lengths.[147] From these examples it becomes more and more apparent that type II polyketide synthases are not just discrete alternate acting domains but are more complex interchanging enzymes with a well organized quaternary structure.

A hallmark of nearly all polyaromatic polyketides is the presence of cyclases and aromatases, proteins that channel in a chaperone like manner the correct folding, cyclisation and aromatisation of the nascent polyketide chain. Two cyclases *antC* and *antH* can be found in the anthraquinone cluster, which can be further divided into two groups. AntH belongs to the didomain group in which two repeats of the ARO/CYC domain are present, whereas

deletion of only one domain leads to a loss of catalytic activity.[77] These types of ARO/CYC enzymes are often associated with ketoreductase containing PKSs and catalyze the first ring cyclisation between C7-C12. This is presumably consistent with the first aromatic ring cyclisation of anthraquinones.[2] The deletion of *antH* in *Photorhabdus* led to the octaketide derived shunt products mutactin and dehydromutactin (Figure 3). Mutactin and dehydromutactin are well known shunt products of the *Streptomyces coelicolor* actinorhodin biosynthesis which accumulate when only minimal PKS and ketroreductase are expressed (Figure 3).[124,201] It is assumed that folding and cyclisation arise mainly by spontaneous aldol condensations, whereas it cannot be eliminated that the minimal PKS favours first ring cyclisation. However, in actinorhodin biosynthesis it was shown that ketoreduction is necessary to yield mutactin, while in the absence of KR two new shunt products SEK4 and SEK4b are formed (Figure 3).[58] Anthraquinone biosynthesis runs congruent in many parts to actinorhodin biosynthesis and one might anticipate that additional deletion of ketoreductase *antA* would lead to the same shunt products. Interestingly, the second cyclase *antC*, which belongs to the group having a single domain, seems to be unable to recognize the intermediate products of the *antH* mutant as a substrate. This can be assumed as in actinorhodin biosynthesis, in which a second cyclase is missing the same shunt product is produced, namely mutactin. One explanation might be that the produced polyketide intermediates are not recognized as the correct precursors. Crystal structure analysis of the monodomain tetracenomycin aromatase/cyclase gave an idea on the functional mode of these enzymes which contain a highly conserved interior pocket of size and shape that is important to orient and regiospecifically fold the polyketide.[3] However an *antC* generated insertion mutant exhibited two novel peaks in the UV chromatogram and the absence of anthraquinones (Figure 2). Unfortunately, to date it was not possible to assign a mass to one of these peaks; therefore it is still elusive if these new shunt products possess an 18 carbon or 16 carbon backbone like the final anthraquinone product. The elucidation of the unknown shunt products will give information on the cyclisation steps that cyclase AntC is involved in.

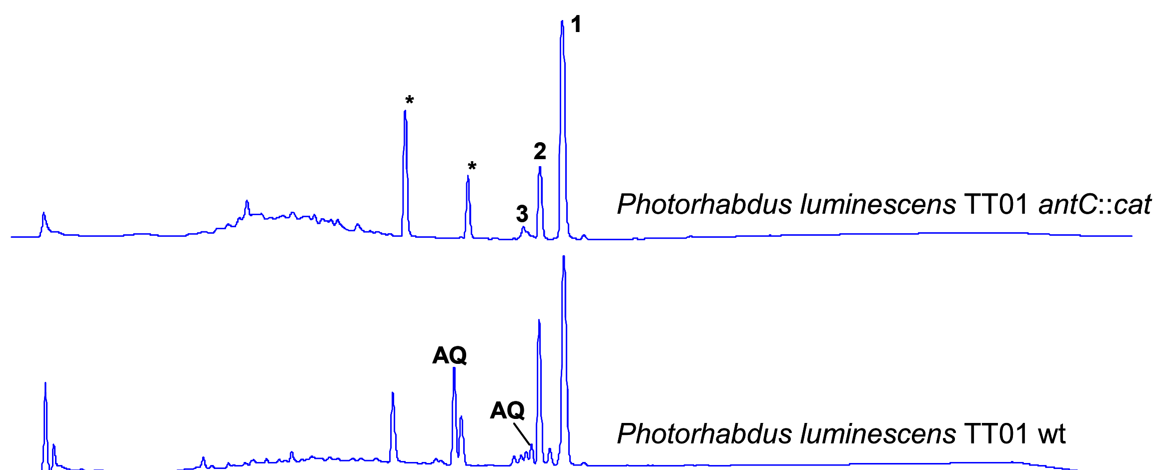


Figure 2. UV chromatograms of *Photorhabdus luminescens* TT01 and a cyclase *antC* insertion mutant. The *antC* mutant is still able to produce stilbenes (1=IPS, 2=eIPS and 3=ES; see chapter 5.1), but not anthraquinones (AQ). Instead, two additional new peaks (marked with an asterisk) are detectable which probably represent anthraquinone biosynthesis shunt products.

Like many other type II PKS clusters the *ant* cluster endows a ketoreductase (AntA). These enzymes with only few exceptions reduce regiospecifically the C9 carbonyl group.[77] In this work it was possible to assign the same specificity to AntA, as the octaketide mutactin is formed instead of the anthraquinone deduced heptaketide (Chapter 2). This finding gave evidence that C9 instead of C7 is reduced. Inhibition kinetics and emodin cocrystal structure of the actinrhodin ketoreductase *act* KR gained insight into structural and functional characteristics.[102] The preference of *act* KR for bicyclic substrates made it obvious that C7-C12 cyclized intermediates are the most likely substrates. Therefore C9 regiospecificity results from the dual constraints of a three point docking in the active site and the C7-C12 ring geometry of the substrate.[102] All this can properly also be assigned to AntA if the first C7-C12 cyclisation in anthraquinone biosynthesis takes place independently of the first acting cyclase AntH. This suggests that the minimal PKS also influences the first ring cyclisation, like it presumably does in the act minimal PKS products of SEK4 and SEK4b (Figure 3).

