Strain development for heterologous expression of secondary metabolite clusters in actinobacteria

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Publications

Complete genome sequence of *Streptomyces fulvissimus*
Journal of biotechnology (2013)
M. Myronovskyi, B. Tokovenko, N. Manderscheid, L. Petzke, A. Luzhetskyy

Insights into the Pamamycin Biosynthesis
Angewandte Chemie (2015)
Dr. Yuriy Rebets, Dr. Elke Brötz, Niko Manderscheid, Dr. Bogdan Tokovenko, Dr. Maksym Myronovskyi, Prof. Dr. Peter Metz, Dr. Lutz Petzke, and Dr. Andriy Luzhetskyy

An influence of the copy number of biosynthetic gene clusters on the production level of antibiotics in a heterologous host.
Niko Manderscheid, Bohdan Bilyk, Thomas Paululat, Lutz Petzke and Andriy Luzhetskyy
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Patents

Gene Cluster for Biosynthesis of Pamamycin
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Conference participation

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Identification and activation of cryptic gene clusters in *Streptomyces fulvissimus*

Niko Manderscheid, Andriy Luzhetskyy

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Engineering precursor supply for polyketide synthesis in *Streptomyces albus*

Niko Manderscheid, Yuriy Rebets, Maksym Myronovskyi, Elke Brötz, Joachim Schmid, Klaus Mauch, Andriy Luzhetskyy

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Engineering precursor supply for Pamamycin biosynthesis in *Streptomyces albus*

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Abbreviations:

%	percent
°C	degree Celsius
µ-	micro-
ACN	Acetonitrile
ad	up to
bidest.	double distilled
BLAST	Basic Local Alignment Search Tool
Bp	base pairs
ca.	circa
CCR	Crotonyl-CoA carboxylase / reductase
DAD	Diode-Array-Detection
DCM	Dichloromethane
dest.	distilled
DNA	Desoxyribonucleic acid
dNTP	2‘-Desoxyribonukleosid-5’triphosphate
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Elektrospray ionization mass spectroscopy
et al.	et alii
FT-ICR-MS	Fourier transformation ion cyclotron resonance mass spectrometry
g	gram (weight unit)
h	hour
HPLC	High performance liquid chromatography
l	Liter (volume unit)
m	Meter (length unit)
m-	milli-
M	Molar
mAU	milli absorbance units
MCM	Methylmalonyl-CoA mutase
MeOH	Methanol
Min	Minutes
MM	Minimal media
MS	Mass spectrometry
MW	Molecular weight
n-	nano-

N	Normal
NL	Nährlösung (cultivation media)
NMR	nuclear magnetic resonance
OD	optical density
OM	Oatmeal
PCC	Propionyl-CoA carboxylase
PCR	polymerase chain reaction
pH	pondus Hydrogenii
PKS	Polyketide synthase
PMM	Pamamycin
rpm	rotations per minute
RT	retention time
SDS	sodium dodecyl sulphate
sek	Second
SGG	Starch-Glucose-Glycerol
TLC	Thin-Layer Chromatography
U	units
UV	ultraviolet
VDH	Valine dehydrogenase

A. Summary

In this study we could show the biosynthetic potential of the genus *Streptomyces* by sequencing the genome of *Streptomyces fulvissimus*, which revealed 32 putative biosynthetic gene clusters. We were able to elucidate the biosynthesis of the macrodiolide antibiotic pamamycin by knocking out responsible genes in the pamamycin biosynthetic cluster and analyzing the resulting intermediates. We could show that succinyl-CoA is utilized as a starter unit and either malonyl-CoA, methylmalonyl-CoA or ethylmalonyl-CoA are used as extender units in pamamycin biosynthesis. The knowledge of the pamamycin biosynthesis was used to engineer our heterologous host, the model organism *Streptomyces albus* J1074, in a specific direction. We focused our efforts to reduce or abolish the cellular concentration of methylmalonyl-CoA and ethylmalonyl-CoA in *S. albus* J1074 to reduce the compound spectrum of produced pamamycins. We could greatly influence the compound spectrum and we could identify the bottleneck of pamamycin production as succinyl-CoA. We could also prove that the metabolism of valine is the sole provider for the biosynthesis of pamamycin. Furthermore we could show the dependency of antibiotic production on gene dosage and we could increase the production of several secondary metabolites by increasing the number of clusters present on the genome.

B. Zusammenfassung

Wir konnten das genetische Potential des Genus *Streptomyces* anhand der kompletten Genom-Sequenz von *Streptomyces fulvissimus*, der 32 putative Biosynthese-Gencluster besitzt, zeigen. Außerdem konnten wir die Biosynthese des Makrodiolid-Antibiotikums Pamamycin aufklären, indem die für die Biosynthese verantwortlichen Gene ausgeknockt und die daraus entstandenen Intermediate nachgewiesen wurden. In der Pamamycin-Biosynthese wird Succinyl-CoA als Startereinheit verwendet, auf welche entweder Malonyl-CoA, Methylmalonyl-CoA oder Ethylmalonyl-CoA als weitere Bausteine folgen können. Dieses Wissen nutzten wir, um unseren heterologen Stamm *Streptomyces albus* J1074 gezielt zu verändern. Unser Ziel war es, die intrazellulären Konzentrationen von Methylmalonyl-CoA und Ethylmalonyl-CoA in *S. albus* zu verringern um das Substanzspektrum der produzierten Pamamycine einzuschränken. Wir konnten das Substanzspektrum der produzierten Pamamycine stark beeinflussen, dadurch Succinyl-CoA als den limitierenden Faktor der Biosynthese identifizieren, und nachweisen, dass sich die Pamamycin-Biosynthese ausschließlich aus dem Metabolismus von Valin speist. Durch das Einbringen mehrerer Biosynthesegencluster in denselben genetischen Hintergrund konnten wir die Produktion verschiedener Antibiotika steigern und dadurch zeigen, dass die Sekundärstoffproduktion von der Anzahl der Gencluster abhängig ist.

I. Introduction

1. Natural products

Natural products are a part of the human society since the early beginning. Either as ointments made from plants, poisons from animals or antibiotics from microorganisms. In general, natural products are substances isolated from various organisms. They are either products of the primary or secondary metabolism. Substances from the primary metabolism (polysaccharides, proteins, nucleic and fatty acids) are present in all biological systems. Substances from the secondary metabolism (secondary metabolites) are often produced exclusively by a certain group of organisms and are usually low-molecular-weight (MW < 3000) and chemically diverse substances with many biological applications. Antibiotics belong to the natural products family originating from the secondary metabolism and are produced mostly by bacteria or fungi (Berdy 2005).

2. Antibiotics

Antibiotics are low-molecular-weight (MW < 2000) metabolites produced by microorganisms in a step-by-step biosynthesis. They inhibit growth of other microorganisms in low concentrations (< 200 µg/ml) (Waksman & Fennes F. 1949). The definition of antibiotics has since then been extended to include also natural products with various structures produced by the secondary metabolism of living organisms and synthetic substances which show inhibitory activity against microorganisms (Lanzini & Lorenzeti 1993). Naturally produced antibiotics derive from the secondary metabolism and are thus not essential for growth and replication. Nevertheless they provide an advantage for the producer under unfavorable living conditions (Davis 1990). Antibiotic production often starts after reaching the stationary phase of growth or after reaching a growth-optimum (Bu" Lock et al. 1965). The genes responsible for production of individual secondary metabolites in bacteria are almost always located together in the genome and are referred to as biosynthetic gene clusters (Doroghazi & Metcalf 2013). The golden age of antibiotics spanned the 1940s and 1950s (Clardy et al. 2006). During this time, nearly all known groups of antibiotics have been discovered: β-lactams, tetracyclins, chloramphenicols, aminoglycosides, macrolides, aminocoumarines, glycopeptides, macrolactams and streptogramines. Since then, the only new classes of antibiotics which have been approved for therapy were avermectins (in 1989) and daptomycin (in 2003) Antibiotics have originally been developed for clinical use to treat infectious diseases. The various types of compounds discovered so far do not often show antibacterial activity, but instead activities against fungi, insects, herbs or even cancer cells. Therefore antibiotics have been applied not only in clinical therapy but also as food additives, as fungicides, herbicides or insecticides for plant protection or in veterinary use.

3. Antibiotic resistance

After having approved a new antibiotic for clinical use, emergence of resistant bacteria to this specific antibiotic is inevitable. This is quite coherent because of the short replication time of bacteria and their typical mutation rate of 1 in 10^7 bacteria. The time period for bacteria to become resistant can vary among one year for penicillin V and up to 30 years for vancomycin (Walsh & Wright 2005). But people still see it as given that antibiotics are present to treat infections (Nathan 2004). Therefore new antibiotics have to be developed continuously. However, the antimicrobial pipeline for new therapeutics is now drying up. Nowadays, the big pharma companies have abandoned the research programs devoted to new antimicrobial compounds, and rather focus on more profitable fields like chronic diseases or affective dysfunctions (Leeb 2004).

The most usual causes for development or spreading of a resistance are the use of antibiotics as a performance-enhancing drug in livestock farming, as a feed additive for poultry to suppress bacterial infections caused by factory farming, the premature termination of an antibiotic therapy, wrong diagnosis of an infection or long term antibiotic treatment after surgery. Infections which could be treated in a few days by antibiotics, like pneumonia, have to be treated for months or in some cases prophylactic nowadays (Zähner & Fiedler 1995).

The most important resistance mechanisms include the inactivation of the drug by enzymes, the modification of the drug target and the activation of drug efflux pumps. Hydrolysis of β-Lactams is an example for drug inactivation by enzymes. Vancomycin resistant enterococci (VRE) protect themselves by modification of the drug target which causes a loss in affinity of the drug to the target. Efflux-pumps from the class of the ABC-transporters are used as active transportation mechanisms to reduce the antibiotic concentration to a sub-toxic level (Walsh & Wright 2005). Because of these problems it is of greatest importance to search continuously for new antibiotics and to approve them for clinical use.

4. Screening for new compounds

Classical screening for antibiotics was very successful between 1950 and 1960, but the chances for success are limited nowadays, because of the high rediscovery rate of already known compounds. Probably every year over a million of microorganisms are screened for antibiotic production, and most of the time the same screening-methods and the same microorganisms (*Streptomyces* and other actinobacteria, lower fungi, *Bacillus*, *Pseudomonas*, etc.) are used. After having applied this screening method for over 50 years, it is not surprising that from a huge pile of known compounds only a few new substances are discovered (Zähner & Fiedler 1995).

Nevertheless, the traditional search for antibiotics can be improved by using taxonomical methods for dereplication of the isolates and by applying taxonomical insights for cultivation and screening for active metabolites (Goodfellow & Fiedler 2010). The endeavor to screen *Streptomyces* species for new antibiotics should pay off, if one considers that only a small amount of antibiotics produced by *Streptomyces* species have been discovered. Watve et al. (2001) predicted that the 3000 known compounds produced by *Streptomyces* roughly account only for about 2 % of the potential products. With this in mind, around 98 % of hidden potential still remains undiscovered. This assumption is supported by the whole genome sequence of the model organism *Streptomyces coelicolor* A3(2) (Bentley et al. 2002) and *Streptomyces avermitilis* (Ikeda et al. 2003). Both genomes encode a large number of putative biosynthetic gene clusters which are not expressed under standard laboratory conditions. These gene clusters are referred to as cryptic or silent. Also other actinobacteria like *Saccharopolyspora erythraea* (Oliynyk et al. 2007) with 25 secondary metabolite gene clusters show high biosynthetic potential. These findings led many researchers to predict a renaissance for antibiotic discovery by genome mining (Baltz 2008; Fischbach & Walsh 2009; Challis 2008). Doroghazi & Metcalf (2013) performed an investigation to determine whether a cryptic biosynthetic gene cluster in one species is also likely to be cryptic in a second species. For this they analyzed and compared six actinomycete genera (*Mycobacterium*, *Corynebacterium*, *Rhodococcus*, *Arthrobacter*, *Frankia* and *Streptomyces*). Their research concluded that the potential to discover novel secondary metabolites is still high and especially for the genus *Streptomyces* the amount of overlapping clusters between genomes is very low. Especially strains which are not closely related share no secondary metabolite clusters aside from the most common ones (Doroghazi et al. 2014).

With these findings and the fact that most of these clusters are not expressed under standard laboratory conditions, the focus shifts to gain access to this potential. Therefore, new methods have to be invented and applied to activate the silent (cryptic) gene clusters. But also known compounds previously discovered should be kept in mind because new applications can still be found in new screening processes.

5. Streptomyces

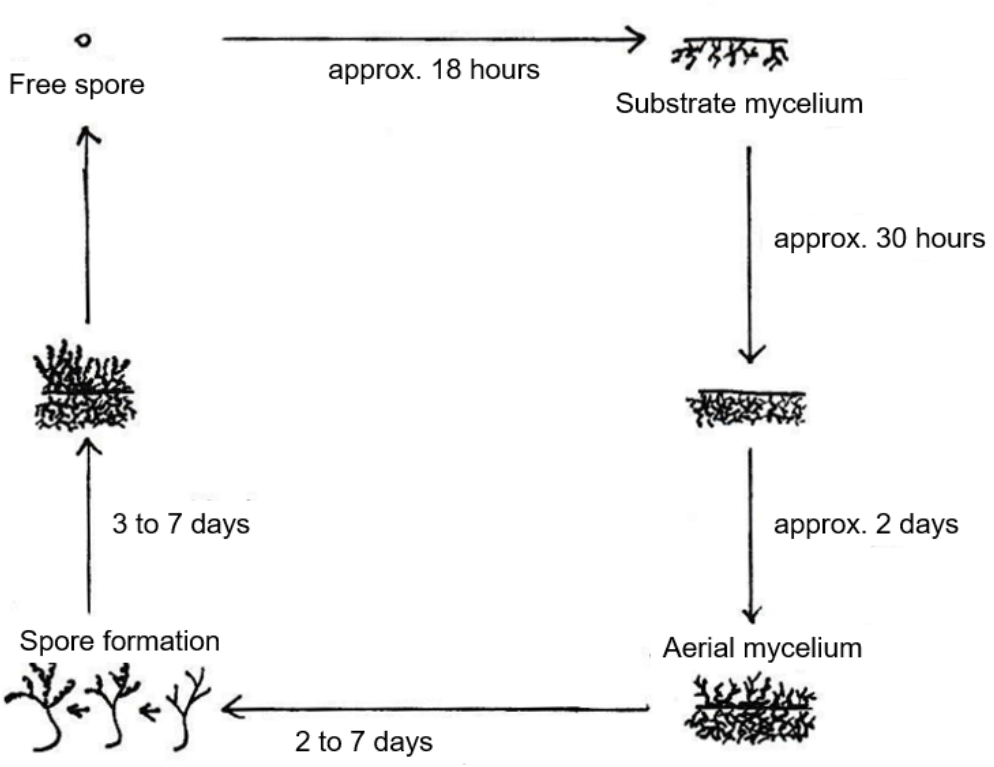


Figure I.1: Life cycle of Streptomyces.

The family *Streptomyces* belongs to the order *Actinomycetales*. They are immotile, Gram-positive, mostly non-pathogenic aerobic soil bacteria with a mycelium-like growth (Stackebrandt & Woese 1981; Wright & Bibb 1992). The GC-content of their DNA is around 74 %. *Streptomyces* usually contain the LL-form of diaminopimelic acid in their peptidoglycan, their fatty acid pattern consists of even-numbered and uneven-numbered fatty acids with a length of 14 to 18 carbon-atoms and they usually contain menachinones with nine isoprenic units (Embley & Stackebrandt 1994). *Streptomyces* are able to degrade insoluble organic matter from the soil like cellulose, chitin, lignin or xylanes and use them as carbon source. These components are utilized by hydrolysis conferred by exo-enzymes like chitinases, ligninases, cellulases, proteases, lipases and nucleases. *Streptomyces* grow on solid media in various different stages of differentiation (Wildermuth 1970; figure I.1). Starting from a spore a branched substrate mycelium is formed, which consists of septa, strongly branched hyphae. When a lack of nutrients occurs, an aerial mycelium is formed by partial lysis of the substrate mycelium. After 3 to 7 days, exospores are formed from the aerial mycelium (Figure I.2). These spores are resistant to dryness and other physical stresses.

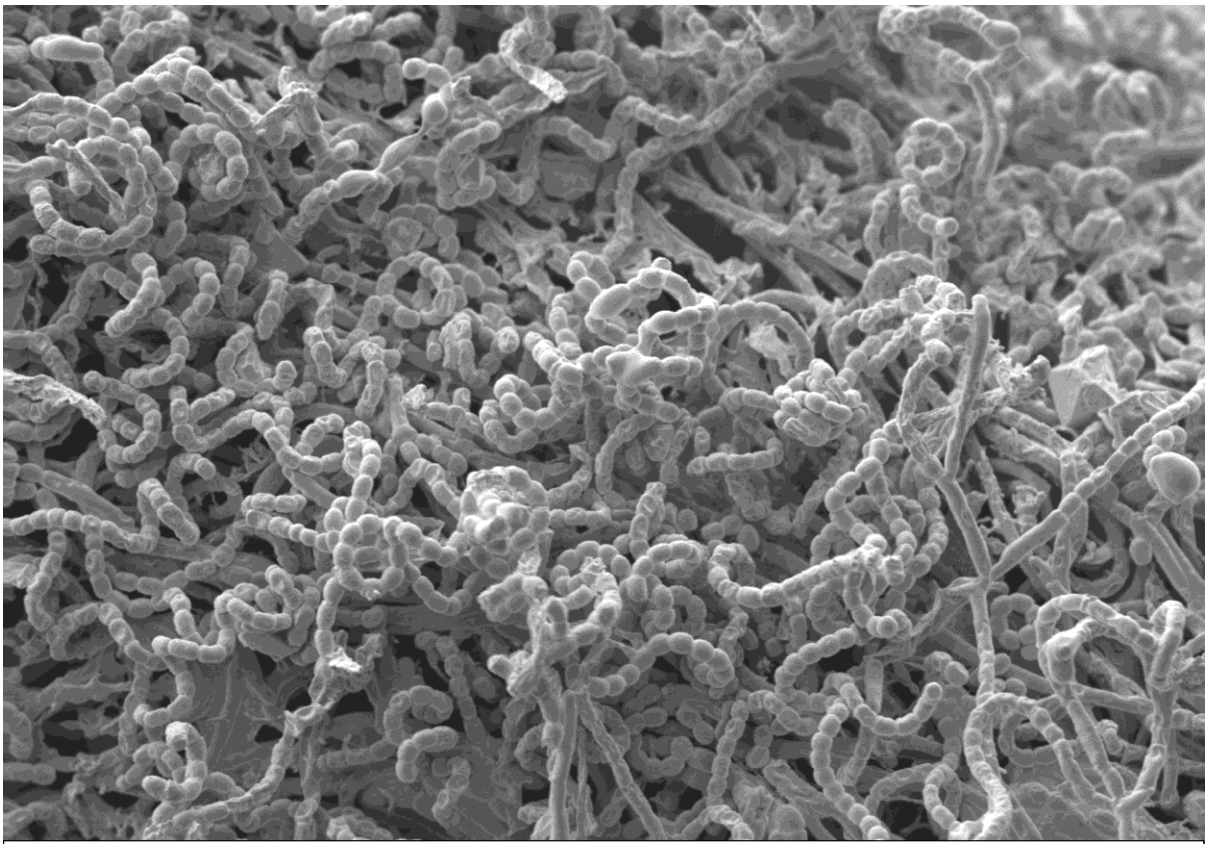


Figure I.2: Spores of Streptomyces sp. Tü 6406 (Diploma thesis Niko Manderscheid).

About 70 % of all known antibiotics are produced by actinomycetes, whereof *Streptomyces* form the biggest part of the producers (Gräfe 1992). Besides antibiotics, which represent the biggest group of bioactive secondary metabolites, also other secondary metabolites produced by *Streptomyces* show biological activities. On the basis of their biological activity secondary metabolites of *Streptomyces* can be classified in 4 groups: (1) inhibitory substances, which include antibacterial, antifungal, antiprotozoal and antiviral compounds; (2) pharmacological substances, which include antitumor-compounds, immunomodulators, neurological substances and enzyme-inhibitors; (3) agricultural substances, which include insecticides, pesticides and herbicides; and (4) regulatory substances like growth-factors, siderophores and morphogenetic substances (Tarkka & Hampp 2008).

6. How to access the hidden potential

New sequencing techniques allow large genomes to be sequenced rapidly and with reasonable costs. With the help of analysis tools like antiSMASH (Medema et al. 2011) these genomes can be screened quickly and efficiently for secondary metabolite clusters, which leaves us with a huge pool of cryptic or poorly expressed gene clusters. The major challenge in the field is therefore to find ways to turn on or turn up the expression of cryptic or poorly expressed pathways to provide material for structure elucidation and biological testing (Baltz 2011). In his review Baltz (2011) introduced five ways of accessing the hidden potential: (1) Disruption of negative regulatory genes; (2) Duplication of

secondary metabolite biosynthetic pathway gene clusters; (3) Deletion or disruption of competing secondary metabolite pathways; (4) Antibiotic selection for mutations that enhance transcription and translation of secondary metabolite genes in stationary phase; (5) Expression of secondary metabolite genes in heterologous hosts. Apart from the methods advertised by Baltz (2011), classical methods like random mutagenesis with chemical or physical agents or variation of fermentation conditions can be used for cluster activation or overproduction. An interesting path to explore is co-cultivation of *Streptomyces* cultures with different bacteria or fungi. Like the studies performed by Schrey et al. (2012) or Onaka et al. (2011) assume that microbial communities are very complex and there may be substances produced only in close proximity to the corresponding partner. The production of certain compounds could also be stimulated by adding extracts of specific organisms to the culture of the producing strain of interest (Beauséjour et al. 1999).

7. Heterologous expression

Since the natural producers of interesting compounds or strains with cryptic clusters of interest are not always genetically accessible, easy to cultivate or show unreliable growth or metabolite production profile, heterologous expression of the target genes or clusters is a commonly used tool. The principle is to identify genes or clusters of interest and express them in a suitable host using an optimized expression system. Thus it is preferred to express these clusters in a well-studied model organism, in which a lot of tools were already applied and for which cultivation conditions, media composition and storage conditions are well described.

There are many examples of successful application of this tool. Heterologous expression was used to express foreign genes from animals or plants in bacteria or fungi (Frommer & Ninnemann 1995) to study gene function or to identify products of gene clusters (Luzhetskyy et al. 2007, Rebets et al. 2015), to elucidate unknown genes or pathways (Baltz 2010), to engineer known or unknown biosynthetic gene clusters (Wenzel et al. 2005), to generate new derivatives of known compounds (Luzhetskyy et al. 2007) or to increase the production of desired metabolites (Baltz 2010).

Despite the huge success of heterologous expression in a vast area of applications, there can be severe drawbacks in using this method. Often the yield of the desired product in the heterologous host is significantly lower than in the parental strain (Binz et al. 2008; Huo et al. 2012). Steps like the introduction of promoters upstream of the cluster to force its expression or the engineering of the precursor supply to ensure sufficient production of the desired metabolite have to be made. These steps are quite often not intuitive and very time consuming. An additional problem which occurs quite often is the toxicity of the produced compound for the heterologous host. To overcome the resistance problem, the corresponding resistance genes which are mostly located in close proximity to the gene cluster, can be co-expressed. Solutions like these rely heavily on deeper knowledge of the corresponding cluster and the produced substance.

8. Streptomyces albus J1074

The *S. albus* G mutant defective in SalI restriction and modification was labeled *S. albus* J1074 (Chater & Wilde 1976). This strain was early on discovered to be a suitable host for cloning and expression of streptomycete genes. It was first used as a cloning host by the Salas group (Fernández et al. 1996; Ma Rodriguez et al. 1993). *S. albus* J1074 has since been used for the heterologous production of steffimycin (Gullón et al. 2006), fredericamycin (Wendt-Pienkowski et al. 2005; Chen et al. 2008), isomigrastatin (Feng et al. 2009) and thiocoraline (Lombó et al. 2006). Since its discovery, *S. albus* J1074 has proven to be a reliable and easy to work with chassis strain for heterologous expression of different kinds of gene clusters from various sources and its genome has been completely sequenced (Zaburannyi et al. 2014).

9. Strain development

Since heterologous expression is a tool of choice for the production of interesting natural products from undeveloped natural producer strains, for the increase of production, and a tool for the production of compounds from cryptic clusters, suitable heterologous hosts need to be developed.

Genome sequencing of producers of interesting secondary metabolites revealed the potential of these strains to produce not only the known compounds but also several other compounds which are mostly cryptic (Bentley et al. 2002; Ikeda et al. 2003; Doroghazi & Metcalf 2013). As a result the idea arose to delete all putative secondary metabolite gene clusters to produce a chassis strain for heterologous production of secondary metabolites. These strains should produce only the products of the expressed clusters and the background noise of naturally produced compounds should be reduced to a minimum. This should result in a much clearer spectrum of the produced compounds, which should lead to a much easier detection of the heterologously produced substances. With a minimized genome, the flux of precursors for biosynthesis should also flow directly into the production of the expressed gene cluster and is not divided between several clusters.

Ikeda et al. (2014) took the industrial producer strain of avermectin, *Streptomyces avermitilis*, which is optimized for precursor supply and is genetically stable, and deleted approximately 20 % of the genome of *S. avermitilis*. This deletion mutant did not produce any endogenous metabolites but showed no sign of growth limitation, because no essential genes were deleted from the chromosome. They showed an increase of production in their minimized strain for streptomycin and cephamycin in comparison to the original producers (Komatsu et al. 2010). This proves the genome-minimized mutant strain to be a versatile and effective host for heterologous expression of secondary metabolite clusters. Gomez-Escribano & Bibb (2011) deleted four endogenous secondary metabolite gene clusters from *Streptomyces coelicolor* M145 and introduced point mutations into *rpoB* and *rpsL*

to pleiotropically increase the secondary metabolite production. These changes led to an increase production of chloramphenicol and congocidine in comparison to the *S. coelicolor* M145 strain.

Deletion of disrupting elements such as unwanted secondary metabolite clusters is one possibility to increase heterologous production of secondary metabolites. Another possibility is to directly divert the precursor production from the primary metabolism to the desired compounds. This can be achieved by overexpression of certain genes in order to increase the flux through a certain pathway, as for example the overexpression of acetyl-CoA carboxylase leads to an increased rate of fatty acid biosynthesis (Davis et al. 2000), by inactivation of certain genes, like the inactivation of the ADP-glucose pyrophosphorylase and the acyl-CoA:diacylglycerol acyltransferase led to an increase of the intracellular concentration of glucose-1-phosphate / glucose-6-phosphate and thus to an increase in mithramycin production (Zabala et al. 2013), or by remodeling or introduction of complete precursor pathways, like remodeling of the glycolytic pathway by deletion of two glyceraldehyde-3-phosphate dehydrogenases led to an increase in clavulanic acid production (Li & Townsend 2006).

The deletion of unwanted clusters and the mutations in *rpoB* and *rpsL* can be generally beneficial for secondary metabolite production, or it can be product specific when the precursor production is channeled to enhance the formation of a certain metabolite.

10. Pamamycin

Pamamycin is a class of natural products which belong to the macrodiolide antibiotics. Pamamycin-607 was first discovered in the culture of *Streptomyces alboniger* ATCC 12461. It is active in vitro against Gram-positive bacteria, *Neurospora* and *Mycobacteria* (McCann & Pogell 1979). Pamamycin-607 was shown to be an aerial mycelium-inducing agent (Kondo et al. 1988) and a secondary metabolite stimulating substance (Hashimoto et al. 2011). There are at least 14 different kinds of pamamycins known. They reach from pamamycin 593 to 649 of which several derivatives share the same mass (Natsume et al. 1995; figure I.3).

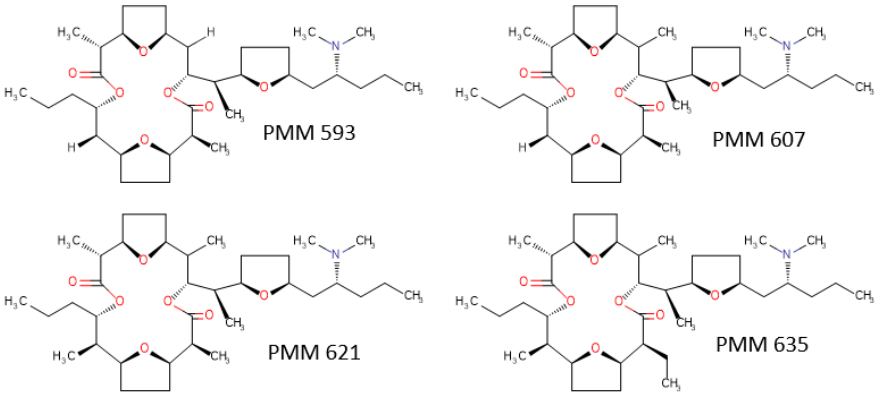


Figure I.3: Different types of pamamycins (not complete).

Pamamycin-607 consists of six acetate, four propionate and three succinate units, and the nitrogen atom derives from the α-amino group of an amino acid (Hashimoto et al. 2005a). The final pamamycin molecule is assembled from two hydroxyl acids, hereafter referred to as hydroxyl acid large and small, with hydroxyl acid small being a stereoisomeric homologue of the building blocks for nonactin (Hashimoto et al. 2005b). Nonactin was first discovered in the extracts of a *Streptomyces* species (Corbaz et al. 1955) and belongs to the macrotetrolides group as well.

11. PKS

Polyketides are a large family of natural products found in bacteria, fungi and plants, and include many clinically important drugs such as tetracycline, daunorubicin, erythromycin, rapamycin and lovastatin. They are biosynthesized from acyl CoA precursors by polyketide synthases (Shen 2003). There are three types of polyketide synthases known to date. Type I PKS are modular proteins of which every module carries out a specific step in the biosynthesis. Type II PKS are commonly multienzyme complexes with a set of enzymes which are used iteratively. Type III PKS consist of a single enzyme which catalyzes the biosynthesis (Shen & Kwon 2002).

Type II PKS are of special interest because of the many clinically useful drugs derived from their products. PKS II types are mostly iteratively used multienzyme complexes with a minimal PKS consisting of the KS_α and KS_β subunits and an acetyl carrier protein (ACP). The minimal PKS catalyses the iterative decarboxylative condensation of malonyl-CoA extender units with an acyl starter unit (Hertweck et al. 2007; figure I.4).

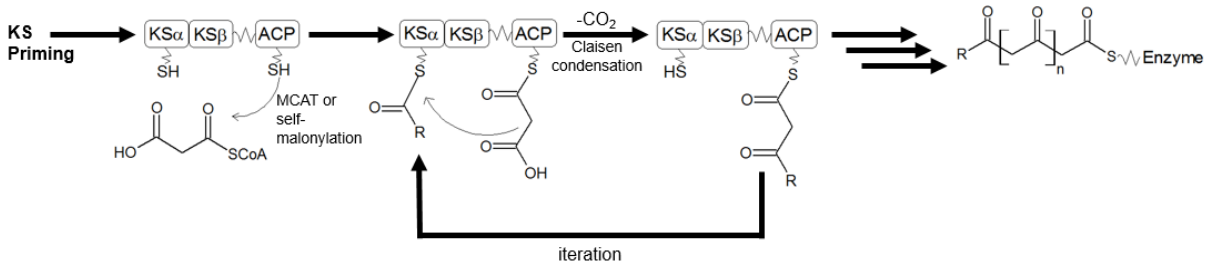


Figure I.4: Basic mechanisms of aromatic polyketide biosynthesis.

Besides the minimal PKS there are quite often other enzymes in polyketide biosynthesis. The keto reductase (KR) catalyzes the stereospecific hydrogen transfer from NAD(P)H onto a keto group, resulting in the formation of a secondary alcohol, cyclases support specific ring formation, aromatases dehydrate cyclic alcohols to yield aromatic ring systems, methyl transferases transfer the activated methyl group from *S*-adenosyl-L-methionine to nitrogen, carbon or oxygen, oxygenates promote the incorporation of oxygen into a substrate, and glycosyltransferases attach sugars to the molecule (Hertweck et al. 2007).

12. Aim of this work

The main focus of this work was to engineer *Streptomyces albus* J1074 for optimized heterologous expression of secondary metabolites coupled with the elucidation of the pamamycin biosynthesis and optimization of the pamamycin production.

A cosmid library of *Streptomyces alboniger* DSM 40043 was created and screened for the pamamycin biosynthetic gene cluster. Heterologous expression of the pamamycin biosynthetic genes was established in *Streptomyces albus* J1074 and the biosynthesis was elucidated by knockout of important genes and measurement of the resulting intermediates. By knocking out several of the genes responsible for precursor supply for pamamycin biosynthesis from the primary metabolism of *Streptomyces albus* J1074, the compound spectrum of produced pamamycins should be shifted into a specific direction. Furthermore the bottleneck for pamamycin production in *Streptomyces albus* J1074 and the source of precursors should be detected.

A different approach for an overproduction of secondary metabolites was performed by integration of additional page ϕC31 attachment sites into the genome of *Streptomyces albus* J1074. The effect of gene dosage on secondary metabolite production should be shown by the expression of four different antibiotic clusters in the panel of produced strains with different amounts of attachment sites.

II. Complete genome sequence of *Streptomyces fulvissimus*

Members of the genus *Streptomyces* served as a source of new natural products and antibiotics in particular for a long time. With the emergence of data indicating that most streptomycetes contain up to 30 gene clusters involved in the synthesis of secondary metabolites in their genomes, it has become clear that their biosynthetic potential was underestimated (Bentley et al. 2002; Ikeda et al. 2003; Ohnishi et al. 2008). Nowadays, lots of efforts are made to estimate this potential and also to get access to natural products whose biosynthetic pathways are encoded in the genomes of the corresponding *Streptomyces* strains. Accumulation of high quality genome sequencing data will strongly contribute to the implementation of this task. Here we present the complete genome sequence of the new *Streptomyces* strain *S. fulvissimus*.

The genome sequencing of *S. fulvissimus* (DSM 40593) was performed with the Roche GS FLX Titanium technology. High molecular mass DNA was extracted from *S. fulvissimus* and an 8 kb paired-end library as well as a shotgun library were constructed. Reads of an approximate length of 350 bp and 2 x 125 bp were obtained for shotgun and paired-end libraries, respectively. Newbler software was used for the genome assembly. Gaps were closed by PCR with subsequent Sanger sequencing.

Annotation was performed by automatically merging 3 annotation sources, with the following manual curation and problems resolution. The three sources used were: Glimmer (Delcher et al. 2007) gene prediction trained on annotated open reading frames of *S. coelicolor* and *S. avermitilis* with BlastP annotation against the NR, Nt and swissProt databases; Prodigal (Hyatt et al. 2010) gene prediction with AutoFACT (Koski et al. 2005) annotation against UniRef90 and COG; xbase (http://xbase.ac.uk/annotation/) annotation against *S. coelicolor*.

The *S. fulvissimus* genome consists of a single linear chromosome of 7,905,758 bp (71,5 % G+C) with no plasmids. The chromosome is of smaller size than the ones of *S. coelicolor* A3(2) (8,7 Mbp), *S. avermitilis* MA-4680 (9,0 Mbp) or *S. griseus* IFO13350 (8,5 MbP) (Bentley et al. 2002; Ikeda et al. 2003; Ohnishi et al. 2008). The analysis of the *S. fulvissimus* genome revealed that its chromosome contains 6925 predicted protein coding sequences as well as 73 tRNA genes (Table II.1). Also, similar to other streptomycetes like *S. coelicolor*, *S. avermitilis*, *S. griseus*, and *S. vattleva* etc (Barbe et al. 2011; Bentley et al. 2002; Ikeda et al. 2003; Ohnishi et al. 2008), the genome of *S. fulvissimus* contains 6 rRNA gene clusters.

Table II.1: Genome statistics

Attribute	Value
Genome size (bp)	7,905,758
DNA coding region (bp)	7,023,792
DNA G+C content (bp)	71,5 %
Number of replicons	1
Extrachromosomal elements	0
Total genes	7,027
rRNA genes	18
rRNA operons	6
tRNA genes	73
Protein coding genes	6,925
Genes with predicted function	5,194
Secondary metabolite clusters	32

We estimated the potential of *S. fulvissimus* to produce secondary metabolites. Preliminary data after the analysis of the genome sequence with the secondary metabolites search tool antiSMASH (Medema et al. 2011) indicates the presence of 32 putative gene clusters involved in the biosynthesis of different natural products (Table II.1). In comparison, genomes of *S. griseus* and *S. avermitilis* contain 34 and 30 secondary metabolite clusters, respectively. Among the clusters of *S. fulvissimus*, 6 encode NRPS, 5 are involved in the biosynthesis of terpenoids, 3 encode hybrid NRPS-PKS, 2 are type III PKS, 2 are type II PKS, 1 is a type I PKS, 2 are involved in siderophore production and 2 in the biosynthesis of lantibiotics. Not surprisingly most of the clusters are located on the terminal arms of the chromosome. This data indicates that *S. fulvissimus* has a high potential to produce secondary metabolites.

It was described before that *S. fulvissimus* contains a NRPS gene cluster responsible for the biosynthesis of the cyclic depsipeptide ionophore antibiotic Valinomycin (Matter et al. 2009). Its structure contains three repeated tetradepsipeptide units of D-a-hydroxyisovaleryl-D-valyl-L-lactyl-L-valyl (Cheng 2006). Indeed, the genome sequence of *S. fulvissimus* contains the respective cluster with two NRPS genes encoding two modules each. On amino acid level products of these two genes are highly homologous to Vlm1 and Vlm2 of *S. tsusimaensis* ATCC 15141 sharing 84 % and 85 % identity, respectively. As in case of *S. tsusimaensis*, the Valinomycin NRPS genes are flanked by a transposase gene and a discrete gene encoding thioesterase (Cheng 2006).

Furthermore, a cluster homologous to the Nonactin biosynthetic cluster was revealed in the genome sequence of *S. fulvissimus*. Nonactin is a macrotetrolide ionophore antibiotic produced by *S. griseus* DSM40695/ ETH A7796 (Smith et al. 2000; Woo et al. 1999). Both gene organization and nucleotide sequence of these clusters are very similar.

III. Insights into the pamamycin biosynthesis

1. Introduction

Pamamycins (**1**) are a group of macrodiolide antibiotics produced by several *Streptomyces* species (Figures III.1, III.S1). They were identified due to their ability to stimulate formation of aerial mycelia in *Streptomyces alboniger* DSMZ40043 and were demonstrated to inhibit the growth of Gram-positive bacteria and fungi (McCann & Pogell 1979; Kondo et al. 1988; Lefevre et al. 2004) . Initially, the compound with the structure designated as pamamycin-607 (R^1 - R^4 = Me, R^5 = H) was isolated from *S. alboniger* (Kondo et al. 1988), however, a reexamination of extracts revealed a broader chemical diversity of **1** differing in their side chain substituents (Figures III.1, III.S1) (Natsume et al. 1991; Natsume et al. 1995; Kozone et al. 1999). **1** are composed of two asymmetrical parts named hydroxy acids small (**3**) and hydroxy acids large (**4**) (Scheme III.1). Due to the pronounced bioactivities and challenging molecular structure, these natural products have stimulated intense synthetic efforts (Metz 2005; Kang & Lee 2005), which have already culminated in several total syntheses of pamamycin-607 (Germay et al. 2001; Lee et al. 2001; Wang et al. 2001; Kang et al. 2002) and some homologues (Fischer et al. 2005; Ren & Wu 2009; Ren et al. 2010; Fischer et al. 2011). Feeding experiments clearly demonstrated the polyketide origin of **1** by the incorporation of ^{13}C labeled acetate, propionate and succinate (Hashimoto et al. 2005a). This fact is especially interesting since succinate cannot directly participate in Claisen condensation. Respective biochemical reaction as well as the enzyme conducting it remained mysterious till now.