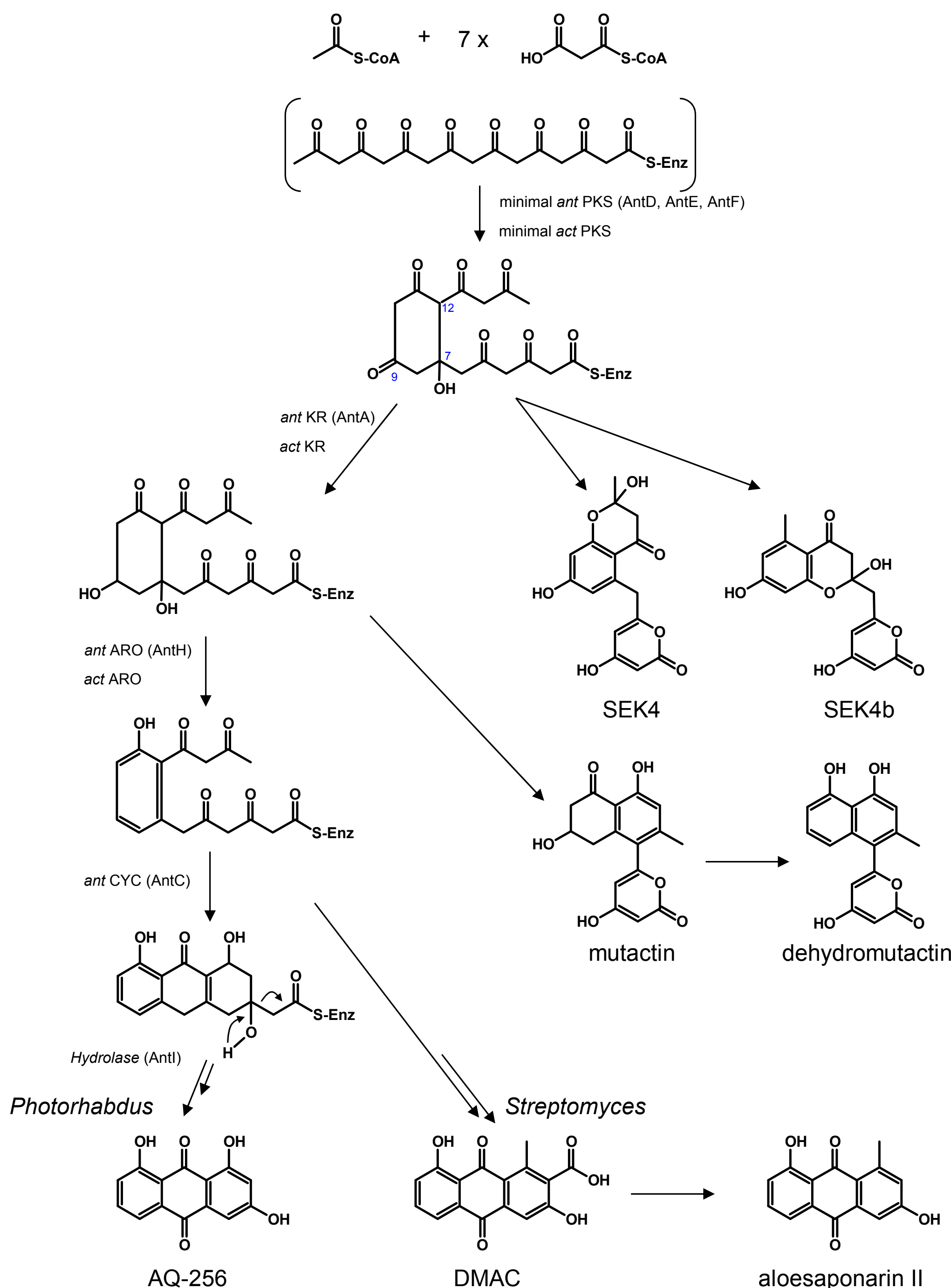


Figure 3. Similarities of anthraquinone and actinorhodin biosynthesis. Both minimal PKS form an octaketide, which is derived from acetyl-CoA and the elongation of seven malonyl-CoA extender units. The minimal PKS might also channel the first ring cyclisation between C7 and C12. SEK4 and SEK4b are shunt products of actinorhodin biosynthesis which occur when no further enzyme activities follow on the minimal PKS polyketide intermediate.[58,59] These products will probably also occur in an only minimal PKS expressing *Photorhabdus* mutant, although this still have to be demonstrated. The ketoreductase (KR) is regiospecifically reducing the polyketide intermediate at C9. An interruption after this point leads to the shunt product mutactin in anthraquinone and actinorhodin biosynthesis.[16] Dehydromutactin is a product which arises after the aromatisation and elimination of water in mutactin. In the presence of the aromatase (ARO) and cyclase (CYC) anthraquinone products are formed in both pathways, AQ-256, 3,8-dihydroxymethylanthraquinonecaboxylic acid (DMAC) and its decarboxylated derivative aloesaponarin II.[6,178] The apparent difference in the number of carbons of the final anthraquinone products may be ascribed to AntI which might be responsible for the shortening and release of the *Photorhabdus* derived polyketide intermediate.

Furthermore, the *ant* cluster of *Photorhabdus* harbours additional biosynthetic genes. Uncommonly, the cluster carries its own phosphopantetheinyltransferase (PPTase) encoded by gene *antB*. Its affiliation was proven by plasmid insertion that showed abolishment of anthraquinone production. Complementation by the second PPTase *ngrA* encoded in the genome, which is required for the activation of enzymes involved in stilbene biosynthesis, could not be detected. These observations suggest that *antB* shows a high specificity to the ACP AntF and which therefore might be the only substrate that is activated by AntB.

Unusual for anthraquinone biosynthesis is the existence of a CoA ligase encoding gene within the *ant* cluster. Normally these proteins are only found in type II PKS when a starter unit other than acetyl-Co or malonyl-CoA is used. For example, CoA ligases involved in frenolicin and enterocin biosyntheses are primed with butyryl-CoA and benzoate-CoA, respectivley.[77] Whether the CoA ligase AntG is involved in priming the anthraquinone PKS with acetyl-CoA or malonyl-CoA has to be verified. Furthermore a gene encoding an acyltransferase (AT) which loads the acyl-CoA onto the ACP AntF is missing within the *ant* cluster. Currently the best hypothesis is that PKSs for elongation with malonyl-CoA recruit the malonyl-CoA:ACP acyltransferase (MAT) of the related FAS. However, there are reports that the MAT is not necessarily needed as at least the *act* ACP is capable of self-malonylation in dependency on the ratio of ACP: KS_{α}: KS_{β}.[80,123] Further deletion mutants and *in vitro* experiments of the relevant anthraquinone biosynthesis enzymes will help to prove the described functions.

A gene encoding for a putative hydrolase/peptidase arouse interest, because it might be responsible for C-C bond cleavage and polyketide chain release. The closest homologue of the respective protein AntI shows similarity to a hydrolase from *Arthrobacter nicotinovorans*, which cleaves an 1,3-diketone hetero aromatic intermediate into its hetero aromatic ring and acyl-rest.[164] A related mechanism on the octaketide intermediate which releases the final 14 carbon anthraquinone and cleaves the redundant two carbons is therefore conceivable. Questionable is the time point of action, either before or after the last ring is cyclised. However, if this hypothesis can be confirmed it would be the first example of a release factor of a type II PKS.[77]

The *ant* cluster is imbedded by two genes for putative transcriptional regulators *plu4195* and *plu4185*. Both proteins depict a typical helix-turn-helix motif of DNA binding proteins and are most likely involved in regulation of anthraquinone production. Strikingly, the cluster includes neither methyltransferases, hydroxylases nor oxygenases although one could expect those based on the modified anthraquinone derivatives. Nevertheless several

methyltranseferase candidates with unknown substrate targets are located within the genome. Hydroxylases might not be required for anthraquinone biosynthesis, as the respective hydroxylated derivatives could only be isolated from extracts together with nematodes or insects.[85] So it is more likely that hydroxylation is not catalysed by the bacteria, but by insect or nematode derived enzymes. In addition it is also not clear yet, if an enzyme is involved in the quinone forming step. A homologue to the actinorhodin ActVA-ORF5 that is supposed to be responsible for C-6 oxygenation in the benzoisochromanequinone polyketides actinorhodin, granaticin and medermycin could not be found in the genome of *Photorhabdus*.[139] However, Plu0947 which shows similarities to tetracenomycin TcmH and actinorhodin ActVA-ORF6 monooxygenases represents a possible candidate for quinone formation.[77] Apart from that, spontaneous oxygenation cannot be excluded.

Xenofuranones

The xenofuranones were the first natural products isolated from the entomopathogenic bacterium *Xenorhabdus szentirmaii*, which displays itself a strong antimicrobial activity.[62] However, the observed bioactivity could not be ascribed to xenofuranones. The later isolated and already known phenazine iodinin is rather supposed to mediate the antibiotic activity against Gram positive bacteria and several fungi.[54] However, for the structural related and recently described allantofuranone from the imperfect ascomycete *Allantophomopsis lycopodina* an activity against filamentous fungi was demonstrated.[166] A similar function of xenofuranones in order to protect the infested insect larvae from saprophytic moulds might be possible and reasonable. Another so far untried investigation is the role of xenofuranones as quorum sensing molecules. These are cell-to-cell signalling molecules which enable bacteria to sense their population density and to respond thereon.[61,68] Some of these compounds have a furanone core structure and are known to be effective inhibitors of important bacterial pathogenesis factors.[119,133] A well known example are the halogenated furanones from the red algae *Delisea pulchra* which display inhibition of virulence factor production in the phytopathogen *Erwinia carotovora* and inhibition on swarming motility in *Proteus mirabilis*.[36,70,120] *X. szentirmaii* has to switch between a symbiotic and pathogenic mode in its life cycle and thereby has to respond on current conditions in the haemolymph of the infected host. Therefore communications signals which help to adapt the appropriate behaviour are conceivable. Xenofuranones might mediate such functions, but also inhibitory effects on bacterial competitors are imaginable. Recently xenofuranones were also identified in *Xenorhabdus stockiae* DSM17904 and *Xenorhabuds mauleonii* DSM17908 indicating that these compounds display an important function in different *Xenorhabdus* species as well (Figure 1).[17] The recently successful generation of a xenofuranone deficient *X. szentirmaii* mutant by an established transposon mutagenesis method,[160] will deliver answers on the biosynthetic origin and the biological function of xenofuranones (unpublished).