The only other natural products known to utilize succinate as a building block are the macrotetrolide antibiotic nonactin (R^1 - R^4 = Me) (**2**) produced by *S. griseus* and *S. fulvissimus* (Figure III.1) (Ashworth et al. 1989; Ashworth & Robinson 1983; Ashworth et al. 1982, 1988; Myronovskyi et al. 2013). The **2** consist of two enantiomers of tetrahydrofuran rings containing hydroxy acids stereospecifically assembled into the final molecule. The gene cluster for the biosynthesis of **2** (*non*) contains 5 ketosynthase KS genes (Kwon et al. 2001; Walczak et al. 2000; Woo et al. 1999). These unusual KSs were classified as non-iterative type II PKS based on sequence similarity despite the lack of ACP proven by the heterologous expression and deletion experiments (Kwon et al. 2001; Walczak et al. 2000). Extensive studies using ^{13}C labeled precursors showed utilization of acetate and succinate as building blocks for **2** assembly (Ashworth et al. 1989; Ashworth & Robinson 1983; Ashworth et al. 1982, 1988) as well as direct incorporation of 3-oxoadipate as a precursor into the **2** (Nelson & Priestley 2002), leading to the idea that the first committed step in biosynthesis is condensation of succinate and malonate. However, the labeling pattern of 3-oxoadipate used in this study did not

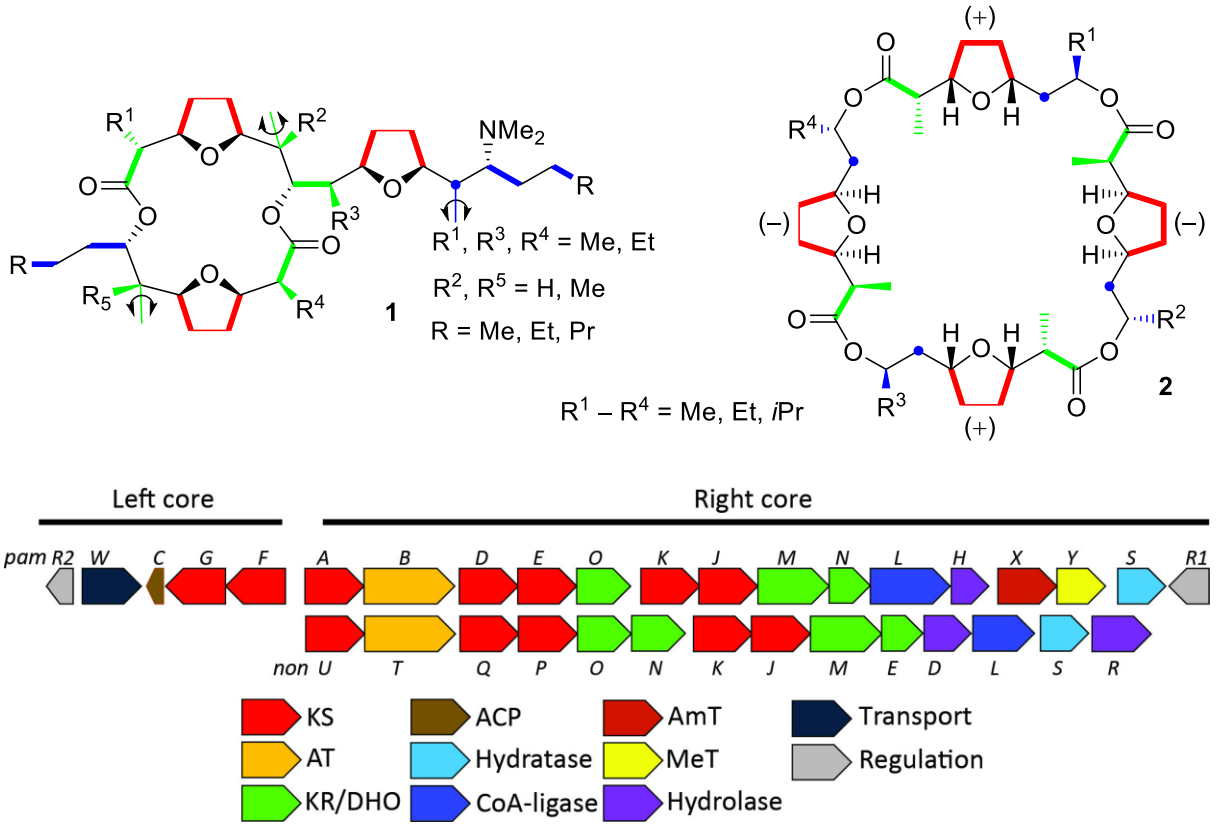


*Figure III.1: Structures of **1** and **2** and the genetic organization of the respective biosynthesis gene clusters. The origin of carbon atoms is color coded: blue – acetate, green – propionate, red – succinate (Hashimoto et al. 2005a; Ashworth et al. 1989; Ashworth & Robinson 1983; Ashworth et al. 1982, 1988). Arrows indicate decarboxylation. The deduced function of genes is based on **2** biosynthesis studies and sequence homology. KS – ketosynthase, AT – acyltransferase, KR/DHO – ketoreductase/dehydrogenase, ACP – acyl-carrier protein, AmT – aminotransferase, MT – methyltransferase.*

explain how this intermediate is made by the **2** biosynthesis machinery. Lastly, the acetate incorporated in positions of 1 and 2 that correspond to the 3-oxoadipate location proceed through the double decarboxylation contributing only one carbon atom to the structure of final molecules. These unusual features distinguish the biosynthesis of **1** and **2** from other polyketides. Despite the excellent insights into **1** and **2** precursor's origins, feeding experiments are not sufficient to establish their biosynthetic route. Moreover, the *non* genes inactivation experiments further puzzled the biosynthetic hypothesis of macrotetrolides (Kwon et al. 2001; Walczak et al. 2001; Kwon et al. 2002). The aim of this project was to elucidate the biosynthetic pathway leading to production of **1** with a focus on the enzyme(s) responsible for incorporation of succinate. We endeavor 1) to identify the enzymes capable to utilize succinate as an intact four-carbon building block in **1** and **2** biosynthesis; 2) to explain the incorporation of only one carbon atom from acetate into polyketide backbone of **1** and **2**; 3) to identify enzyme(s) responsible for elongation of **3** to **4** by addition of the second succinate unit; 4) to establish the entire biosynthetic route leading to assembly of **1**.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

Strains and plasmids used are listed in Table III.S1. *E. coli* strains were grown in LB with appropriate antibiotics as necessary. TSB media was used to grow streptomycetes strains for routine applications. MS media was used for genetic manipulations of *Streptomyces* strains and spore stock preparations (Kieser 2000). For pamamycin in production *Streptomyces* strains were grown in SGG medium (Starch soluble 10 g, Glucose 10 g, Glycerol 10 g, Cornsteep Powder 2,5 g, Bacto Peptone 5 g, Yeast extract 2 g, NaCl 1 g, $CaCO_3$ 3 g, Tap water 1 L, pH 7,3).

2.2. Preparation and manipulation of DNA

DNA extraction and manipulation, *E. coli* transformation and *E. coli* / *Streptomyces* conjugation were performed according to standard procedures (Kieser 2000; Chong 2001). The hot start KOD DNA polymerase (Novagen) was used to amplify fragments used for gene expression. Dream Taq polymerase (Fermentas) was used for PCR for the verification of gene deletions in cosmids or *Streptomyces* strains. DNA fragments were purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen). Plasmid and chromosomal DNA were purified with QIAprep Spin miniprep kit and DNeasy Blood and Tissue Kit (Qiagen). DNA processing enzymes used in this work were obtained from New England Biolabs. Oligonucleotides used in this study are listed in Table III.S2.

2.3. Sequencing of genomes of *S. alboniger* DSMZ and *S. sp.* HKI118 and their analysis

Streptomyces alboniger (DSM40043) was obtained as a lyophilized culture from DSMZ (Braunschweig, Germany). Genomic DNA was isolated from 30 mL cultures grown in TSB at 28 °C for 24 hours. Total DNA isolation was performed according to the salting out procedure followed by RNase treatment (Kieser 2000). Isolated DNA was sent to Baseclear (Leiden, Netherlands) for Illumina sequencing. Two libraries were constructed, PE (insert size 250 bp, average total coverage 225) and MP (insert size 4,000 bp, used only for scaffolding). Assembly of the reads was performed with the Mira assembler software version 3.9 (Chevreux et al. 2004) resulting in 330 contigs longer than 500 bp. Contigs were reviewed and edited in Gap4 from the Staden package (Staden 1996), scaffolded using SSPACE Pro (Baseclear), gaps closed using Soapdenovo GapCloser. The final assembly has 7965650 bp (including 1629 Ns) in 10 scaffolds. The longest scaffold has 3,041,941 bp, the shortest –5,131 bp, scaffold N50 is 1,406,014. *Streptomyces sp*. HKI 118 was obtained from Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knöll-Institute (Jena, Germany) and sequenced by LGC genomics (Berlin, Germany), resulting in 10,817,059 bp in 1,664 scaffolds. Both genomes were annotated using prokka (Seemann 2014), secondary metabolite clusters

were predicted with the help of antiSMASH 2.0 (Blin et al. 2013). To identify pamamycin gene cluster 1664 scaffolds of *Streptomyces sp*. HKI 118 were mapped using Geneious (Biomatters Ltd, Auckland, New Zealand) to 10 scaffolds of *Streptomyces alboniger* DSM40043. Sequences of *pam* clusters from both *S. alboniger* DSM40043 and *Streptomyces sp*. HKI 118 were deposited in GenBank (accession numbers: KM923741 and KM923742).

2.4. Deletion of the *pamD* gene in the *S. alboniger* genome

Regions flanking *pamD* gene were amplified using Pmm00734F1Bam/Pmm00734R1EcV (L) and Pmm00734F2EcV/Pmm00734R2Xba (R) primer pairs and cloned into pSTBlue-1 AccepTor vector (Novagen). The resulting plasmids pSTL and pSTR were digested with BamHI and EcoRV and the deletion construct was assembled by cloning the L fragment into pSTR. A spectinomycin resistance cassette was cloned into the EcoRV site of the resulting plasmid to give pSTRLaadA. pSTRLaadA was digested with BamHI and XbaI and the deletion construct was sub-cloned into pKC1139. The final construct was introduced into *S. alboniger* via intergeneric conjugation. The obtained strain was grown in 20 ml of liquid TSB media without antibiotics at 39 °C for 3 days, plated on MS agar supplemented with spectinomycin to obtain a single cross-over strain. Plasmid integration was proven by resistance marker inheritance after growth for several passages in liquid medium without selective antibiotics. After the last cycle in non-selective conditions the strain was plated on MS media and spore dilutions were prepared and plated on MS with spectinomycin. The secondary cross-over strains were selected by screening for apramycin sensitive and spectinomycin resistant colonies. Seven of such colonies have been obtained. The deletion of *pamD* was proven by PCR analysis.

2.5. Pamamycin production analysis

For pamamycin production 2 ml of a 2 day old pre-culture was inoculated into 50 ml of SGG media and grown for 3 days at 30 °C with agitation at 250 rpms. The biomass was separated from supernatant by centrifugation. Metabolites were extracted with ethyl acetate from cultural liquid and with aceton:methanol (1:1) mixture from biomass. Samples were evaporated, dissolved in 200 µl of methanol:DMSO (1:1) and subjected to LC-MS analysis. For quantification chemically synthesized pamamycin 607 was used as standard. Metabolites were separated on Waters BEH C18 column (100 mm x 2.1 mm, 1.7 µm, column temperature 45 °C) using an UPLC-ESI-MS instrument (Dionex Ultimate 3000, Thermo Fisher Scientific GmbH and amaZon Speed ETD, Bruker). Samples were eluted with solvent A: ammonium formate buffer 90 mM and solvent B: 80:20 acetonitrile/100 mM ammonium formate buffer in a multistep gradient. 0.2 min 20 % B, 20 % B to 97 %B in 2.8 min, 97 % B to 100 % B in 6 min, 2 min 100 % B, 100 % B to 20 % B in 1 min, 3 min equilibration at 20 % B. Flow 0.55 mL/min. Alternatively, longer protocol was used with multistep gradient: 0.2 min 10 % B, 10 % B to 69 % B in 4 min, 69 % B to 87.5 % B in 26 min, 87.5 % B to 95 % B in 0.5 min, 1 min 95 % B, 100 % B to 10 % B in 0.5 min, 4 min equilibration at 10 % B. Detection for quantitative analysis

in srm-mode on Amazon MS-spectrometer using the ms2 fragment m/z = 396 from parent ion of Pamamycin m/z = 608 [M+H]+. The reference sample concentrations of Pamamycin 607 were c = 15, 10, 5, 2.5 and 1 µg/ ml. In the case of extracts from *S. albus* R2Δ*pamX* and *pamY* strains the samples were separated on the same UPLC-ESI-MS system with the Waters BEH C18 column (100 mm x 2.1 mm, 1.7 µm, column temperature 45 °C). 0.1 % formic acid was used as solvent A and 100 acetonitrile as solvent B. Samples were eluted with gradient of solvent B from 5 to 95 % in 18 minutes. The flowrate was 0.5 mL/min.

2.6. Gene library preparation and cloning of the *pam*-gene cluster

S. alboniger DSM 40043 chromosomal DNA was purified with the chloroform method (Kieser 2000). DNA was partially digested with Sau3A. Digested DNA was size-fragmented by agarose gel. The 27-39 kb DNA fragments were selected, purified and ligated into pre-cut BamHI and HpaI and CIAP-treated cosmid pOJ436 vector. Ligation was packaged with package extracts as instructed by the SuperCos1 Cosmid Vector Kit (Stratagene). The primary library was titrated on *E. coli* XL-Blue MRA cells. Since the vector has two Cos sites, only colonies containing cosmid plasmid with inserts of 27-39 kb can be packaged and grown on LB-apramycin plates. Colonies were screened by hybridization with the two probes (480 bp each) that correspond to the 5' and 3' of the pam gene cluster, respectively. The hybridization was performed as described by manufacturer of ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare). Cosmids hybridized with both probes were isolated and end sequenced from T7 and T3 primers to prove the cloning of the respective region of the *S. alboniger* chromosome.

2.7. Construction of the mutant strains

The cosmids with deletions of the pam gene were constructed using the PCR-based Red/ET technology and a hygromycin resistance marker from the iterative markers system (Myronovskyi et al. 2014a; Zhang et al. 1998). Gene deletions were confirmed by PCR. The mutagenized cosmids were introduced into *S. albus* J1074 by RP4-based conjugation from *E. coli* ET12567 containing pUZ8002 and selected for with apramycin (Flett et al. 1997). The hygromycin cassette was removed during conjugation due to the activity of the ϕC31 integrase of pOJ436 based cosmid. Integration of cosmids into the genome of the recipient strain and loss of the marker were proved by PCR.

2.8. Complementation of *pam* gene mutations

Complementation of strains with a deleted *pam* gene was performed with *pam* genes and their nonactin counterparts. For this, *pam* and *non* genes were amplified using the primers described in table III.S2 from genomic DNA of *S. alboniger* and *S. fulvissimus* (Myronovskyi et al. 2013) respectively. PCR products were digested with HindIII/BamHI or HindIII/BglII and cloned into HindIII/BamHI digested pUWLFLPhyg (Fedoryshyn et al. 2008) under control of the ErmE* promoter. Generated

plasmids were introduced into *S. albus* strains containing the respective mutated variants of R2 cosmid and selected by hygromycin resistance. Metabolites production was tested as described above.

2.9. Feeding of mutant strains with hydroxyl acids S and L

Strains of *S. albus* R2 with deletions of *pamL* and *pamB* genes were grown in SGG media for 2 days and mixture of hydroxyl acids S and L mixture was added. Strains were cultivated for two more days, metabolites were extracted and production of pamamycins was analyzed as described above.

2.10. Deletion of 3-oxoadipate:succinyl-CoA transferase gene in *S. albus* J1074

Genes encoding both subunits of the 3-oxoadipate:succinyl-CoA transferase XNR_0219 and XNR_0220 were replaced with a hygromycin resistance cassette within the 1G14 BAC library clone using PCR-based Red/ET technology (Zhang et al. 1998; Gust et al. 2004). Recombinant BAC was introduced into *S. albus* J1074 by conjugation and clones were selected with hygromycin. Ten colonies were checked for secondary cross-over by PCR and three of them were found to have XNR_0219 and XNR_0220 replaced with a hygromycin cassette.

2.11. Cloning, expression, purification and characterization of PamA

pamA was amplified with the primers 0726ETFNcoI and 0726ETRXhoI using *S. alboniger* chromosomal DNA as a template. The obtained fragment was digested with NcoI and XhoI, and inserted into a similarly digested pET28b vector to generate pET28bPamA. pET28bPamA was transformed into *E. coli* BL21(DE3) for overexpression. A flask containing 1.5 L of LB was inoculated with overnight BL21 culture containing PamA expression plasmid, and was grown at 37 °C until an OD600 of 0.6, followed by a shift to 20 °C. Expression was induced through the addition of 0.5 mM IPTG. The culture was incubated at 20 °C overnight, and then the cells were harvested by centrifugation (5000 x g, 4 °C, 20 min), lysed in lysis buffer (10 mM TrisHCl, pH 7.2; 400 mM NaCl, 0.1 % Triton X100) containing benzonase nuclease and lysozyme (Novagen). Proteins were purified with Ni-NTA agarose (Qiagen), elution buffer replacement and concentration of the protein was done using Amicon Ultra spin columns with a cut off at 10 kDa (Merk Millipore). The proteins were analyzed by SDS PAGE and stored at -80 °C in 10 % glycerol. The assay was performed in reaction buffer containing 10 mM TrisHCl (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$ and 1 mM DTT in total volume of 40 µl. 40 mM of succinyl-CoA and malonyl-CoA (or methyl-malonyl-CoA) were used in reaction mixture as substrates. In competition reactions malonyl-CoA and methyl-malonyl-CoA were used in concentration 20 mM each or 40 mM of malonyl-CoA and 4 mM of methylmalonyl-CoA. Reaction was started by adding 1 mM of purified enzyme. The reaction mixture was incubated for 30

min at RT, stopped by adding an equal volume of methanol. 20 µl of the reaction mixture was analyzed by HPLC on Dionex Ulitmate 3000 HPLC system using conditions described in (Kaschabek et al. 2002). CoA-esters were detected by UV absorption at 260 nm. Fractions containing new peaks were collected, evaporated, dissolved in methanol and subjected to HILIC LC-MS analysis on HILIC column (Phenomenex Luna 3µ Hilic 200A; 100 x 2.00 mm) using UPLC-ESI-MS system (Dionex Ultimate 3000, Thermo Fisher Scientific GmbH and amazon speed, Bruker). As solvent, linear gradient of water with 0.1 % ammonium formate (pH4.7) from 95 to 50% against acetonitrile in the course of 12 min was used. The flow rate was 0.5 ml/min.

Table III.S1: Strains and plasmids used in this work.

Plasmid/Strain	**Description**	**Source/ reference**
E. coli DH5α	General cloning host, supE44 ΔlacU169(φ80lacZΔM15)	(Hanahan 1983)
E. coli BL21 (DE3)	expression host	Novagen
E. coli ET12567/ pUZ8002	Host strain for conjugation from *E. coli* to *Streptomyces*	(Flett et al. 1997)
E. coli BW25113/ pIJ790	Strain used for λ RED-mediated recombination	(Gust et al. 2004)
S. alboniger DSM 40043	*S. alboniger* wild type strain, **1** producer	(Hesseltine et al. 1954)
S. sp. HKI 118	Pamamycin producing strain	(Kozone et al. 2008)
S. albus J1074	Host for heterologous expression	(Zaburannyi et al. 2014)
S. alboniger Δ*pamD*-7	DSM40043 derivative with *pamD* gene replaced by spectinomycin resistance cassette, **1** non-producing strain	This study
S. albus R2	R2 cosmid integrated into J1074 genome	This study
S. albus AdCoA	*S. albus* J1074 derivative with deleted 3-oxoadipate:succinyl-CoA transferase gene	This study
pSTBlue-1	T/A PCR cloning plasmid	Novagen
pET28b	Protein expression and purification vector	Novagen
pUWLFLPhyg	*E. coli*-Streptomyces replicative shuttle vector derivative of pUWLoriT, Hyg®	(Fedoryshyn et al. 2008)
pKC1139	*E. coli-Streptomyces* replicative shuttle vector with temperature sensitive replicon of pSG5	(Kieser 2000)
phyg-OK	ApR, HygR; source of hygR6Kγ and oriT for λ RED recombination	(Myronovskyi et al. 2014a)
pKC39pamDLRaadA	pKC1139 derivative with construct for *pamD* replacement	This study
R2	pOJ436 derivative cosmid containing entire pam gene cluster	This study
R2Δ*pamC*	R2 derivative with deletion of *pamC* gene	This study
R2Δ*pamG*	R2 derivative with deletion of *pamG* gene	This study
R2Δ*pamF*	R2 derivative with deletion of *pamF* gene	This study
R2Δ*pamA*	R2 derivative with deletion of *pamA* gene	This study
R2Δ*pamB*	R2 derivative with deletion of *pamB* gene	This study
R2Δ*pamO*	R2 derivative with deletion of *pamO* gene	This study
R2Δ*pamK*	R2 derivative with deletion of *pamK* gene	This study
R2Δ*pamJ*	R2 derivative with deletion of *pamJ* gene	This study

Plasmid/Strain	Description	Source/reference
R2Δ*pamL*	R2 derivative with deletion of *pamL* gene	This study
R2Δ*pamX*	R2 derivative with deletion of *pamX* gene	This study
R2Δ*pamY*	R2 derivative with deletion of pamY gene	This study
pUWL*pamC*	pUWLhyg derivative with *pamC* gene cloned under control of P_{ErmE}	This study
pUWL*lanC*	pUWLhyg derivative with *lanC* gene cloned under control of P_{ErmE}	This study
pUWL*pamA*	pUWLhyg derivative with *pamA* gene cloned under control of P_{ErmE}	This study
pUWL*nonU*	pUWLhyg derivative with *nonU* gene cloned under control of P_{ErmE}	This study
pUWL*pamB*	pUWLhyg derivative with *pamB* gene cloned under control of P_{ErmE}	This study
pUWL*nonT*	pUWLhyg derivative with *nonT* gene cloned under control of P_{ErmE}	This study
pUWL*pamJ*	pUWLhyg derivative with *pamJ* gene cloned under control of P_{ErmE}	This study
pUWL*nonJ*	pUWLhyg derivative with *nonJ* gene cloned under control of P_{ErmE}	This study
pUWL*pamX*	pUWLhyg derivative with *pamX* gene cloned under control of P_{ErmE}	This study

Table III.S2: Primers used in this study

Primer name	Sequence	Description
0729DF	CCGACGCAGCGTCCCCCGGAGGAAACGGTGTA CGAATCGTCGACCCGGTACCGGAGTA	Deletion of *pamC*
0729DR	CCGTGCGCACCGCGCTCTCCAGGGACCTGGGC ACGGCTCTGACTACGCCCCCAACTGAGAG	
0730DF	ATGGACCACAACAGGACACGCGCGGCCGTGCT GGCGGGCGTCGACCCGGTACCGGAGTA	Deletion of *pamG*
0730DR	CGCGGTACGTATGCACGGCCAGGGCCGTGACG TCCGGCCACACTACGCCCCCAACTGAGAG	
0731DF	ACAGCGGCACGCAACACAGGGAGTCTCATGG CGCATCAGTCGACCCGGTACCGGAGTA	Deletion of *pamF*
0731DR	CCATGTCCGCGGGCCTCACGCCTTCCCCAGCA GGATCGCTGACTACGCCCCCAACTGAGAG	
0732DF	ATTCCCGGCGGATAGCTTTCCCGTATGGCTGC ACAGGCTTCGACCCGGTACCGGAGTA	Deletion of *pamA*
0732DR	GTCCGCGCGGGTTCGGCACCGGTCATGCGGCG CCGAGTGTGACTACGCCCCCAACTGAGAG	
0733DF	GGTGCCGAACCCGCGCGGACCGAACCCCTGAA GGAGACCTCGACCCGGTACCGGAGTA	Deletion of *pamB*
0733DR	TCACCTGGTGGGATCCTGCGGATCGCGTCACC TGTAGAATGACTACGCCCCCAACTGAGAG	
0737DF	GTGAACGGCCATGTCCGATTCCAGGAATGCGC TGGTGACCTCGACCCGGTACCGGAGTA	Deletion of *pamO*
0737DR	GGCGTCGTTACGGAACCCAGCCGGGCGGCCCG CCGCCGTCACTACGCCCCCAACTGAGAG	
0738DF	GTGAACTACGGCTTTTTCGCGGGGTACTTCGA GTACCCGCTCGACCCGGTACCGGAGTA	Deletion of *pamK*

Primer name	Sequence	Description
0738DR	TCAGATCCGGTACAGGAGGCTGCCCCAGCACC AGACGCCGACTACGCCCCCAACTGAGAG	
0740DF	ATGCGGTGACCACGACCAAGGGCCCCCGGGA CGCACTCGTCGACCCGGTACCGGAGTA	Deletion of *pamJ*
0740DR	CGGGCCCGGCGCCGGGCGGCGTGTGCTCAGTC ATCGGTGTGTGTAGGCTGGAGCTGCTTC	
0743DF	GCCCGGGATCCCTGAATGACGGGCCCCACGAC GTCAGCGTCGACCCGGTACCGGAGTA	Deletion of *pamL*
0743DR	GTCACGGGTGACCTCGATCCCGGCCGCCACGC GGTCCCAACTACGCCCCCAACTGAGAG	
0745DF	GCCCGGGATCCCTGAATGACGGGCCCCACGAC GTCAGCGTCGACCCGGTACCGGAGTA	Deletion of *pamX*
0745DR	GTCACGGGTGACCTCGATCCCGGCCGCCACGC GGTCCCAACTACGCCCCCAACTGAGAG	
0746DF	GTGAGTGACGACATGTCCAATGCGCACACCGA TCGCAAGTCGACCCGGTACCGGAGTA	Deletion of *pamY*
0746DR	TCACTGCTTGCGGTAGACGGACACCGCGTCCC TGCTGTCGACTACGCCCCCAACTGAGAG	
0729CF	CCGGAGGAAACGGTGTACG	Verification *pamC* deletion
0729CR	CTCTCCAGGGACCTGGGCA	
0730CF	ATGGACCACAACAGGACACG	Verification *pamG* deletion
0730CR	GACCGATTCGTACACCGTTT	
0731CF	TGTCGACAGCGGCACGCAA	Verification *pamF* deletion
0731CR	TGTCCTGTTGTGGTCCATGT	
0732CF	GGTACTTCAAGTACCACAGG	Verification *pamA* deletion
0732CR	AGGACGATGGTCCGCTCCT	
0733CF	TTCGCCATCGGCAGCCAGA	Verification *pamB* deletion
0733CR	GCTCCTCACCTGGTGGGAT	
0737CF	GTGAACGGCCATGTCCGAT	Verification *pamO* deletion
0737CR	GGCGTCGTTACGGAACCCA	
0738CF	TATATCACCGGATCGGTCA	Verification *pamK* deletion
0738CR	CTTCGCTCAGATCCGGTACA	
0740CF	TGTACCGGATCTGAGCGAAG	Verification *pamJ* deletion
0740CR	AGGGAGCCCGTTTCGAGCA	
0743CF	TTGGTCAACTGGGAGCAGTA	Verification *pamL* deletion
0743CR	GGTCACGGGTGACCTCGAT	
0745CF	ACAGAACCAGCACGTATCCA	Verification *pamY* deletion
0745CR	CATTGGACATGTCGTCACTC	
0746CF	GATGACTCGTCAGGGCCAC	Verification *pamX* deletion
0746CR	ATGCCTGTACGGGCAGGTT	

Primer name	Sequence	Description
adCoAtraABDF	ATTCGTACGCGCCGAGGAGCTGCCTGCCGTGG AACGATCCGTCGACCCGGTACCGGAGTA	Deletion of XNR_0219
adCoAtraABDR	TCAGCGGAGGTAGACCCGGCCCGGGTCGACGT CCTCGCGCAGCACTACGCCCCCAACTGAGAG	
adCoAtraABCF	CGTTTCCGATTCGTACGCG	Verification XNR_0219 deletion
adCoAtraABCR	ACGACTCTGATGCGCATGA	
00734F1Bam	ATTGGATCCGGGATGCATCTGCACCTGG	Deletion of *pamD*, left flanking region
00734R1EcV	ATTGATATCCACCTGTAGAAACCTTGCGG	
00734F2EcV	ATTGATATCGCAACACATCGAGTGTGGGA	Deletion of *pamD*, right flanking region
00734R2Xba	ATTTCTAGAGTGGAACACGGAGTCGATGA	
0734CheckF	TGCGCAGTCTCGATCCGCAA	Verification *pamD* deletion
0734CheckR	GTCACCGACGTATTCGGAGA	
0729ComFHind	ATAAGCTTGCATACGTACCGCGGCTGA	*pamC* complementation of *pamC* mutation
0729ComRBam	ATGGATCCCCTGACCACAGGAAGCAGC	
lanCFHind	ATAAGCTTGATGACGCCACCCACTTCC	*lanC* complementation of *pamC* mutation
lanCRBam	ATGGATCCTCTGTATCTCTTCTCCAGTCT	
0732ComFHind	ATAAGCTTATGAGACTCCCTGTGTTGC	*pamA* complementation of *pamA* mutation
0732ComRBam	ATGGATCCGTGCTCATGGTCTCCTTCA	
0733ComFHind	ATAAGCTTGCAGCCAGACCTCGGTACT	*pamB* complementation of *pamB* mutation
0733ComRBam	CCTGGTGGGATCCTGCGGAT	
0737ComHind	ATAAGCTTTTCCGGTACCAGTGAACGGC	*pamO* complementation of *pamO* mutation
0737ComBam	ATGGATCCGCCGTAGTTCACCCACGGGT	
0738ComHind	ATAAGCTTGAATGCCGACGCCACTAGTT	*pamK* complementation of *pamK* mutation
0738ComBam	ATGGATCCTTGGTCGTGGTCACCGCATT	
0740ComFHind	ATAAGCTTAGCCTCCTGTACCGGATCT	*pamJ* complementation of *pamJ* mutation
0740ComRBam	ATGGATCCAGGGAGCCCGTTTCGAGCA	
0743ComHind	ATAAGCTTCCGGCGGTCACATGGTCGA	*pamL* complementation of *pamL* mutation
0743ComBgII	ATAGATCTCAGACGTCGACGACACGGTG	
0745ComFHind	ATAAGCTTACCTCTGGCTGTACGGCAA	*pamX* complementation of *pamX* mutation
0745ComRBam	ATGGATCCCTCACCTTCCATCACGTGGA	
0746ComFHind	ATAAGCTTTTCGCCAAGGCCATGGGATG	*pamY* complementation of *pamY* mutation
0746ComRBam	ATGGATCCTACCTACGCCCTGGAAGTAC	
nonKS32FHind	ATAAGCTTCCGGAAATCGAGTACTGGCA	*nonU* complementation of *pamA* mutation
nonKS32RbglII	ATAGATCTTTCGGGGGCTGTGGTCATG	
nonATHind	ATAAGCTTGATCGCAGACCTCCGTCAT	*nonT* complementation of *pamB* mutation
nonATbglII	ATAGATCTTGGTGGTCGTCATCGGTAG	

Primer name	Sequence	Description
nonOCBam	ATAAGCTTATGACCGGCCAGGCCGTCAA	*NonO* complementation of *pamO* mutation
nonOCHind	ATGGATCCAGAAGGTGTCCAGGTCGGTG	
nonNCBam	ATAAGCTTCTCCCCGAGAAAGCGGAGAA	*nonN* complementation of *pamO* mutation
nonNCHind	ATGGATCCAAGCCCATCCCGGTGACCAG	
nonKCBam	ATAAGCTTTGGGCTCGGGCTGGTACTAC	*nonK* complementation of *pamK* mutation
nonKCHind	ATGGATCCACCGGTCAGAAAGGTGGTCC	
nonJHind	ATAAGCTTCAACAGCGCCCTGCTGCT	*nonJ* complementation of *pamJ* mutation
nonJBam	ATGGATCCTGCTGTGAACGTGGGTCAT	
nonLCHind	ATAAGCTTTACGAGAGTGTGCTCCAGCT	*nonL* complementation of *pamL* mutation
nonLCBam	ATGGATCCTGGCCCGTCGGAAATGCTTC	
0732ETNcoF	ATCCATGGCTGCACAGGCTGAAT	Cloning and expression of *pamA*
0732ETXhoR	CTCGAGTGCGGCGCCGAGTGCCAG	

3. Results and discussion

To identify the biosynthetic genes for **1**, the genomes of two producing strains, *S. alboniger* DSMZ40043 and *S. sp.* HKI 118, were sequenced, searched for secondary metabolism gene clusters with AntiSMASH (Blin et al. 2013) and aligned to identify clusters present in both strains. Only two regions coding for PKS and terpenoid biosynthesis were identical in *S. alboniger* and *S. sp*. HKI118. The PKS gene cluster from the *S. alboniger* contains 20 genes with counterparts in the *S. sp* HKI118 and share a high degree of homology with the *non* clusters of *S. fulvissimus* (Myronovskyi et al. 2013) and *S. griseus subsp. griseus* (Kwon et al. 2001; Walczak et al. 2000; Woo et al. 1999) (Figure III.1, Table III.S3). This cluster (*pam*-cluster) consists of two "cores" of KS genes. The "right core" highly resembles the *non* gene clusters and includes 5 KS (*pamA,D,E,K,J*), 3 KR/DHO (*pamO,M,N*), AT (*pamB*), *nonS*-like enoyl-CoA hydratase (*pamS*, putatively involved in tetrahydrofuran ring closure) (Woo et al. 1999), acyl-CoA ligase (*pamL*) and putative resistance (*pamH*) genes (Figure III.1). In addition, genes for an aminotransferase (*AmT*) and a methyltransferase (MT) (*pamX* and *Y*) are located in this part of the cluster. The "left core" includes two KS (*pamF* and *G*) and ACP (*pamC*) genes. Genes encoding a putative transporter protein and two transcriptional regulators of the TetR and LuxR families were also found flanking the biosynthetic genes. All KS in the cluster, except for PamA, possess characteristic CHN active site triad with N predicted as acyl-CoA binding site (Table III.S4). This architecture of catalytic residues is more typical for type III PKS enzymes rather than for type II where CHH motif is conserved (van Lanen & Shen 2008).

The *pamD* gene, encoding a KS from the "right core" of the cluster (Figure III.1), was deleted from the chromosome of S. *alboniger*. This mutant failed to produce **1**, proving that the identified region is indeed responsible for its biosynthesis (Figure III.S2). An *S. alboniger* cosmid library was created and screened for clones containing the entire set of *pam* genes using two probes flanking the cluster. Expression of one of the positive clones, termed R2, in *S. albus* J1074 resulted in the production of **1** (Figure III.S3).

To determine the sequential biosynthetic steps resulting in **1** production, a set of mutant cosmids with deletions of individual pam genes was created. Metabolites produced by recombinant *S. albus* strains harboring these cosmids were analyzed by LC-MS (Table III.1). In contrast to the deletions of *pamB, pamD* and *pamO*, which led to the complete cessation of biosynthesis, *S. albus* containing cosmids with mutations in *pamK, pamJ* and *pamL* retained the ability to produce **3** and **4** (Table III.1, Figure III.S4). This indicates that PamB, D and O are responsible for the initial steps of **1** biosynthesis, which are shared for both **3** and **4**, while PamK, PamJ and PamL are involved in the final steps of **1** formation. Deletion of aminotransferase and methyltransferase genes *pamX* and *pamY* resulted in accumulation of **3** and hydroxy acid K (**5**) (Table III.1, Figure III.S5) (Hashimoto et al. 2005b), the *non*-aminated precursor of **4**. This proves that amination and methylation occur before the closure of

the macrodiolide ring as proposed previously (Hashimoto et al. 2005b). In contrast, deletion of the KS genes *pamF* and *pamG* from the "left core" of the cluster caused accumulation of **3**, whereas neither **1** nor **4** were found, indicating that these enzymes are necessary for the extension of **3** to give **4**. Deletion of *pamC*, which encodes ACP, perturbed the structural range of **1**, with a shift toward accumulation of lower molecular weight compounds (Table III.1, Figure III.S6). This result indicates that PamC participates in the delivery of starter units as described in several cases of bacterial type III PKS (Song et al. 2006).

Table III.1: Accumulation of 1 and their precursors produced by S. albus strains harboring cosmids with various pam gene deletions.

Cosmid\Compound	1	3	4	5
R2	+	+	+	-
pamC [a]	+	+	+	-
pamG	-	+	-	-
pamF	-	+	-	-
pamA [a]	+	+	+	-
pamB	-	-	-	-
pamD	-	-	-	-
pamO	-	-	-	-
pamK	-	+	+	-
pamJ	-	+	+	-
pamL	-	+	+	-
pamX	-	+	-	+
pamY	-	+	-	+

Deletion of *pamA* caused a significant decrease in **1** production as well as changes in the structural range of accumulated compounds (Table III.1, Figure III.S7, II.S8). Because PamA was proposed to participate in the first condensation step of the pathway, any perturbation of its function should lead to the complete cessation of **1** production. PamA catalyzed condensation of succinate and malonate should result in the production of 3-oxoadipyl-CoA that is one of the key intermediates in the degradation of aromatic compounds in bacteria (Díaz et al. 2013). A KEGG COMPOUND search (Hattori et al. 2003) of the *S. albus* genome revealed genes that could cause accumulation of this metabolite including the gene for 3-oxoadipate:succinyl-CoA transferase. Thus, in the absence of PamA, the biosynthesis machinery for **1** draws 3-oxoadipyl-CoA from the primary metabolism resulting in the residual accumulation of **1**. *S. albus* lacking the 3-oxoadipate:succinyl-CoA transferase gene was generated. This mutant remained able to produce **1** when the *pamA* deficient cosmid was

introduced (Figure III.S8). However, the level of production was severely reduced. Thus, 3-oxoadipyl-CoA is an intersection point for primary metabolism and the **1** biosynthesis pathway. The fact that the mutant strain was still able to produce **1** indicates existence of an alternative mechanism of 3-oxoadipate production via a 3-oxoadipate:succinyl-CoA transferase independent process, most likely through the phenylacetic acid degradation pathway (Díaz et al. 2013).

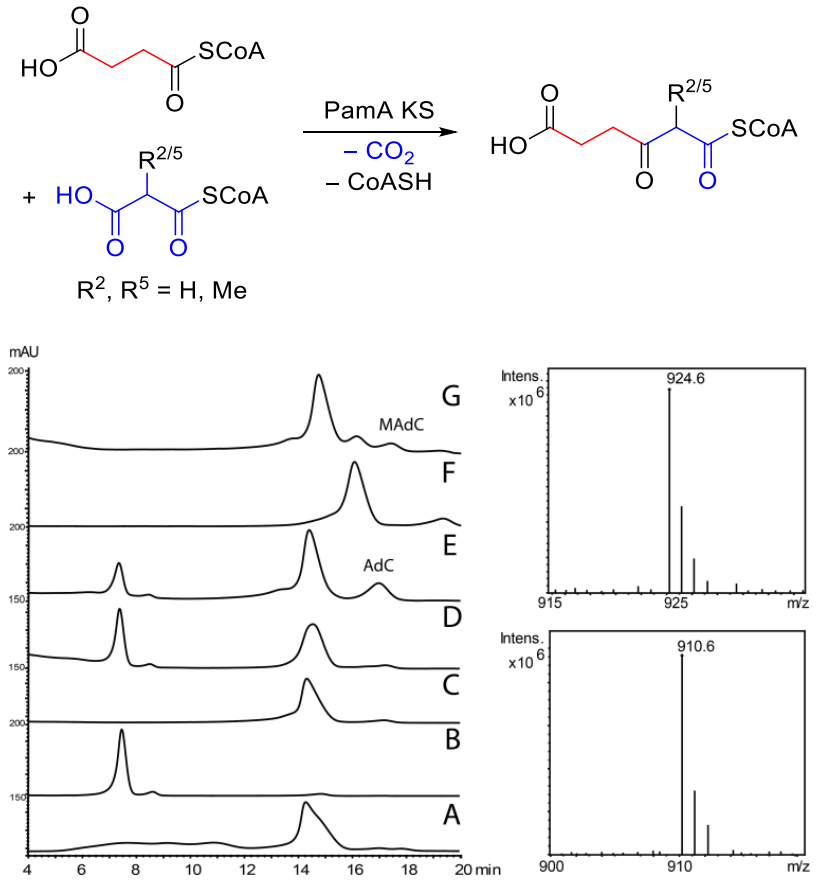
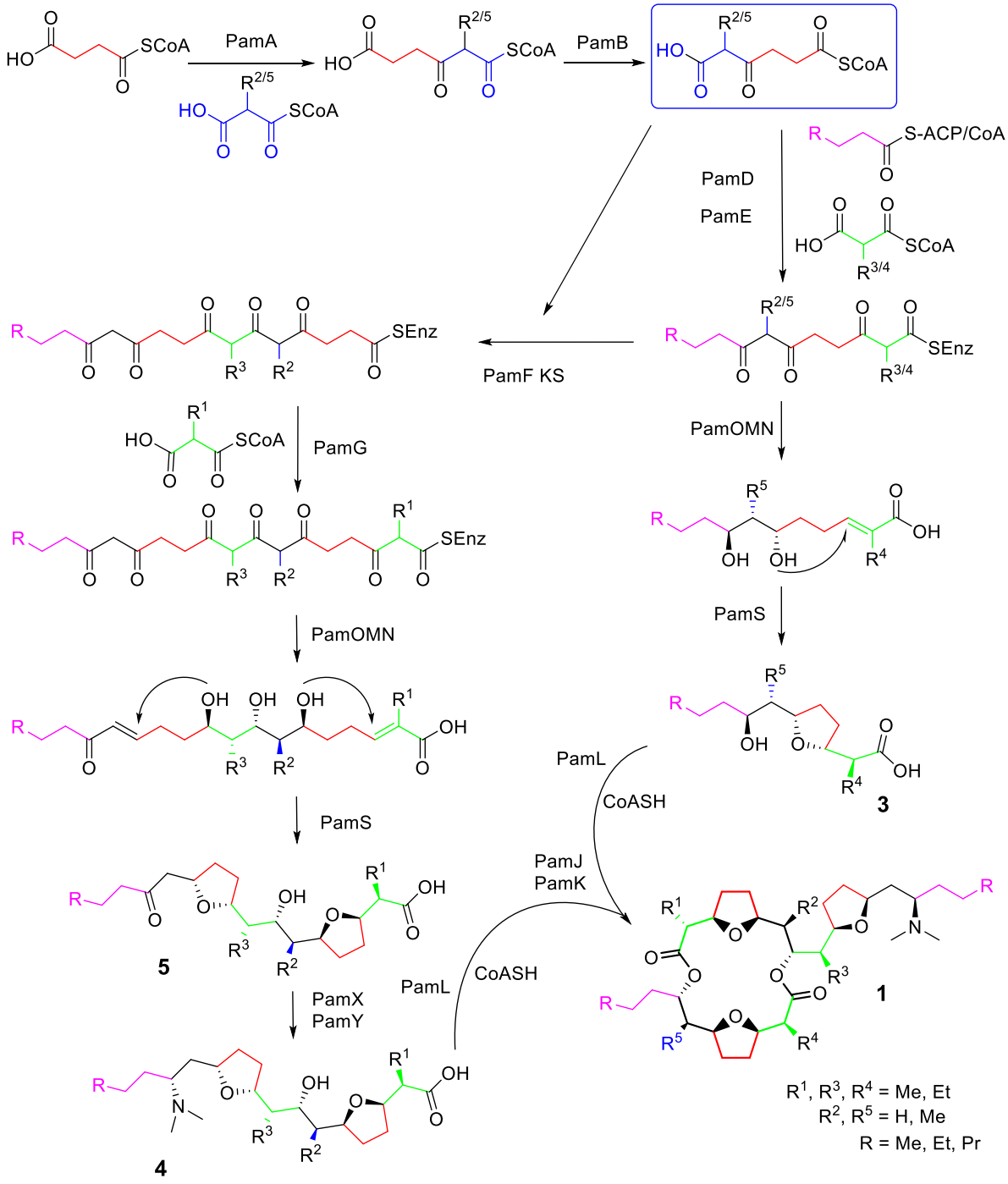


Figure III.2: HPLC profiles of CoA-esters standards and reaction mixtures containing PamA protein. A. CoA-SH, B. Malonyl-CoA, C. Succinyl-CoA, D. Reaction mixture with heat-inactivated PamA. E. Reaction mixture with PamA. F. Methylmalonyl-CoA. G. Reaction mixture with PamA and malonyl-CoA substituted with methylmalonyl-CoA. AdC - 3-oxoadipyl-CoA, MAdC - 2-methyl-3-oxoadipyl-CoA.

The function of PamA in pamamycin biosynthesis was further demonstrated by in vitro reconstitution. PamA was overexpressed in *E. coli* and purified (Figure III.S9). The enzyme's activity was tested in an assay containing malonyl-CoA and succinyl-CoA. The reaction was monitored by HPLC (Figure III.2). Fractions corresponding to the detected peaks were collected and subjected to LC-MS analysis using an HILIC approach (Jandera 2011). Formation of a new compound with an HPLC retention time (RT) of 16.2 min and an absorption spectrum typical of CoA-esters was observed in the reaction mixture after 30 min of incubation (Figure III.2E), and the amount of this compound was further increased with increasing the reaction time. HILIC-LC- MS analysis of this product yielded an m/z of 909.6, which corresponds to the mass of 3-oxoadipyl-CoA (calculated m/z: 909.61) (Figure III.2,

III.S10). The side chains R2 and R5 in several **1** contain methyl groups (Figure III.1) that are predicted to be incorporated by the PamA reaction, suggesting enzyme-substrate promiscuity.



*Scheme III.1: Proposed pathway for the biosynthesis of **1**.*

Accordingly, PamA incubated with succinyl-CoA and methylmalonyl-CoA instead of malonyl-CoA produced a new compound with an RT of 17.1 min and an m/z of 924. (Figure III.2, III.S11). This m/z corresponds to the predicted mass of 2-methyl-3-oxoadipyl-CoA (calculated m/z: 924.64). When the reaction was performed in the presence of both malonyl- and methylmalonyl-CoA substrates, the ratio of the products was found to depend on the initial ratio of the substrates (Figure III.S12). Thus, unlike

NonU, which appears to be specific to malonate because no version of **2** with side chains substituents is known, PamA is more flexible in its choice of substrates. Furthermore, introduction of *nonU* into the *pamA* mutant strain did not restore the native range of **1** produced, due to NonU inability to produce 2-methyl-3-oxoadipate (Table III.S5). The ability of NonU to synthesize 3-oxoadipyl-CoA is masked by its supply from the primary metabolism.

Using obtained genetic and biochemical data the entire pathway leading to the formation of **1** was proposed (Scheme III.1). The first pathway reaction is the PamA-catalyzed condensation of succinyl-CoA with malonyl- or methylmalonyl-CoA. The resulting compounds are then rotated by PamB acyltransferase as proposed by Rong and co-authors (Rong et al. 2010). The resulting 4-oxoadipyl-CoA and 5-methyl-4-oxoadipyl-CoA are key intermediates in the biosynthetic pathway for **1** and are used as extenders for a Claisen condensation facilitating the incorporation of succinate. PamD catalyzes the first extension of a short chain acyl starter unit with one of these compounds followed by the PamE-catalyzed addition of malonyl-CoA. The starter units are most likely supplied as ACP-esters (Song et al. 2006). After this step, the biosynthetic pathway divides into two branches. In one, the activity of the KRs PamO, M and N coupled with the closure of the tetrahydrofuran ring by PamS, results in the formation of **3**. In the other branch, PamF adds the second molecule of adipate followed by the final extension with malonate catalyzed by PamG. Ketoreduction and closure of the tetrahydrofuran rings by PamO, M, N and S result in the formation of **5**, which is further reductively aminated and methylated by PamX and PamY, respectively, to produce **4**. Both **3** and **4** are re-activated by PamL, an acyl-CoA ligase. The feeding of free acids mixture to the *S. albus* culture expressing the *pamL*, *pamJ* and *K* genes produced **1** (Figure III.S13). The closure of the **1** macrodiolide ring is performed by PamJ and PamK KSs, which catalyze an unusual C-O condensation reaction (Kwon et al. 2002).

4. Conclusion

In summary, the biosynthetic gene cluster for the unusual polyketide secondary metabolite **1** was cloned, and the steps leading to its production were elucidated. In particular, the mechanism of succinate incorporation into the polyketide backbone was demonstrated to occur through 3-oxoadipyl-CoA, which represents a new node intermediate between the secondary and primary metabolism. To the best of our knowledge, PamA is the first studied enzyme responsible for the incorporation of succinate into a polyketide molecule. The same condensation reaction occurs in the biosynthesis of the **2** and macrodiolide compound that consists of two molecules of homononactic acid (Jois et al. 1986). The biosynthetic genes responsible for the production of the latter compound may be early predecessors of the *pam* and *non* gene clusters. The biosynthesis of these compounds is an interesting example of the utilizing of unusual substrates to increase the structural diversity of polyketide natural products. In addition, the characterized *pam* genes will greatly expand the toolbox for combinatorial biosynthesis of new polyketides.

IV. Strain development of *Streptomyces albus* J1074 for pamamycin production

1. Introduction

Pamamycin is a macrodiolide antibiotic of polyketide origin. Its biosynthesis utilizes several building blocks from the primary metabolism including succinyl-CoA, malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA and 3-oxoadipyl-CoA (Rebets et al. 2015). The ketoacyl synthases act promiscuously and incorporate building blocks based on their abundance. This leads to a variety of different compounds of which 14 have already been described (Natsume et al. 1995; figure IV.1).

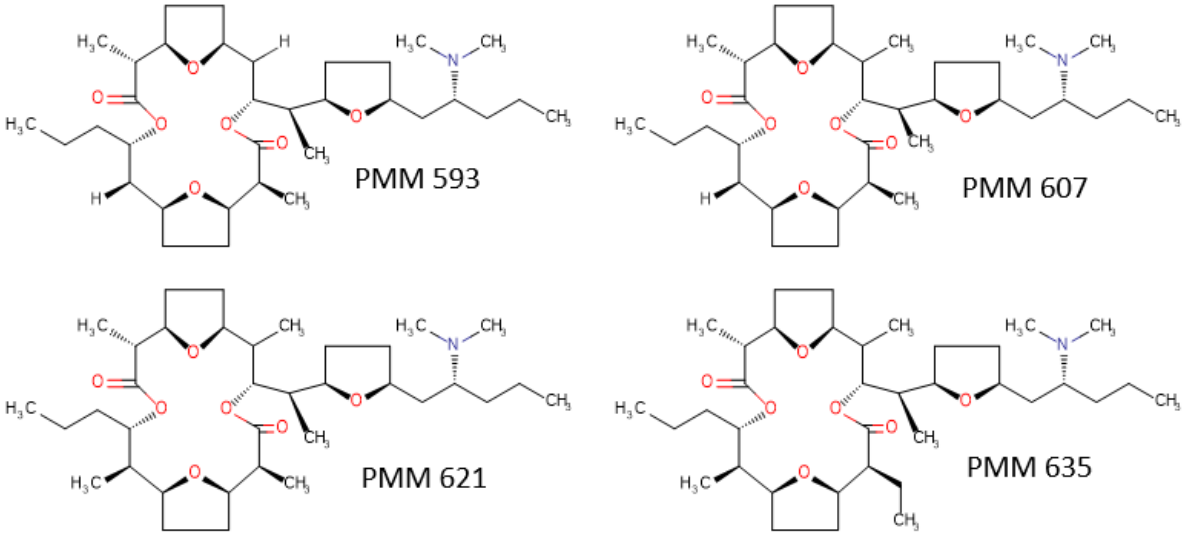


Figure IV.1: Different types of pamamycins (not complete).

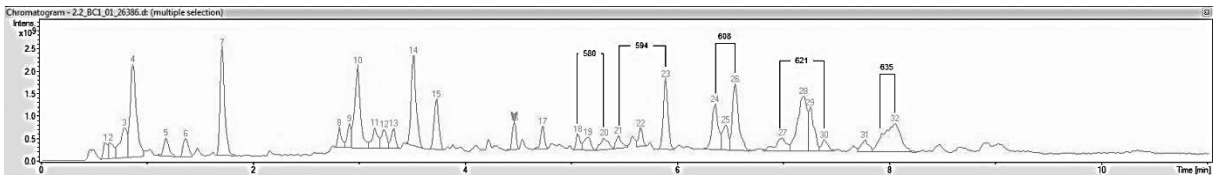


Figure IV.2: HPLC-MS chromatogram of a S. albus J1074 / R2 extract. Different types of pamamycins marked by their corresponding m/z.

Many different compounds produced by a single biosynthetic cluster lead to a complex HPLC-profile as shown in figure IV.2. This does not only result in difficulties in the analysis of the spectra but also leads to more pressuring problems like overall production yields for single compounds and problems in purification of specific products.

Since different pamamycins are a result of the incorporation pattern of the utilized building blocks, the hypothesis is that the compound spectrum could be heavily influenced by manipulation of the building block production levels.

All utilized building blocks are products of the primary metabolism of the producer strain and are either intermediates in energy generation and precursor supply like succinyl-CoA, intermediates of degradation processes like 3-oxoadipyl-CoA (Díaz et al. 2013), or building blocks for cellular structures like malonyl-CoA, methylmalonyl-CoA or ethylmalonyl-CoA. The biosynthetic pathways to these compounds are well studied (Chan et al. 2009) and they are thus excellent targets for manipulation.

Pamamycin biosynthetic studies revealed succinyl-CoA and 3-oxoadipyl-CoA as the essential building blocks, which leaves malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA prone to manipulation.

Extender units like malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA are common for polyketide biosynthesis. Malonyl-CoA is incorporated into the type I PKS associated compounds e.g. lovastatin (Kennedy et al. 1999; Ma & Tang 2007), into type II PKS associated compounds e.g. daunorubicin, doxorubicin and griseorhodin (Strohl et al. 1997; Otten et al. 1990; Li & Piel 2002) and into the type III PKS associated compounds e.g. 3,5-dihydroxyphenylglycine (Chen et al. 2001). Methylmalonyl-CoA is most often incorporated into the type I PKS associated compounds e.g. erythromycin (Rawlings 2001) and rarely into the type II associated compound e.g. nonactin (Ashworth et al. 1989). PKS I derived compounds incorporating ethylmalonyl-CoA include compounds like monensin A (Le Day et al. 1973), elaiophylin (Gerlitz et al. 1992), concanamycin (Bindseil & Zeeck 1994), tylosin (Omura et al. 1977), leptomycin B (Hamamoto et al. 1985) and spiramycin (Inoue et al. 1983).

Malonyl-CoA and methylmalonyl-CoA derive from the primary metabolism and are mainly used for fatty acid biosynthesis, macrolide and polyether antibiotic formation (Zhang et al. 1999). Straight-chain and branched-chain fatty acids of streptomycetes are formed from the catabolism of L-valine, L-isoleucine and L-leucine (Massey et al. 1976; Wallace et al. 1995). Malonyl-CoA can either be used for branched-chain fatty acids (Kaneda 1991) or for *n*-fatty acids in mycobacteria whereas methylmalonyl-CoA is used for branched-chain fatty acids in mycobacteria (Kolattukudy et al. 1997).

Ethylmalonyl-CoA is an intermediate in the ethylmalonyl-CoA pathway used for acetyl-CoA assimilation and hereby provision of cell constituents in bacteria that lack isocitrate lyase activity (Erb et al. 2007). Isocitrate lyase is a key enzyme in the glyoxylate cycle. The crotonyl-CoA carboxylase / reductase (CCR) which forms ethylmalonyl-CoA by carboxylation of crotonyl-CoA is not only used by bacteria lacking the isocitrate lyase but also in bacteria like streptomycetes to supply extender units

for polyketide biosynthesis (Quade et al. 2012; Wilson & Moore 2012; Alber 2011; Liu & Reynolds 2001).

The starter unit succinyl-CoA is very rare and is only found in one other compound class besides pamamycin which is nonactin (Nelson & Priestley 2002) shown in figure IV.3. In fact, pamamycin and nonactin biosynthesis have very similar gene clusters (figure IV.4), with nonactin having one additional ketoreductase / dehydrogenase gene and pamamycin having two additional ketosynthase genes respectively (Rebets et al. 2015).

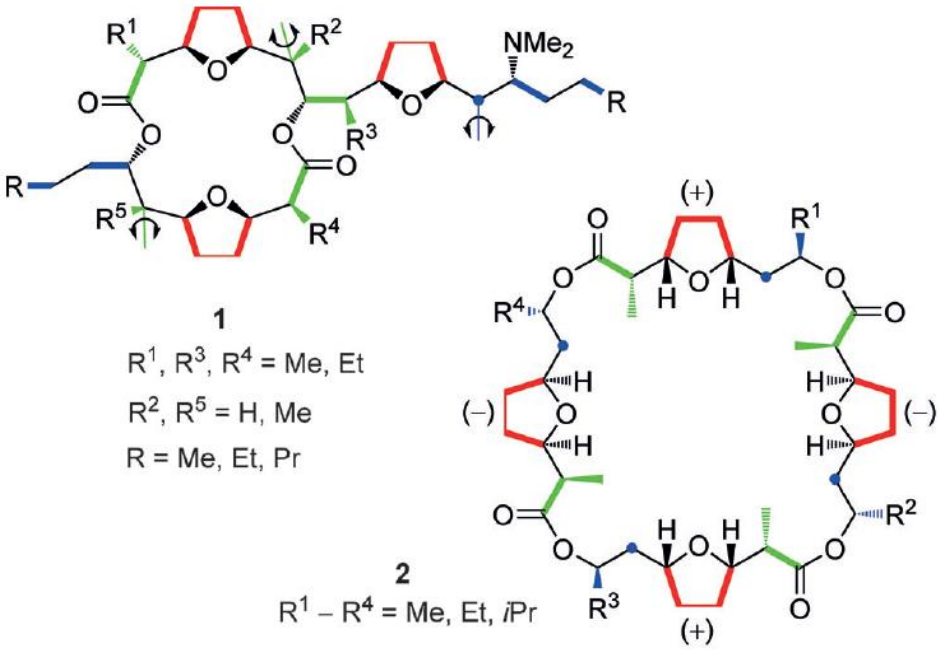


Figure IV.3: Structures of pamamycin (1) and nonactin (2). The origin of carbon atoms is color coded: blue–acetate, green–propionate, red–succinate. Arrows indicate decarboxylation (Rebets et al. 2015).

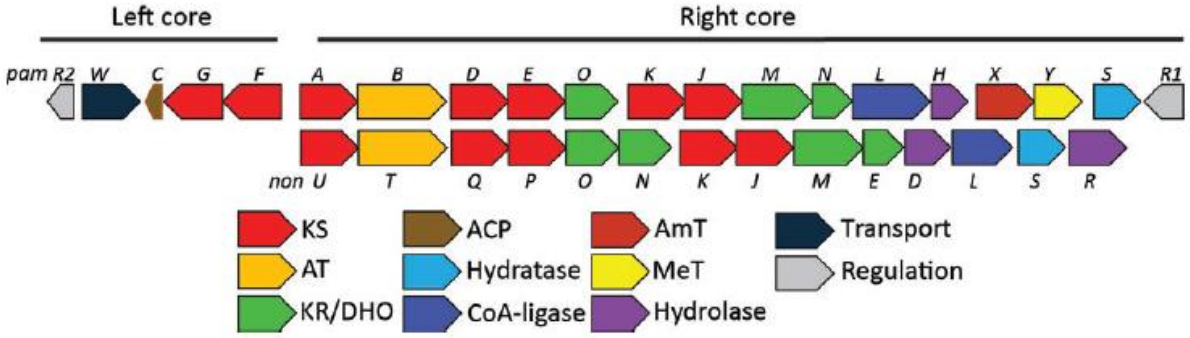


Figure IV.4: Genetic orientation of the pamamycin and nonactin gene clusters. KS: ketosynthase, AT: acyltransferase, KR/DHO: ketoreductase/dehydrogenase, ACP: acyl-carrier protein, AmT: aminotransferase, MT: methyltransferase (Rebets et al. 2015).

The knowledge of the promiscuity of the ketosynthases in pamamycin biosynthesis and their many different derivatives led to the idea to simplify the compound spectrum by tuning the precursor supply.

Ethylmalonyl-CoA is incorporated into all pamamycins bigger than pamamycin 621 and in several derivatives of pamamycin 607 and 621. Methylmalonyl-CoA is incorporated into all known derivatives of pamamycin.

It was hypothesized that a knockout of the ethylmalonyl-CoA producing genes should lead to a decrease or disappearance of the pamamycins bigger than pamamycin 621 and of several derivatives of pamamycin 607 and 621. This should lead to a simplified compound spectrum and an increase in production of the remaining pamamycins. Knockout of the genes responsible for methylmalonyl-CoA production should either completely abolish pamamycin production or reveal the presence of small pamamycins without methylmalonyl-CoA.

A panel of several different combinations of the knocked out genes with the final mutant lacking all of the identified genes was created and the pamamycin cosmid with the biosynthetic gene cluster was brought into these strains and pamamycin production was measured.

2. Materials and Methods

2.1. Materials

2.1.1. Equipment

Table IV.1: Devices used in this work

Device	Identifier	Manufacturer
Biological Safety Cabinet	NU-437-600E	Nuaire, Plymouth, USA
Centrifuges	Centrifuge 517R MF 48-R Micro Centrifuge AL 220VAC MiniStar silverline	Eppendorf, Hamburg Awel centrifugation, BLAIN, France Carl Roth, Karlsruhe VWR, Erlangen
Electroporator	Eporator	Eppendorf, Hamburg
Gel documentation system	Gel Stick “Touch”	INTAS, Göttingen
Gel elektrophoresis-equipment	Elektrophoresis chamber MIDI 1 Power Supply EV 261	CarlRoth, Karlsruhe Consort, Turnhout, Belgium
HPLC-System	Dionex Ultimate 3000 ESI-MS amazon	Thermo Fischer Scietific, Braunschweig Bruker, Billerica, USA
Incubators	Heraeus Function line Infors HT Multitron Standard	Thermo Fischer Scientific, Braunschweig Infors, Einsbach
Magnet stirrer	Heat-stir CB162	Stuart, Staffordshire, UK
pH-meter	MP230	Mettler Toledo, Columbus, USA
Photometer	BioPhotometer plus	Eppendorf, Hamburg
Pipets	Pipetman 0,2 – 2 µl, 1 – 10 µl, 2 – 20 µl, 20 – 100 µl, 50 – 200 µl, 200 – 1000 µl	Gilson, Villiers le Bel
Plate reader	POLARstar Omega	BMG labtech, Ortenberg
Sample concentrator	Dri-Bock heater DB-3, 25 - 100 °C Sample concentrator SC-400	Biostep, Burkhardtsdorf
Scales	M-power Practum® Analysenwaage PRACTUM 124-1S	Sartorius, Göttingen
Thermocycler	peqSTAR 2x Gradient	VWR, Erlangen
Thermomixer	Thermomixer F 1.5	Eppendorf, Hamburg
Ultrapure water	MilliQ Water Purification System	Millipore, Schwalbach
Vortex	Vortex-Genie 2	Scientific Industries, New York, USA
Water bath	WnB1 Waterbath	Memmert, Schwabach

2.1.2. Consumables

Table IV.2: Consumables used in this work

Description	Manufacturer
Disposable cuvettes 1,5 ml	Brand, Wertheim
HPLC-vials TopSertTPX	neoLab, Heidelberg
Microtube 1,5 ml	Sarstedt, Nümbrecht
Multi-Pro PCR tubes	Sarstedt, Nümbrecht
Parafilm	American National Can, Chicago
Petri dishes 94/16 mm	Greiner bio-one, Frickenhausen
Pipet tips	Sarstedt, Nümbrecht
Rotilabo-syringe filter CME	Carl Roth, Karlsruhe
Safe seal microtube 2 ml	Sarstedt, Nümbrecht
Syringe Omnifix 10 ml	B. Braun, Melsungen
Tube 15ml, 50 ml	Greiner bio-one, Frickenhausen

2.1.3. Chemicals

If not specifically mentioned, all used chemicals were p.a. from Sigma-Aldrich Chemie, Steinheim.

Table IV.3: Chemicals used in this work

Chemicals	Manufacturer
6X DNA loading dye	Fischer Scientific, Schwerte
Acetone	Fischer Scientific, Schwerte
Agarose, molecular grade	Bioline, Luckenwalde
Ampicillin sodium salt	Carl Roth, Karlsruhe
Bacto Casaminoacids	Difco Laboratories, Augsburg
Bacto Peptone	Difco Laboratories, Augsburg
$CaCl_2$	VWR, Erlangen
$CaCO_3$	Carl Roth, Karlsruhe
$CaCO_3$	Carl Roth, Karlsruhe
CH_3COOK	Carl Roth, Karlsruhe
Chloramphenicol	Carl Roth, Karlsruhe
dNTPs (100 mM)	Fischer Scientific, Schwerte
Ethylacetate	Fischer Scientific, Schwerte
GeneRuler™ 1 kb DNA Ladder	Fischer Scientific, Schwerte
Glacial acetic acid	Merck, Darmstadt
Glycerol	VWR, Erlangen
HCl	Carl Roth, Karlsruhe
Hygromycin B	Carl Roth, Karlsruhe
Kanamycin sulfate	Carl Roth, Karlsruhe
Lincomycin	Alfa Aesar, Karlsruhe
L-rhamnose	Carl Roth, Karlsruhe
Maltose monohydrate	Carl Roth, Karlsruhe

Chemicals	Manufacturer
Methanol	Fischer Scientific, Schwerte
$MgCl_2$	VWR, Erlangen
NaCl	VWR, Erlangen
Nalidixic acid	Carl Roth, Karlsruhe
NaOH	Carl Roth, Karlsruhe
Oatmeal (Holo Hafergold)	Neuform, Zarretin
Polypeptone	Carl Roth, Karlsruhe
SDS	Carl Roth, Karlsruhe
Soymeal fat-reduced / fat	Henselwerk, Magstadt Sobo Naturkost, Köln-Marsdorf Spielberg, Brackenheim
Starch soluble	Carl Roth, Karlsruhe

2.1.4. Cultivation Media

pH was adjusted accordingly with HCl or NaOH. All media were autoclaved for 20 minutes at 120 °C. 1.5 % Agar was added prior to autoclaving to solid media unless stated otherwise.

Table IV.4: Cultivation media used in this work

Description	Composition	
LB (Sambrook et al. 1989)	NaCl	5 g
	Yeast extract	5 g
	Tryptone	10 g
	deionized water	ad 1.0 l
MS (Kieser 2000)	Mannitol	20 g
	Soymeal fat	20 g
	Agar	20 g
	deionized water	ad 1.0 l
NL 410 (pH 7,0)	Glucose	10 g
	Glycerol	10 g
	Oatmeal	5 g
	Soymeal	10 g
	Yeast extract	5 g
	Bacto Casaminoacids	5 g
	$CaCO_3$	1 g
	Tap water	ad 1.0 l
NL SGG (pH 7,3)	Starch soluble	10 g
	Glucose	10 g
	Glycerol	10 g
	Cornsteep Powder	2,5 g
	Bacto Peptone	5 g
	Yeast extract	2 g
	NaCl	1 g
	$CaCO_3$	3 g
	Tap water	ad 1.0 l

Description	Composition	
PPM (Hashimoto et al. 2005a)	Maltose monohydrate	15 g
	Polypeptone	10 g
	Yeast extract	3 g
	NaCl	3 g
	$CaCO_3$	2 g
	deionized water	ad 1.0 l

2.1.5. Solutions and buffers

Table IV.5: Solutions and buffers used in this work

Description	Composition	
Agarose	in 1 x TAE-Puffer	1%
TAE-buffer (50 x)	Tris/HCl (pH 7,6)	242.2 g
	EDTA	18.62 g
	Glacial acetic acid	57.1 ml
	deionized water	ad 1.0 l
Ethidiumbromide (1 % w/v) for staining		5 µg/ml in 1 l deionized water
Conservation solution	Glycerol	20 g
	Sucrose	10 g
	deionized water (autoclave)	ad 70 ml
TE-Puffer (pH 8,0)	1 M Tris	12.5 ml
	0.5 M EDTA	25 ml
	deionized water	ad 500 ml
Buffer 1 (Plasmid isolation)	1 M Tris-HCl	12.5 ml
	0.5 M EDTA	10 ml
	deionized water	ad 500 ml
Buffer 2 (Plasmid isolation)	10 % SDS	50 ml
	1 N NaOH	100 ml
	deionized water	ad 500 ml
Buffer 3 (Plasmid isolation)	5 M CH_3COOK	300 ml
	glacial acetic acid	57.5 ml
	deionized water	500 ml
Buffer 1 (MaxBac kit)	1 M glucose	10 ml
	1 M Tris (pH 8)	5 ml
	0.5 M EDTA	4 ml
	deionized water	ad 200 ml
Buffer 2 (MaxBac kit)	10 % SDS	20 ml
	1 M NaOH	40 ml
	deionized water	ad 200 ml
Buffer 3 (MaxBac kit)	CH_3COOK	58.8 g
	glacial acetic acid	23 ml
	deionized water	ad 200 ml

2.1.6. Enzymes and kits

As long, as not stated otherwise, all enzymes used in this work were from Fischer Scientific, Schwerte.

Table IV.6: Enzymes and kits used in this work

Enzyme or Kit	Manufacturer
Lysozyme	Carl Roth, Karlsruhe
Wizard ® SV Gel and PCR Clean-Up System	Promega, Madison, USA
Pure Yield ™ Plasmid Midiprep System	Promega, Madison, USA
Wizard ® Plus SV Minipreps DNA Purification System	Promega, Madison, USA
BACMAX ™ DNA Purification Kit	Epicentre, Madison, USA

2.1.7. Antibiotic solutions

All antibiotic stock solutions were stored at – 20 °C.

Table IV.7: Antibiotics with stock solution and working concentration used in this work

Antibiotic	Solvent	Stock solution	Working concentration	Target
Ampicillin	H_2O	100 mg/ml	100 µg/ml	*E. coli*
Apramycin	H_2O	100 mg/ml	50 µg/ml	*E. coli / Streptomyces*
Chloramphenicol	Ethanol	25 mg/ml	50 µg/ml	*E. coli*
Hygromycin	H_2O	50 mg/ml	100 µg/ml	*E. coli / Streptomyces*
Kanamycin	H_2O	30 mg/ml	50 µg/ml	*E. coli*
Nalidixic Acid	1 N NaOH	100 mg/ml	50 µg/ml	*E. coli*
Lincomycin	H_2O	100 mg/ml	100 µg/ml	*Streptomyces*

2.1.8. Synthetic oligonucleotides

Oligonucleotides used in this work were synthesized by Eurofins Genomics, Ebersberg. Concentration of the primers in the PCR reaction was 10 µM.

Table IV.8: Primers used in this work

Primer name	Primer sequence (5' → 3')
ccr1SalChckF	TCAGCCCTCACTACGCCCTT
ccr1SalCheckR	TTCTGGCGCTCTGTCATGGA
ccr1SalDF	CACTACGCCCTTCGCCGGAGGCACCACCGTGAAGGAAATATTCCG GGGATCCGTCGACC
ccr1SalDR	TGGCGCTCTGTCATGGAGGGAACCTCAGATGTTCCGGAATGTGTA GGCTGGAGCTGCTTC
MCM_redET_for	CCGTCAGCGGCTCACAGCTCGTGGCCGAGCGACGCGGCAAGCCGC TCGACTTCCGGGGATCCGTCGACCC

Primer name	Primer sequence (5' → 3')
MCM_redET_rev	GGGCAAGGTCACAGGCGCGGGTACTGCTGCACGCCTGCCGCGCTG TCCTATGTAGGCTGGAGCTGCTTCG
MCM_seq_for	GATCGATCGTCGCCGCCAT
MCM_seq_rev	ATCAGCAGCGTTGCGGAGA
PCC1_redET_for	TCATGCCTGGGCCTCCTCGGCGGGGGTGGCGGGAGCCTGCCGTAC GACGGTTCCGGGGATCCGTCGACCC
PCC1_redET_rev	TCATTAACGCCGGAGCGGTCGCCCCACCGGAGGCCGCGCCGGTCA CGGCATGTAGGCTGGAGCTGCTTCG
PCC1_seq_for	GTGGCGGGGGTGGGTGATT
PCC1_seq_rev	GGTCGTTAGCGGTCGTTAA
PCC2_redET_for	GGCTGCGCGCCCGCCACCTCGACATCCCCGAAGCCGTCCTCAGCC AGGAGTTCCGGGGATCCGTCGACCC
PCC2_redET_rev	TCACGCGGTTTCCTCCTGGACGACGGCGAGCAGGGCACCGACCTC GACCTTGTAGGCTGGAGCTGCTTCG
PCC2_seq_for	ATCAGGGTGGTGGCGGTGG
PCC2_seq_rev	CTGCTCGGTGACGGTGTCG
VDH_redET_for	CACCTTTACGGACTCCGCCCATCCGCCGCATCTCACCGGGAGTCAC CACCTTCCGGGGATCCGTCGACCC
VDH_redET_rev	CGGCGACGGGGGAAAATTGGCCGGAAATGATCCTCCGTCCGGGGT GCGGGTGTAGGCTGGAGCTGCTTCG
VDH_seq_for	CTCATGCCACGGCGTCCGC
VDH_seq_rev	AATCCCCCGCCGCACACCT

2.1.9. Plasmids and Cosmids

Table IV.9: Plasmids and Cosmids used in this work.

Name	Marker	Description
patt-saac-OriT	*aac(3)IV*	Plasmid containing the IMES (Myronovskyi et al. 2014a), used for gene replacement by redET
pR2	*Aac(3)IV*	Cosmid containing the pamamycin gene cluster (Rebets et al. 2015).
pSMARTgus	*cat*	Cosmid used for S. albus J1074 BAC library, containing the GUS gene
pSMARTgus_1M11	*cat*	Cosmid containing the genomic regions of the PCC 2 gene
pSMARTgus_1M11_ΔPCC 2	*cat/ aac(3)IV*	PCC 2 gene replaced with IMES
pSMARTgus_2E5	*cat*	Cosmid containing the genomic regions of the PCC 1 gene
pSMARTgus_2E5_ΔPCC 1	*cat/ aac(3)IV*	PCC 1 gene replaced with IMES
pSMARTgus_2J19	*cat*	Cosmid containing the genomic regions of the VDH gene
pSMARTgus_2J19_ΔVDH	*cat/ aac(3)IV*	VDH replaced with IMES
pSMARTgus_3D17	*cat*	Cosmid containing the genomic regions of the CCR 1 gene
pSMARTgus_3D17_ErythAmp	*bla/strep*	*cat* replaced by *bla/strep*

Name	Marker	Description
pSMARTgus_3D17_ΔCCR	*cat/ aac(3)IV*	CCR replaced with IMES
pSMARTgus_3J4	*cat*	Cosmid containing the genomic regions of the MCM gene
pSMARTgus_3J4_ΔMCM	*cat/ aac(3)IV*	MCM replaced with IMES

2.1.10. Bacterial strains

Table IV.10: Bacterial strains (E. coli and Streptomyces) used in this work

Strain	Description
Del1	*S. albus* J1074 lacking the CCR 2 gene (Myronovskyi et al. 2014b)
Del1ΔccrD1-1	Del1 strain with the CCR 1 gene knocked out by IMES
Del1ΔccrD1-1 / R2	Strain with deleted CCR and integrated pamamycin R2 cosmid
Del1ΔccrD1-1 ΔMCM	CCR 1 + 2 and MCM knocked out by IMES
Del1ΔccrD1-1 ΔMCM / R2	Strain with deleted CCR and MCM and integrated pamamycin R2 cosmid
Del1ΔccrD1-1 ΔMCM ΔPCC 1	CCR 1 + 2, MCM and PCC 1 knocked out by IMES
Del1ΔccrD1-1 ΔPCC 1	CCR 1 + 2 and PCC 1 knocked out by IMES
Del1ΔccrD1-1 ΔPCC 2	CCR 1 + 2 and PCC 2 knocked out by IMES
Del1ΔccrD1-1 ΔPCC 2 ΔMCM	CCR 1 + 2, PCC 2 and MCM knocked out by IMES
Del1ΔccrD1-1 ΔPCC 2 ΔMCM ΔPCC 1	CCR 1 + 2, PCC 1 + 2 and MCM knocked out by IMES
Del1ΔccrD1-1 ΔPCC 2 ΔMCM ΔPCC 1 / R2	Strain with deleted CCR, PCC, MCM and integrated pamamycin R2 cosmid
Del1ΔccrD1-1 ΔPCC 2 ΔMCM ΔPCC 1 ΔVDH	CCR 1 + 2, PCC 1 + 2, MCM and VDH knocked out by IMES
Del1ΔccrD1-1 ΔPCC 2 ΔMCM ΔPCC 1 ΔVDH / R2	Strain with deleted CCR, PCC, MCM, VDH and integrated pamamycin R2 cosmid
Del1ΔccrD1-1 ΔPCC 2 ΔPCC 1	CCR 1 + 2, PCC 1 and 2 knocked out by IMES
ET12567 pUB307	*E. coli* strain ET12567 (MacNeil et al. 1992) transformed with pUB307 (Bennett et al. 1977) used for conjugation
GB05red	*E. coli* strain derived from GB2005 by integration of genes necessary for λ-mediated recombination
S. albus J1074	Isoleucine-plus-valine auxotrophic derivative of *S. albus* G (Chater & Wilde 1976b), lacking *Sal*I-restriction activity
S. albus J1074 / R2	*S. albus J174* with integrated pamamycin R2 cosmid
S. albus J1074 ΔVDH	*S. albus* J1074 with VDH knocked out by IMES
S. albus J1074 ΔVDH / R2	*S. albus* J1074 strain with deleted VDH and integrated pamamycin R2 cosmid

2.2. Methods

2.2.1. Methods in microbiology

2.2.1.1. Strain maintenance

Streptomyces strains were stored at – 80 °C. For this, the strains were grown in a 500 ml-Erlenmeyer flask with one buffle filled with 100 ml of NL410 (Table IV.4). The flask was inoculated with spores and grown for at least 72 hours at 28 °C, 180 rpm. 0.8 ml of the culture was mixed with 0.8 ml of the sterile conservation solution (Table IV.5) mixed and frozen.

2.2.1.2. Cultivation conditions

2.2.1.2.1. Preculture

100 ml of NL 410 were inoculated with spores and cultivated for 48 hours at 28 °C, 180 rpm in a 500 ml-Erlenmeyer flask.