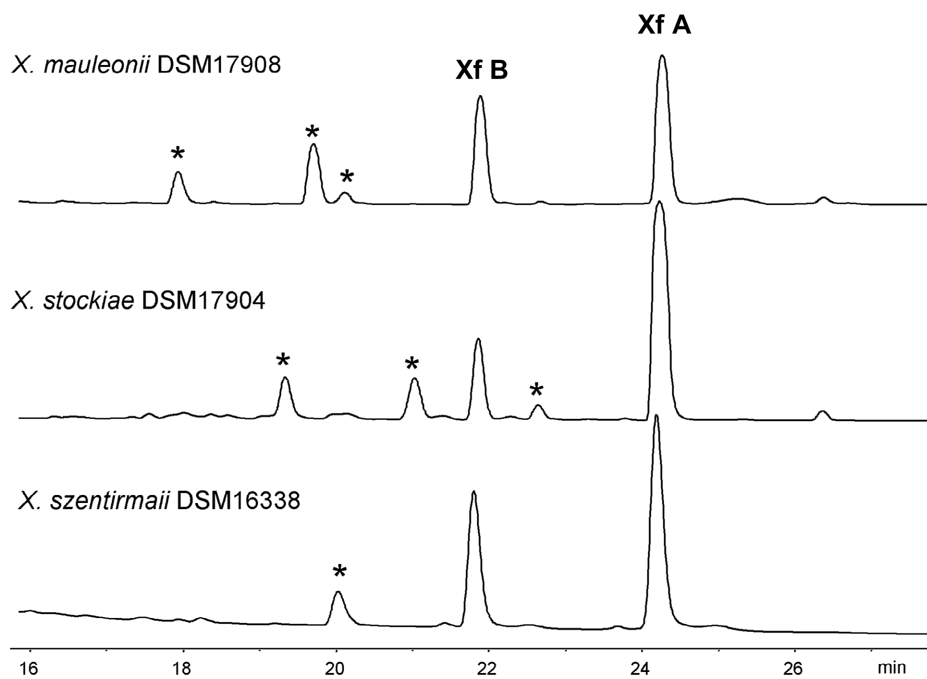


Figure 1. Production of xenofuranones (Xf) and so far unknown compounds (*) by different *Xenorhabdus* species.[17]

Reference List

The list only includes the citations of the introduction and the discussion, the single chapters contain their own reference list.

1. **Akhurst, R. J. and N. E. Boemare**. 1988. A numerical taxonomic study of the genus *Xenorhabdus* (Enterobacteriaceae) and proposed elevation of the subspecies of *X. nematophilus* to species. Journal of General Microbiology **134**:1835-1845.

2. **Alvarez, M. A., H. Fu, C. Khosla, D. A. Hopwood, and J. E. Bailey**. 1996. Engineered biosynthesis of novel polyketides: properties of the whiE aromatase/cyclase. Nat Biotechnol **14**:335-338.

3. **Ames, B. D., T. P. Korman, W. J. Zhang, P. Smith, T. Vu, Y. Tang, and S. C. Tsai**. 2008. Crystal structure and functional analysis of tetracenomycin ARO/CYC: Implications for cyclization specificity of aromatic polyketides. P. Natl. Acad. Sci. USA **105**:5349-5354.

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

5. **Bale, J. S., J. C. van Lenteren, and F. Bigler**. 2008. Biological control and sustainable food production. Philos. Trans. R. Soc Lond B Biol Sci. **363**:761-776.

6. **Bartel, P. L., C. B. Zhu, J. S. Lampel, D. C. Dosch, N. C. Connors, W. R. Strohl, J. M. Beale, and H. G. Floss**. 1990. Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthesis genes in Streptomycetes - clarification of actinorhodin gene functions. J. Bacteriol. **172**:4816-4826.

7. **Baur, M. E., H. K. Kaya, and D. R. Strong**. 1998. Foraging ants as scavengers on entomopathogenic nematode-killed insects. Biological Control **12**:231-236.

8. **Belas, R., R. Schneider, and M. Melch**. 1998. Characterization of *Proteus mirabilis* precocious swarming mutants: Identification of rsbA, encoding a regulator of swarming behavior. J. Bacteriol. **180**:6126-6139.

9. **Bennett, H. P. J. and D. J. Clarke**. 2005. The pbgPE operon in *Photorhabdus luminescens* is required for pathogenicity and symbiosis. J. Bacteriol. **187**:77-84.

10. **Bird, A. F. and R. J. Akhurst**. 1983. The nature of the intestinal vesicle in nematodes of the family Steinernematidae. Int. J. Parasitol. **13**:599-606.

11. **Bisang, C., P. F. Long, J. Cortés, J. Westcott, J. Crosby, A. L. Matharu, R. J. Cox, T. J. Simpson, J. Staunton, and P. F. Leadlay**. 1999. A chain initiation factor common to both modular and aromatic polyketide synthases. Nature **401**:502-505.

12. **Bode, H. B.** 2009. Entomopathogenic bacteria as a source of secondary metabolites. Curr Opin Chem Biol.**13**:224-230

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

14. **Boemare, N. E., R. J. Akhurst, and R. G. Mourant**. 1993. DNA relatedness between *Xenorhabdus spp*. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes,

and a proposal to transfer *Xenorhabdus luminescen*s to a new genus, *Photorhabdus* gen. nov. Int. J. Syst. Bacteriol. **43**:249-255.

15. **Brachmann, A. O., S. Forst, G. M. Furgani, A. Fodor, and H. B. Bode**. 2006. Xenofuranones A and B: phenylpyruvate dimers from *Xenorhabdus szentirmaii*. J. Nat. Prod. **69**:1830-1832.

16. **Brachmann, A. O., S. A. Joyce, H. Jenke-Kodama, G. Schwär, D. J. Clarke, and H. B. Bode**. 2007. A type II polyketide synthase is responsible for anthraquinone biosynthesis in *Photorhabdus luminescens*. ChemBioChem **8**:1721-1728.

17. **Brachmann, A. O., G. Schwär, and H. B. Bode**. 2008. *Photorhabdus* and *Xenorhabdus*: potent secondary metabolite producers. IOBC/wprs Bulletin **31**:151-156.

18. **Brunel, B., A. Givaudan, A. Lanois, R. J. Akhurst, and N. Boemare**. 1997. Fast and accurate identification of *Xenorhabdus* and *Photorhabdus* species by restriction analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. **63**:574-580.

19. **Burson, K. K. and C. Khosla**. 2000. Dissecting the Chain Length Specifcity in Bacterial Aromatic Polyketide Synthases using Chimeric Genes. Tetrahedron Tetrahedron **56**:9401-9408.

20. **Cane, D. E. and C. T. Walsh**. 1999. The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases. Chem. Biol. **6**:R319-R325.

21. **Chalabaev, S., E. Turlin, S. Bay, C. Ganneau, E. Brito-Fravallo, J. F. Charles, A. Danchin, and F. Biville**. 2008. Cinnamic acid, an autoinducer of its own biosynthesis, is processed via hca enzymes in *Photorhabdus luminescens*. Appl. Environ. Microbiol. **74**:1717-1725.

22. **Challis, G. L. and J. H. Naismith**. 2004. Structural aspects of non-ribosomal peptide biosynthesis. Curr. Opin. Struct. Biol. **14**:748-756.

23. **Chandra, H., P. Khandelwal, A. Khattri, and N. Banerjee**. 2008. Type 1 fimbriae of insecticidal bacterium *Xenorhabdus nematophila* is necessary for growth and colonization of its symbiotic host nematode *Steinernema carpocapsiae*. Environ. Microbiol. **10**:1285-1295.

24. **Ciche, T.** 2007. The biology and genome of *Heterorhabditis bacteriophora*. WormBook.1-9.

25. **Ciche, T. A., M. Blackburn, J. R. Carney, and J. C. Ensign**. 2003. Photobactin: a catechol siderophore produced by *Photorhabdus luminescens*, an entomopathogen mutually associated with *Heterorhabditis bacteriophora* NC1 nematodes. Appl. Environ. Microbiol. **69**:4706-4713.

26. **Ciche, T. A. and J. C. Ensign**. 2003. For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? Appl. Environ. Microbiol. **69**:1890-1897.

27. **Ciche, T. A., K. S. Kim, B. Kaufmann-Daszczuk, K. C. Q. Nguyen, and D. H. Hall**. 2008. Cell invasion and matricide during *Photorhabdus luminescens* transmission by *Heterorhabditis bacteriophora* nematodes. Appl. Environ. Microbiol. **74**:2275-2287.

28. **Clarke, D. J.** 2008. *Photorhabdus*: a model for the analysis of pathogenicity and mutualism. Cell Microbiol **10**:2159-2167.

29. **Clemo, G. R. and A. F. Daglish**. 1948. Structure of the pigment of *Chromobacterium iodinum*. Nature **162**:776.

30. **Clemo, G. R. and A. F. Daglish**. 1950. The Phenaxine Series. Part VIII. The Constitution of the Pigment of *Chromobacterium iodinum*. J. Chem. Soc.1481-1485.

31. **Copping, L. G. and S. O. Duke**. 2007. Natural products that have been used commercially as crop protection agents. Pest Management Science **63**:524-554.

32. **Cowles, K. N., C. E. Cowles, G. R. Richards, E. C. Martens, and H. Goodrich-Blair**. 2007. The global regulator Lrp contributes to mutualism, pathogenesis and phenotypic variation in the bacterium *Xenorhabdus nematophila*. Cellular Microbiology.

33. **Cragg, G. M., P. G. Grothaus, and D. J. Newman**. 2009. Impact of natural products on developing new anti-cancer agents. Chem Rev. **109**:3012-3043.