2.2.1.2.2. Main culture

100 ml of the respective media was inoculated with 6 % of the preculture medium and cultivated for 72 hours at 28 °C, 180 rpm.

2.2.1.2.3. Optimization of NL SGG for pamamycin production

The influence of different carbon / nitrogen sources on pamamycin production was tested by modifying the NL SGG medium.

Table IV.11: Media composition for the media variation test

	NL SGG	Variation I	Variation II	Variation III	Variation IV
Starch soluble	10 g	---	---	---	---
Cornsteep powder	2.5 g	---	---	---	---
Yeast extract	2 g	---	---	---	---
NaCl	1 g	---	---	---	---
CaCO3	3 g	---	---	---	---
Glucose	10 g	Glucose 15 g	Maltose 20 g	Glucose 15 g	Maltose 20 g
Glycerol	10 g	Glycerol 15 g	---	Glycerol 15 g	---
Bacto peptone	15 g	---	---	Soymeal 10 g	Soymeal 10 g
Tap water	1.0 l	---	---	---	---
pH	7.3	---	---	---	---

2.2.1.2.4. Pamamycin production dependency on O_2 level

Pamamycin production in dependency on O_2 level was determined by using different shaking flasks, amount of medium and rotation speed. There were 10 different combinations, sorted by O_2 level.

1. 100 ml medium; flask without buffle; 120 rpm
2. 100 ml medium; flask without buffle; 180 rpm
3. 50 ml medium; flask without buffle; 120 rpm
4. 50 ml medium; flask without buffle; 180 rpm
5. 150 ml medium; flask with 1 buffle; 120 rpm
6. 150 ml medium; flask with 1 buffle; 180 rpm
7. 100 ml medium; flask with 1 buffle; 120 rpm
8. 100 ml medium; flask with 1 buffle; 180 rpm
9. 100 ml medium; flask with 4 buffles; 120 rpm
10. 100 ml medium; flask with 4 buffles; 180 rpm

2.2.1.2.5. Pamamycin production curve

To determine when pamamycin production reaches its maximum during cultivation, the *S. albus* J1074 / R2 strain was cultivated as described in IV.2.2.1.2.1 and IV.2.2.1.2.2 and samples were taken at 0 h, 3.5 h, 7 h, 10 h, 14 h, 24 h, 27 h, 31 h, 34 h, 38 h, 48 h, 51 h, and 53 h.

2.2.1.2.6. Influence of ammonium ions and L-Valine on VDH activity

To test the influence of ammonium ions or L-Valine on the activity of the VDH, 50 mM ammonium acetate or 50 mM L-Valine were added to the cultivation medium after sterilization.

2.2.1.2.7. Influence of succinate on pamamycin production

The influence of succinate on pamamycin production was tested by adding 100 µM, 500 µM, 10 mM and 50 mM sodium succinate to the medium after sterilization.

2.2.2. Secondary metabolite analytics

2.2.2.1. Extraction of pamamycin

10 ml of the main cultures were collected in a 15 ml tube and separated by centrifugation for 10 minutes at 8000 rpm. 5 ml of supernatant were transferred to a new 15 ml tube and extracted with 5 ml ethyl acetate for 30 minutes on a shaking incubator. The remaining biomass was extracted with 10 ml of a mixture of methanol and acetone (1+1) for 30 minutes on a shaking incubator. Both the supernatant and biomass extracts were evaporated to dryness using a sample concentrator. The

supernatant extract was then resolved in 500 µl of methanol. The biomass extract was resolved in 1000 µl of methanol. Both kinds of extracts were diluted with DMSO (1+1) prior to HPLC measurement.

2.2.2.2. Dry weight calculation

To determine the dry weight, 1 ml of the main culture was collected in a previously weighted 1.5 microtube and dried at 65 °C for 24 h. After the liquid has evaporated, the tube was weighed again.

2.2.2.3. HPLC Data Analysis

Measurements were performed using a 100 mm BEH C18 column (Waters, Milford, USA) with a flow rate of 0.55 ml/min and the following gradient: 0 min 20 % D, linear gradient to 3 min 97 % D, linear gradient to 11 min 100 % D (solvent C: 90 mM ammoniumformiate, solvent D: acetonitrile/100 mM ammoniumformiate (80 + 20)).

Pamamycin production was measured by the change in adsorption of the derivative PMM 607, which was chosen because of the high abundance of this compound and the presence of a synthesized standard for this compound. Several other pamamycin derivatives could be detected and behaved accordingly

2.2.3. Methods in molecular biology

2.2.3.1. Plasmid isolation

Plasmids and cosmids were isolated by scratching *E. coli* cells containing the corresponding vector from well grown LB-agar plates by adding 12 ml of sterile H_2O to the plates, scratching the cells from the surface and transferring the cells into new 1.5 ml micro tubes. The tubes were centrifuged at 1000 rpm and the supernatant was discarded. The cells were resuspended in 100 µl of Buffer 1 (Table IV.5). Buffer 1 maintains the pH, protects the DNA by inactivation of nucleases and makes the *E. coli* cell wall more sensitive. 200 µl of Buffer 2 (Table IV.5) were added and the solution was mixed by inverting. Buffer 2 is used for lysis of the cells. NaOH denatures the DNA and SDS lyses the cells. 150 µl of Buffer 3 (Table IV.5) were added and the solution was mixed by inverting the tubes. Buffer 3 contains acetic acid which neutralizes NaOH and potassium acetate to precipitate the protein fraction. This mixture was then incubated for 10 minutes at – 20 °C. After incubation, the tubes were centrifuged at 15.000 rpm and the supernatant was transferred to new 1.5 ml microtubes containing 600 µl of 2-propanol. After mixing, the tubes were centrifuged for 20 minutes at 15000 rpm at 4 °C to recover the plasmid DNA. The supernatant was discarded and 600 µl of 70 % ethanol were added to remove the salts from the DNA. After centrifugation for 5 minutes at 15000 rpm, the supernatant was discarded and the washing step with ethanol was repeated once more. The remains of ethanol after discarding the supernatant were removed by pipetting with a 200 µl tip and the pellet was dried at

37 °C for 3 minutes. To dissolve the DNA, 30 µl of water was added to the tubes. After dissolving of the DNA, the concentration was measured with an Eppendorf photometer.

2.2.3.2. BAC isolation by MAX BAC

An overnight (37 °C; 180 rpm) culture of the corresponding *E. coli* cells containing the BAC of interest was inoculated from glycerol stock. 2 ml of the over overnight culture was transferred to a 2 ml micro-tube and centrifuged at 10000 rpm for 1 minute. The supernatant was discarded and this procedure was repeated twice until 6 ml of the culture were processed. The cells were resuspended in 250 µl of buffer 1 (Table IV.5) and then 250 µl of buffer 2 (Table IV.5) were added to the mixture. The solution was carefully mixed and 350 µl of buffer 3 (Table IV.5) were added and the solution was carefully mixed again. The tube was incubated on ice for 4 minutes and then centrifuged at 15000 rpm and 4 °C for 4 minutes. The supernatant was transferred into new 2 ml micro tubes containing 1 ml of 2-propanol. The solution was mixed and then incubated at room temperature for 4 minutes. After incubation the tubes were centrifuged at 15000 rpm; 4 °C for 10 minutes. The supernatant was discarded and 1 ml of 70 % ethanol was added to the pellet. The pellet was gently washed and then centrifuged at 15000 rpm for 1 minute. This procedure was repeated once more and then the pellet was dried at room temperature for 4 minutes. The pellet was then resolved in 50 µl of H_2O.

2.2.3.3. Measurement of DNA concentration

DNA concentration was measured according to the Beer-Lambert Law. Due to this, the nucleic acids reach their characteristic absorption maximum at 260 nm. Extinction in one unit corresponds to 50 µg / mg of DNA concentration. DNA purity was measured at 280 nm, corresponding to the absorption maximum of aromatic amino acids. The ratio between 260 nm and 280 nm may not exceed 1.8. The measurements were carried out photometrically with an Eppendorf photometer (Table IV.1).

2.2.3.4. Chemical transformation

2.2.3.4.1. Preparation of chemically competent *E. coli* cells

For preparation of chemically competent *E. coli* cells, a glycerol stock of the corresponding cells was resuspended in 1 ml of LB, dilutions (1:100 – 1:1000000) were plated on LB-agar and incubated at 37 °C overnight. A single clone was chosen, picked in 100 ml of LB and incubated at 37 °C, 180 rpm overnight. 8 flasks filled with 100 ml of LB were inoculated with 1 ml of the overnight culture and cultivated at 37 °C, 180 rpm until an OD_{600} of 0.7 ($^{+}/_{-}$ 0.1) was reached. Two flasks were distributed on 4 50 ml tubes and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the procedure was repeated until all flasks were distributed on the 4 50 ml tubes. The following steps were performed on ice or with centrifuge a precooled to 4°C. The remaining cell pellet was resuspended by adding 15 ml of ice-cool sterile 0.1 M $MgCl_2$ and vortexing. The tubes were then filled up to 40 ml

with ice-cooled 0.1 M $MgCl_2$, mixed and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded afterwards. 15 ml of ice-cooled sterile 0.1 M $CaCl_2$ was added into two of the 50 ml tubes, the cell pellets were resuspended and then transferred into the remaining two tubes and the pellets there were also resuspended. The tubes were then filled up to 40 ml with ice-cooled 0.1 M $CaCl_2$, mixed and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded. 15 ml of ice-cooled 0.1 M $CaCl_2$ / 15 % glycerol was added into one 50 ml tube and the cell pellet was resuspended. The resuspended cells were transferred into the remaining 50 ml tube and the cells were resuspended. The tube was filled up to 40 ml with ice-cooled 0.1 M CaCl2/15 % glycerol, mixed and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the remaining cell pellet was resuspended in 5 ml of 0.1 M $CaCl_2$ / 15 % glycerol. 60 µl aliquots of this cell solution were transferred into new pre cooled 1.5 ml micro tubes and then frozen at – 80 °C.

2.2.3.4.2. Transformation of chemically competent cells by heat shock

An aliquot of the previously prepared chemically competent cells was thawed on ice. 5 µl of the desired vector were added to the cells and mixed gently. This mixture was incubated for 30 minutes on ice. After incubation the cells were heat shocked at 42 °C for 1 minute. After heat shock, the cells were incubated on ice for 1 minute. 1 ml of 37 °C LB medium was added to the cells and then incubated at 37 °C while shaking for 1 hour. Afterwards the cells were plated on the appropriate selective medium and incubated at 37 °C for 12 hours.

2.2.3.5. Intergenic Conjugation of *E. coli* and *Streptomyces*

To introduce the desired vector into the genome of *Streptomyces albus* J1074, the method of intergeneric conjugation was used. This method is based on the RP1 conjugation system with a relaxed recipient specificity and imply transfer of DNA from the non methylating *E. coli* ET 12567 donor strain containing the RP1 derivative plasmid pUB307 (Piffaretti et al. 1988) to the recipient *Streptomyces* strain.

2.2.3.5.1. Strain Preparation

The desired vector was introduced into the chemically competent *E. coli* ET 12567 / pUB307 cells by heat shock transformation (IV.2.2.3.4.2). The cells were then spread on LB-agar containing 30 µg/ml kanamycin for pUB307 and an additional antibiotic matching the resistance of the corresponding vector. One of the obtained double resistant clones was picked and spread on a new plate of LB-agar containing the corresponding antibiotics and was incubated at 37 °C for 12 hours.

Streptomyces strains were picked from a well grown agar plate and spread onto a new MS-agar plate. The plates were incubated at 28 °C for 72 hours or until sporulation was obtained.

2.2.3.5.2. Conjugation

For intergeneric conjugation between *E. coli* and *Streptomyces*, 8 ml of sterile H_2O was added to a plate of *Streptomyces*, the spores were scratched and 5 1.4 ml micro tubes were filled with the spore suspension. The spore suspension was incubated at 50 °C for 10 minutes. During the 10 minutes incubation, 4 ml of sterile H_2O were added on each of two plates of *E. coli* and the cells were scratched. The cells suspension was distributed on two 1.5 ml micro tubes per plate and centrifuged at 1000 rpm for 1 minute. The supernatant was discarded but approximately 60 µl were left in the tube and the cells were resuspended in the remaining H_2O. After incubation, the *Streptomyces* spore suspensions were centrifuged at 15000 rpm for 5 minutes. The supernatant was discarded and approximately 60 µl were left in the tube and the spores were resuspended in the remaining H_2O. For conjugation, 1 tube of *E. coli* and 1 tube of *Streptomyces* spores were mixed and spread on a new MS-agar plate. These plates were incubated at 28 °C. As a control the *Streptomyces* spores were plated without *E. coli*. After 10 hours of incubation, the conjugations were overlaid with nalidixic acid to kill the *E. coli* and the antibiotic of which the resistance should be transferred by the vector.

2.2.3.6. Agarose gel electrophoresis

For agarose gel electrophoresis, a 1 % agarose gel in 1 x TAE buffer (Table IV.5) was prepared by melting 1 g agarose in 100 ml of 1 x TAE buffer. 20 µl of the DNA of interest was mixed with 3 µl of loading dye and filled in the gel pockets. The DNA was separated at 120 V in 1 x TAE buffer at room temperature. A 1 kb DNA ladder was used to identify the fragment size. After electrophoresis, the gel was stained for 15 minutes in a 2 µg / ml ethidium bromide containing water bath. The DNA fragments were visualized with a Gel documentation system (Table IV.1).

2.2.3.7. DNA purification from agarose gels

After staining the gel in a 0.6 % methylenium blue solution for 30 minutes, the desired fragment was cut out of the gel with a scalpel and transferred to a new 1.5 ml micro tube. DNA from this fragment was isolated using the Promega ™ Wizard ® Gel and PCR Clean-Up System according to the manufacturer's instructions and eluted in 50 µl of nuclease free water and stored at – 20 °C for further use.

2.2.3.8. Polymerase chain reaction (PCR)

To amplify desired DNA fragments, polymerase chain reaction was used. The *phusion* polymerase was used to amplify fragments for homologous recombination purposes while *dreamtaq* polymerase was used to amplify fragments for analytical needs. The annealing temperature (T_A) was determined specifically with a gradient PCR from 62 °C to 72 °C. The standard reaction mixtures and protocols used for fragment amplification are shown in tables IV.12 and IV.13.

Table IV.12: Standard reaction mixture for PCR used in this work

	Dream Taq	**Phusion**
Buffer	5 µl	10 µl
dNTPs	1 µl	1 µl
Primer 1	0.5 µl	0.5 µl
Primer 2	0.5 µl	0.5 µl
Template	0.5 µl	0.5 µl
Polymerase	0.5 µl	0.5 µl
DMSO	2.5 µl	2.5 µl
H_2O	38.5 µl	34.5 µl

Table IV.13: PCR methods used in this work

	Dream Taq		**Phusion**		**Cycle**
	Temperature	**Time**	**Temperature**	**Time**	
Initial denaturation	95 °C	3 min	98 °C	30 sec	1 x
Denaturation	95 °C	45 sec	98 °C	30 sec	30 x
Annealing	T_A	30 sec	T_A	45 sec	
Extension	72 °C	30 sec	70 °C	30 sec	
Final extension	72 °C	10 min	72 °C	10 min	1 x
Storage	4 °C	∞	4 °C	∞	∞

2.2.3.9. Red / ET Recombineering

2.2.3.9.1. Fragment preparation for cosmid targeting

For fragment deletion the primers from table IV.8 labeled Red / ET were used. These primers contain a 50 bp long sequence on the 5'-end matching the chromosome, flanking the region which is going to be deleted. The 20 bp 3'-end is matching either the right or left shoulder of the disruption cassette.

To amplify the disruption cassette with the corresponding genome match, a PCR was performed with the plasmid containing the disruption cassette as a template and the primers for the corresponding gene. These fragments were then purified by gel electrophoresis and eluted from the gel as previously described (IV.2.2.3.7).

2.2.3.9.2. Δ-red mediated recombination in *E. coli* GB05red

The vector containing the region to be disrupted was purified using the MaxBAC protocol (IV.2.2.3.2). 5 µl of the purified vector were then transformed by chemical transformation into chemical competent *E. coli* GB05red cells and incubated overnight.

On the next day, one clone was picked into 14 ml of LB medium, with the corresponding antibiotic, and incubated at 37 °C; 180 rpm overnight. From this overnight-culture 300 µl were used to inoculate 14 ml of LB medium. This culture was incubated at 37 °C and 180 rpm until an OD_{600} of 0.43 was

reached. When the OD was reached, the expression of the recombinases was induced by addition of 400 µl of 10 % L-rhamnose. Afterwards the strain was incubated until an OD_{600} of 0.8 was reached.

1.5 ml of the culture was added into a 1.5 ml micro-tube and centrifuged at 10000 rpm for 1 minute at 2 °C. The supernatant was discarded and again 1.5 ml of the culture were added, centrifuged, the supernatant was discarded and the tubes were placed on ice. On ice, the cells were resuspended in 1 ml of ice-cold H_2O and then centrifuged at 10000 rpm for 1 minute at 2 °C. The supernatant was discarded and the cells were again resuspended in 1 ml of ice-cold H_2O. This procedure was repeated twice. After washing the cells three times, the supernatant was discarded and approximately 20 - 30 µl were left in the tube. 2 µl of the previously obtained PCR product were added to the cells and mixed. The mixture was then transferred into electroporation cuvettes (1 mm) and incubated at room temperature for 3 minutes. After incubation the cells were electroporated at 1800 V. Then 1 ml of LB medium was added and the cells were transferred into a new 1.5 ml micro-tube. The transformed cells were incubated at 37 °C; 950 rpm for 70 minutes and then spread on LB-agar containing the corresponding antibiotics.

After incubation of the plates at 37 °C for 24 hours, single colonies of transformants were detected and one colony was selected. 100 ml of the selective LB-medium were inoculated with this colony and incubated at 37 °C and 180 rpm for 12 hours. The cosmid DNA of this colony was isolated using the plasmid isolation protocol (IV.2.2.3.1). The recombination was verified by amplification of the disrupted fragment by PCR and sequencing.

2.2.3.9.3. Transfer of the recombined cosmid into *S. albus* J1074 and selection of recombinants

The cosmids containing the disruption cassette were introduced into *E. coli* ET12567 / pUB307 by chemical transformation (IV.2.2.3.4.2.) and thereafter transferred into *S. albus* J1074 (or the corresponding knockout strains based on *S. albus* J1074) by intergeneric conjugation (IV.2.2.3.5.2). Some of the obtained exconjugants were overlaid with X-Gluc (70 µl per 100 ml) to test for β-Glucuronidase activity. β-Glucuronidase activity (blue color) is a sign, that a single crossover event has occurred. 8 exconjugants were chosen randomly and transferred to new selective MS-agar plates (4 exconjugants per plate). After sporulation was achieved, a drop of X-Gluc was placed in between the patches to check for β-Glucuronidase activity. One patch with β-Glucuronidase activity was chosen and dilutions (1:100000 – 1:10000000) were plated on new selective MS-agar plates containing X-Gluc. The appearance of white colonies is a sign for a double crossover event. One white colony was selected randomly and spread on a new selective MS-agar plate. Intergeneric conjugation of pUWLint31 and the corresponding *S. albus* J1074 knockout strain was performed. This was done to express the recombinase present on pUWLint31 in the knockout strain to excise the disruption cassette with the resistance marker (Myronovskyi et al. 2014a). 50 of the resulting exconjugants were picked

on new unselective MS-agar. This should result in a loss of pUWLint31 and the resistance it mediated. These patches were picked on LB-agar containing the corresponding antibiotic to screen for sensitive clones which successfully lost their disruption cassette. Any clone which was not able to grow on the selective LB-agar had the resistance cassette successfully excised.

To check whether the disruption cassette was correctly excised, a PCR was performed with isolated chromosomal DNA of the recombined *Streptomyces* strains and the _seq_ primers from table IV.8. If the recombination happened successfully, a PCR product of the "scar" left on the genome should be obtained, which is about 300 bp in length. This fragment was separated by gel-electrophoresis (IV.2.2.3.6) and isolated from the gel (IV.2.2.3.7). The fragment was sequenced to obtain the exact sequence left on the genome.

The cosmid containing the PCC2 gene did not contain the GUS-gene. Instead of selecting by β-Glucuronidase activity, the resulting exconjugants were therefore checked by PCR using primers for the chloramphenicol gene. The chloramphenicol gene is located on the backbone of the cosmid and is still present after a single crossover event but lost after a double crossover event. Except for the determination of the crossover events, the exconjugants were treated as previously described.

3. Results

3.1. Pamamycin production in *Streptomyces albus* J1074

3.1.1. Heterologous expression of pamamycin cosmids R1 – R4

The pamamycin cosmids R1 – R4 were brought into *S. albus* J1074 via intergeneric conjugation (IV.2.2.3.5.). Two exconjugants for each cosmid were cultivated in NL SGG and PPM (Hashimoto et al. 2005a) (IV.2.1.4.). The extraction and determination of the pamamycin production was carried out as described in (IV.2.2.1.2.). The synthesized standard of pamamycin 607 (Wang et al. 2001) was used for comparison. *S. albus* J1074 without a cosmid was used as a negative control to rule out any pamamycin production by the native *S. albus* J1074 host.

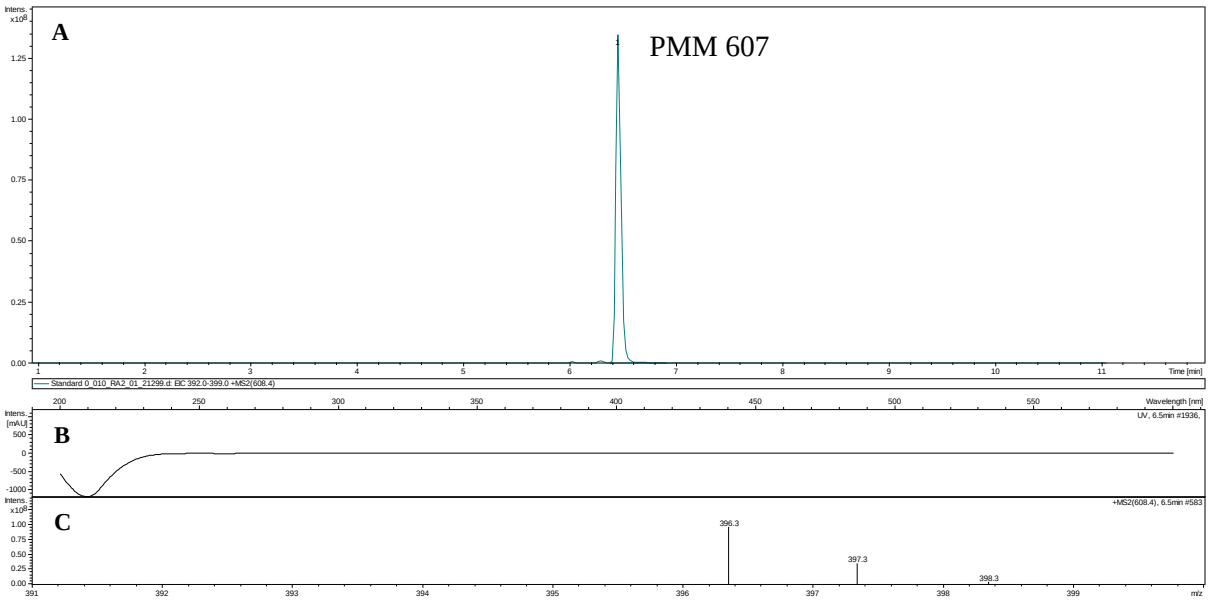


Figure IV.5: HPLC-MS chromatogram of the synthesized pamamycin 607. A compound pamamycin 607 with a retention time of 6.5 minutes. B the UV-chromatogram of pamamycin 607. C the fractionation pattern of pamamycin 607 in positive mode.

Figure IV.5 shows the synthesized pamamycin 607 measured with the method described in IV.2.2.2.3. This method is an optimized version of the original pamamycin measurement method used for the first detection of pamamycin. The two methods only differ in the length of the gradient. The original method had a gradient of 30 minutes and the optimized version was shortened to 15 minutes.

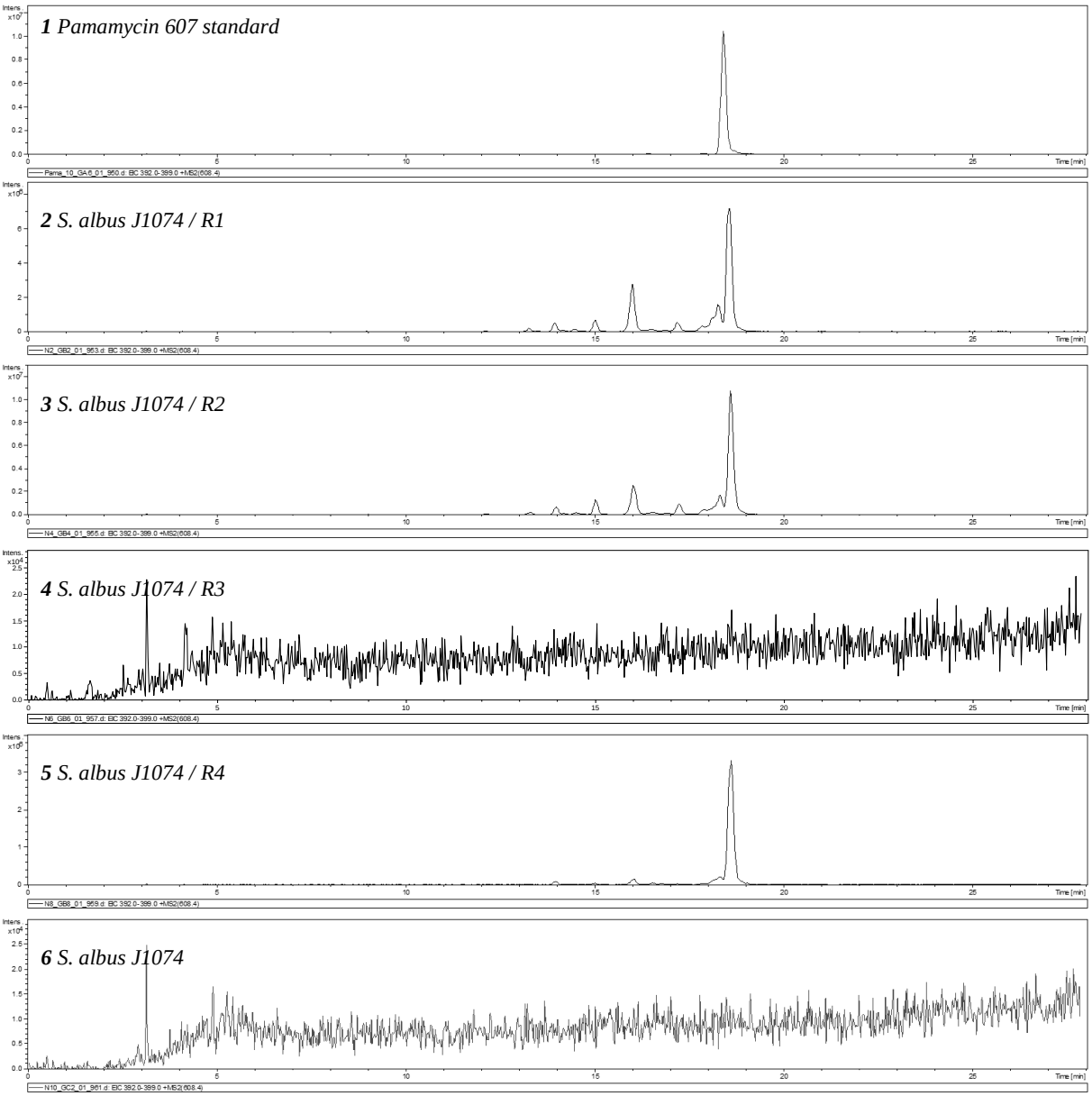


Figure IV.6: HPLC-chromatograms of the synthesized pamamycin 607, the supernatant extracts of S. albus J1074 with the pamamycin cosmids R1 - R4 and S. albus J1074 without the pamamycin cosmid.

Pamamycin production could be detected in the extracts of three of the four tested cosmids as shown in figure IV.6 (Figure IV.6 2, 3 + 5). Only the extracts of cosmid R3 (Figure IV.6 4) did not show production of pamamycin 607. The negative control *S. albus* J1074 without a cosmid (Figure IV.6 6) also showed no sign of pamamycin production.

The major peak in all extracts in comparison to the standard has a retention time shift of + 0.2 minutes. A peak with the same retention time as the standard could also be detected but it is much smaller in comparison. Since both peaks show the same mass, they could be derivatives of pamamycin 607 produced by *Streptomyces albus* J1074.

Based on these results, the strain with the integrated cosmid R2 and the medium NL SGG were selected for further experiments because this combination led to the highest production of pamamycin 607.

3.1.2. Optimization of NL SGG for pamamycin production

To further increase the pamamycin production, the carbon and nitrogen sources of NL SGG were increased or replaced by different ones. The changes are noted in table IV.11 (IV.2.2.1.2.3.).

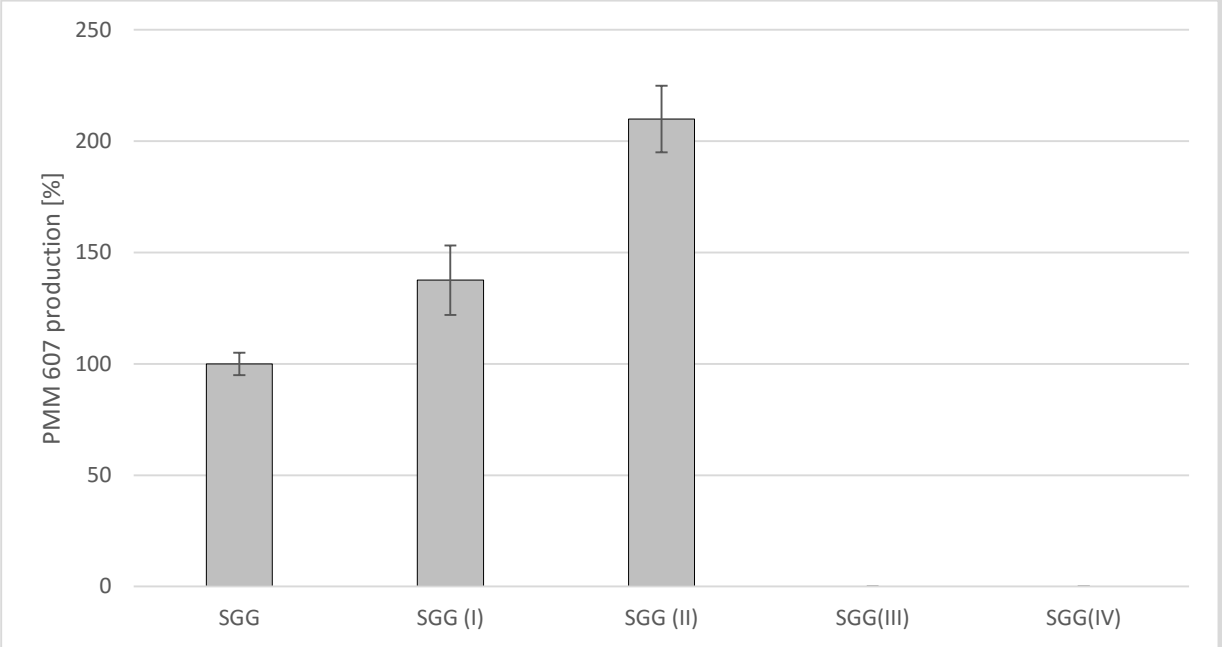


Figure IV.7: Production of pamamycin 607 in NL SGG and the modified versions (I - IV).

The increase of the carbon sources glucose and glycerol from 10 g to 15 g (SGG (I)) increased the pamamycin production by 37 % as shown in figure IV.7. The change from 10 g glucose to 20 g maltose (SGG (II)) increased the production by 109 %. The change of the nitrogen source from Bacto peptone to soymeal leads to a complete loss in production. SGG (II) was chosen for further experiments because of the increased pamamycin production.

3.1.3. Pamamycin production dependency on O_2 level

To test the pamamycin production in dependency of the O_2 level, several flasks with different numbers of buffles and different volumes of media were cultivated either at 120 rpm or 180 rpm as described in IV.2.2.1.2.4.

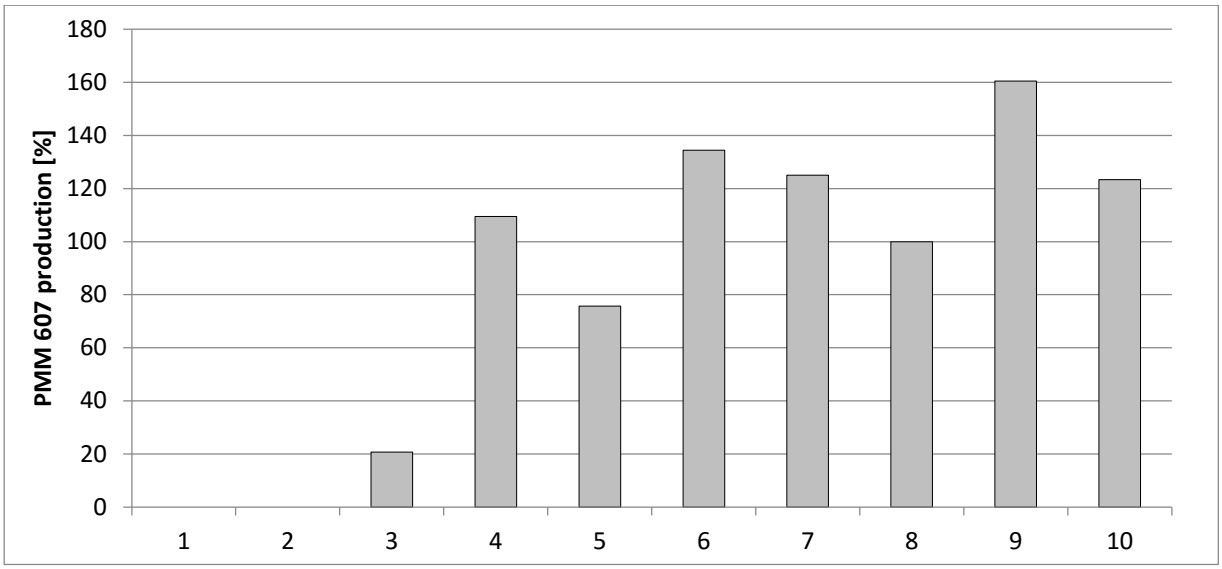


Figure IV.8: Production of pamamycin 607 in NL SGG (II) from little O_2 concentration (1) to high O_2 concentration (10).

Pamamycin production is strongly dependent on the O_2 level. Concentration 8 in figure IV.8 corresponds to the standard cultivation conditions and is thus set to 100 %. Below a certain concentration of O_2, pamamycin production stopped. The different tested conditions did not influence pamamycin production fundamentally and thus the standard cultivation conditions were kept unchanged.

3.1.4. Pamamycin production curve

To determine the optimal cultivation duration, a production curve for pamamycin 607 and 621 was created as described in IV.2.2.1.2.5.

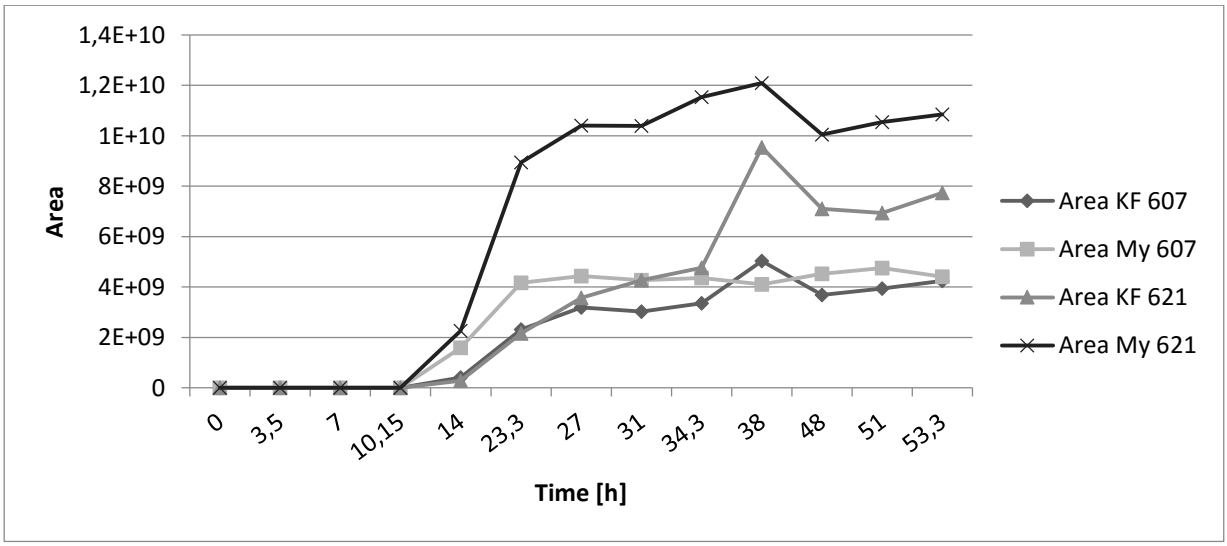


Figure IV.9: Production curve for pamamycin 607 and 621. Pamamycin quantities are each displayed by the calculated peak area. Supernatant (KF) and biomass (My) were extracted separately.

Pamamycin production starts between 10 and 14 hours after inoculation of the production medium and reaches a plateau at around 24 hours as shown in figure IV.9. Production of pamamycin 607 is stable

after 24 hours and does not increase significantly from this point on. Production of pamamycin 621 increases rapidly from 10 hours to 24 hours but keeps increasing afterwards. The ideal cultivation duration for production of pamamycin 607 and 621was determined to be around 48 hours. Other pamamycins could also be detected and increased even after cultivation for 48 hours. Thus for further studies a cultivation duration of 72 hours was chosen, to ensure sufficient production of all pamamycins.

3.2. *Streptomyces albus* J1074 strain development for pamamycin production

As described by Rebets et al. (2015), pamamycin biosynthesis utilizes succinyl-CoA, malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA from the primary metabolism of the producer strain to produce a wide variety of different pamamycins. To deepen the understanding of the pamamycin biosynthesis and its precursor supply, several genes, from the primary metabolism responsible for extender unit formation, as described by Chan et al. (2009) were knocked out and the pamamycin production in these strains was analyzed.

3.2.1. Identified genes in *S. albus* J1074

The genes involved in the formation of methylmalonyl-CoA and ethylmalonyl-CoA were chosen to be knocked out to reduce the number of pamamycin derivatives without those extender units.

Four genes were identified as essential for the production of these extender units:

The crotonyl-CoA carboxylase / reductase (CCR) which facilitates the formation of ethylmalonyl-CoA from crotonyl-CoA.
The methylmalonyl-CoA mutase, which is responsible for formation of *(2R)*-methylmalonyl-CoA from succinyl-CoA.
The propionyl-CoA carboxylase which forms *(2S)*-methylmalonyl-CoA from propionyl-CoA.
The valine dehydrogenase which is the starting point for the formation of methylmalonyl-CoA from L-valine.
These genes were identified by a blast (http://blast.ncbi.nlm.nih.gov) analysis with the corresponding gene from *Streptomyces coelicolor*.

CCR 1:	XNR_5889	Crotonyl-CoA reductase
CCR 2:	XNR_0456	Crotonyl-CoA carboxylase/reductase, ethylmalonyl-CoA producing
PCC 1:	XNR_2273	Acetyl/propionyl CoA carboxylase alpha subunit
	XNR_2274	Acetyl/propionyl CoA carboxylase, beta subunit
PCC2:	XNR_4211	Acetyl/propionyl CoA carboxylase alpha subunit
	XNR_4212	Acetyl/propionyl CoA carboxylase

MCM: XNR_4665 Methylmalonyl-CoA mutase large subunit
XNR_4666 Methylmalonyl-CoA small subunit
VDH: XNR_2839 Valine dehydrogenase

Six genes could be identified on the genome of *S. albus* J1074. These are, two genes for CCR, two genes for PCC one MCM and one VDH.

3.2.2. InSilico simulations

InSilico Biotechnology AG (Meitnerstraße 9, 70563 Stuttgart) performed a knockout simulation for some of the corresponding genes with regard to the pathway products of interest and the feasibility of the resulting knockout mutants shown in figure IV.10.

Knockout	Genes	Malonyl-CoA	Methylmalonyl-CoA	Ethylmalonyl-CoA	Growth
-	-	Feasible	Feasible	Feasible	Feasible
ACC	XNR_4019, XNR_2648	**Abolished**	Feasible	Feasible	**Abolished**
CCR	XNR_5889, XNR_0456	Feasible	Feasible	Feasible	Feasible
PCC	XNR_4019, XNR_4023, XNR_4024, XNR_2273, XNR_2274, XNR_4211, XNR_4212	Feasible	Feasible	Feasible	Feasible
BCC	XNR_4019, XNR_4023, XNR_4024, XNR_2273, XNR_2274, XNR_4211, XNR_4212	Feasible	Feasible	Feasible	Feasible
MSC	XNR_0457, XNR_0458, XNR_0459, XNR_0460, XNR_1439	Feasible	Feasible	Feasible	Feasible
MCM	XNR_4665, XNR_4666	Feasible	Feasible	Feasible	Feasible
PCC+MCM	See above	Feasible	**Abolished**	Feasible	Feasible
CCR+BCC	See above	Feasible	Feasible	**Abolished**	Feasible
CCR+PCC+BCC+ MSC+MCM	See above	Feasible	**Abolished**	**Abolished**	Feasible
ACC+CCR+PCC+ BCC+MSC+MCM	See above	**Abolished**	**Abolished**	**Abolished**	**Abolished**

Figure IV.10: Knockout simulation for the S. albus J1074 strain development for pamamycin production project by InSiIico Biotechnology. ACC, acetyl-CoA carboxylase; BCC, butyryl-CoA carboxylase; CCR, crotonyl-CoA carboxylase / reductase; MCM, methylmalonyl-CoA mutase; MSC, methylsuccinyl-CoA carboxylase; PCC, propionyl-CoA carboxylase.

The simulations support the assumption that by knockout of CCR, MCM and PCC the pools of methylmalonyl-CoA and ethylmalonyl-CoA should be significantly reduced. InSilico also identified additional genes which could be involved in formation of these extender units.

3.2.3. Knockout of the identified genes

Knockouts of the identified genes (IV.3.2.1.) were performed with the IMES system (Myronovskyi et al. 2014a) as described in IV.2.2.3.9. Except for the knockout of the VDH gene in *Streptomyces albus* J1074, all knockouts were done in the Del1 strain described in Myronovskyi et al. 2014b.

A panel of strains with different combinations of knockouts was created:

S. albus J1074	(wildtype strain)
S. albus J1074 ΔVDH	(1 knockout)
Del1	(1 knockout)
S. albus J1074 Δccr	(1 knockout)
Del1ΔccrD1-1	(2 knockouts)
Del1ΔccrD1-1 ΔPCC1	(3 knockouts)
Del1ΔccrD1-1 ΔPCC2	(3 knockouts)
Del1ΔccrD1-1 ΔMCM	(3 knockouts)
Del1ΔccrD1-1 ΔPCC2 ΔPCC1	(4 knockouts)
Del1ΔccrD1-1 ΔPCC2 ΔMCM	(4 knockouts)
Del1ΔccrD1-1 ΔMCM ΔPCC1	(4 knockouts)
Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1	(5 knockouts)
Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 ΔVDH	(6 knockouts)

Proof of knockouts can be found in the appendix.

3.2.4. Heterologous expression of the pamamycin producing cosmid R2 in selected knockout mutants

3.2.4.1. Measurements of pamamycin production in the CCR mutants

To determine the influence of the different CCR genes on the pamamycin production, the pamamycin cosmid R2 was brought into *S. albus* J1074, Del1, *S. albus* J1074 ΔCCR1 and Del1ΔCCRD1-1 by conjugation (IV.2.2.3.5.). *S. albus* J1074 is the wildtype strain with both CCR genes present, the Del1 strain is lacking the CCR 2 gene, in *S. albus* J1074 ΔCCR1 the CCR1 gene was knocked out and the Del1ΔCCRD1-1 strain is missing both CCR genes. The strains were cultivated as described in IV.2.2.1.2. and extracted as described in IV.2.2.2. The different pamamycins are labeled corresponding to their molecular weight.

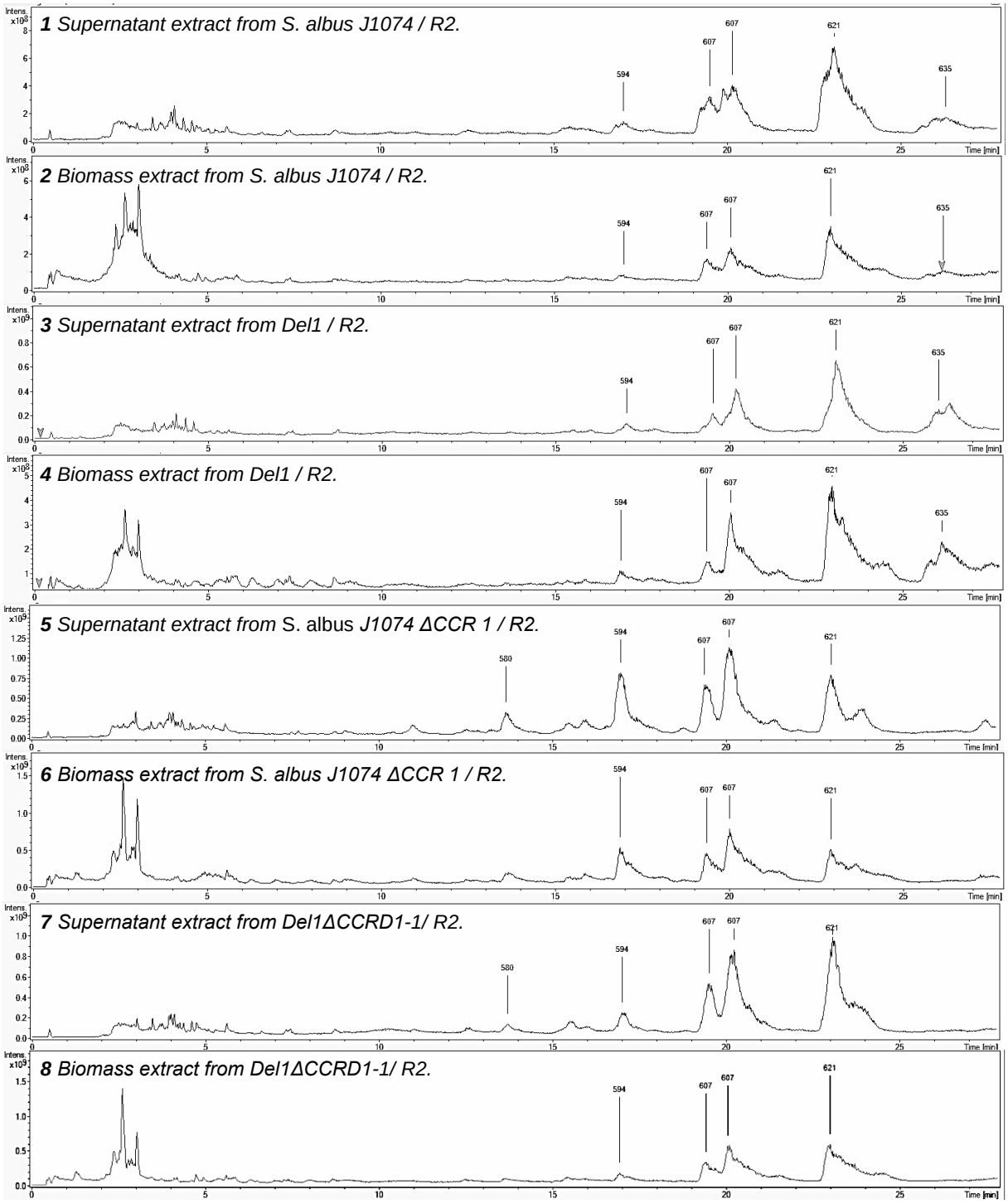


Figure IV.11: HPLC-MS chromatograms of the supernatant and biomass extracts from S. albus J1074 and the CCR knockout mutants with integrated pamamycin cosmid R2.

The wildtype strain *S. albus* J1074 (Figure IV.11 1 + 2) as well as the Del1 strain (Figure IV.11 3 + 4) show no significant difference in pamamycin production levels compared to each other. Different types of pamamycins can be detected (594, 607, 621 and 635) whereas 607 and 621 are the major products.

S. albus J1074 ΔCCR1 (Figures IV.11 5 + 6) and the Del1ΔCCRD1-1 strain (Figure IV.11 7 + 8) also show similar traits except for the increased production of pamamycin 594 in *S. albus* J1074 ΔCCR1 compared to Del1ΔCCRD1-1. These strains differ greatly from *S. albus* J1074 and Del1 with their detectable production of pamamycin 580, their increased production of pamamycin 594, 607 and 621 as well as the loss of pamamycin 635 production.

To compare the production levels of the different strains, pamamycin 607 production was quantified.

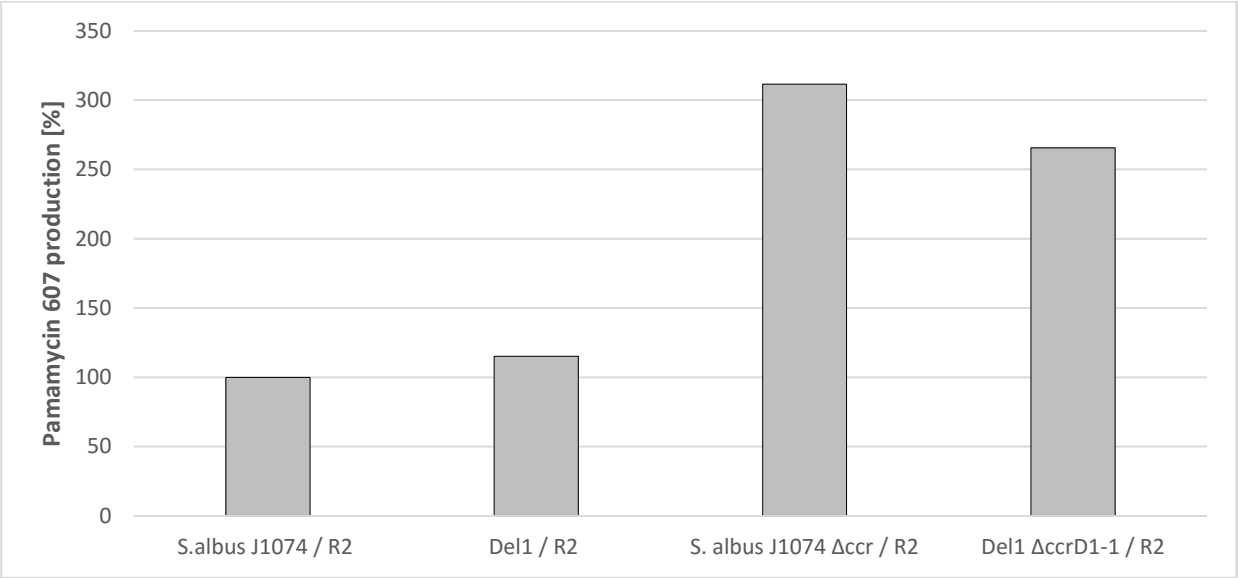


Figure IV.12: Production of pamamycin 607 in S. albus J1074, Del1, S. albus J1074 ΔCCR1 and Del1 ΔCCRD1-1. Pamamycin production of strain S. albus J1074 was set to 100 %.

Pamamycin 607 production in *S. albus* J1074 and Del1 show no significant difference as shown in figure IV.12, whereas the production in *S. albus* J1074 ΔCCR1 and Del1ΔCCRD1-1 was increased up to 3 times, to 311 %.

3.2.4.2. Measurement of pamamycin production in the triple knockout mutants

The Del1 ΔCCRD1-1 strain was used as a basis for further knockouts because of its lacking both CCR genes. In this background the MCM, PCC1 and PCC2 genes were knocked out (IV.2.2.3.9.) and the pamamycin biosynthetic gene cluster was introduced into these strains via conjugation (IV.2.2.3.5.). The strains were cultivated as described in IV.2.2.1.2. and pamamycin production was determined as described in IV.2.2.2.

For a better comparison of the effects of the knockouts, the LC-MS chromatograms of the mutants were overlaid with the *S. albus* J1074 / R2 chromatogram.

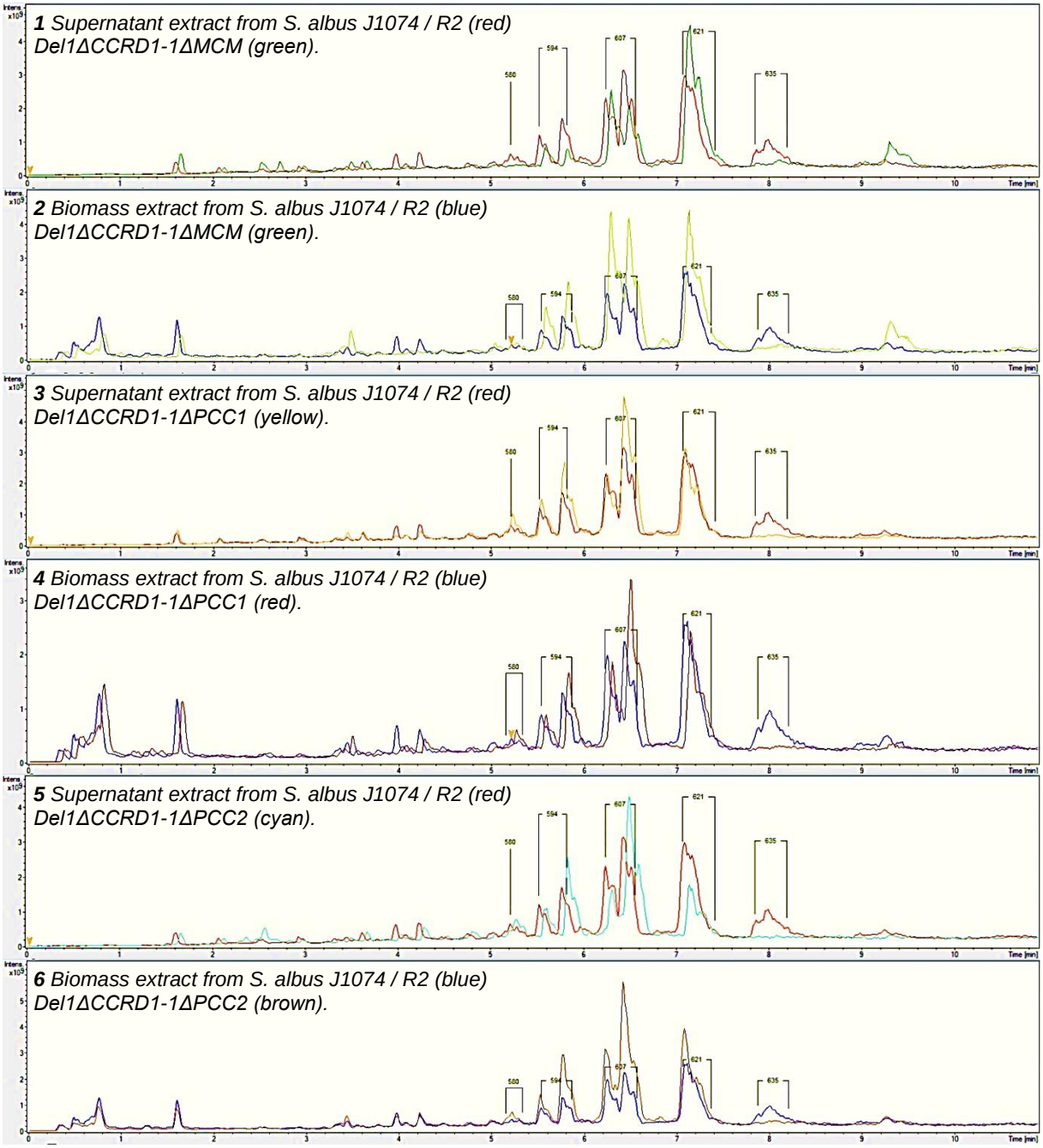


Figure IV.13: HPLC-MS chromatograms of S. albus J1074 and the triple knockout mutants with integrated pamamycin cosmid R2.

All knockouts shown in figure IV.13 have similar traits, like the lack of pamamycin 635 already seen in the ΔCCR1 strains in IV.3.2.4.1. Additionally, most knockout mutants show an increase of pamamycin 594, 607 or 621 production. Interestingly, the PCC1 knockout mutant (Figure IV.13 3+ 4) shows no increase in pamamycin 621 production like the MCM (Figure IV.13 1 + 2) or PCC2 (Figure IV.13 5 + 6) mutants.

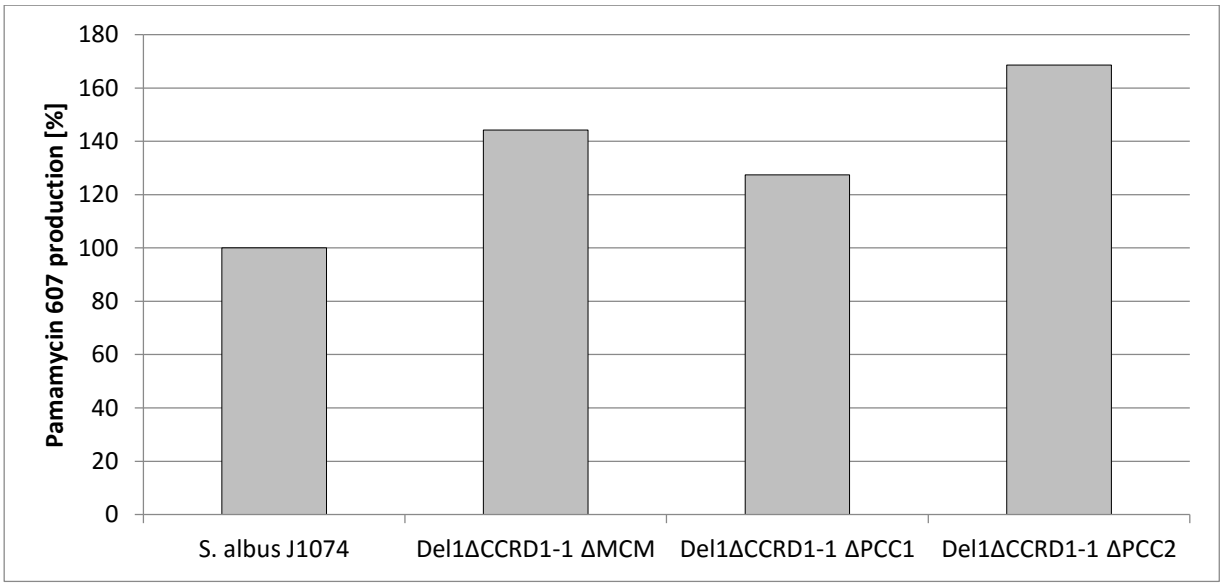


Figure IV.14: Pamamycin 607 production in S. albus J1074 / R2, Del1ΔCCRD1-1ΔMCM / R2, Del1ΔCCRD1-1ΔPCC1 / R2 and Del1ΔCCRD1-1ΔPCC2 / R2. Production of S. albus J1074 / R2 is set to 100 %.

Pamamycin 607 production is increased in all knockout mutants, as shown in figure IV.14. The PCC2 knockout mutant with a production increase of approximately 70 % shows the biggest difference.

3.2.4.3. Measurement of pamamycin production in the penta knockout mutant

Based on the results of the triple knockouts (IV.3.2.4.2) all knockouts were combined in one strain (Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1). The pamamycin gene cluster was brought into this strain by conjugation (IV.2.2.3.5). The strain was cultivated (IV.2.2.1.2) and the pamamycin production was measured (IV.2.2.2).

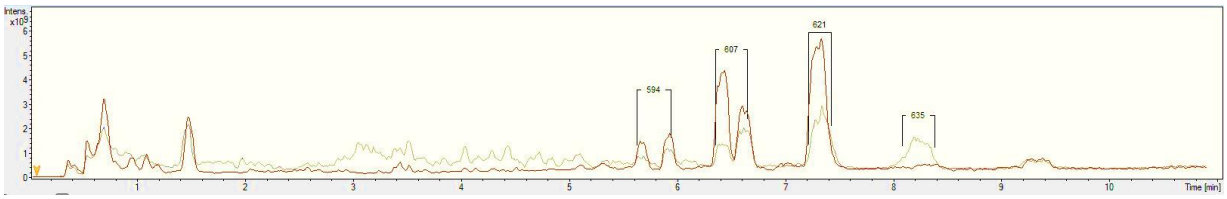


Figure IV.15: HPLC-MS chromatogram of the biomass extract from S. albus J1074 / R2 (grey) and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 / R2 (brown).

The Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1, shown in figure IV.15, strain shows a significant increase in pamamycin 594, 607 and 621 production, whereas production of pamamycin 635 ceased below detection level in comparison to *S. albus* J1074.

3.2.4.4. Ammonium feeding to *S. albus* J1074 / R2 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 / R2

To determine the role of valine in the precursor supply for pamamycin biosynthesis, ammonium was fed to the cultures of *S. albus* J1074 / R2 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 / R2. Ammonium was shown to inhibit the valine dehydrogenase activity (Tang et al. 1994) and thus

pamamycin production should be blocked or decreased when valine is used as a source for precursor production.

Ammonium was fed prior to inoculation of the main culture as described in IV.2.2.1.2.6., the strains were cultivated (IV.2.2.1.2.) and the pamamycin production was determined (IV.2.2.2.).

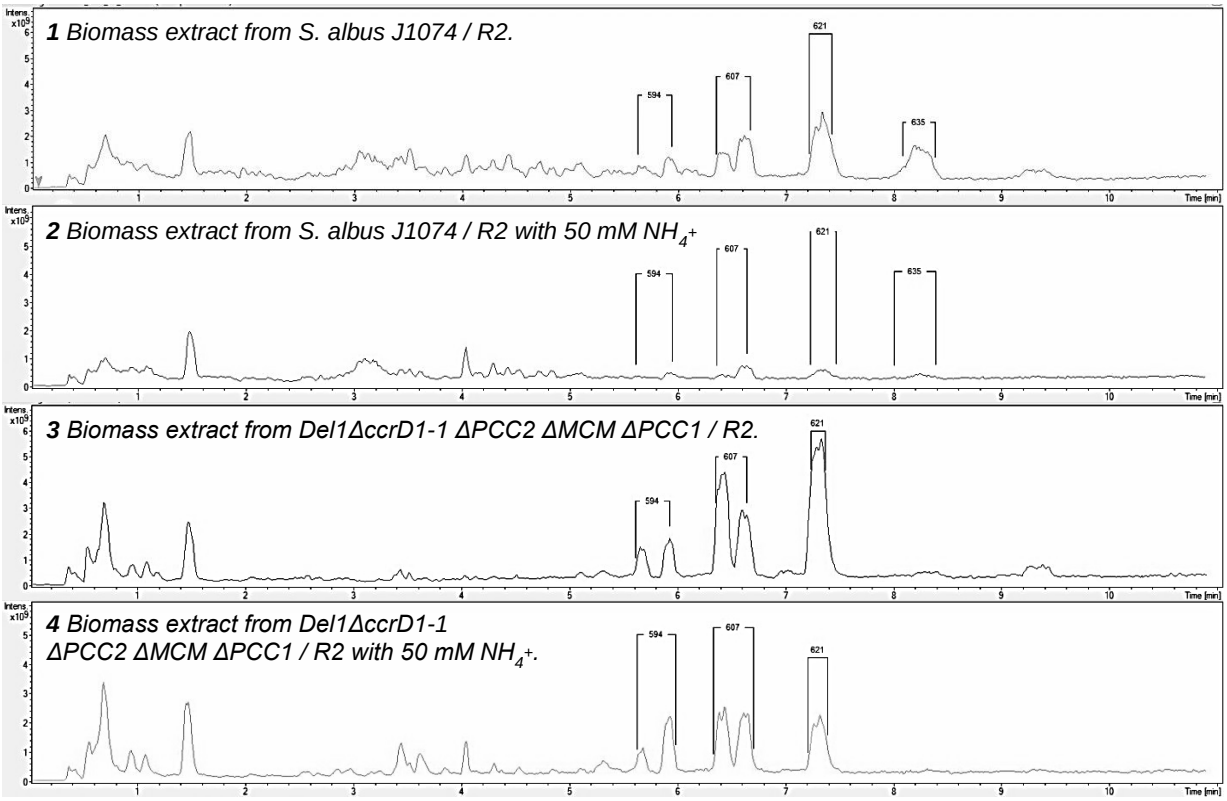


Figure IV.16: HPLC-MS chromatograms of S. albus J1074 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 with integrated pamamycin cosmid R2 with and without addition of 50 mM of NH_{4+}

The influence of NH_{4+} ions on pamamycin production is shown in figure IV.16. Addition of 50 mM ammonium inhibits (Figure IV.16 2) or decreases greatly (Figure IV.16 4) pamamycin production in comparison to the cultures without addition of ammonium (Figure IV.16 1 + 3).

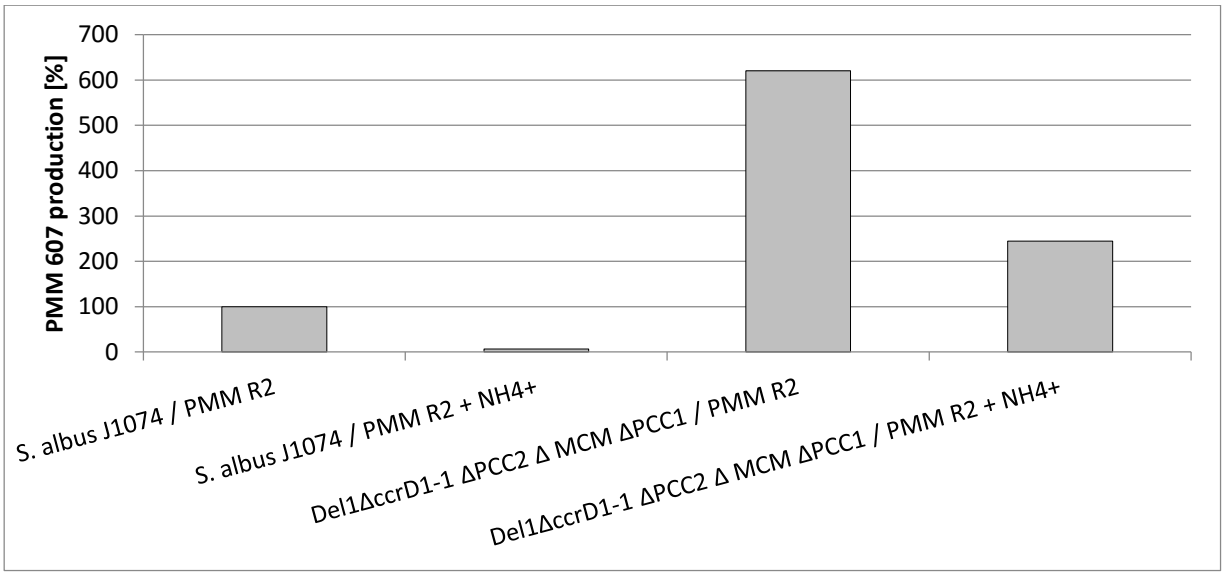


Figure IV.17: Production of pamamycin 607 in strains S. albus J1074 / R2 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 / R2 with and without addition of 50 mM ammonium. Production of S. albus J1074 / R2 is set to 100 %.

As shown in figure IV.17, production of pamamycin 607 is increased approximately 500 % in Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 / R2 in comparison to *S. albus* J1074 / R2. Addition of ammonium ions reduced pamamycin production almost completely in *S. albus* J1074 and about 350 % in Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 / R2.

3.2.4.5. Measurement of pamamycin production in the VDH knockout mutants

The role of the VDH gene in precursor supply for pamamycin production was analyzed by knockout of this gene in *S. albus* J1074 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1. The pamamycin cosmid was brought into these strains via conjugation (IV.2.2.3.5.). The strains were cultivated (IV.2.2.1.2.) and pamamycin production was determined (IV.2.2.2.).

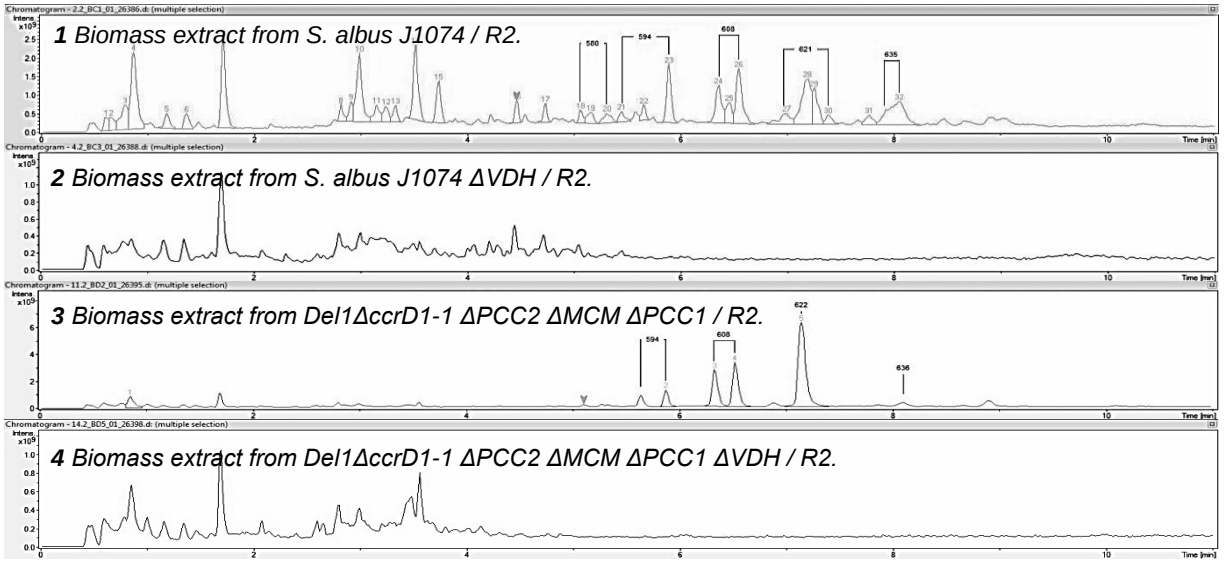


Figure IV.18: HPLC-MS chromatograms of S. albus J1074 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 with integrated pamamycin cosmid R2 with and without knocked out VDH gene.

As shown in Figure IV.18, pamamycin production is completely blocked by the knockout of the VDH gene in either strain (Figure IV.18 2 + 4).

3.2.4.6. Influence of succinate on pamamycin production

The influence of succinate on the production of pamamycins was tested by feeding of sodium succinate to *S. albus* J1074 with the integrated pamamycin R2 cosmid prior to inoculation to the main culture as described in IV.2.2.1.2.7., cultivated (IV.2.2.1.2.) and the pamamycin production was determined (IV.2.2.2.). Baking soda was used as a control to exclude the influence of sodium ions on the production of pamamycins.

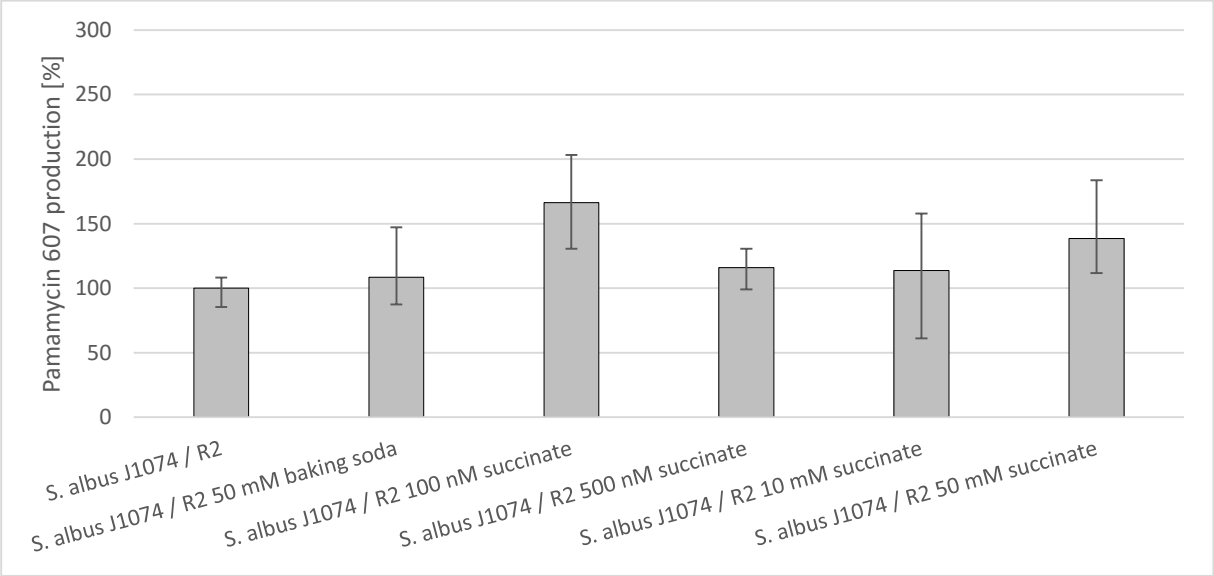


Figure IV.19: Production of pamamycin 607 in S. albus J1074 / R2 with and without addition of different concentrations of succinate or baking soda. Production of S. albus J1074 / R2 without any additive is set to 100 %.

As shown in figure IV.19, pamamycin 607 production is increased about 70 % when 100 nM succinate are fed to the culture. Higher concentrations of succinate also show an increase in production, however they do not show further enhancement of production. The control with 50 mM baking soda shows no significant effects on pamamycin production.

4. Discussion

4.1. Pamamycin production in *Streptomyces albus* J1074

4.1.1. Heterologous expression of pamamycin cosmids R1 – R4

Heterologous expression is a versatile tool to study antibiotic production. Pamamycin production was detected in *Streptomyces alboniger* DSM 40043 and a cosmid library was created and screened for clones containing the entire set of *pam* genes using two probes flanking the cluster (Rebets et al. 2015). Several cosmids were obtained and tested for the production of pamamycins in *Streptomyces albus* J1074. Previous experiments with *S. alboniger* DSM 40043 showed optimal pamamycin production in the media NL SGG and PPM, so these media were used for pamamycin production in *S. albus* J1074. To identify possible pamamycins, the synthesized standard pamamycin 607 (Wang et al. 2001) was used as a reference.

Pamamycin production was observed in *S. albus* J1074 after introducing the three cosmids (R1, R2 and R4). Additional compounds similar to known pamamycins could be detected in the three cosmids (R1, R2 and R4), what can be explained by the biosynthetic potential of the pamamycin gene cluster to produce several compounds (Natsume et al. 1995). The compound with the same retention time as the synthesized standard was the minor peak in all extracts and corresponded perfectly to pamamycin 607. With a retention time shift of 0.2 minutes, an additional compound could be detected which also corresponded to pamamycin 607. This compound was the major product in all extracts.

The cosmid R2 and the medium NL SGG have been chosen for further experiments because their combination yields the highest production of pamamycins.

4.1.2. Optimization of NL SGG for pamamycin production

Media composition influences secondary metabolite production greatly (Higashide 1984). To increase pamamycin production, different combinations of various carbon and nitrogen sources as well as different amounts of them were varied with NL SGG as a starting point. An increase of the carbon sources glucose and glycerol from 10 g/l to 15 g/l each also led to an increase of pamamycin production by 30 %. An increase of the carbon source and a switch from glucose to maltose (10 g/l glucose to 20 g/l maltose) further increased the production of pamamycin by 100 %. Glucose is generally used in rich media as a fast available carbon source for rapid biomass formation, because glucose can be consumed without the need of specific exoenzymes. But glucose is not the only carbon source in these media, because of its ability to inhibit antibiotic

production (Sanchez et al. 2010). The switch from glucose to maltose as a rapidly available carbon source can explain the increase of production.

Changing the nitrogen source from Bacto® peptone to soymeal completely abolished the production of pamamycins. This can be explained by the increase of nitrogen which has a negative effect on antibiotic production like in case of cephamycin and streptomycin (Srinivasan et al. 1991).

Because of its increased pamamycin production, NL SGG (variation II) has been chosen for further studies.

4.1.3. Pamamycin production dependency on the O_2 level

Aeration is very important for the production of secondary metabolites in aerobic organisms. Pfefferle et al. (2000) showed that ε-rhodomycinone-production in the fermentation process of *Streptosporangium* sp. NN 22303 increases with higher aeration rate, but drops after a certain level. To determine the influence of aeration on pamamycin production in *Streptomyces albus* J1074, several different flasks with different volumes of production medium and different rotations speeds were used. The two cultivations without a buffle and 100 ml medium in a 300 ml flask showed no pamamycin production, which indicates that pamamycin biosynthesis requires extensive aeration. The highest pamamycin production was achieved in a 4 buffle flask with 120 rpm with an increase of 60 % in comparison to the standard conditions (1 buffle flask, 180 rpm). An increase of the rotation speed to 180 rpm in the 4 buffle flask resulted in an increase of approximately 20 % in comparison to the standard conditions.

4.1.4. Pamamycin production curve

Secondary metabolite production generally starts after the stationary phase of growth or after the optimal growth is reached (Bu" Lock et al. 1965). Pamamycin production in *S. albus* J1074 starts between 10 and 14 hours of inoculation and the production maximum for pamamycin 607 and pamamycin 621 in the biomass is reached after 30 hours, whereas the pamamycin concentration in the supernatant slowly increases over time until the cultures were harvested. Since *S. albus* J1074 is a fast growing organism (personal experience) that needs 72 hours to achieve full sporulation in comparison to *Streptomyces lividans* that needs 168 hours, these observations are in accordance with the literature. Pamamycin production starts after an optimal growth phase is reached and continues to increase over time afterwards until the cultures are harvested. 72 hours was determined to be the optimal harvest time, since pamamycin production has reached a sufficient production level until then and the cultivation time is considered acceptable.

4.2. *Streptomyces albus* J1074 strain development for pamamycin production

4.2.1. Identified genes in *S. albus* J1074

Several important genes for methylmalonyl-CoA and ethylmalonyl-CoA biosynthesis have been identified according to the review of Chan et al. (2009)(Figure IV.20) in the fully annotated genome of *S. albus* J1074 (Zaburannyi et al. 2014).