34. **Daborn, P. J., N. Waterfield, C. P. Silva, C. P. Y. Au, S. Sharma, and R. H. ffrench-Constant**. 2002. A single Photorhabdus gene, makes caterpillars floppy (mcf), allows *Escherichia coli* to persist within and kill insects. P. Natl. Acad. Sci. USA **99**:10742-10747.

35. **Daniel, R.** 2004. The soil metagenome - a rich resource for the discovery of novel natural products. Curr. Opin. Biotechnol. **15**:199-204.

36. **de Nys, R., M. Givskov, N. Kumar, S. Kjelleberg, and P. D. Steinberg**. 2006. Furanones. Prog. Mol. Subcell. Biol. **42**:55-86.

37. **Demain, A. L.** 1999. Pharmaceutically active secondary metabolites of microorganisms. Appl Microbiol Biotechnol **52**:455-463.

38. **Demain, A. L.** 2006. From natural products discovery to commercialization: a success story. J. Ind. Microbiol. Biotechnol. **33**:486-495.

39. **Derzelle, S., E. Duchaud, F. Kunst, A. Danchin, and P. Bertin**. 2002. Identification, characterization, and regulation of a cluster of genes involved in carbapenem biosynthesis in *Photorhabdus luminescens*. Appl. Environ. Microbiol. **68**:3780-3789.

40. **Derzelle, S., S. Ngo, E. Turlin, E. Duchaud, A. Namane, F. Kunst, A. Danchin, P. Bertin, and J. F. Charles**. 2004. AstR-AstS, a new two-component signal transduction system, mediates swarming, adaptation to stationary phase and phenotypic variation in *Photorhabdus luminescens*. Microbiology-(UK) **150**:897-910.

41. **Diaz, E., A. Ferrandez, M. A. Prieto, and J. L. Garcia**. 2001. Biodegradation of aromatic compounds by *Escherichia coli*. Microbiol Mol Biol Rev. **65**:523-69, table.

42. **Dowling, A. J., N. R. Waterfield, M. C. Hares, G. Le Goff, C. H. Streuli, and R. H. ffrench-Constant**. 2007. The Mcf1 toxin induces apoptosis via the mitochondrial pathway and apoptosis is attenuated by mutation of the BH3-like domain. Cellular Microbiology **9**:2470-2484.

43. **Du, L. and B. Shen**. 2001. Biosynthesis of hybrid peptide-polyketide natural products. Curr Opin Drug Discov Devel **4**:215-228.

44. **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.

45. **Duchaud, E., C. Rusniok, L. Frangeul, C. Buchrieser, A. Givaudan, S. Taourit, S. Bocs, C. Boursaux-Eude, M. Chandler, J. F. Charles, E. Dassa, R. Derose, S. Derzelle, G. Freyssinet, S. Gaudriault, C. Medigue, A. Lanois, K. Powell, P. Siguier, R. Vincent, V. Wingate, M. Zouine, P. Glaser, N. Boemare, A. Danchin, and F. Kunst**. 2003. The genome

sequence of the entomopathogenic bacterium Photorhabdus luminescens. Nat. Biotechnol. **21**:1307-1313.

46. **Easom, C. A. and D. J. Clarke**. 2008. Motility is required for the competitive fitness of entomopathogenic *Photorhabdus luminescens* during insect infection. Bmc Microbiology **8**.

47. **Ehlers, R. U.** 2001. Mass production of entomopathogenic nematodes for plant protection. Appl. Microbiol. Biotechnol. **56**:623-633.

48. **Eisner, T., S. Nowicki, M. Goetz, and J. Meinwald**. 1980. Red Cochineal Dye (Carminic Acid) - Its Role in Nature. Science **208**:1039-1042.

49. **Farmer, J. J.** 1984. Other genera of the family Enterobacteriaceae, p. 506-516. *In* N. N. Krieg and J. G. Holt (eds.), Bergey's Manual of systematic Bacteriology, Williams & Williams. Baltimore, MD.

50. **Farmer, J. J., III, J. H. Jorgensen, P. A. Grimont, R. J. Akhurst, G. O. Poinar, Jr., E. Ageron, G. V. Pierce, J. A. Smith, G. P. Carter, and K. L. Wilson**. 1989. *Xenorhabdus luminescens* (DNA hybridization group 5) from human clinical specimens. J Clin Microbiol **27**:1594-1600.

51. **Fieseler, L., U. Hentschel, L. Grozdanov, A. Schirmer, G. P. Wen, M. Platzer, S. Hrvatin, D. Butzke, K. Zimmermann, and J. Piel**. 2007. Widespread occurrence and genomic context of unusually small polyketide synthase genes in microbial consortia associated with marine sponges. Appl. Environ. Microbiol. **73**:2144-2155.

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

53. **Fischer-Le Saux, M., V. Viallard, B. Brunel, P. Normand, and N. E. Boemare**. 1999. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. Int J Syst Bacteriol **49 Pt 4**:1645-1656.

54. **Fodor, A., S. Forst, L. Haynes, M. Hevesi, J. Hogan, M. G. Klein, A. Mathe-Fodor, E. Stackebrandt, A. Szentirmai, F. Sztaricskai, T. Érsek, and M. Zeller**. 2008. New perspectives of *Xenorhabdus* antibiotics research. Insect Pathogens and Insect Parasitic Nematodes IOBC/wrps Bulletin **31**:157-164.

55. **Forst, S., B. Dowds, N. E. Boemare, and E. Stackebrandt**. 1997. *Xenorhabdus* and *Photorhabdus* spp.: Bugs that kill bugs. Annu Rev Microbiol **51**:47-72.

56. **Foucault, C. and P. Brouqui**. 2007. How to fight antimicrobial resistance. FEMS Immunol. Med. Microbiol. **49**:173-183.

57. **Fremont, L.** 2000. Minireview - Biological effects of resveratrol. Life Sci. **66**:663-673.

58. **Fu, H., S. Ebert-Khosla, D. A. Hopwood, and C. Khosla**. 1994. Engineered Biosynthesis of Novel Polyketides: Dissection of the Catalytic Specificity of the act Ketoreductase. J Am Chem Soc **116**:4166-4170.

59. **Fu, H., D. A. Hopwood, and C. Khosla**. 1994. Engineered biosynthesis of novel polyketides: evidence for temporal, but not regiospecific, control of cyclization of an aromatic polyketide precursor. Chem. Biol. **1**:205-210.

60. **Funabashi, M., N. Funa, and S. Horinouchi**. 2008. Phenolic lipids synthesized by type III polyketide synthase confer penicillin resistance on *Streptomyces griseus*. J. Biol. Chem. **283**:13983-13991.

61. **Fuqua, C. and E. P. Greenberg**. 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat Rev. Mol Cell Biol **3**:685-695.

62. **Furgani, G., E. Boszormenyi, A. Fodor, A. Mathe-Fodor, S. Forst, J. S. Hogan, Z. Katona, M. G. Klein, E. Stackebrandt, A. Szentirmai, F. Sztaricskai, and S. L. Wolf**. 2008. *Xenorhabdus* antibiotics: a comparative analysis and potential utility for controlling mastitis caused by bacteria. J. Appl. Microbiol. **104**:745-758.

63. **Gaugler, R. and H. K. Kaya**. 1990. Entomopathogenic nematodes in biological control. CRC Press, Inc..

64. **Gerrard, J. G., S. A. Joyce, D. J. Clarke, R. H. ffrench-Constant, G. R. Nimmo, D. F. M. Looke, E. J. Feil, L. Pearce, and N. R. Waterfield**. 2006. Nematode symbiont for *Photorhabdus asymbiotica*. Emerg. Infect. Dis. **12**:1562-1564.

65. **Gerrard, J. G., S. McNevin, D. Alfredson, R. Forgan-Smith, and N. Fraser**. 2003. *Photorhabdus* species: bioluminescent bacteria as emerging human pathogens? Emerg. Infect. Dis. **9**:251-254.

66. **Gerritsen, L. J., R. G. de, and P. H. Smits**. 1992. Characterization of form variants of *Xenorhabdus luminescens*. Appl. Environ. Microbiol **58**:1975-1979.

67. **Givaudan, A., S. Baghdiguian, A. Lanois, and N. Boemare**. 1995. Swarming and Swimming Changes Concomitant with Phase Variation in *Xenorhabdus nematophilus*. Appl. Environ. Microbiol. **61**:1408-1413.

68. **Gonzalez, J. E. and N. D. Keshavan**. 2006. Messing with bacterial quorum sensing. Microbiol. Mol. Biol. Rev. **70**:859-875.

69. **Goodrich-Blair, H. and D. J. Clarke**. 2007. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. Mol. Microbiol. **64**:260-268.