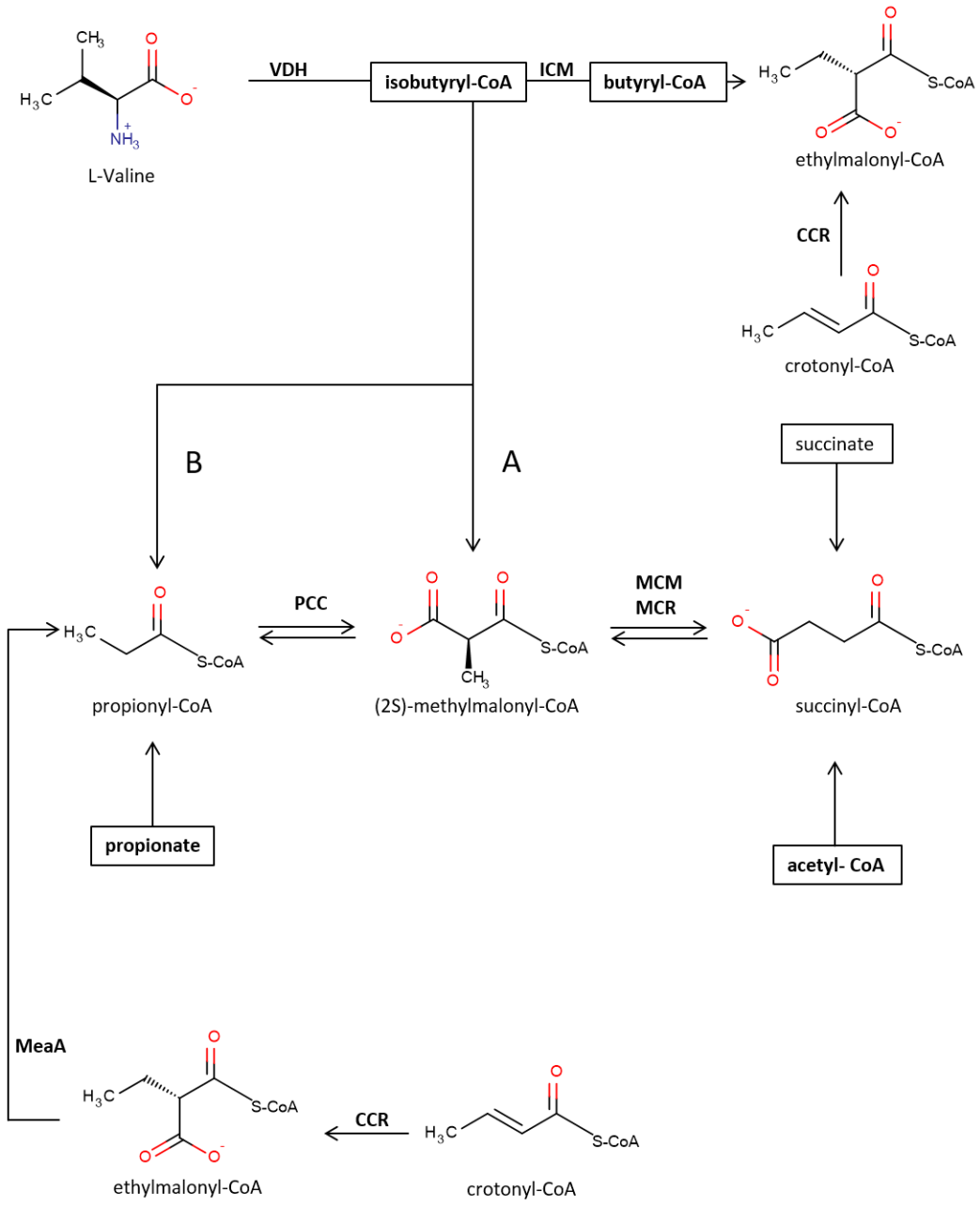


Figure IV.20: Metabolic pathways leading to the formation of (2S)-methylmalonyl-CoA and (2S)-ethylmalonyl-CoA. Abbreviations: ICM, isobutyryl-CoA mutase; PCC, propionyl-CoA carboxylase; CCR, crotonyl-CoA carboxylase/reductase; MCR, methylmalonyl CoA racemase; MCM, methylmalonyl-CoA mutase; VDH, valine dehydrogenase.

As the most important pathways for (*2S*)-methylmalonyl-CoA biosynthesis the transformation of succinyl-CoA to (*2R*)-methylmalonyl-CoA by the methylmalonyl-CoA mutase (MCM) and subsequent rearrangement to (*2S*)-methylmalonyl-CoA by the methylmalonyl-CoA racemase (MCR), the carboxylation of propionyl-CoA to (*2S*)-methylmalonyl-CoA by the propionyl-CoA carboxylase (PCC) and the catabolism of L-valine induced by the valine dehydrogenase (VDH) have been identified.

Ethylmalonyl-CoA is formed by the carboxylation of crotonyl-CoA mediated by the crotonyl-CoA carboxylase / reductase (CCR).

A blast search with the corresponding genes from *Streptomyces coelicolor* revealed one MCM gene, two PCC genes, a VDH gene and two CCR genes.

4.2.2. InSilico simulations

InSilico Biotechnology AG (Meitnerstraße 9, 70563 Stuttgart) performed knockout simulations for the corresponding knockouts based on the metabolomic model of *S. albus* J1074 they created (unpublished data). Their simulations were focused on the production of malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA and the liveliness of the cells after the knockouts.

Their simulations supported the previous decision on target genes for the specific knockouts.

4.2.3. Knockout of the identified genes

All identified genes could be knocked out separately or in different combinations of several or all target genes and the cells were still viable. As one would suspect, knockout of several genes from the primary metabolism responsible for important functions resulted in a reduced fitness of the cells but overall the biomass accumulation and growth speed were not impaired significantly. In contrast, the time until full sporulation was achieved even decreased slightly. This could be due to the reduced fitness and limited supply of cell constituents caused by the knockouts.

4.2.4. Heterologous expression of the pamamycin producing cosmid R2 in selected knockout mutants

4.2.4.1. Measurements of pamamycin production in the CCR mutants

There are two CCR genes (CCR 1 and CCR 2) present on the genome of *S. albus* J1074. To determine the function of each of these CCR genes on the production of ethylmalonyl-CoA for pamamycin biosynthesis, both genes have been knocked out independently and in combination.

The Del1 strain (Myronovskyi et al. 2014b) lacks the CCR 2 gene and the CCR1 gene was knocked out in *S. albus* J1074 resulting in *S. albus* J1074 ΔCCR 1. The knockout strain with both genes deleted was termed Del1ΔCCRD1-1.

Pamamycin production in the Del1 strain is not significantly altered in comparison to *S. albus* J1074. Both show a comparable compound spectrum and both produce pamamycin 635 which utilizes ethylmalonyl-CoA. The CCR 1 knockout mutant *S. albus* J1074 ΔCCR 1 and the double knockout mutant Del1ΔCCRD1-1 show a significantly altered compound spectrum. Not only is the production of pamamycin 635 reduced to levels where it is barely detectable, but also the production of the remaining pamamycins is increased. In this case pamamycin 607 production is increased by about 200 % in the CCR 1 knockout mutant and by about 160 % in the double knockout mutant. Pamamycin 580 is also clearly detectable in these strains in comparison to *S. albus* J1074 and the CCR 2 knockout strain.

These results support our assumption that the keto synthase genes from the pamamycin biosynthetic gene cluster are promiscuous and incorporate the extender units based on their availability. A strain, in which the supply of ethylmalonyl-CoA is reduced or absent, produces less or even none of the bigger pamamycins. The increase of production can also be explained by this observation. Because of the lack of ethylmalonyl-CoA, malonyl-CoA and methylmalonyl-CoA are used for the biosynthesis and thus the products using these extender units are produced in greater amounts.

Interestingly the knockouts show that only the CCR 1 gene participates in ethylmalonyl-CoA formation. There are bacterial strains which naturally lack CCR activity like *Saccharopolyspora erythraea* (Stassi et al. 1998) and strains like *Streptomyces lividans* which have CCR activity (Hu et al. 2005). In addition, there are several cases reported of cluster associated CCR genes (Haydock et al. 2004; Gandecha et al. 1997; Karray et al. 2007; Haydock et al. 2005) which seem to provide additional ethylmalonyl-CoA for biosynthetic production. The genome sequence of *S. albus* J1074 (Zaburannyi et al. 2014) reveals that the CCR 1 gene is located near the core of the genome and is not associated to any putative biosynthetic gene cluster, and that the CCR 2 gene is located on the outer right arm of the genome and in close proximity to a putative biosynthetic gene cluster. *S. albus* J1074 is one of the strains naturally able to produce ethylmalonyl-CoA via the ethylmalonyl-CoA pathway which contains a cryptic cluster that needs CCR activity for precursor supply.

The CCR knockouts also showed that the supply of malonyl-CoA or methylmalonyl-CoA is not the bottleneck for pamamycin production since the production is increased when the pool of ethylmalonyl-CoA is reduced. Additionally unlike monensin biosynthesis in *Streptomyces*

cinnamonensis (Li et al. 2004) CCR activity does not provide methylmalonyl-CoA for antibiotic production in *S. albus* J1074 under the tested conditions.

4.2.4.2. Measurement of pamamycin production in the triple knockout mutants

On the basis of the double CCR knockout strain Del1 ΔCCRD1-1 knockouts of MCM, PCC1 and PCC2 were performed, to determine the relevance of these genes on methylmalonyl-CoA biosynthesis for pamamycin biosynthesis.

Like the Del1 ΔCCRD1-1 strain, the triple knockout strains Del1 ΔCCRD1-1ΔMCM, Del1 ΔCCRD1-1ΔPCC1 and Del1 ΔCCRD1-1ΔPCC2 showed reduced production of pamamycin 635 and an increase in production of pamamycins 580, 593, 607 and 621. The increase in production of pamamycin 607 was ranging from 130 % for the PCC1 knockout to 170 % for the PCC2 knockout. These results are in the range of the double CCR knockout.

None of the knocked out genes showed a significant effect on pamamycin production, which means that a knockout of one methylmalonyl-CoA producing gene can be compensated by the remaining genes.

4.2.4.3. Measurement of pamamycin production in the penta knockout mutant

Since a single knockout of a putative methylmalonyl-CoA producing gene did not show an influence on pamamycin production, all identified major genes for methylmalonyl-CoA biosynthesis were knocked out in the same background. This strain lacks both CCR, the MCM, PCC1 and PCC2 genes resulting in a penta knockout strain termed Del1ΔCCRD1-1 ΔPCC2 ΔMCM ΔPCC1.

Pamamycin production was measured in this strain and compared to *S. albus* J1074.

Like the CCR double knockout mutant or the triple mutants with both CCR genes and either MCM, PCC1 or PCC2 knocked out, the penta knockout showed reduced production of pamamycin 635 and increased production of the remaining pamamycins. The production of pamamycin 607 and 621 in the penta knockout strain was even higher than in any previously obtained knockout mutant.

Since *Streptomyces albus* J1074 has the biological potential to form (*2S*)-methylmalonyl-CoA either from the carboxylation of propionyl-CoA (PCC), the rearrangement and epimerization of succinyl-CoA (MCM) or the catabolism of valine (VDH), the knockout of MCM, PCC1 and PCC2 in the background of the CCR knockouts should either lead to a decrease of pamamycin production if methylmalonyl-CoA was supplied by any of these pathways, or pamamycin production should be left unaltered in comparison to the double CCR knockout if methylmalonyl-CoA was solely provided by the catabolism of valine.

The increase of pamamycin production in the penta knockout mutant can be explained by the fact that a knocked out gene blocks a pathway which indirectly leads to the accumulation of a precursor for pamamycin biosynthesis, or some unspecific effects force the bacteria to channel methylmalonyl-CoA biosynthesis in one pathway with the result that methylmalonyl-CoA biosynthesis is unspecifically increased which in turn facilitates pamamycin biosynthesis.

The observed increase of precursor supply for pamamycin production is most probably due to the knockout of MCM. Knockout of MCM, which rearranges succinyl-CoA to (*2R*)-methylmalonyl-CoA, could lead to an increase of the succinyl-CoA pool in the cell. Since three succinyl-CoA units are incorporated into the final pamamycin molecule, this could lead to an increase of production, if succinyl-CoA was the bottleneck of pamamycin production.

4.2.4.4. Ammonium feeding to *S. albus* J1074 / R2 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 / R2

To clarify the source of methylmalonyl-CoA for pamamycin production, ammonium was fed to the cultures of *S. albus* J1074 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 containing the pamamycin gene cluster. Ammonium was chosen because of its potential to block VDH activity (Tang et al. 1994).

Feeding of 50 mM NH_4^+ showed a nearly complete cessation of pamamycin production in *S. albus* J1074 and a reduction of more than 50 % in the penta knockout strain. This indicates that valine catabolism is the source of methylmalonyl-CoA for pamamycin production.

4.2.4.5. Measurement of pamamycin production in the VDH knockout mutants

The VDH gene was knocked out in *S. albus* J1074 and in the penta knockout strain to verify the results from the ammonium feeding and to see whether *S. albus* J1074 was able to compensate the loss of methylmalonyl-CoA biosynthesis from the catabolism of valine.

The knockout of the VDH gene led to a complete cessation of pamamycin production both in *S. albus* J1074 and the penta knockout strain.

This result shows that the catabolism of valine is the sole provider of methylmalonyl-CoA for pamamycin production, and that the strain is not able to compensate the loss of the VDH gene for secondary metabolite production. The catabolism of valine has already been described to be important for secondary metabolite production (Stevens & Chester 1958; Tang et al. 1994), and pamamycin biosynthesis appears to be completely dependent of this pathway under the tested conditions.

4.2.4.6. Influence of succinate on pamamycin production

To check if succinyl-CoA is the bottleneck for pamamycin biosynthesis, succinate was fed to *S. albus* J1074 containing the pamamycin biosynthetic gene cluster.

Feeding of 100 nM sodium succinate showed a significant increase in pamamycin 607 production of 66 % which indicates that succinyl-CoA is a limiting factor for the production of pamamycins.

The higher concentrations of fed succinate also showed a slight increase in production of 38 %, but not as much as the lower concentration did.

Since succinate is an important intermediate of the citric acid cycle, excess of succinate could interfere with its balance and in turn meddle with cellular processes. This could have negative effects on pamamycin production, what explains the production increase for lower concentrations of succinate.

5. Summary of the strain development of *Streptomyces albus* J1074 for pamamycin production

We could prove pamamycin production in *S. albus* J1074 after the heterologous expression of 3 of 4 cosmids obtained by screening for the biosynthetic gene cluster. We optimized the production medium, checked O_2 consumption and production characteristics of pamamycin. We identified the pathways leading to methylmalonyl-CoA and ethylmalonyl-CoA for pamamycin production by knocking out the responsible genes. We could determine the level of succinyl-CoA as a bottleneck for pamamycin production. We demonstrated this by knocking out the MCM gene, which leads to an increase of succinyl-CoA, and by feeding of succinate directly to cultures of *S. albus* J1074 / R2. A panel of knockout strains was created resulting in a final mutant strain which combines six knockouts in the same genetic background. The compound profile for pamamycin was heavily influenced and the production of some pamamycin derivatives was increased by knocking out specific genes from the primary metabolism. In this study we could clearly show the connection between certain pathways of the primary metabolism for precursor supply of secondary metabolites. By knocking out certain genes we could exploit the promiscuous PKS of the pamamycin biosynthesis to shift the compound spectrum into a specific direction.

V. An influence of the copy number of biosynthetic gene clusters on the production level of antibiotics in a heterologous host

1. Introduction

Heterologous expression, the expression of foreign genes in a suitable host, has been shown to be a powerful tool in biotechnology. The principle is to identify genes or clusters of interest and express them in a suitable host using an optimized expression system. This method is mostly used if the native producer strains or organisms are not genetically accessible and/or show unreliable growth and metabolite production profiles. Thus these clusters are preferably expressed in a well-studied model organism, where a lot of tools were already successfully applied and cultivation conditions, media composition and storage conditions are well described. In fact, heterologous expression has already been used to express foreign genes from animals or plants in bacteria or fungi (Frommer & Ninnemann 1995), to study the function of genes or clusters (Luzhetskyy et al. 2007; Rebets et al. 2015), to elucidate unknown genes or pathways (Baltz 2010), to engineer known or unknown biosynthetic gene clusters (Wenzel et al. 2005), to generate new derivatives of known compounds (Luzhetskyy et al. 2007), to increase the production of desired metabolites (Baltz 2010) or to create unnatural pathways (Gomez-Escribano & Bibb 2014).

It is also a tool of choice to study secondary metabolite production and is widely used in that field for many purposes. Some fundamental works base on *Actinobacteria*. *Actinobacteria* have been shown to not only be a prolific source of natural products (Baltz 2008; Hodgson 2000), but also to be suitable hosts for their expression. Several model organisms like *Streptomyces albus* J1074, *Streptomyces coelicolor*, *Streptomyces lividans* or *Streptomyces griseofuscus* have been shown to be fitted for specific tasks whereas also industrial derived strains could be useful for heterologous expression (Baltz 2010). There are even great efforts undertaken to optimize some of these model organisms to be better hosts (Komatsu et al. 2010; Gomez-Escribano & Bibb 2011; Komatsu et al. 2013). The advantage in using model organism for heterologous expression is, that these strains are well understood and they are genetically accessible.

Despite a vast range of available expression hosts, heterologous expression often does not result in the desired outcome. Often the yield of the desired product in the heterologous host is significantly lower

than in the parental strain (Binz et al. 2008; Huo et al. 2012). To address this problem, additional steps have to be made, like introducing promoters upstream of the cluster to force its expression or to engineer the precursor supply (Rebets et al. 2015). These steps are quite often not intuitive and very time consuming. Toxicity is also a great issue, because the expression hosts are often more susceptible to the heterologously produced compounds than the natural producers. This problem can be solved by the co-expression of corresponding resistance genes, which also needs time and knowledge about the produced substance.

To address problems like product yield, we integrated additional *attB* attachment sites of the phage ϕC31 into the genome of our heterologous host *Streptomyces albus* J1074 via Tn5 transposon mutagenesis (Petzke & Luzhetskyy 2009). The integration system of this phage (Lomovskaya et al. 1971) promotes the recombination between the *attB* site present in the genome of the host and the *attP* site present on the vector (Thorpe & Smith, Margaret C. M. 1998). After incorporation of the additional attachment sites into the genome of *S. albus* J1074 we aim to increase the copy number of the integrated gene cluster, and thus to enhance the production of the desired compound.

Here we present a nonspecific approach to increase the product yield of expressed secondary metabolite clusters by addition of *attB* attachment sites in our heterologous host *Streptomyces albus* J1074. We selected several antibiotic clusters and expressed them in a range of *S. albus* mutants with different numbers of *attB* attachment sites to study the effect of gene cluster dosage in these strains. Furthermore we were able to identify a new aranciamycin derivative produced by one of our mutants.

2. Materials and methods

2.1. Construction of plasmids for transposon mutagenesis

The *hph* gene was amplified using pAL1 as a template, Fr-MI-attB-hph as a forward primer, carrying *attB* and the MunI restriction site, and Rs-XI-hph as a reverse primer carrying the XbaI site. The amplified fragment was cloned into the MunI and XbaI sites of pTn5Oks resulting in pTn5OksattBhph(II). The EcoRV fragment from pTn5OksattBhph(II), containing the transposon, was ligated into pNLHim, linearized by EcoRV, to result in pHAT(II)3.

To verify the obtained construct, analytical restriction mapping with EcoRV was performed. The obtained 1,9 kb fragment corresponds to the minitransposon construct cloned from pTn5OksattBhph(II).

Table V.1: Existing plasmid constructs

Name	**Description**	**Reference or Source**
pNLTn5	Replicative vector for actinomycetes containing pSG5-rep, *ori*T, and *tn5* gene under the *tipA* promoter	Dissertation of Dr. Lutz Petzke
p41-2C-06	pOJ436 derivative, containing aranciamycin biosynthetic cluster	(Luzhetskyy et al. 2007)
cos2	pOJ436 derivative, containing the mensacarcin biosynthetic cluster	(Yan et al. 2012)
DJ380pUp31	pOJ436 derivative, containing the griseorhodin biosynthetic cluster	(Li & Piel 2002)
PMM R2	pOJ436 derivative, containing the pamamycin biosynthetic cluster	(Rebets et al. 2015)

2.2. Introduction of additional *attB*-sites into *S. albus* genome

Additional *attB* attachment sites were introduced by conjugation of the plasmid pHAT(II)3 into the *S. albus* J1074 chromosome (Protocol for transposon mutagenesis see (Bilyk et al. 2013)).

The integration was proven by Southern blot.

Table V.2: New Plasmid constructs

Name	Vector	Size, kb	Tns	Marker	Description
pHAT(II)3	pNLTn5	11,5	*tn5*	*hph*	Minitransposon with *hph*, ϕC31-*attB*, R6Kγ origin, flanked by MEs
pNLHim	pNLTn5	9,2	*himar1*	*aac(3)IV*	Plasmid derived from pNL1, containing *himar1(a)* gene
pTn5Oks	n/a	3,6	*himar1, tn5*	*bla*	PCR-derivative containing R6Kγ origin flanked by two MEs and two ITRs (Shine Gene, PRC)

Table V.3: Primers used for plasmid construction

Name	Primer sequence (in 5'->3')	Features
Fr-*MI*-*attB*-*hph*	ccccc*caattg*CGGGTGCCAGGGCGTGCCCTTGG GCTCCCCGGGCGCGTACccgtatttgcagtaccagcgt-3	*Mun*I (in italics), *attB* (in capitals)
Rs-*X*I-*hph*	ccccc*tctaga*gaataggaacttcggaatagg	*Xba*I (in italics)

2.3. Heterologous expression of the secondary metabolite clusters

The PMM R2 cluster was obtained by previous work in our group. The cos2 (didesmethylmensacarcin) and the aranciamycin cosmid 41–2C-06 were obtained from Andreas Bechthold. The griseorhodin encoding gene cluster on the cosmid DJ380pUp31 was obtained from Jörn Piel.

All cosmids were brought into *E. coli* ET 12567 (pUB307) by transformation. The transfer of the clusters from *E. coli* ET 12567 (pUB307) into *S. albus* J1074, *S. albus* T1, *S. albus* T11 and *S. albus* ΔpseB4 was accomplished by intergeneric conjugation (Sambrook & Russell 2001), to obtain *S. albus*::PMM R2, *S. albus*::cos2, *S. albus*::41-2C-06, *S. albus*::DJ380pUp31, *S. albus* T1::PMM R2, *S. albus* T1::cos2, *S. albus* T1::41-2C-06, *S. albus* T1::DJ380pUp31, *S. albus* T11::PMM R2, *S. albus* T11::cos2, *S. albus* T11::41-2C-06, *S. albus* T11::DJ380pUp31, *S. albus* ΔpseB4::PMM R2, *S. albus* ΔpseB4::cos2, *S. albus* ΔpseB4::41-2C-06, *S. albus* ΔpseB4::DJ380pUp31.

Table V.4: Used strains

Name	Description	Reference or Source
S. albus ΔpseB4	*S. albus* J1074 strain with deleted ΔpseB4 attachment site	(Bilyk & Luzhetskyy 2014)
S. albus J1074	*S. albus* G mutant	(Chater & Wilde 1976)
S. albus T1	*S. albus* J1074 strain with additional *attB* attachment site introduced by transposon mutagenesis	This work
S. albus T11	*S. albus* J1074 strain with additional *attB* attachment site introduced by transposon mutagenesis	This work

2.3.1. Pamamycin production

The strains containing the PMM R2 cosmid were cultivated in a preculture medium containing 1 % glucose, 1 % glycerol, 0.5 % oatmeal, 1 % soymeal, 0.5 % yeast extract, 0.5 % Bacto casaminoacids and 0.1 % CaCO3 in tap water; the pH was adjusted to 7.0 (1M NaOH) prior to sterilization. The production media consisted of glucose 1 %, glycerol 1 %, soluble starch 1 %, corn steep powder 0.25 %, Bacto peptone 0.5 %, yeast extract 0.2 %, NaCl 0.1 % and CaCO3 0.3 % in tap water; the pH was adjusted to 7.3 (5 M NaOH) prior to sterilization. The preculture medium was inoculated using 1/8 of a well grown agar plate. The production medium was inoculated with 6 % of the preculture medium. The strains were cultivated for 48 h in the preculture medium, transferred to the production medium and harvested after 72 h.

2.3.2. Didesmethylmensacarcin and griseorhodin production

The strains containing the cos2 or DJ380pUp31 cosmid were cultivated for 48 h in tryptic soy broth (TSB, Sigma, Germany) as a preculture medium. The production medium contained 4 % dextrin, 0.75 % soytone, 0.5 % baker's yeast, 2.1 % MOPS in deionized water; the pH was adjusted to 6.8 (1 M NaOH) prior to sterilization. 100 ml of the preculture medium were inoculated using 1/8 of a well grown agar plate. The production medium was inoculated with 6 % of the preculture medium. The strains were cultivated for 48 h in the preculture medium, transferred to the production medium and harvested after 120 h. All strains were grown at 28 °C; 180 rpm.

2.3.3. Aranciamycin production

The strains containing the 41–2C-06 cosmid were cultivated for 216 h on 30 ml solid medium containing 2 % mannitol, 2 % soymeal, 2 % agar in distilled water. The plates were inoculated using 100 µl of a spore suspension consisting of 1/3 of a well grown plate harvested in 1 ml H_2O.

2.4. Determination of production

One clone of each strain with the corresponding, integrated secondary metabolite cluster was chosen and a preculture was inoculated from spores. From this preculture three flasks of the main culture were inoculated to produce three technical replicates for each exconjugant. Cultivation of the pre and main culture were performed as previously described in V.2.3.1., V.2.3.2. and V.2.3.3.

The cultures were harvested, separated by centrifugation and 5 ml of supernatant was extracted with ethylacetate. The remaining biomass was extracted with a mixture of methanol and acetone (1+1). Both the supernatant and biomass extracts were evaporated to dryness using a rotary evaporator (RV10, VWR, Germany). The supernatant extract was then resolved in 500 µl of methanol. The biomass extract was resolved in 1000 µl of methanol. The extracts were then measured using a 100 mm BEH C18 column (Waters, Milford, USA) with a flow rate of 0.55 ml/min and the following gradient: 0 min 5 % B, 9 min 95 % B (solvent A: water + 0.1 % formic acid, solvent B: acetonitrile + 0.1 % formic acid).

The peak area of the corresponding compound was measured, normalized to biomass and used for comparison. For all secondary metabolites, the production of the ΔpseB4 strain with one attachment site was set as 100 %.

2.4.1. Determination of pamamycin production

The samples were extracted as previously described. Measurement was performed using a 100 mm BEH C18 column (Waters, Milford, USA) with a flow rate of 0.55 ml/min and the following gradient: 0 min 20 % D, linear gradient to 3 min 97 % D, linear gradient to 11 min 100 % D (solvent C: 90 mM ammoniumformiate, solvent D: acetonitrile/100 mM ammoniumformiate (80 + 20)).

Pamamycin production was measured by analysis of the compound area of the derivative PMM 607, which was chosen because of the high abundance of this compound and the presence of a synthesized standard (Wang et al. 2001). Several other pamamycin derivatives could be detected and behaved accordingly.

2.4.2. Determination of demethoxyaranciamycinone production

The agar of each plate was cut into pieces and put into a 50 ml Falcon tube. 10 ml of ethylacetate was added and shaken at 180 rpm at 28 °C for 15 minutes. The organic phase was separated from the water phase by centrifugation and evaporated. This procedure was repeated three times and the corresponding fractions were combined. The extracts were resolved in 1.5 ml of a mixture of Methanol and DMSO (2+1) and measured using a 100 mm BEH C18 column (Waters, Milford, USA) with a flow rate of 0.55 ml/min and the following gradient: 0 min 5 % B, 9 min 95 % B (solvent A: water + 0.1 % formic acid, solvent B: acetonitrile + 0.1 % formic acid).

2.5. Production and isolation of 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone

The preparative production of 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone was carried out on 120 x 120 x 17 mm square petri dishes (Greiner bio-one, Germany) containing approx. 50 ml of MS medium (1 % mannitol, 1 % soymeal, 1 % agar in deionized water). The plates were inoculated with 1 ml of spores of *S. albus* T11::41-2C-06 and cultivated at 28 °C for 168 h. 58 plates were prepared.

For extraction, the agar was cut and filled into 2 two L bottles (Schott). The agar was extracted three times with 750 ml of ethylacetate. After each step the agar was filtered to remove the ethylacetate. The resulting extracts were combined yielding 4.5 L of the extract. This extract was reduced to dryness on a rotary evaporator and resolved in 13 ml of methanol. This methanol solution was further separated using our semiprep HPLC system using a NUCLEODUR C18 HTec column (Waters, Milford, USA) with a flow rate of 5.6 ml/min and a linear gradient: 0 min 5 % B to 20 min 100 % B (solvent A: water + 0.1 % formic acid, solvent B: acetonitrile + 0.1 % formic acid).

2.6. Structure elucidation of 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone

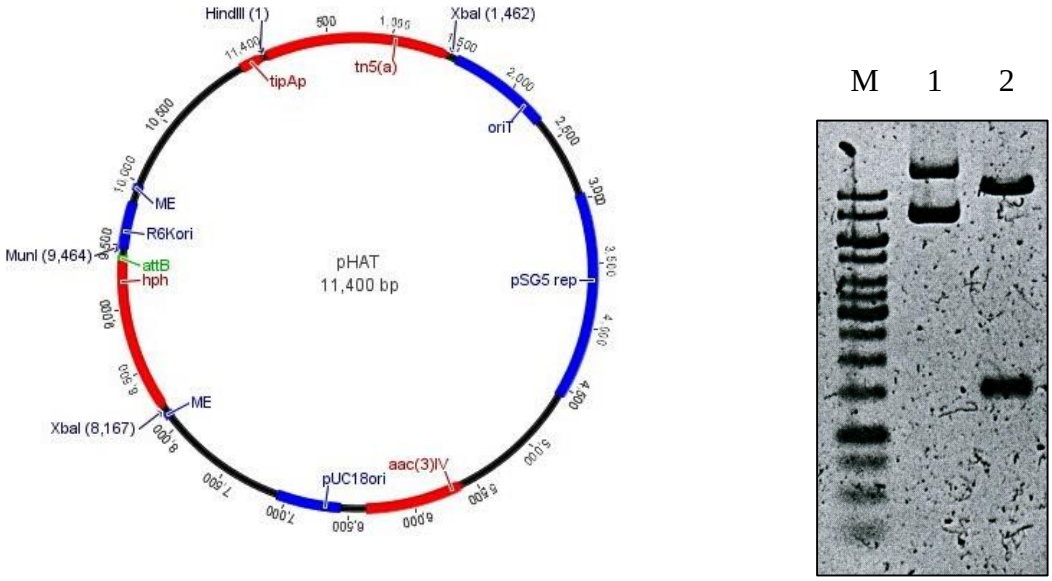
Mass spectra were recorded using a Thermo Finnigan LTQ Orbitrap mass spectrometer. NMR spectra were measured using a Varian VNMR-S 600 equipped with 3 mm triple resonance and 3 mm dual broadband probes in $CDCl_3$ at T = 25 °C. The residual solvent signals were used as internal reference (δ_H = 7.25 ppm, δ_C = 77.0 ppm).

3. Results and Discussion

3.1. Introduction of additional *attB*-sites into the genome of *Streptomyces albus* J1074

Heterologous expression of biosynthetic gene clusters in streptomycetes very often involves the ϕC31-based integration system. Integration vectors should contain the *attP* site for integration and the integrase encoding gene. The recipient chromosome typically carries an attachment *attB* site for successful integration of the vector. In our previous studies (Bilyk & Luzhetskyy 2014), we have shown that *S. albus* J1074 contains two highly active *attB* sites, which partially explain its excellent features as a heterologous host for the expression of biosynthetic gene clusters. The heterologous production of aranciamycin is significantly reduced when one *attB* site was deleted from the chromosome of *S. albus* J1074. In order to investigate the correlation between the copy number of an antibiotic cluster and the heterologous production of the corresponding antibiotic, we decided to introduce additional copies of the *attB* site into the chromosome of *S. albus* J1074 using transposon integration. With this aim a primer containing *attB* was used for the amplification of the hygromycin resistance gene from pAL1. The obtained PCR-fragment was cloned into pTN5Oks synthesised previously (Table V.3). Thus, a Tn5-transposon containing *attB* and the hygromycin resistance gene was obtained. This transposon was cloned into EcoRV-linearized pNLTn5, resulting in pHAT(II)3. The plasmid was introduced into *S. albus* J1074 by intergeneric conjugation, transposon mutagenesis was carried out according to the protocol described previously (Bilyk et al. 2013) and eleven mutants with randomly inserted *attB* sites were isolated and analysed by Southern hybridization. The hygromycin resistance gene was used as a selection marker. According to the results (Figure V.1) it was speculated that mutant #11 has more than one integration of the transposon into the genome. Thus, this mutant, marked as T11 and one mutant with one copy of the transposon, marked as T1, were taken for further analysis. To summarize, a set of four strains with the number of attachment sites ranging from one (pseB4-strain) to four (T11-strain) was obtained.

Figure V.1: Southern hybridization of S. albus J1074 with integrated pHAT(II)3



A B

Figure V.2: The map (A) and analytical restriction (B) of pHAT. (A) Plasmid contains following features: oriT – origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon; tn5(a) – synthetic transposase gene, under control of tipAp – thiostrepton inducible promoter; aac(3)IV – apramycin resistance marker; hph – hygromycin resistance marker; ME – mosaic end recognition sequence for transposase; R6Kγ-ori – origin for rescue cloning; attB – ϕC31 phage attachment site. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with HindIII and XbaI. The transposase fragment is visible as 1,5 kbp and the backbone as 10 kbp.

3.2. Heterologous expression of pamamycin, aranciamycin, mensacarcin and griseorhodin

Four secondary metabolite clusters for the biosynthesis of pamamycin, mensacarcin, aranciamycin and griseorhodin (Figure V.3) were brought into all four *S. albus* J1074 mutant host strains, delta pseB4, wild type, T1 and T11 by intergenic conjugation. The integration of the corresponding secondary metabolite cluster was confirmed by apramycin resistance conferred by the integrated cosmids, PCR and HPLC-MS profile.

During work with these strains, several observations were made. Conjugation efficiency in the ΔpseB4 strain was slightly increased whereas it was severely reduced in T1 and T11 in comparison to the *S. albus* J1074 wild type strain. In our previous study, we have shown that the integration of the ϕC31 based constructs occurred in all available *attB* sites within the *S. albus* chromosome. Since integration into additional sites is harder to achieve for the strain, the integration efficiency dropped significantly in the T1 and T11 strains in comparison to the *S. albus* J1074 strain and increased for the ΔpseB4 strain (Bilyk & Luzhetskyy 2014).

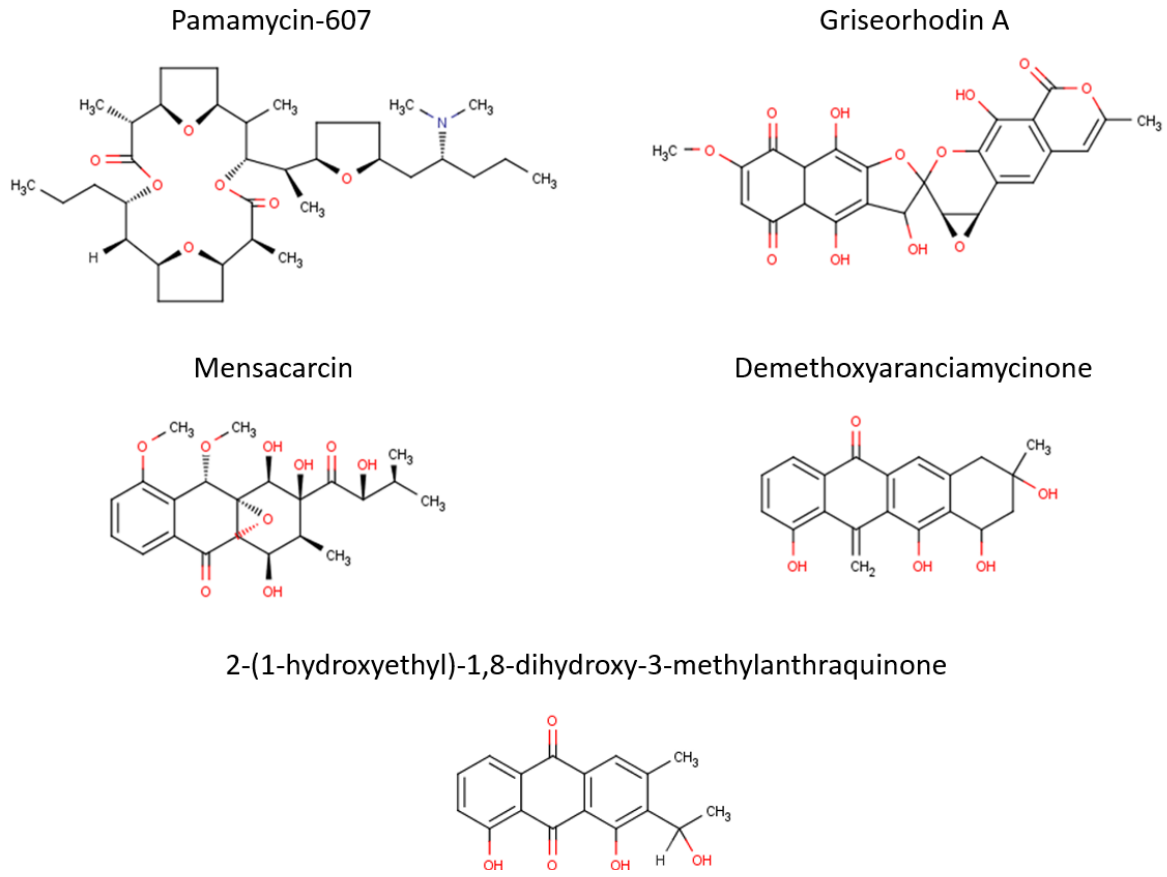


Figure V.3: Structures of the secondary metabolites used in this work. Products with the corresponding cosmid: Pamamycin-607 (PMM R2), Griseorhodin A (DJ380pUp31), Mensacarcin (cos2), Demethoxyaranciamycinone and 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone (41-2C-06)

The conjugation efficiency also was influenced by the type of the inserted secondary metabolite cluster. The integration of the pamamycin biosynthetic gene cluster was less efficient in comparison to the other clusters, which could be due to the fact, that pamamycin is a highly active antimicrobial compound. The exconjugants with the aranciamycin and griseorhodin clusters could be checked by phenotype for their production, since both compounds have a red color, which can be seen around the producing clones. The production of all four compounds was confirmed by HPLC-MS of several independent clones containing the corresponding gene clusters. All tested exconjugants produced pamamycin, whereas in case of aranciamycin, griseorhodin and mensacarcin we have observed clones with completely abolished production of the corresponding compound. The *S. albus* T11::41-2C-06

(aranciamycin gene cluster) also differed from the wild type by its lack of spore formation on solid medium and the inability to grow in liquid medium.

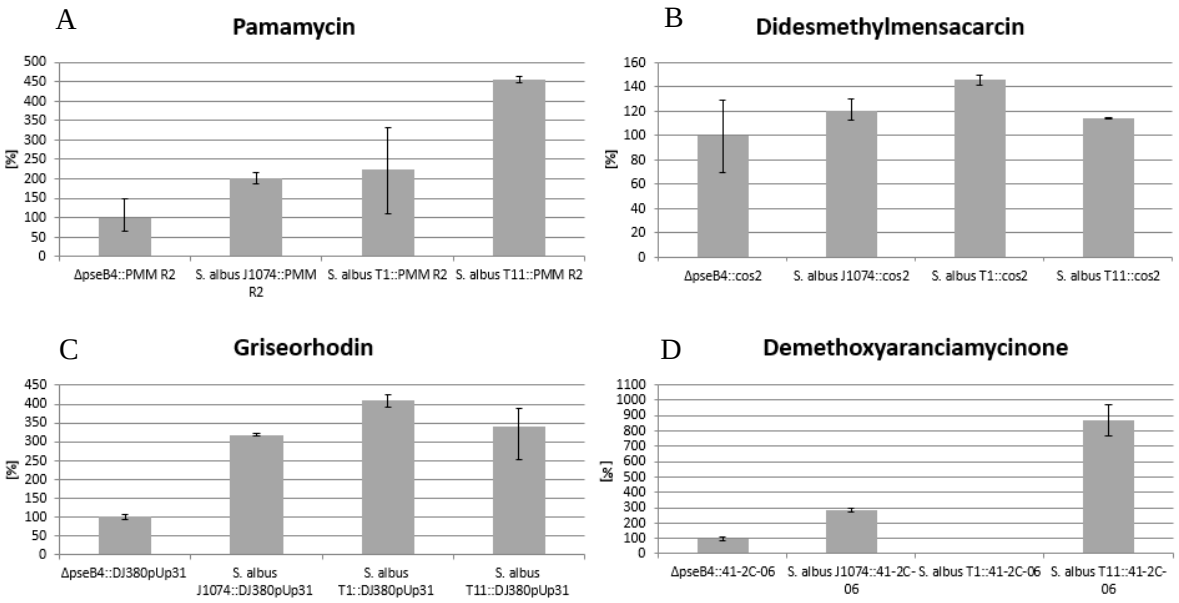


Figure V.4: Antibiotic production by the corresponding cosmids (PMM R2 for pamamycin, cos2 for DDMM, DJ380pUp31 for griseorhodin A and 41-2C-06 for demethoxyaranciamycinone) in ΔpseB4 (one attB site), J1074 (two attB sites), T1 (three attB sites), T11 (four attB sites). The pamamycin production increases roughly about 100 % for each additional attB site. The technical replicates of Strain T1 showed a high diversity ranging from 100 % to 300 % of the ΔpseB4 level. DDMM production was increased about 20 % in J1074 and about 40 % in T1. T11 showed a minor increase of about 10 % in comparison to ΔpseB4. Production increase for griseorhodin A from one to two attB sites was about 200 %, increase from one to three attB sites was 300 %. Strain T11 showed a high diversity between the three replicates and production increase in comparison to ΔpseB4 was around 250 %. Production increase for demethoxyaranciamycinone from one to two attB sites was about 180 %, increase from one to four attB sites in T11 was 770 %. Strain T1 showed no production of demethoxyaranciamycinone.

The production of pamamycin proved to be greatly influenced by the copy number of the gene cluster present in the genome. As seen in figure V.4.A, production increased by approx. 100 % from one to two attachment sites. The strains with the additional attachment sites showed even greater increase with T11 of about 350 % in comparison to *S. albus* Δpse4. Strain T1 did not seem to be completely stable because of the great fluctuation in production. One of the three technical replicates showed the same production level as the ΔpseB4 strain with one *attB* site, another replicate showed a similar production level as the *S. albus* J1074 strain with two sites, and the third replicate produced amounts of pamamycin as expected from a strain with three attachment sites. Production levels were accordingly 100%, 200 % and about 350 %. Arguably, some of these replicates may have lost one or more clusters during the cultivation.

Didesmethylmensacarcin (DDMM) production, as seen in figure V.4.B, increased roughly about 20 % for two attachment sites. The strains with additional attachment sites showed an increase of 40 % for T1 and only a minor increase of 10 % for T11.

Griseorhodin A production, as seen in figure V.4.C, increased from one to two attachment sites around 200 %. With additional attachment sites the production increased about 300 % for T1 and between 150 % and 280 % for T11 in comparison to the ΔpseB4 strain.

Demethoxyaranciamycinone, the product of the aranciamycin gene cluster, was produced on agar plates because of the inability of strain T11 to grow in liquid media. This inability could be the result of the very high demethoxyaranciamycinone production, which was observed later. The strain T11 showed weak growth on agar plates and was not able to produce spores even after 14 days of cultivation. The other tested strains showed spore formation after 3 days. The production, as seen in figure V.4.D, increased around 180 % for *S. albus* J1074 and around 770 % for strain T11. Strain T1 did not show any production of demethoxyaranciamycinone but of several byproducts, which could also be detected in approximately the same amount in the other strains (data not shown).

3.3. Production and isolation of 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone

During analysis of the *S. albus* T11::41-2C-06 extracts, we detected a distinct compound with the corresponding molecular ion m/z = 299.09085 ($[M+H]^+$) and a characteristic UV-spectrum of known aranciamycins. After comparison with the known aranciamycins and a search in the Dictionary of Natural Products, we could not assign this mass to a previously known compound. Probably due to the huge increase of the demethoxyaranciamycinone production level in the mutant T11, we could observe a significant amount of the previously unknown compound. We decided to purify the unknown compound in order to elucidate its structure.

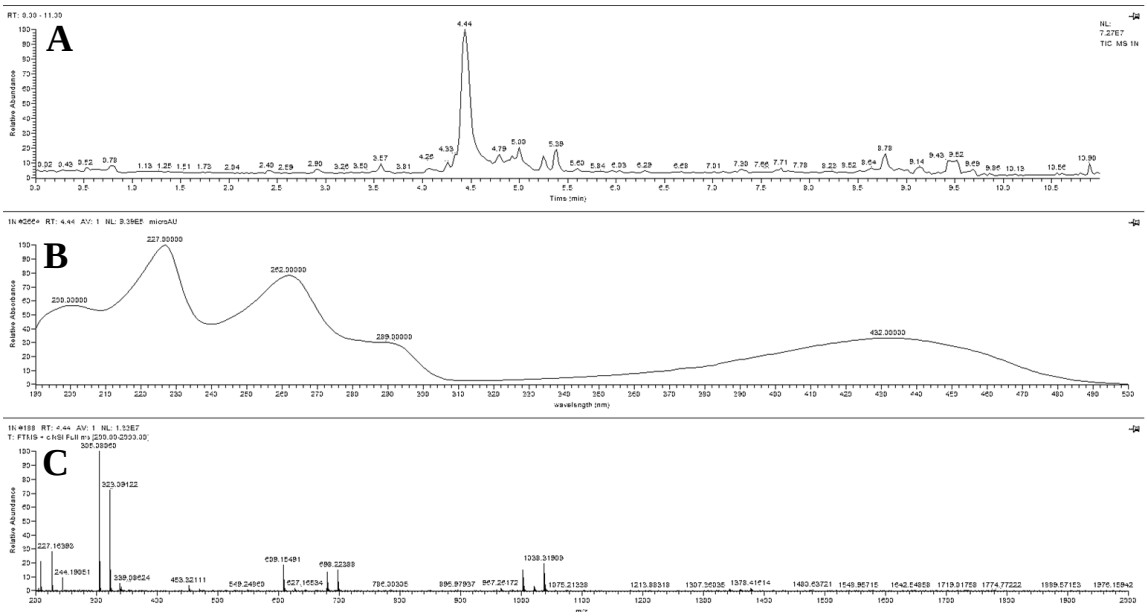


Figure V.5: HPLC-MS run of compound 1. (A) shows the HPLC-MS chromatogram, (B) shows the UV-chromatogram of the compound with a retention time of 4.44 min, (C) shows the MS pattern of compound 1. The molecular ion m / z = 339.09122 $[M+H]^+$ shows the molecular mass of the compound to be 338 gmol^{-1}

Since this compound was only a byproduct of demethoxyaranciamycinone, we cultivated *S. albus* T11::41-2C-06 on 58 plates to be able to purify enough material for structure elucidation. After extraction and purification by semiprep HPLC we were able to obtain 8 mg of demethoxyaranciamycinone and 4 mg of our unknown compound. Both compounds were measured by HPLC-MS to determine their exact mass. Figure V.5 shows the HPLC-MS run of demethoxyaranciamycinone (Luzhetskyy et al. 2007) with the retention time of 4.44 min. Figure V.6 shows the HPLC-MS run of the previously unknown compound with a retention time of 5.76 min.

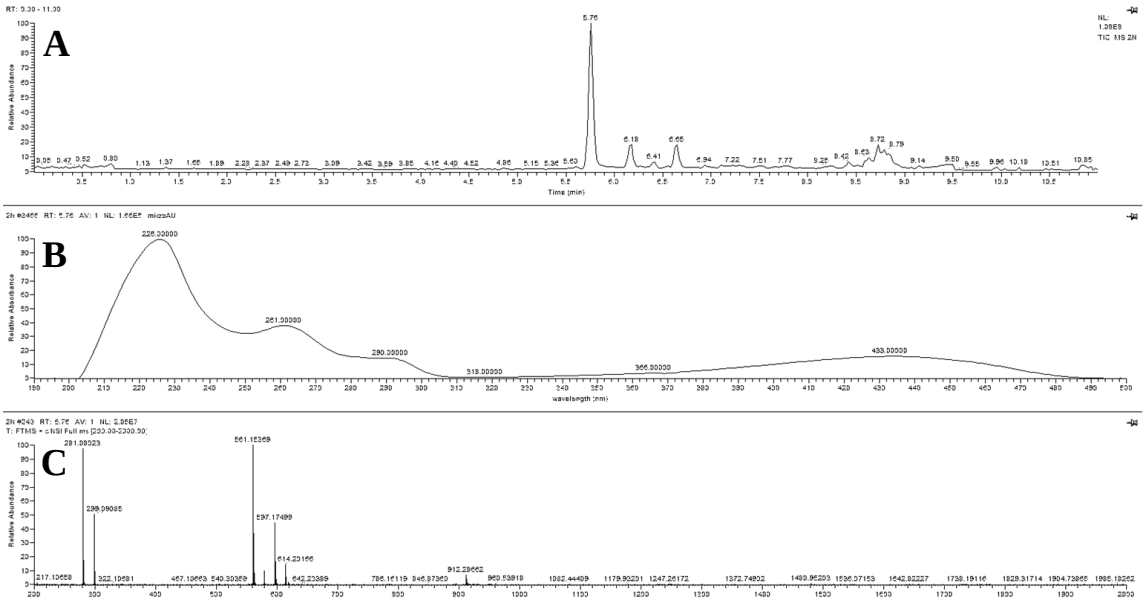


Figure V.6: HPLC-MS run of compound 2. (A) shows the HPLC-MS chromatogram, (B) shows the UV-chromatogram of the compound with a retention time of 5.76 min, (C) shows the MS pattern of compound 2. The molecular ion m / z = 299.09085 $[M+H]^+$ shows the molecular mass of the compound to be 298 $gmol^{-1}$

3.4. Structure elucidation of 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone

The mass spectrum of the second compound at rt = 5.76 min show ions with m / z = 299.1 $[M+H]^+$, 597.2 $[2M+H]^+$ and 619.2 $[2M+Na]^+$ proving the molecular mass to be 298 $gmol^{-1}$. The molecular formula $C_{17}H_{14}O_5$ could be determined from high resolution mass spectrometry (m/z = 299.09085 form $[M+H]^+$). Combination of ^{13}C NMR and HSQC spectra show 2 methyl groups, 5 methins and 10 quarternary carbon atoms to be present in the molecule. 2 carbonyl groups (δ_C = 193.1 and 181.6 ppm) show typical values for quinone systems. The ^1H NMR spectrum shows 2 chelated phenols (δ_H = 11.96 and 12.88 ppm) supporting an anthraquinone substructure. Ring A of the anthraquinone is substituted by a hydroxyethyl at C-2 and a methyl group at C-3 proven by HMBC correlations C-2 / 1-OH, C-2 / 4-H, C-2 / 12-H_3, C-2 / 13-H_3 and C-3 / 13-H_3, C-4 / 13-H_3. An isolated methin in position C-4 is proven by the singulet at δ_H = 7.65 ppm supported from HMBC couplings C-10 / 4-H, C-9a / 4-H and C-13 / 4-H. Ring C of the anthraquinone show one hydroxyl group at C-8, and a ABC system

C7-C5, deduced from COSY couplings (5-H / 6-H, 6-H /7-H) and the HMBC signal C-10 / 5-H. Therefore the compound could be identified as 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone. Tietze et al. (2007) synthesized 2-(1-Hydroxyethyl)-1,8-dimethoxy-3-methylantharquinone as an intermediate of the chrysophanol analogs.

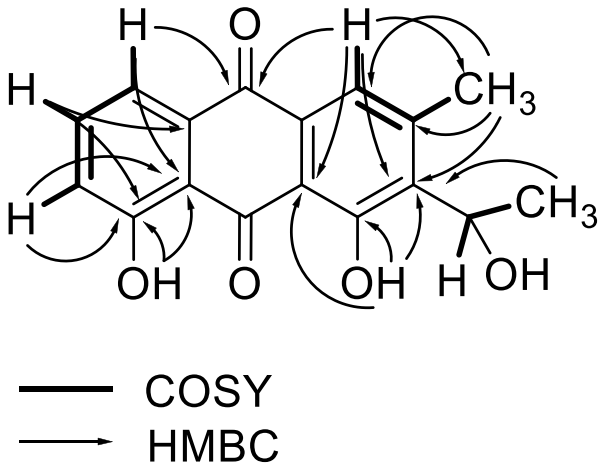


Figure V.7: Structure elucidation of 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone

Since the demethoxyaranciamycinone seems to be very toxic to *S. albus*, we believe that the new compound resulted from the host enzymatic activity in the *S. albus* T11 strain as a part of a detoxification process, because of the high production of demethoxyaranciamycinone.

4. Summary

We were able to show a clear correlation between attachment sites / number of clusters and production level of secondary metabolites. We created a panel of strains for different approaches. Strain ΔpseB4 could be used to tightly control the expression level of the desired compound or to specifically produce only minor amounts of compound if the compound is toxic. For some metabolites it seems that gene dosage should not be the only approach to increase the production. For others the number of clusters has an accumulative effect and the production can be increased significantly. This approach could be used in addition to other approaches to create a range of different expression hosts.

VI. Final conclusion

In this study we were able to sequence the genome of the *Streptomyces* strain *Streptomyces fulvissimus* and to identify 32 putative secondary metabolite clusters. Besides valinomycin we could also identify several different derivatives of nonactin in the extracts of *S. fulvissimus* cultures and we could also link these compounds to the corresponding gene clusters present in the genome. The nonactin gene cluster helped to elucidate the pamamycin biosynthesis. Pamamycin derivatives were found in the extracts of *S. alboniger* DSM40043 and we were able to obtain several cosmids carrying the biosynthetic gene cluster. The cosmids were brought into *S. albus* J1074 and we were able to heterologously express the pamamycin gene cluster. We were able to elucidate the pamamycin biosynthesis by knocking out the responsible biosynthetic genes on the producing cosmid and measuring the resulting intermediates in the extracts of *S. albus* J1074 / R2. We could show that pamamycin biosynthesis uses 3-oxoadipyl-CoA and either malonyl-CoA or methylmalonyl-CoA as starter units and that pamamycin biosynthesis utilizes the unusual starter unit succinyl-CoA. Furthermore we showed the promiscuity of several KS which either utilize methylmalonyl-CoA or ethylmalonyl-CoA which results in a complex compound spectrum. To simplify the compound spectrum, to increase the production and to identify the supplying pathways for pamamycin precursors, we identified several pathways from the primary metabolism with the corresponding key genes for methylmalonyl-CoA and ethylmalonyl-CoA biosynthesis. We abolished production of the bigger pamamycins by knocking out the genes responsible for ethylmalonyl-CoA formation and could observe an increase of production of the remaining pamamycin derivatives. The knockouts of the CCR genes also revealed the metabolic potential of *S. albus* J1074 which can either use the glyoxylate cycle or the ethylmalonyl-CoA pathway for the biosynthesis of cell constituents. The knockout of several genes identified as responsible for methylmalonyl-CoA biosynthesis did not lead to a loss or decrease of the produced pamamycin derivatives which utilize methylmalonyl-CoA. Only the knockout of the MCM gene resulted in an increase of pamamycin production. We could link this production yield to the increased amount of available succinyl-CoA present in the cell due to this knockout. We could also show that pamamycin production is increased by feeding succinate to producing cultures of *S. albus* J1074, which indicates that succinyl-CoA is the bottleneck for pamamycin production. To clarify the source of methylmalonyl-CoA for pamamycin production, ammonium ions, which are shown to block the valine dehydrogenase activity, were fed to pamamycin producing cultures of *S. albus* J1074 / R2. Under these conditions, pamamycin biosynthesis was almost completely blocked and we could confirm the catabolism of valine as the single source of methylmalonyl-CoA for pamamycin production by knocking out the VDH gene. This knockout resulted in a complete cessation of pamamycin production. We created several different strains with different amounts of knockouts, what finally led to a strain with six knockouts of genes of the primary metabolism in the same genetic

background. This knockout strain also showed that there are no pamamycins built without methylmalonyl-CoA. Interestingly, the strain with six knockouts was still viable and showed only reduced biomass accumulation. Additionally it needed less time until full sporulation was reached. Lastly we created a panel of strains with different amounts of attachment sites for heterologous expression of secondary metabolite clusters and we could show a clear correlation between the number of attachment sites / number of clusters and the production level of secondary metabolites.

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Appendix

1. Complete genome sequence of *Streptomyces fulvissimus*

Streptomyces fulvissimus HPLC-chromatogram (ALL MS+) (Amazon-System):

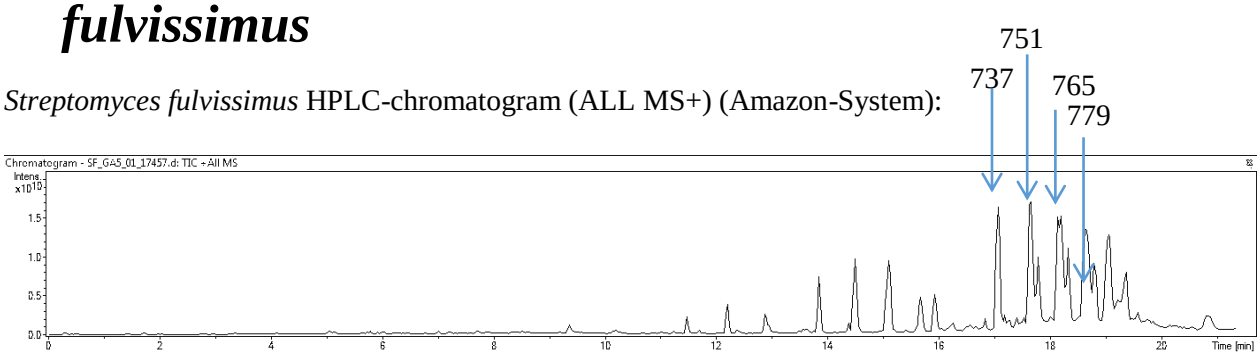


Figure II.S1: Streptomyces fulvissimus HPLC-chromatogram with distinct masses (nonactins) highlighted

Nonactin from the Dictionary of Natural Products:

Nonactin	Formula: C40H64O12	MW: 736.938	→	Peak 737
Monactin	Formula: C41H66O12	MW: 750.965	→	Peak 751
Dinactin	Formula: C42H68O12	MW: 764.992	→	Peak 765
Isodinactin	Formula: C42H68O12	MW: 764.992	→	Peak not detected
Trinactin	Formula: C43H70O12	MW: 779.019	→	Peak 779
Isotrinactin	Formula: C43H70O12	MW: 779.019	→	Peak not detected
Tetranactin	Formula: C44H72O12	MW: 793.046	→	Peak not detected

Orbitrap measurements:

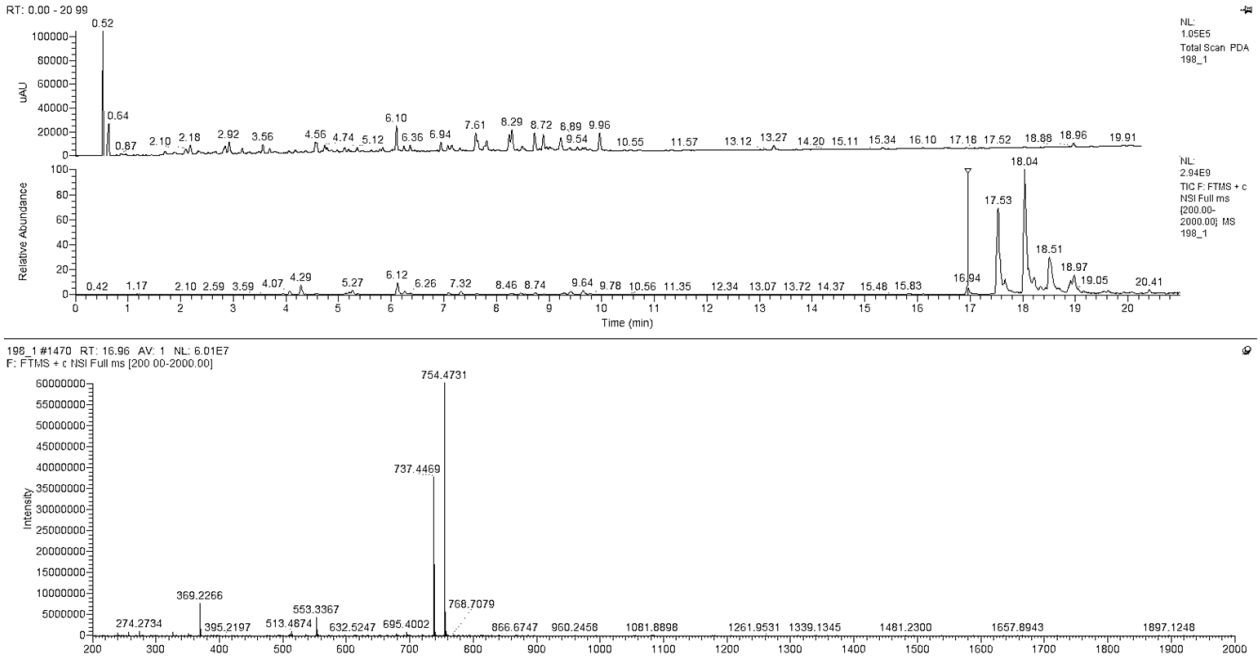


Figure II.S2: Nonactin.

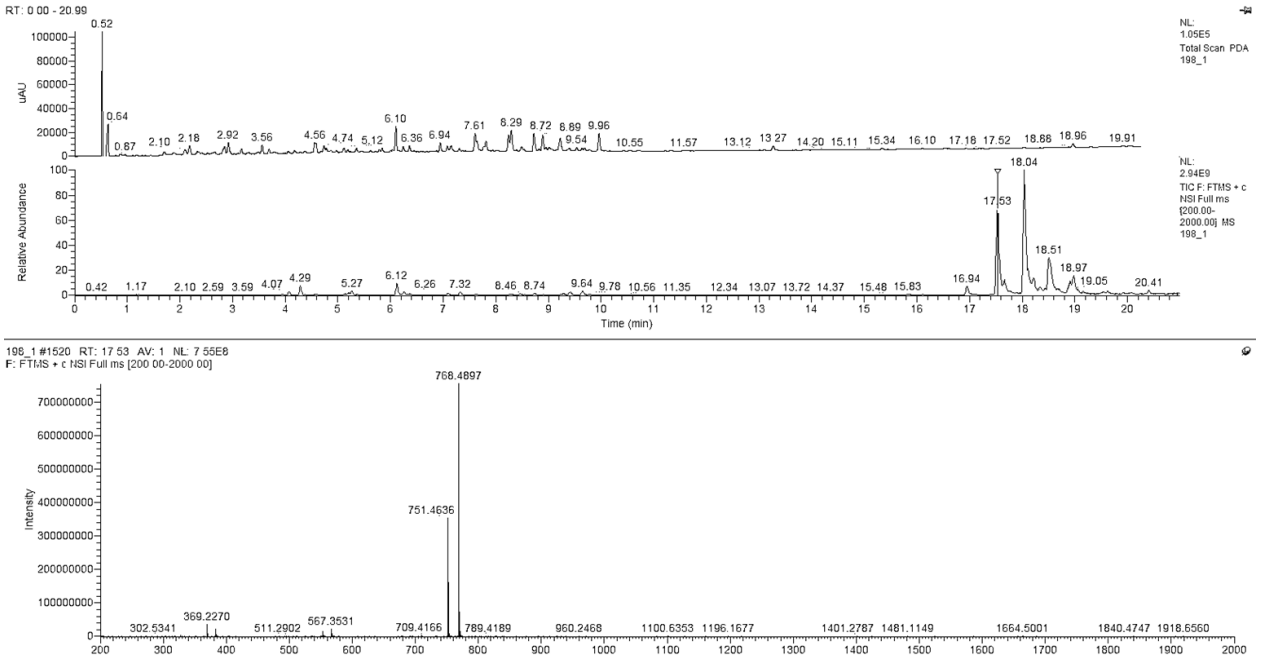


Figure IIS.3: Monactin.

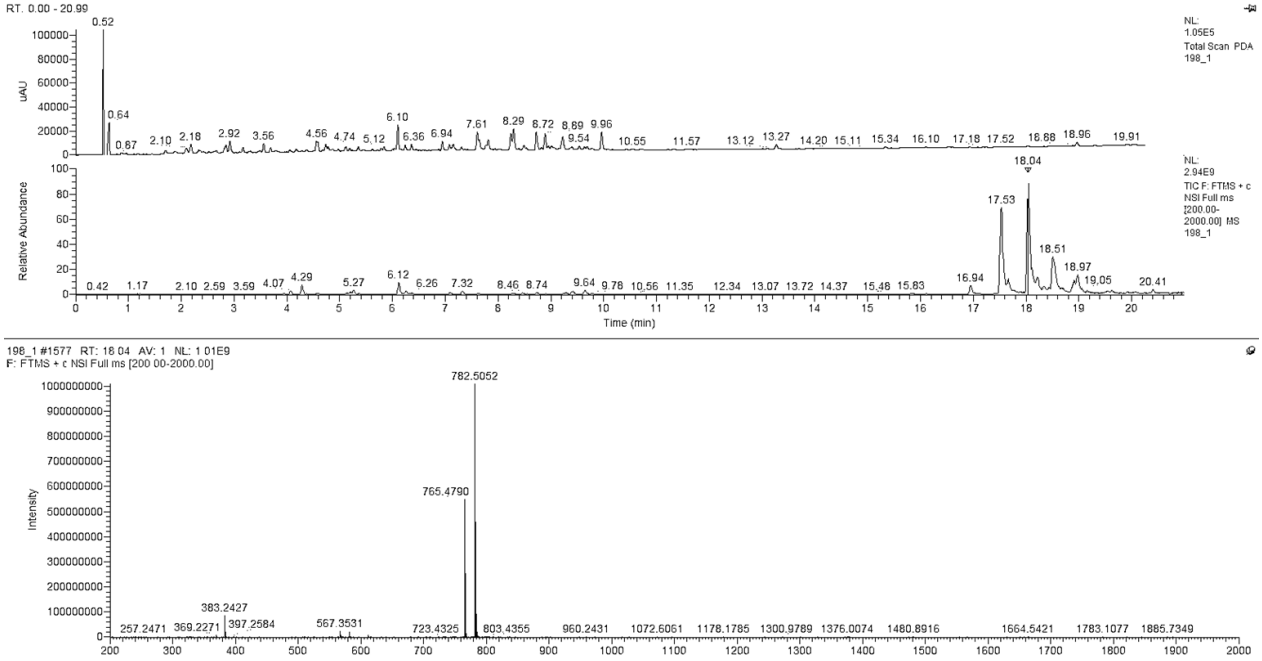


Figure II.S4: Dinactin.

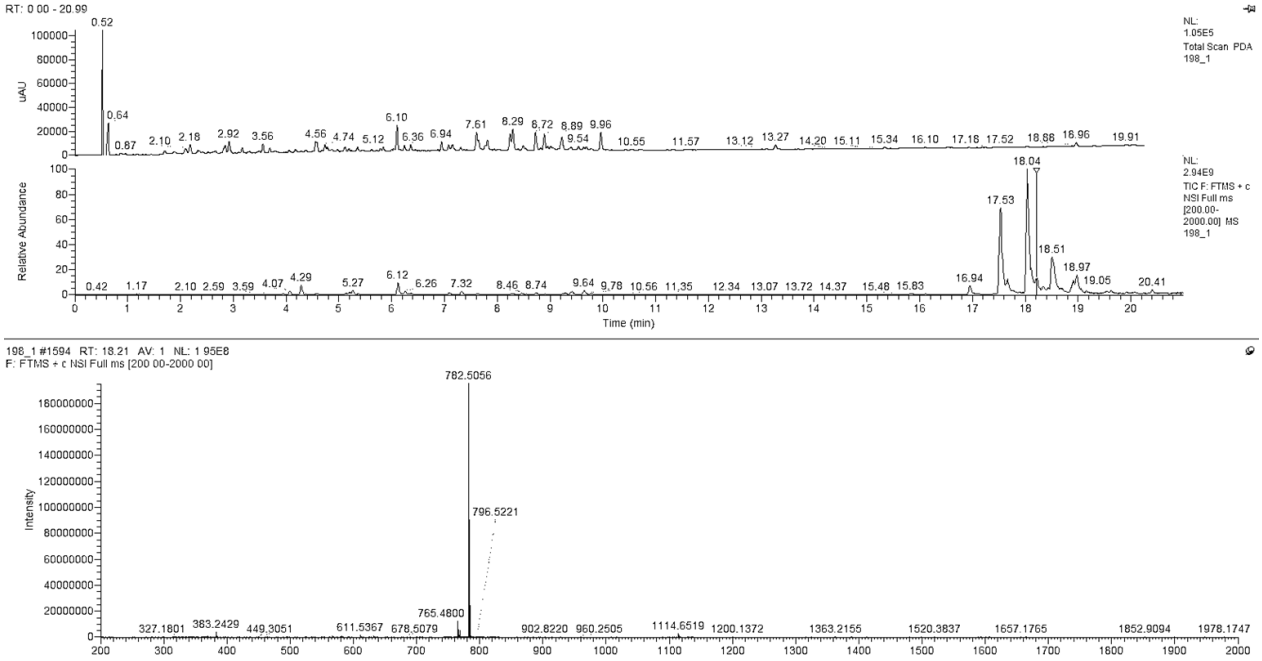


Figure II.S5: Isodinactin.

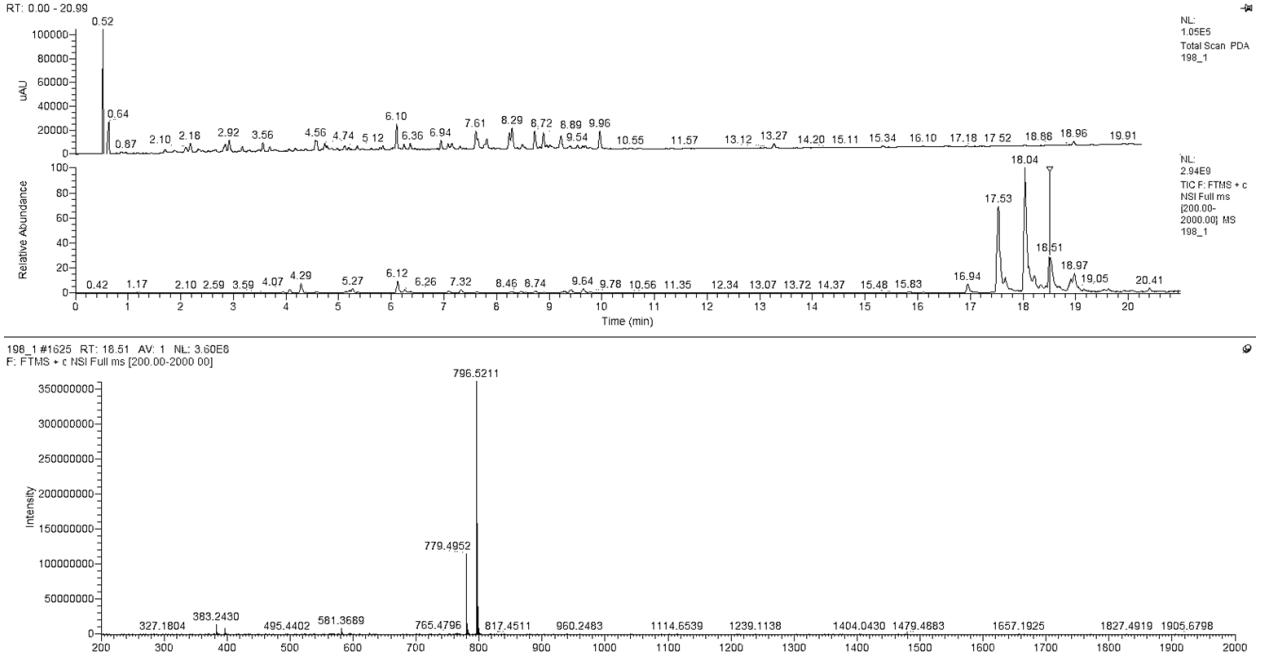


Figure II.S6: Trinactin.

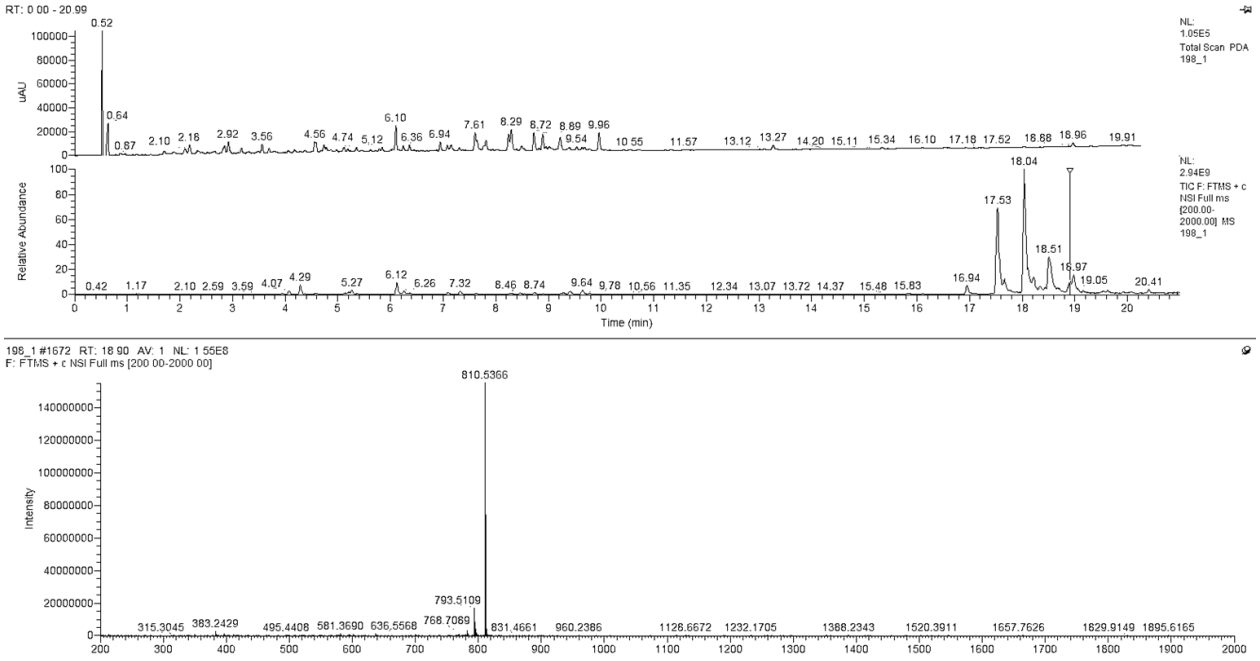


Figure IIS.7: Tetranactin.

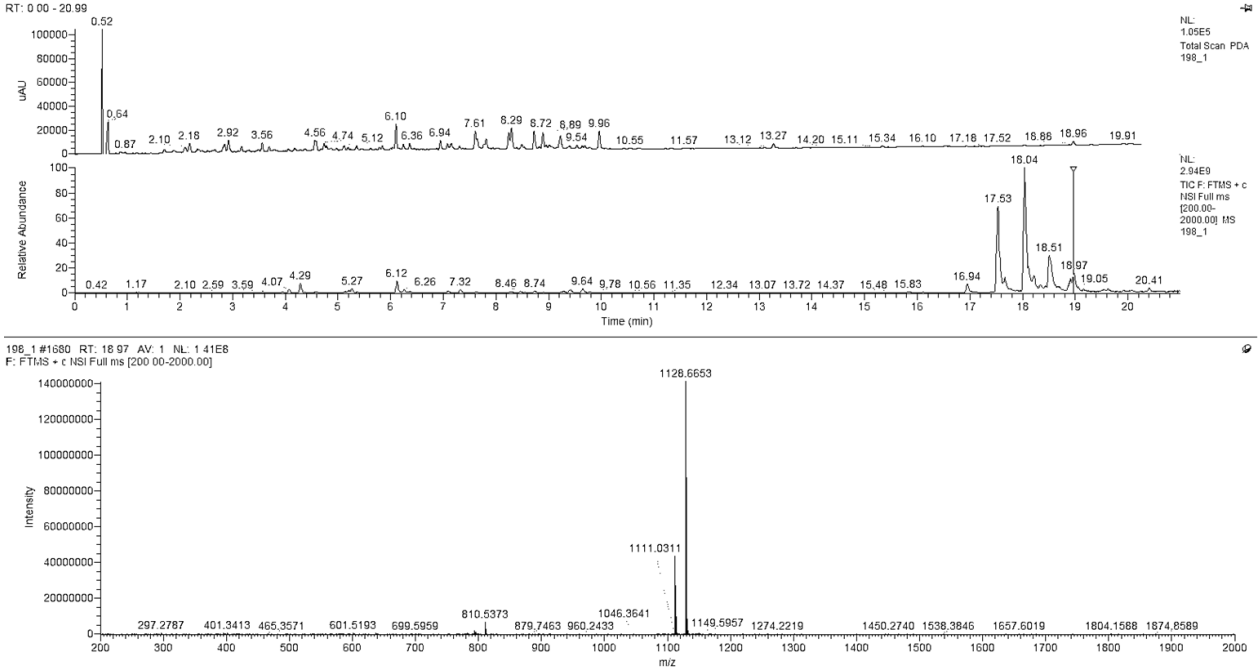


Figure II.S8: Valinomycin.

2. Insights into the pamamycin Biosynthesis

Table III.S3: pam genes, and their counterparts in non-gene cluster and their predicted function.

Gene name	Nonactin counterpart	Protein function
pamR2	-	TetR family transcriptional regulator, absent in non-cluster.
pamW	-	Drug resistance transporter, EmrB/QacA subfamily, absent in non-cluster.
pamC	-	Acyl carrier protein, absent in non-cluster.
pamG	-	Ketoacyl-acyl carrier protein synthase III, no homologues in *non*-cluster.
pamF	-	Ketoacyl-acyl carrier protein synthase III, similar to nonK (i46%, p57%).
pamA	*nonU*	Ketoacyl-acyl carrier protein synthase II, similar to nonU (i52%, p64%).
pamB	*nonT*	3-ketoacid (adipate/glutaconate) CoA transferase, similar to nonT (i54%, p64%).
pamD	*nonQ*	Ketoacyl-acyl carrier protein synthase III, similar to nonP (i43%, p61%).
pamE	*nonP*	Ketoacyl-acyl carrier protein synthase III, similar to nonQ (i54%, p68%).
pamO	*nonO, nonN*	Ketoacyl-(acyl-carrier-protein) reductase, similar to nonO (i58%, p70%).
pamK	*nonK*	Ketoacyl-acyl carrier protein synthase III, similar to nonP (i40%, p62%).
pamJ	*nonJ*	Ketoacyl-acyl carrier protein synthase II, similar to nonK (i49%, p62%).
pamM	*nonM*	Ketoacyl-(acyl-carrier-protein) reductase, similar to nonM (i43%, p52%).
pamN	*nonE*	Ketoacyl-(acyl-carrier-protein) reductase, similar to nonE (i48%, p60%).
pamL	*nonL*	Hydroxyl acids CoA ligase, similar to nonL (i53%, p63%).
pamH	*nonC*	Metal-dependent hydrolase (resistance), similar to nonC (i42%, p53%).
pamX	-	Aminotransferase, no homologues in *non*-cluster.
pamY	-	Methyltransferase, no homologues in *non*-cluster.
pamS	*nonS*	Enoyl-CoA hydratase, similar to nonL (i41%, p50%).
pamR1	-	Response regulator LuxR family, no homologues in *non*-cluster.

i - % of identical amino acids

p - % of positive amino acids

Table III.S4: Active site catalytic triad composition of Pam and Non KS enzymes.