70. **Gram, L., N. R. de, R. Maximilien, M. Givskov, P. Steinberg, and S. Kjelleberg**. 1996. Inhibitory Effects of Secondary Metabolites from the Red Alga *Delisea pulchra* on Swarming Motility of *Proteus mirabilis*. Appl. Environ. Microbiol **62**:4284-4287.

71. **Handelsman, J., M. R. Rondon, S. F. Brady, J. Clardy, and R. M. Goodman**. 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem. Biol. **5**:R245-R249.

72. **Hendrie, M. S., A. J. Holding, and J. M. Shewan**. 1974. Emended descriptions of the genus *Alcaligenes* and of *Alcaligenes faecalis* and proposal that the generic name *Achromobacter* be rejected - status of the named species of *Alcaligenes* and *Achromobacter* - request for an opinion. Int. J. Syst. Bacteriol. **24**:534-550.

73. **Herbert, E. E., K. N. Cowles, and H. Goodrich-Blair**. 2007. CpxFA regulates mutualism and pathogenesis in *Xenorhabdus nematophila*. Appl. Environ. Microbiol. **73**:7826-7836.

74. **Herbert, E. E. and H. Goodrich-Blair**. 2007. Friend and foe: the two faces of *Xenorhabdus nematophila*. Nat. Rev. Microbiol. **5**:634-646.

75. **Hertweck, C.** 2009. Hidden biosynthetic treasures brought to light. Nat Chem Biol **5**:450-452.

76. **Hertweck, C.** 2009. The biosynthetic logic of polyketide diversity. Angew. Chem Int. Ed Engl. **48**:4688-4716.

77. **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.

78. **Hertweck, C., L. Xiang, J. A. Kalaitzis, Q. Cheng, M. Palzer, and B. S. Moore**. 2004. Context-dependent behavior of the enterocin iterative polyketide synthase; a new model for ketoreduction. Chem. Biol. **11**:461-468.

79. **Hilker, M. and S. Schulz**. 1991. Anthraquinones in different developmental stages of *Galeruca tanaceti* (Coleoptera, Chrysomelidae). J. Chem. Ecol. **17**:2323-2332.

80. **Hitchman, T. S., J. Crosby, K. J. Byrom, R. J. Cox, and T. J. Simpson**. 1998. Catalytic self-acylation of type II polyketide synthase acyl carrier proteins. Chem. Biol. **5**:35-47.

81. **Hjelmgaard, T., M. Givskov, and J. Nielsen**. 2007. Expedient total synthesis of pyrrothine natural products and analogs. Org. Biomol. Chem. **5**:344-348.

82. **Hopwood, D. A.** 1997. Genetic contributions to understanding polyketide synthases. Chem Rev **97**:2465-2497.

83. **Hori, Y., S. Takase, N. Shigemastsu, T. Goto, M. Okuhara, and M. Kohsaka**. 1993. R1128 substances, novel non-steroidal estrogen-receptor antagonists produced by a *Streptomyces*. II. Physico-chemical properties and structure determination. J Antibiot **46**:1063-1068.

84. **Hu, K. J., J. X. Li, B. Li, J. M. Webster, and G. H. Chen**. 2006. A novel antimicrobial epoxide isolated from larval *Galleria mellonella* infected by the nematode symbiont, *Photorhabdus luminescens* (Enterobacteriaceae). Bioorganic & Medicinal Chemistry **14**:4677-4681.

85. **Hu, K. J., J. X. Li, W. J. Wang, H. M. Wu, H. Lin, and J. M. Webster**. 1998. Comparison of metabolites produced in vitro and in vivo by *Photorhabdus luminescens*, a bacterial symbiont of the entomopathogenic nematode *Heterorhabditis megidis*. Can J Microbiol Can J Microbiol **44**:1072-1077.

86. **Huang, Y. H., L. Ferrieres, and D. J. Clarke**. 2006. The role of the Rcs phosphorelay in Enterobacteriaceae. Res. Microbiol **157**:206-212.

87. **Hubbard, B. K. and C. T. Walsh**. 2003. Vancomycin assembly: nature's way. Angew. Chem. Int. Ed. **42**:730-765.

88. **Hwang, S. Y., S. Paik, S. H. Park, H. S. Kim, I. S. Lee, S. P. Kim, W. K. Baek, M. H. Suh, T. K. Kwon, J. W. Park, J. B. Park, J. J. Lee, and S. I. Suh**. 2003. N-phenethyl-2-phenylacetamide isolated from *Xenorhabdus nematophilus* induces apoptosis through caspase activation and calpain-mediated Bax cleavage in U937 cells. Int J Oncol **22**:151-157.

89. **Janse, J. D. and P. H. Smits**. 1990. Whole cell fatty acid patterns of *Xenorhabdus* species. Lett Appl Microbiol **10**:131-135.

90. **Ji, D., Y. Yi, G. H. Kang, Y. H. Choi, P. Kim, N. I. Baek, and Y. Kim**. 2004. Identification of an antibacterial compound, benzylideneacetone, from *Xenorhabdus nematophila* against major plant-pathogenic bacteria. FEMS Microbiol. Lett. **239**:241-248.

91. **Johnigk, S. A. and R. U. Ehlers**. 1999. *Endotokia matricida* in hermaphrodites of *Heterorhabditis* spp. and the effect of the food supply. Nematology **1**:717-726.

92. **Joyce, S. A. and D. J. Clarke**. 2003. A hexA homologue from *Photorhabdus* regulates pathogenicity, symbiosis and phenotypic variation. Mol. Microbiol. **47**:1445-1457.

93. **Joyce, S. A., R. J. Watson, and D. J. Clarke**. 2006. The regulation of pathogenicity and mutualism in *Photorhabdus*. Curr. Opin. Microbiol. **9**:127-132.

94. **Kaya, H. K. and R. Gaugler**. 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. **38**:181-206.

95. **Keating, T. A., C. G. Marshall, C. T. Walsh, and A. E. Keating**. 2002. The structure of VibH represents nonribosomal peptide synthetase condensation, cyclization and epimerization domains. Nat. Struct. Biol. **9**:522-526.

96. **Keatinge-Clay, A. T., D. A. Maltby, K. F. Medzihradszky, C. Khosla, and R. M. Stroud**. 2004. An antibiotic factory caught in action. Nat Struct Mol Biol **11**:888-893.

97. **Kleine-Gunk, B.** 2007. Resveratrol Schlüssel für ein langes Leben? Pharmazeutische Zeitung **152**:16-22.

98. **Knaggs, A. R.** 2001. The biosynthesis of shikimate metabolites. Nat. Prod. Rep. **18**:334-355.

99. **Kniazeva, M., Q. T. Crawford, M. Seiber, C. Y. Wang, and M. Han**. 2004. Monomethyl branched-chain fatty acids play an essential role in *Caenorhabditis elegans* development. Plos Biology **2**:1446-1459.

100. **Kniazeva, M., T. Euler, and M. Han**. 2008. A branched-chain fatty acid is involved in post-embryonic growth control in parallel to the insulin receptor pathway and its biosynthesis is feedback-regulated in C. elegans. Genes Dev. **22**:2102-2110.

101. **Kohli, R. M. and C. T. Walsh**. 2003. Enzymology of acyl chain macrocyclization in natural product biosynthesis. Chem. Commun.297-307.

102. **Korman, T. P., Y. H. Tan, J. Wong, R. Luo, and S. C. Tsai**. 2008. Inhibition kinetics and emodin cocrystal structure of a type II polyketide ketoreductase. Biochemistry-US **47**:1837-1847.

103. **Kwon, B. and Y. Kim**. 2008. Benzylideneacetone, an immunosuppressant, enhances virulence of *Bacillus thuringiensis* against beet armyworm (Lepidoptera: Noctuidae). J Econ. Entomol. **101**:36-41.

104. **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.

105. **Lang, G., T. Kalvelage, A. Peters, J. Wiese, and J. F. Imhoff**. 2008. Linear and cyclic peptides from the entomopathogenic Bacterium *Xenorhabdus nematophilus*. J. Nat. Prod. **71**:1074-1077.

106. **Lanois, A., G. Jubelin, and A. Givaudan**. 2008. FliZ, a flagellar regulator, is at the crossroads between motility, haemolysin expression and virulence in the insect pathogenic bacterium *Xenorhabdus*. Mol. Microbiol. **68**:516-533.

107. **Lee, S. K., H. J. Lee, H. Y. Min, E. J. Park, K. M. Lee, Y. H. Ahn, Y. J. Cho, and J. H. Pyee**. 2005. Antibacterial and antifungal activity of pinosylvin, a constituent of pine. Fitoterapia **76**:258-260.

108. **Lefevre, F., P. Robe, C. Jarrin, A. Ginolhac, C. Zago, D. Auriol, T. M. Vogel, P. Simonet, and R. Nalin**. 2008. Drugs from hidden bugs: their discovery via untapped resources. Res. Microbiol **159**:153-161.

109. **Leistner, E.** 1973. Biosynthesis of Morindone and Alizarin in intact plants and cell suspension cultures of *Morinda citrifolia*. Phytochemistry Phytochemistry **12**:1669-1674.

110. **Leistner, E. and M. H. Zenk**. 1967. [A new pathway for biosynthesis of anthraquinones: incorporation of shikimic acid into 1,2-dihydroxyanthraquinone (alizarin) and 1,2,4-trihydroxyanthraquinone (purpurin) in *Rubia tinctorum* L]. Z. Naturforsch. B **22**:865-868.

111. **Lengyel, K., E. Lang, A. Fodor, E. Szallas, P. Schumann, and E. Stackebrandt**. 2005. Description of four novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. Syst. Appl. Microbiol. **28**:115-122.

112. **Li, J., G. Chen, and J. M. Webster**. 1996. N-(Indol-3-ylethyl)-2'-hydroxy-3'-methylpentanamide, a Novel Indole Derivative from *Xenorhabdus nematophilus*. J. Nat. Prod. **59**:1157-1158.

113. **Li, J., G. Chen, J. M. Webster, and E. Czyzewska**. 1995. Antimicrobial metabolites from a bacterial symbiont. J. Nat. Prod. **58**:1081-1086.

114. **Li, J., K. Hu, and J. M. Webster**. 1998. Antibiotics from *Xenorhabdus* spp. and *Photorhabdus* spp. (Enterobacterialceae). Chemistry of Hereocyclic Compounds **34**:1331-1339.

115. **Li, J. X., G. H. Chen, and J. M. Webster**. 1997. Nematophin, a novel antimicrobial substance produced by *Xenorhabdus nematophilus* (Enterobactereaceae). Can J Microbiol Can J Microbiol **43**:770-773.

116. **Li, J. X., G. H. Chen, and J. M. Webster**. 1997. Synthesis and antistaphylococcal activity of nematophin and its analogs. Bioorg Med Chem Lett Bioorg Med Chem Lett **7**:1349-1352.

117. **Li, J. X., G. H. Chen, H. M. Wu, and J. M. Webster**. 1995. Identification of two pigments and a hydroxystilbene antibiotic from *Photorhabdus luminescens*. Appl. Environ. Microbiol. **61**:4329-4333.

118. **Liu, S. Y., F. Sporer, M. Wink, J. Jourdane, R. Henning, Y. L. Li, and A. Ruppel**. 1997. Anthraquinones in *Rheum palmatum* and *Rumex dentatus* (Polygonaceae), and phorbol esters in *Jatropha curcas* (Euphorbiaceae) with molluscicidal activity against the schistosome vector snails *Oncomelania, Biomphalaria* and *Bulinus*. Tropical Medicine & International Health **2**:179-188.

119. **Manefield, M., N. R. de, N. Kumar, R. Read, M. Givskov, P. Steinberg, and S. Kjelleberg**. 1999. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology **145 (Pt 2)**:283-291.

120. **Manefield, M., M. Welch, M. Givskov, G. P. Salmond, and S. Kjelleberg**. 2001. Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. FEMS Microbiol Lett. **205**:131-138.

121. **Mansour, S. A.** 2004. Pesticide exposure--Egyptian scene. Toxicology **198**:91-115.

122. **Martens, E. C., K. Heungens, and H. Goodrich-Blair**. 2003. Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria. J. Bacteriol. **185**:3147-3154.

123. **Matharu, A. L., R. J. Cox, J. Crosby, K. J. Byrom, and T. J. Simpson**. 1998. MCAT is not required for in vitro polyketide synthesis in a minimal actinorhodin polyketide synthase from *Streptomyces* coelicolor. Chem. Biol. **5**:699-711.

124. **McDaniel, R., S. Ebert-Khosla, H. Fu, D. A. Hopwood, and C. Khosla**. 1994. Engineered biosynthesis of novel polyketides: influence of a downstream enzyme on the catalytic specificity of a minimal aromatic polyketide synthase. P. Natl. Acad. Sci. USA **91**:11542-11546.

125. **McInerney, B. V., R. P. Gregson, M. J. Lacey, R. J. Akhurst, G. R. Lyons, S. H. Rhodes, D. R. Smith, L. M. Engelhardt, and A. H. White**. 1991. Biologically active metabolites from *Xenorhabdus* spp., Part 1. Dithiolopyrrolone derivatives with antibiotic activity. J. Nat. Prod. **54**:774-784.

126. **McInerney, B. V., W. C. Taylor, M. J. Lacey, R. J. Akhurst, and R. P. Gregson**. 1991. Biologically active metabolites from *Xenorhabdus* spp., Part 2. Benzopyran-1-one derivatives with gastroprotective activity. J. Nat. Prod. **54**:785-795.

127. **Mendez, C. and J. A. Salas**. 2001. Altering the glycosylation pattern of bioactive compounds. Trends Biotechnol. **19**:449-456.

128. **Miller, D. A. and C. T. Walsh**. 2001. Yersiniabactin synthetase: Probing the recognition of carrier protein domains by the catalytic heterocyclization domains, Cy1 and Cy2, in the chain-initiating HMWP2 subunit. Biochemistry-US **40**:5313-5321.

129. **Milne, J. C. and J. M. Denu**. 2008. The Sirtuin family: therapeutic targets to treat diseases of aging. Curr. Opin. Chem. Biol. **12**:11-17.

130. **Milne, J. C., P. D. Lambert, S. Schenk, D. P. Carney, J. J. Smith, D. J. Gagne, L. Jin, O. Boss, R. B. Perni, C. B. Vu, J. E. Bemis, R. Xie, J. S. Disch, P. Y. Ng, J. J. Nunes, A. V. Lynch, H. Y. Yang, H. Galonek, K. Israelian, W. Choy, A. Iffland, S. Lavu, O. Medvedik, D. A. Sinclair, J. M. Olefsky, M. R. Jirousek, P. J. Elliott, and C. H. Westphal**. 2007. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature **450**:712-716.

131. **Mofid, M. R., R. Finking, and M. A. Marahiel**. 2002. Recognition of hybrid peptidyl carrier proteins/acyl carrier proteins in nonribosomal peptide synthetase modules by the 4 '-phophopantetheinyl transferases AcpS and Sfp. J. Biol. Chem. **277**:17023-17031.

132. **Moore, B. S. and C. Hertweck**. 2002. Biosynthesis and attachment of novel bacterial polyketide synthase starter units. Nat. Prod. Rep. **19**:70-99.

133. **Ndagijimana, M., M. Vallicelli, P. S. Cocconcelli, F. Cappa, F. Patrignani, R. Lanciotti, and M. E. Guerzoni**. 2006. Two 2[5H]-furanones as possible signaling molecules in *Lactobacillus helveticus*. Appl. Environ. Microbiol **72**:6053-6061.

134. **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.

135. **Nguyen, K. B. and D. J. Hunt**. 2007. Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts. Brill, Leiden, Bosten.

136. **Nicholson, G. M.** 2007. Fighting the global pest problem: preface to the special Toxicon issue on insecticidal toxins and their potential for insect pest control. Toxicon **49**:413-422.

137. **Nishimura, Y., A. Hagiwara, T. Suzuki, and S. Yamanaka**. 1994. *Xenorhabdus japonicus* Sp-Nov Associated with the Nematode *Steinernema kushidai*. World Journal of Microbiology & Biotechnology **10**:207-210.

138. **Nowak-Thompson, B., P. E. Hammer, D. S. Hill, J. Stafford, N. Torkewitz, T. D. Gaffney, S. T. Lam, I. Molnar, and J. M. Ligon**. 2003. 2,5-dialkylresorcinol biosynthesis in *Pseudomonas aurantiaca*: novel head-to-head condensation of two fatty acid-derived precursors. J. Bacteriol. **185**:860-869.

139. **Okamoto, S., T. Taguchi, K. Ochi, and K. Ichinose**. 2009. Biosynthesis of actinorhodin and related antibiotics: discovery of alternative routes for quinone formation encoded in the *act* gene cluster. Chem Biol **16**:226-236.

140. **O´Halloran, D. M. and A. M. Burnell**. 2002. Olfaction and odour discrimination in the insect parasitic nematode *Heterorhabditis bacteriophora*. Nematology **4**:206.

141. **Paik, S., Y. H. Park, S. I. Suh, H. S. Kim, I. S. Lee, M. K. Park, C. S. Lee, and S. H. Park**. 2001. Unusual cytotoxic phenethylamides from *Xenorhabdus nematophilus*. Bull. Korean Chem. Soc. **22**:372-374.

142. **Park, Y., Y. Kim, S. M. Putnam, and D. W. Stanley**. 2003. The bacterium *Xenorhabdus nematophilus* depresses nodulation reactions to infection by inhibiting eicosanoid biosynthesis in tobacco hornworms, *Manduca sexta*. Arch. Insect Biochem. Physiol. **52**:71-80.

143. **Park, Y., Y. Kim, and D. Stanley**. 2004. The bacterium *Xenorhabdus nematophila* inhibits phospholipases A2 from insect, prokaryote, and vertebrate sources. Naturwissenschaften **91**:371-373.

144. **Paul, V. J., S. Frautschy, W. Fenical, and K. H. Nealson**. 1981. Antibiotics in microbial ecology - Isolation and structure assignment of several new anti-bacterial compounds from the insect-symbiotic bacteria *Xenorhabdus* Spp. J. Chem. Ecol. **7**:589-597.

145. **Peel, M. M., D. A. Alfredson, J. G. Gerrard, J. M. Davis, J. M. Robson, R. J. McDougall, B. L. Scullie, and R. J. Akhurst**. 1999. Isolation, identification, and molecular characterization of strains of *Photorhabdus luminescens* from infected humans in Australia. J Clin Microbiol **37**:3647-3653.

146. **Peleman, R. A.** 2004. New and re-emerging infectious diseases: epidemics in waiting. Curr. Opin. Anaesthesiol. **17**:265-270.

147. **Petkovic, H., A. Thamchaipenet, L. H. Zhou, D. Hranueli, P. Raspor, P. G. Waterman, and I. S. Hunter**. 1999. Disruption of an aromatase/cyclase from the oxytetracycline gene cluster of *Streptomyces rimosus* results in production of novel polyketides with shorter chain lengths. J Biol Chem **274**:32829-32834.

148. **Piel, J.** 2004. Metabolites from symbiotic bacteria. Nat. Prod. Rep. **21**:519-538.

149. **Ramia, S., E. Neter, and D. J. Brenner**. 1982. Production of enterobacterial common antigen as an aid to classification of newly identified species of the families *Enterobacteriaceae* and *Vibrionaceae*. Int. J. Syst. Bacteriol. **32**:395-398.

150. **Rasmann, S., T. G. Kollner, J. Degenhardt, I. Hiltpold, S. Toepfer, U. Kuhlmann, J. Gershenzon, and T. C. Turlings**. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature **434**:732-737.

151. **Reimer, D.** 2008. Masterarbeit. Untersuchungen zur Biosynthese der Xenocoumacine und Identifizierung weiterer Sekundärstoffe aus *Xenorhabdus nematophila*. Naturwissenschaftlich-Technische Fakultät III Chemie, Pharmazie, Bio- und Werkstoffwissenschaften der Universität des Saarlandes

152. **Reimer, D., E. Luxenburger, A. O. Brachmann, and H. B. Bode**. 2009. A New Type of Pyrrolidine Biosynthesis Is Involved in the Late Steps of Xenocoumacin Production in *Xenorhabdus nematophila*. Chembiochem.**10**:1997-2001

153. **Reverchon, S., C. Rouanet, D. Expert, and W. Nasser**. 2002. Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi* and role of this blue pigment in pathogenicity. J. Bacteriol. **184**:654-665.

154. **Richards, G. R., E. E. Herbert, Y. Park, and H. Goodrich-Blair**. 2008. *Xenorhabdus nematophila* lrhA is necessary for motility, lipase activity, toxin expression, and virulence in *Manduca sexta* insects. J. Bacteriol. **190**:4870-4879.

155. **Richardson, W. H., T. M. Schmidt, and K. H. Nealson**. 1988. Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. Appl. Environ. Microbiol. **54**:1602-1605.

156. **Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Liles, K. A. Loiacono, B. A. Lynch, I. A. MacNeil, C. Minor, C. L. Tiong, M. Gilman, M. S. Osburne, J. Clardy, J. Handelsman, and R. M. Goodman**. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol. **66**:2541-2547.

157. **Rudman, P., E. W. B. Costa, F. J. Gay, and A. H. Wetherly**. 1958. Relationship of Tectoquinone to Durability in *Tectona grandis*. Nature **181**:721-722.

158. **Samel, S. A., M. A. Marahiel, and L. O. Essen**. 2008. How to tailor non-ribosomal peptide products - new clues about the structures and mechanisms of modifying enzymes. Molecular Biosystems **4**:387-393.

159. **Sandmann, A., J. Dickschat, H. Jenke-Kodama, B. Kunze, E. Dittmann, and R. Müller**. 2007. A type II polyketide synthase from the gram-negative bacterium *Stigmatella aurantiaca* is involved in aurachin alkaloid biosynthesis. Angew. Chem. Int. Ed. **46**:2712-2716.

160. **Sandmann, A., F. Sasse, and R. Müller**. 2004. Identification and analysis of the core biosynthetic machinery of tubulysin, a potent cytotoxin with potential anticancer activity. Chemistry & Biology **11**:1071-1079.

161. **Schäfer, B.** 2007. Naturstoffe der chemischen Industrie. Elsevier, München.

162. **Scherlach, K. and C. Hertweck**. 2009. Triggering cryptic natural product biosynthesis in microorganisms. Org. Biomol. Chem **7**:1753-1760.

163. **Scherlach, K., L. P. Partida-Martinez, H. M. Dahse, and C. Hertweck**. 2006. Antimitotic rhizoxin derivatives from a cultured bacterial endosymbiont of the rice pathogenic fungus *Rhizopus microsporus*. J. Am. Chem. Soc. **128**:11529-11536.

164. **Schleberger, C., P. Sachelaru, R. Brandsch, and G. E. Schulz**. 2007. Structure and action of a C-C bond cleaving alpha/beta-hydrolase involved innicotine degradation. Journal of Molecular Biology **367**:409-418.

165. **Schröder, J.** 1997. A family of plant-specific polyketide synthases: facts and predictions. Trends Plant Sci. **2**:373-378.

166. **Schüffler, A., D. L. J. C. Kautz, T. Opatz, and T. Anke**. 2009. Allantofuranone, a new antifungal antibiotic from *Allantophomopsis lycopodina* IBWF58B-05A. J Antibiot **62**:119-121.

167. **Schwarzer, D., R. Finking, and M. A. Marahiel**. 2003. Nonribosomal peptides: From genes to products. Nat. Prod. Rep. **20**:275-287.

168. **Shen, Y., P. Yoon, T. W. Yu, H. G. Floss, D. Hopwood, and B. S. Moore**. 1999. Ectopic expression of the minimal whiE polyketide synthase generates a library of aromatic polyketides of diverse sizes and shapes. P. Natl. Acad. Sci. USA **96**:3622-3627.

169. **Sicard, M., K. Brugirard-Ricaud, S. Pages, A. Lanois, N. E. Boemare, M. Brehelin, and A. Givaudan**. 2004. Stages of infection during the tripartite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts. Appl. Environ. Microbiol. **70**:6473-6480.

170. **Sieber, S. A. and M. A. Marahiel**. 2005. Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. Chem Rev **105**:715-738.

171. **Singh, D. N., N. Verma, S. Raghuwanshi, P. K. Shukla, and D. K. Kulshreshtha**. 2006. Antifungal anthraquinones from *Saprosma fragrans*. Bioorg Med Chem Lett Bioorg Med Chem Lett **16**:4512-4514.

172. **Smart, Jr. G. C.** 1995. Entomopathogenic Nematodes for the Biological Control of Insects. Journal of Nematology **27**:529-534.

173. **Smith, S. and S. C. Tsai**. 2007. The type I fatty acid and polyketide synthases: a tale of two megasynthases. Nat. Prod. Rep. **24**:1041-1072.

174. **Soleas, G. J., E. P. Diamandis, and D. M. Goldberg**. 1997. Resveratrol: a molecule whose time has come? And gone? Clin. Biochem. **30**:91-113.

175. **Somvanshi, V. S., E. Lang, S. Ganguly, J. Swiderski, A. K. Saxena, and E. Stackebrandt**. 2006. A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. Systematic & Applied Microbiology **29**:519-525.

176. **Song, L., F. Barona-Gomez, C. Corre, L. Xiang, D. W. Udwary, M. B. Austin, J. P. Noel, B. S. Moore, and G. L. Challis**. 2006. Type III polyketide synthase beta-ketoacyl-ACP starter unit and ethylmalonyl-CoA extender unit selectivity discovered by *Streptomyces coelicolor* genome mining. J. Am. Chem. Soc. **128**:14754-14755.

177. **Stachelhaus, T., H. D. Mootz, and M. A. Marahiel**. 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. **6**:493-505.

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

179. **Strauch, O. and R. U. Ehlers**. 1998. Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. Appl. Microbiol. Biotechnol. **50**:369-374.

180. **Streit, W. R. and R. A. Schmitz**. 2004. Metagenomics - the key to the uncultured microbes. Curr. Opin. Microbiol. **7**:492-498.

181. **Sundar, L. and F. N. Chang**. 1992. The role of guanosine-3',5'-bis-pyrophosphate in mediating antimicrobial activity of the antibiotic 3,5-dihydroxy-4-ethyl-trans-stilbene. Antimicrob. Agents Chemother. **36**:2645-2651.

182. **Suzuki, T., S. Yamanaka, and Y. Nishimura**. 1990. Chemotaxonomic study of *Xenorhabdus* species - cellular fatty acids, ubiquinone and DNA-DNA-hydridization. J Gen Appl Microbiol **36**:393-401.

183. **Sztaricskai, F., Z. Dinya, G. Y. Batta, E. Szallas, A. Szentirmai, and A. Fodor**. 1992. Anthraquinones produced by Enterobacters and nematodes. Acta Chimica Hungarica-Models in Chemistry **129**:697-707.

184. **Tailliez, P., S. Pages, N. Ginibre, and N. Boemare**. 2006. New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. International Journal of Systematic and Evolutionary Microbiology **56**:2805-2818.

185. **Thomas, G. M. and G. O. Poinar**. 1979. *Xenorhabdus* gen. nov., a genus of entomopathogenic nematophilic bacteria of the family *Enterobacteriaceae*. Int. J. Syst. Bacteriol. **29**:352-360.

186. **Thomas, G. M. and G. O. Jr. Poinar**. 1965. A new bacterium, *Achromobacter nematophilus* sp. nov. (Achromobacteriaceae: Eubacteriales) associated with a nematode. International Bulletin Of Bacteriological Nomenclature And Taxonomy **15**:249-254.

187. **Vallet-Gely, I., B. Lemaitre, and F. Boccard**. 2008. Bacterial strategies to overcome insect defences. Nat. Rev. Microbiol. **6**:302-313.

188. **Velisek, J., J. Davidek, and K. Cejpek**. 2007. Biosynthesis of Food Constituents: Natural Pigments. Part 1 - a Review. Czech J. Food Sci. **25**:291-315.

189. **Vigneux, F., R. Zumbihl, G. Jubelin, C. Ribeiro, J. Poncet, S. Baghdiguian, A. Givaudan, and M. Brehelin**. 2007. The xaxAB genes encoding a new apoptotic toxin from the insect pathogen *Xenorhabdus nematophila* are present in plant and human pathogens. J. Biol. Chem. **282**:9571-9580.

190. **Walsh, C. T., H. W. Chen, T. A. Keating, B. K. Hubbard, H. C. Losey, L. S. Luo, C. G. Marshall, D. A. Miller, and H. M. Patel**. 2001. Tailoring enzymes that modify nonribosomal peptides during and after chain elongation on NRPS assembly lines. Curr. Opin. Chem. Biol. **5**:525-534.

191. **Wang, W. B., H. C. Lai, P. R. Hsueh, R. Y. Y. Chiou, S. B. Lin, and S. J. Liaw**. 2006. Inhibition of swarming and virulence factor expression in *Proteus mirabilis* by resveratrol. J. Med. Microbiol. **55**:1313-1321.

192. **Wang, Y., A. L. Bilgrami, D. Shapiro-Ilan, and R. Gaugler**. 2007. Stability of entomopathogenic bacteria, *Xenorhabdus nematophila* and *Photorhabdus luminescens*, during in vitro culture. J Ind. Microbiol Biotechnol **34**:73-81.

193. **Warnecke, F., P. Luginbuhl, N. Ivanova, M. Ghassemian, T. H. Richardson, J. T. Stege, M. Cayouette, A. C. McHardy, G. Djordjevic, N. Aboushadi, R. Sorek, S. G. Tringe, M. Podar, H. G. Martin, V. Kunin, D. Dalevi, J. Madejska, E. Kirton, D. Platt, E. Szeto, A. Salamov, K. Barry, N. Mikhailova, N. C. Kyrpides, E. G. Matson, E. A. Ottesen, X. N. Zhang, M. Hernandez, C. Murillo, L. G. Acosta, I. Rigoutsos, G. Tamayo, B. D. Green, C. Chang, E. M. Rubin, E. J. Mathur, D. E. Robertson, P. Hugenholtz, and J. R. Leadbetter**. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature **450**:560-565.

194. **Waterfield, N., M. Hares, G. Yang, A. Dowling, and R. ffrench-Constant**. 2005. Potentiation and cellular phenotypes of the insecticidal toxin complexes of *Photorhabdus* bacteria. Cellular Microbiology **7**:373-382.

195. **Waterfield, N. R., D. J. Bowen, J. D. Fetherston, R. D. Perry, and R. H. ffrench-Constant**. 2001. The tc genes of *Photorhabdus*: a growing family. Trends Microbiol Trends Microbiol **9**:185-191.

196. **Waters, C. M. and B. L. Bassler**. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol **21**:319-346.

197. **Watson, R. J., S. A. Joyce, G. V. Spencer, and D. J. Clarke**. 2005. The exbD gene of *Photorhabdus temperata* is required for full virulence in insects and symbiosis with the nematode *Heterorhabditis*. Mol. Microbiol. **56**:763-773.

198. **Webster, J. M., G. Chen, and J. Li**. 1998. Parasitic worms: An ally in the war against the superbugs. Parasitol Today Parasitol Today **14**:161-163.

199. **Williams, J. S., M. Thomas, and D. J. Clarke**. 2005. The gene stlA encodes a phenylalanine ammonia-lyase that is involved in the production of a stilbene antibiotic in *Photorhabdus luminescens* TT01. Microbiology **151**:2543-2550.

200. **Wright, G. D.** 2000. Resisting resistance: new chemical strategies for battling superbugs. Chem. Biol. **7**:R127-R132.

201. **Zhang, H. L., X. G. He, A. Adefarati, J. Gallucci, S. P. Cole, J. M. Beale, P. J. Keller, C. J. Chang, and H. G. Floss**. 1990. Mutactin, a novel polyketide from *Streptomyces coelicolor* - Structure and biosynthetic relationship to actinorhodin. J Org. Chem. **55**:1682-1684.

Danksagung

Mein ganz besonderer Dank gilt Prof. Dr. Helge B. Bode, der es mir ermöglichte in seiner damals gerade gegründeten Arbeitsgruppe an einem völlig neuen wissenschaftlichen Thema zu arbeiten. Dankbar bin ich ihm auch für sein stetes Interesse an meiner Arbeit, seiner Hilfsbereitschaft zu jeglicher Problematik und seinem Optimismus, der mich stets motivierte.

Mein Dank gilt auch Prof. Dr. Rolf Müller für die uneingeschränkte Nutzung aller zur Verfügung stehenden Ressourcen, sowie der gleichberechtigten Aufnahme in seine Arbeitsgruppe.

Darüber hinaus gilt mein Dank auch dem ganzen Arbeitskreis von Prof. Dr. Rolf Müller für die freundliche und fruchtbare Zusammenarbeit im Labor, im speziellen auch Birgitta Lelarge für die Erledigung der vielen kleinen bürokratischen Formalitäten. Dr. Daniel Krug möchte ich besonders für seine Hilfsbereitschaft im Bereich der Analytik danken.

Besonders bedanken möchte ich mich auch bei Dr. Peter Meiser, Michael Ring und Prof. Dr. Helge B. Bode für ihre Kollegialität und die teils sehr amüsante Zeit im Büro. Mein Dank für ein sehr angenehmes Arbeitsklima und stets vorhandene Hilfsbereitschaft gilt allen ehemaligen Laborkollegen, im speziellen aus dem Arbeitskreis Bode: Gertrud Schwär, Daniela Reimer, Eva Luxenburger, Wolfram Lorenzen und Katharina Schulz.

Mein größter Dank allerdings gilt Kathrin Buntin für ihre unzähligen Aufmunterungen und Vorschläge zu meiner Arbeit und molekularbiologischen Problemen. Aber auch insbesondere für die schöne Zeit außerhalb der Arbeit und dafür dass sie auch einen deprimierenden und ernüchternden Arbeitstag wieder in einen schönen Tag verwandeln kann (und auch immer noch kann).

Ein ebenso großer Dank gilt auch meiner Familie, ganz besonders meiner Mutter Gabriele Brachmann, die stets ein offenes Ohr für alle Probleme des Lebens und Alltags hat und mich selbstlos in allen Dingen unterstützt.

Ein kleiner Dank gilt allerdings auch meiner Katze Yersenia für manch stimmungsfördernden und erheiternden Moment.