PamKS, *S. alboniger*	Active site	PamKS, *S. sp. HKI118*	Active site	Non KSs, S. fulvissimus	Active site	Non KS, S. griseus	Active site
pamA	CHH	pamA	CHH	nonU	CHH	nonU	CHH
pamD	CHN	pamD	CHN	nonQ	CHN	nonQ	CHN
pamE	CHN	pamE	CHN	nonP	CHN	nonP	CHN
pamG	CHN	pamG	CHN	–			
pamF	CHN	pamF	CHN				
pamK	CHN	pamK	CHN	nonK	CGH	nonK	CGH
pamJ	CAH	pamJ	CAH	nonJ	CYH	nonJ	CYH

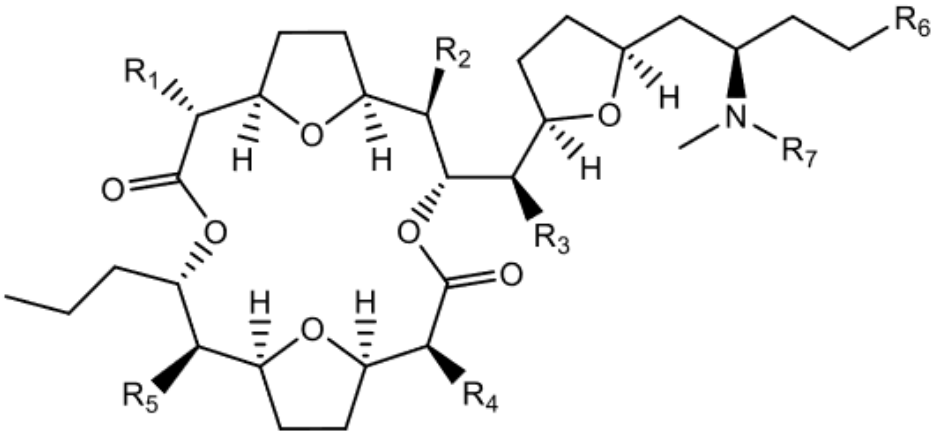
Table III.S5: Complementation of pam-genes mutations with pam-genes and their counterparts from non-gene cluster.

Mutation	Complementation with *pam* genes		Complementation with *non* genes	
ΔpamC	*pamC*	+	NT	
ΔpamA	*pamA*	+	*nonU*	UNSPECIFIED
ΔpamB	*pamB*	+	*nonT*	+
ΔpamO	*pamO*	+	*nonO*	+
ΔpamO			*nonN*	–
ΔpamK	*pamK*	+	*nonK*	–
ΔpamK			*nonJ*	–
ΔpamJ	*pamJ*	+	*nonJ*	–
ΔpamJ			*nonK*	–
ΔpamX	*pamX*	+	NT	
ΔpamY	*pamY*	+	NT	
ΔpamL	*pamL*	+	*nonL*	–

+ restored pamamycin production

– absence of pamamycin production

NT – not tested since gene is absent in *non*-cluster.



1

Pamamycin	R1	R2	R3	R4	R5	R6	R7
593	Me	H	Me	Me	H	Me	Me
De-N-Methyl 579	Me	H	Me	Me	H	Me	H
De-N-methyl 593A	Me	Me	Me	Me	H	Me	H
De-N-methyl 593B	Et	H	Me	Me	H	Me	H
607	Me	Me	Me	Me	H	Me	Me
621A	Me	Me	Me	Me	Me	Me	Me
621B	Et	H	Me	Me	Me	Me	Me
621C	Me	Me	Et	Me	H	Me	Me
621D	Et	Me	Me	Me	H	Me	Me
635A	Me	Me	Et	Me	Me	Me	Me
635B	Me	Me	Me	Et	Me	Me	Me
635C	Et	Me	Me	Me	Me	Me	Me
635D	Et	Me	Me	Et	H	Me	Me
635E	Me	Me	Et	Et	H	Me	Me
635F	Et	Me	Et	Me	H	Me	Me
649A	Et	Me	Et	Et	H	Me	Me
649B	Et	Me	Et	Me	Me	Me	Me
Bishomopamamycin-621A	Me	Me	Me	Me	H	Et	Me
Bishomopamamycin-635A	Me	Me	Me	Me	H	Pr	Me
MS-282A	Me	Me	Me	Me	H	Pr	Me
MS-282A	Me	Me	Et	Me	H	Et	Me

Figure III.S1: Structural features of described pamamycins

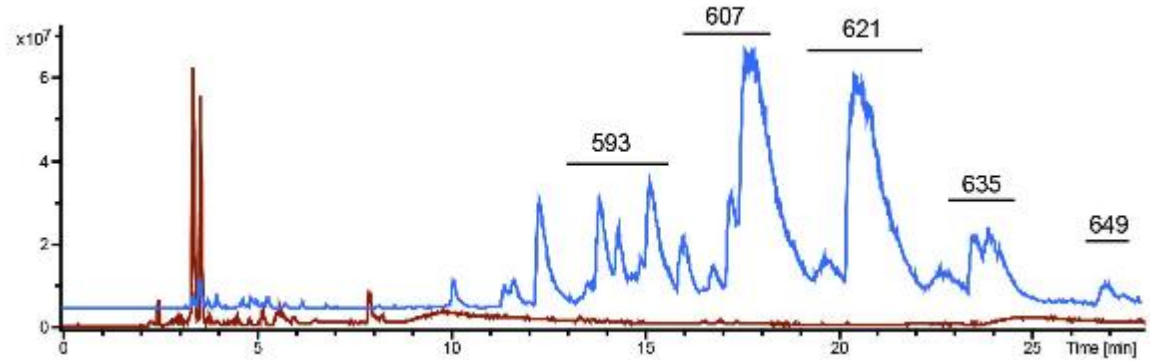


Figure III.S2: LC-MS traces of extracts from S. alboniger DSM40043 wild type (blue) and ΔpamD mutant (brown). Metabolites were extracted and analyzed as described previously. The ΔpamD strain was not able to produce pamamycins. Different groups of pamamycins are marked by their molecular mass.

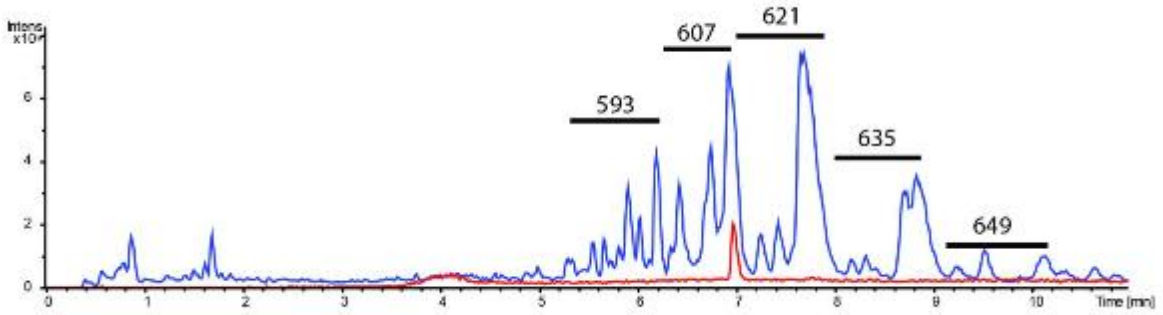
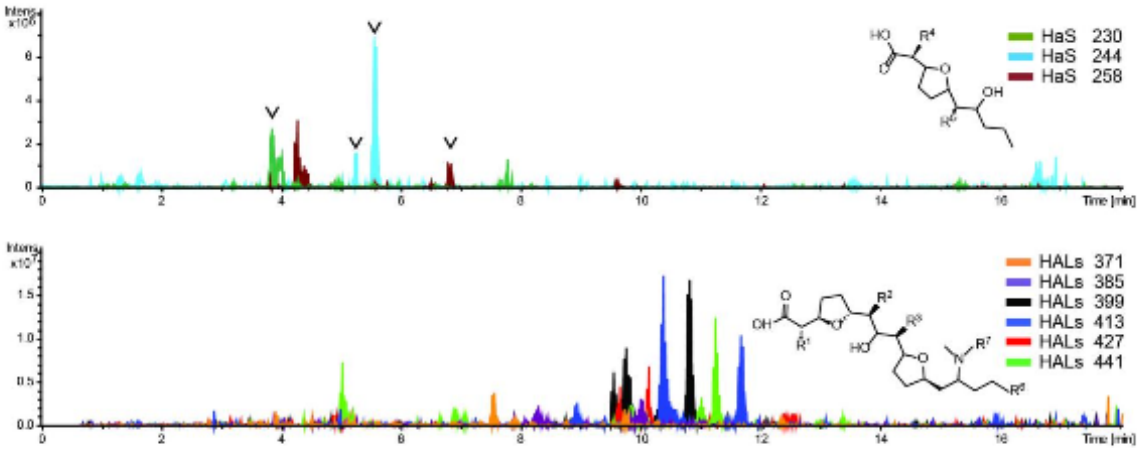


Figure III.S3: Pamamycins production in S. albus J1074 (blue). Pamamycins are marked by their molecular mass. chemically synthesized pamamycin 607 (0.015 μM) was used as standard (red). LC-MS analysis of combined extract from biomass and cultural liquid is shown.



Hydroxy acid S	R4	R5
S230	Me	H
S244	Me	Me
S244	Et	H
S258	Et	Me

Hydroxy acid S/K	R1	R2	R3	R6	R7
L371/ND	Me	H	H	Me	H
L385/K370	Me	H	Me	Me	H
L399/K384	Me	Me	Me	Me	H
	Et	H	Me	Me	H
	Me	H	Et	Me	H
	Me	H	Me	Me	Me
L413/K398	Me	Me	Me	Me	Me
	Et	H	Me	Me	Me
	Me	H	Et	Me	Me
L427/K412	Me	Me	Et	Me	Me
	Et	Me	Me	Me	Me
	Me	Me	Et	Me	Me
	Me	Me	Me	Et	Me
L441/ND	Et	Me	Et	Me	Me
	Me	Me	Me	Pr	Me
	Me	Me	Et	Et	Me

ND – not detected

Figure III.S4: LC-MS analysis of metabolites accumulated by S. albus R2 ΔpamJ, ΔpamK and ΔpamL strains. These enzymes are involved in the last steps of pamamycin biosynthesis, thus deletion of any of them led to accumulation of the intermediates of the pathway hydroxy acids S and L. Different hydroxy acids S and L are marked by their molecular mass. Structures of different species of hydroxy acids S, L and K accumulated by different pam gene mutants are predicted based on structures of known pamamycins

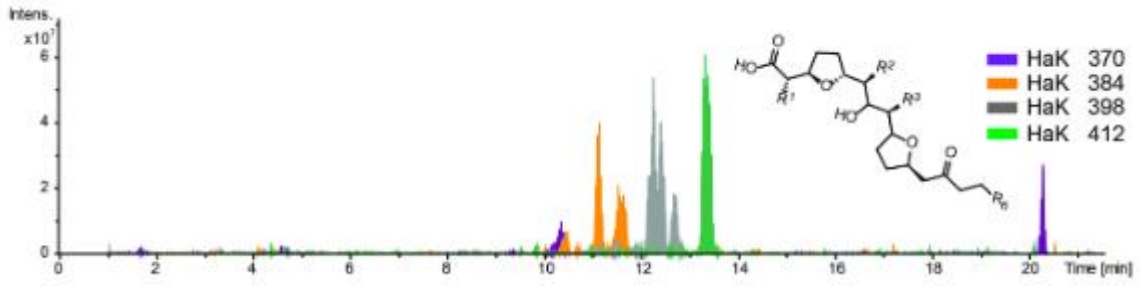


Figure III.S5: LC-MS analysis of metabolites accumulated by S. albus R2 ΔpamY and ΔpamX strains. These enzymes are performing amination and methylation steps in pamamycin biosynthesis. Accumulation of hydroxyl acids K rather than non-aminated pamamycins indicates that these reactions take place before cyclization of final compound but after the formation of hydroxyl acid K. Different hydroxy acids K are marked by their molecular mass. Explanation of structural feature of identified hydroxy acids K are showed on Figure S4.

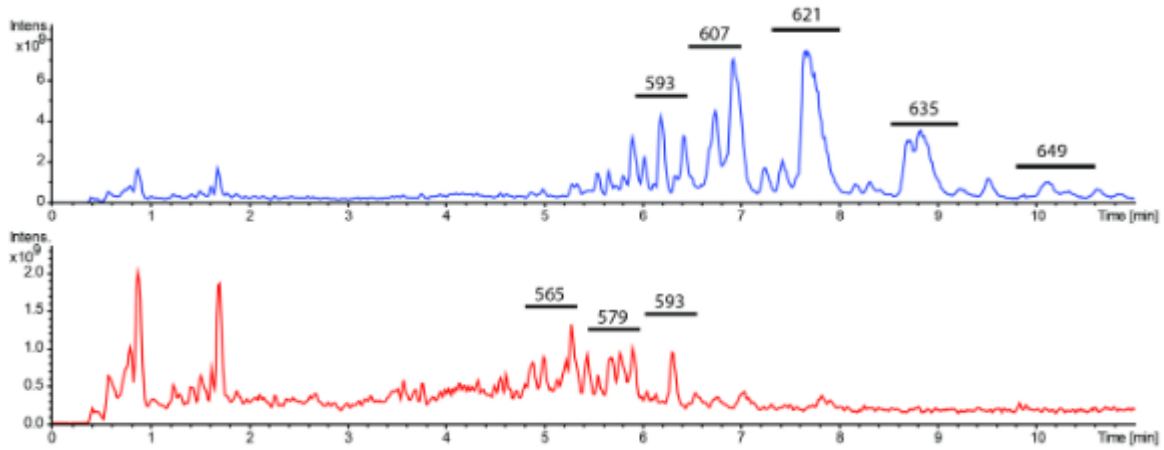


Figure III.S6: LC-MS analysis of metabolites accumulated by S. albus R2 (upper panel) and ΔpamC (lower panel) strains. Deletion of pamC gene led to decrease in pamamycins production as well as caused changes in the structural range of accumulated metabolites.

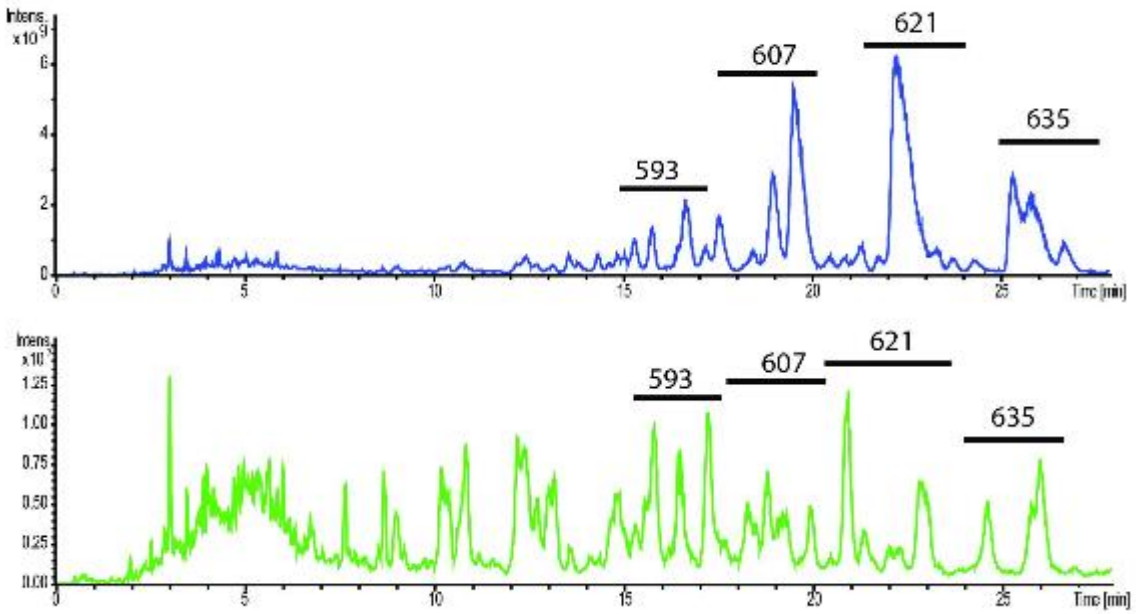


Figure III.S7: Pamamycin production by S. albus R2 and S. albus R2ΔpamA strains. Deletion of pamA did not lead to complete cessation of pamamycins production but affects the level of production and spectra of produced compounds. This is caused by supply of 3-oxoadipyl-CoA from the primary metabolism of S. albus. Production of several pamamycins implicates methyl-adipyl-CoA that cannot be supplied by the host strain. This causes perturbations in spectra of pamamycins synthesized by the mutant strain.

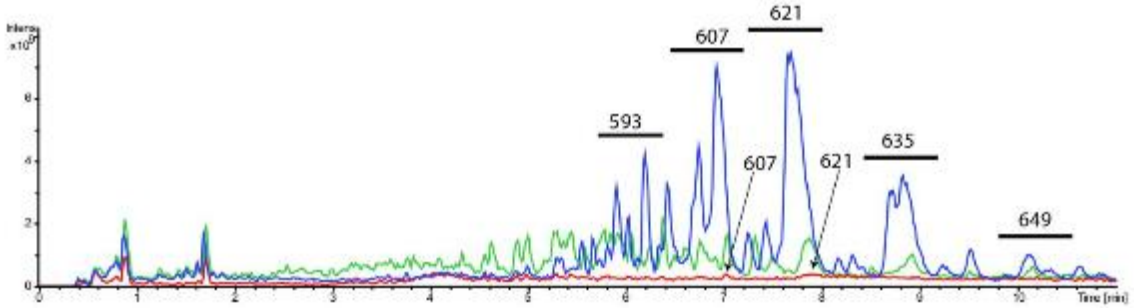


Figure III.S8: Production of pamamycins by S. albus AdCoA R2 (blue), S. albus J1074 R2ΔpamA (green) and S. albus AdCoA R2ΔpamA (red). Since deletion of XNR_0219 and XNR_0220 blocked supply of 3-oxoadipyl-CoA from the host primary metabolism, only trace amounts of pamamycin 607 and two 621 were found in extracts from S. albus AdCoA R2ΔpamA.

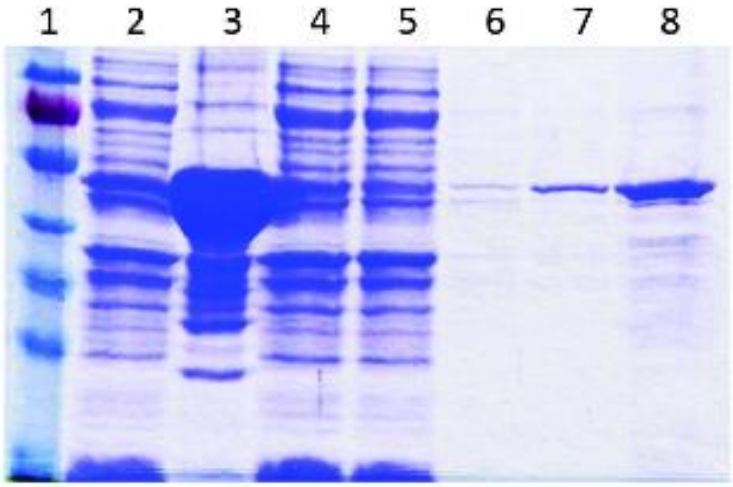


Figure III.S9: SDS PAGE of PamA protein. Lines: 1 Protein marker; 2. Soluble fraction; 3. Insoluble fraction; 4. Column flow (unbound proteins); 5. Washing, step 1 (no imidazole); 6. Washing, step 2 (80mM imidazole); 7. Eluted protein; 8. Protein samples after concentration and buffer exchange.

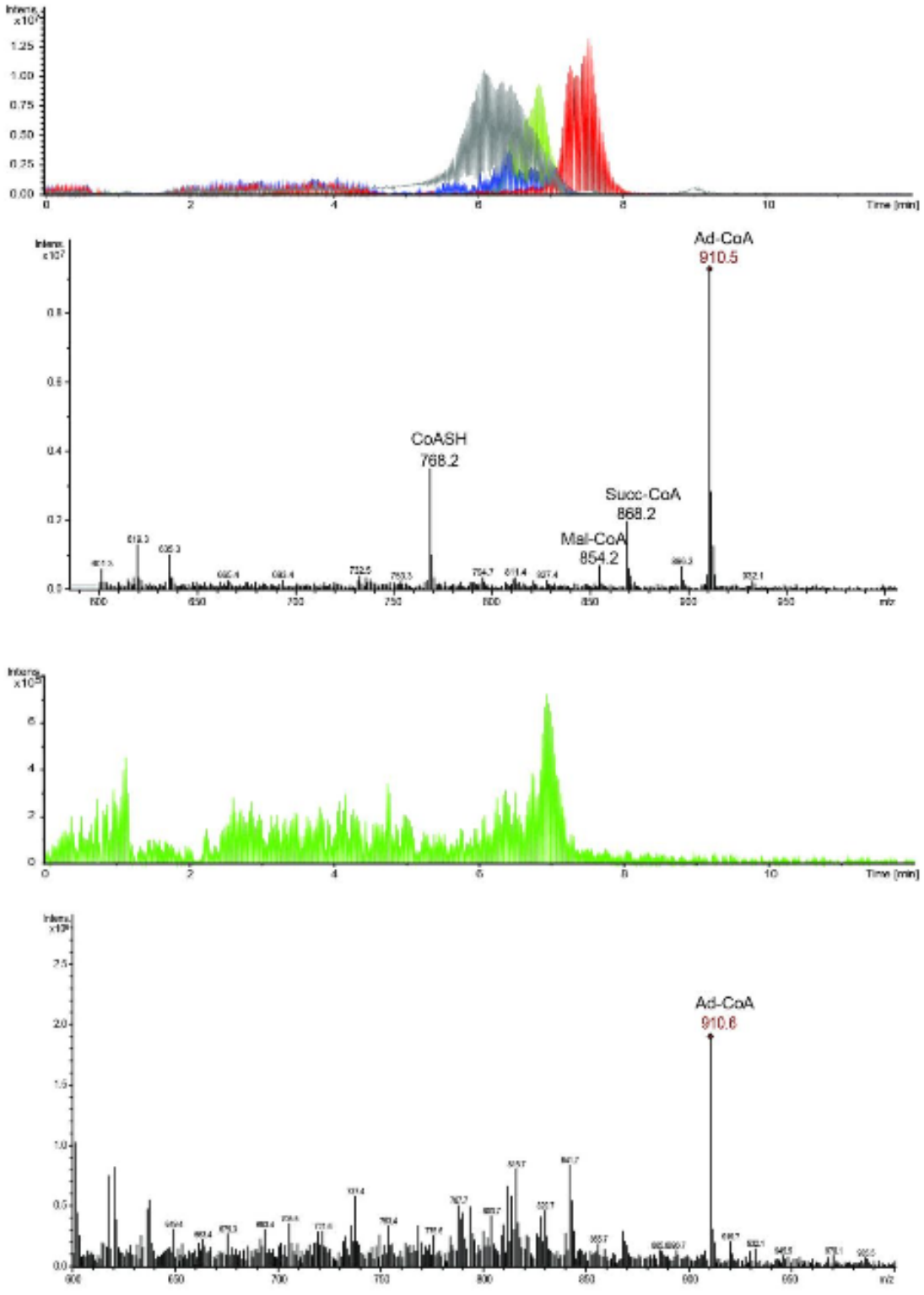


Figure III.S10: HILIC-MS analysis of PamA reaction mixture and purified product of reaction. Substrates and products are marked as CoASH (grey), Mal-CoA–malonyl-CoA (red), Succ-CoA–succinly-CoA (blue) and Ad-CoA–adipyl-CoA (green).

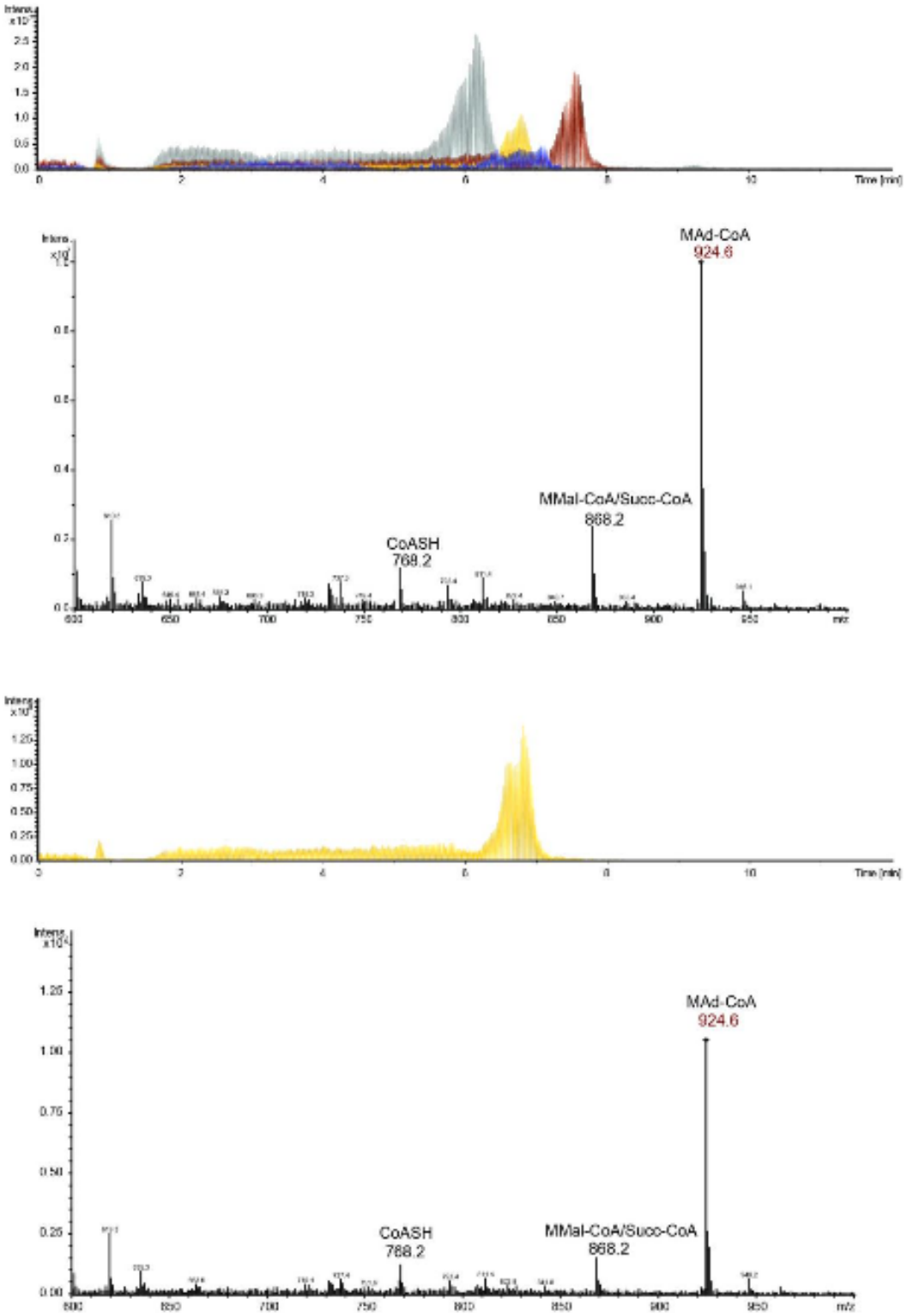


Figure III.S11: HILIC-MS analysis of PamA reaction mixture and purified product of reaction. Substrates and products are marked as CoASH (grey), MMal-CoA–methylmalonyl CoA (brown), Succ-CoA–succinly-CoA (blue) and MAd-CoA–2-methyl-3oxoadipyl-CoA (yellow).

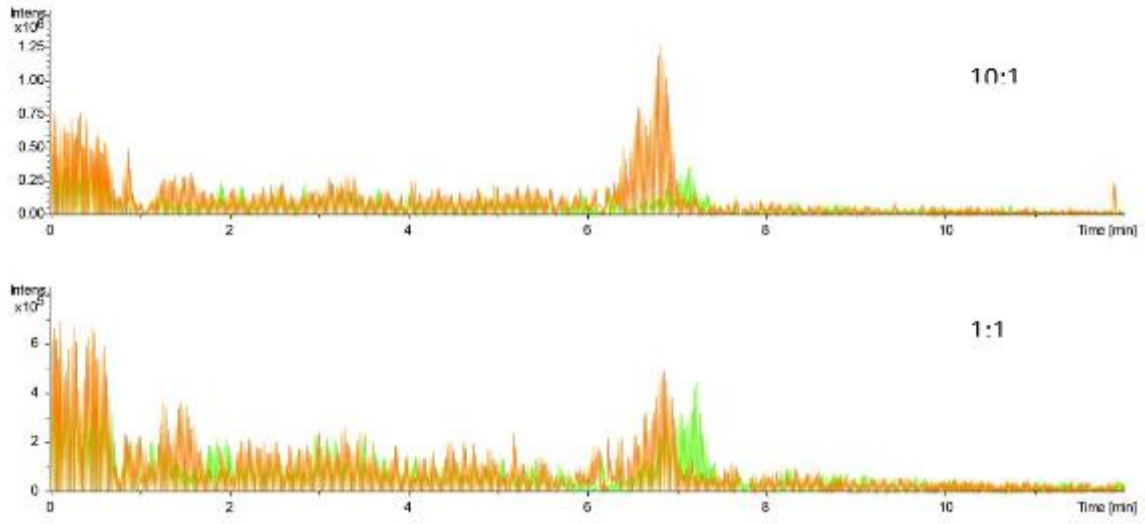


Figure III.S12: HLLC-MS analysis of PamA competition reaction with methylamalonyl-CoA and malonyl-CoA ration 10:1 (top) and 1:1 (bottom). The ratio of 3-oxoadipyl-CoA (green) and 2-methyl-3oxoadipyl-CoA (orange) products was found to depend on initial ratio of methylamalonyl-CoA and malonyl-CoA when succinily-CoA is not limiting factor (60 mM in reaction).

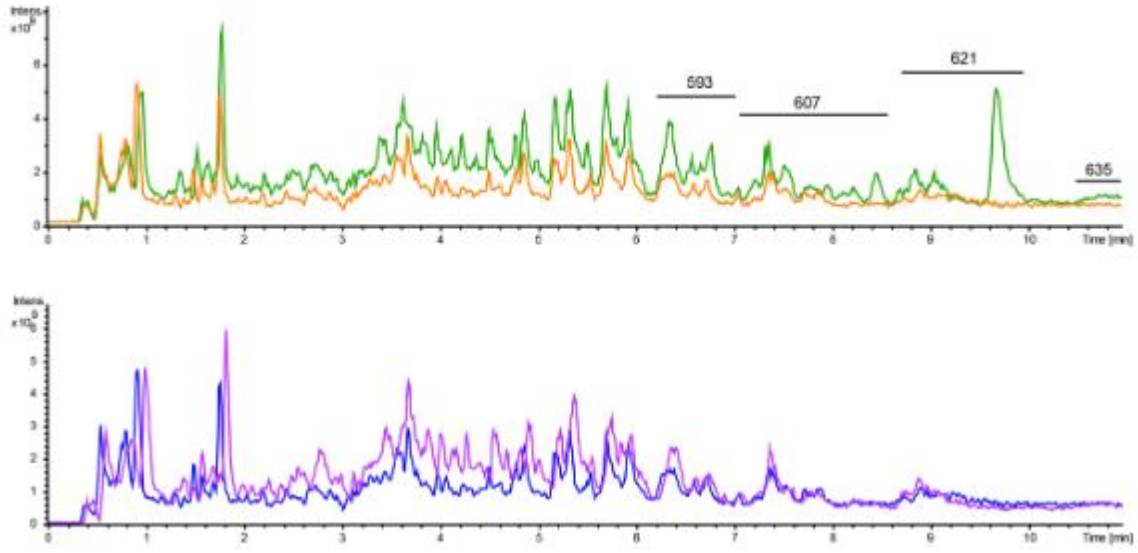
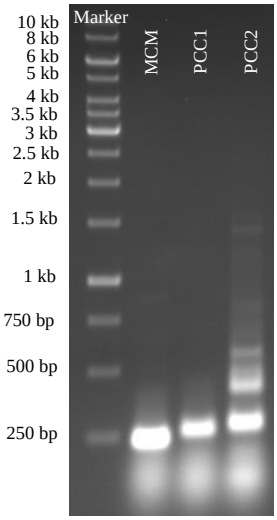


Figure III.S13: Feeding of hydroxy acids S and L to S. albus R2ΔpamB (green) and S. albus R2 ΔpamL (purple). As control extracts of S. albus R2ΔpamB (orange) and S. albus R2 ΔpamL (blue) were used. Feeding of mixture of hydroxy acids S and L to the strain expressing pamL S. albus R2ΔpamB resulted in accumulation of pamamycins. In the same time, strain with deleted pamL S. albus R2 ΔpamL gene was not able to activate free hydroxyl acids to complete the biosynthesis of antibiotics.

3. Strain development of *Streptomyces albus* J1074 for pamamycin production

Gel picture for Del1ΔccrD1-1 ΔPCC1, Del1ΔccrD1-1 ΔPCC2 and Del1ΔccrD1-1 ΔMCM



FigureIV.S1: Gel picture of the PCR products for MCM (240 bp), PCC1 (265 bp) and PCC2 (281 bp) knockout sind the Del1ΔccrD1-1 strain after marker excision.

Sequencing results for Del1ΔccrD1-1 ΔPCC1, Del1ΔccrD1-1 ΔPCC2, Del1ΔccrD1-1 ΔMCM, Del1ΔccrD1-1 ΔPCC2 ΔPCC1, Del1ΔccrD1-1 ΔPCC2 ΔMCM and Del1ΔccrD1-1 ΔMCM ΔPCC1 and Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1

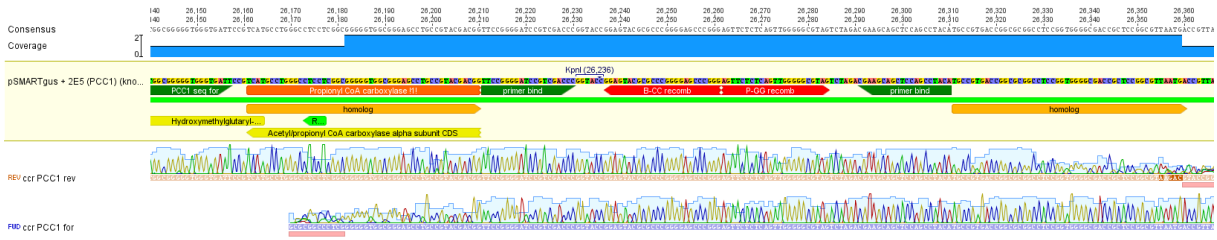


Figure IV.S2: Alignment of the Del1ΔccrD1-1 ΔPCC1 sequencing results to the corresponding genome sequence.

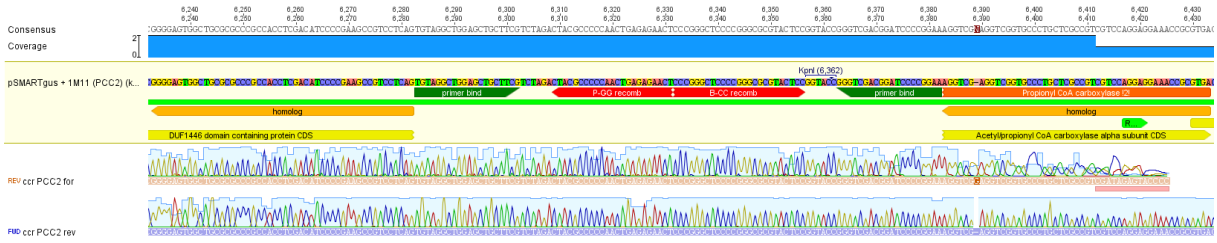


Figure IV.S3: Alignment of the Del1ΔccrD1-1 ΔPCC2 sequencing results to the corresponding genome sequence.

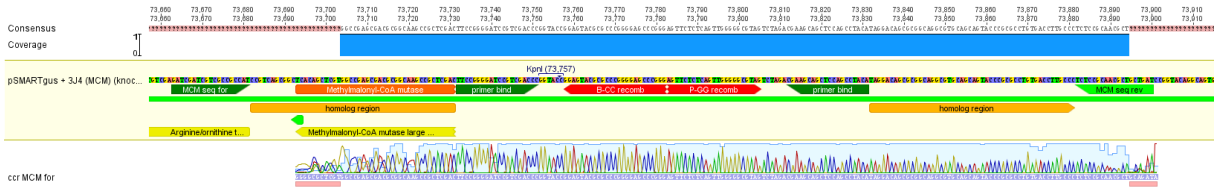


Figure IV.S4: Alignment of the Del1ΔccrD1-1 ΔMCM sequencing results to the corresponding genome sequence.

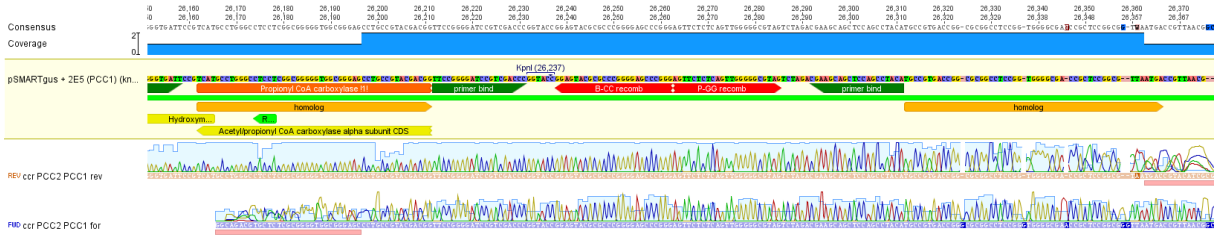


Figure IV.S5: Alignment of the Del1ΔccrD1-1 ΔPCC2 ΔPCC1 sequencing results to the corresponding genome sequence.

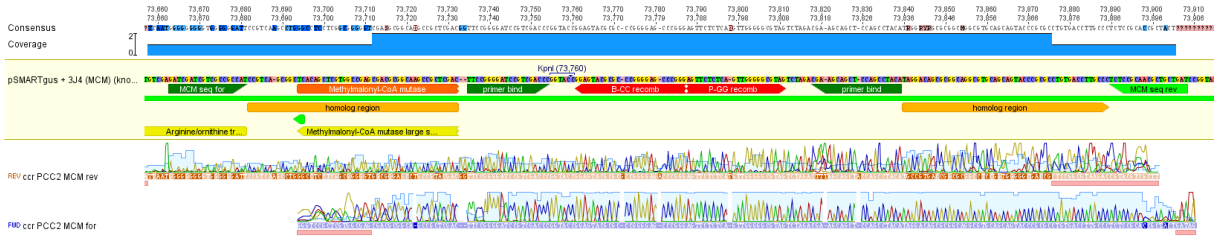


Figure IV.S6: Alignment of the Del1ΔccrD1-1 ΔPCC2 ΔMCM sequencing results to the corresponding genome sequence.

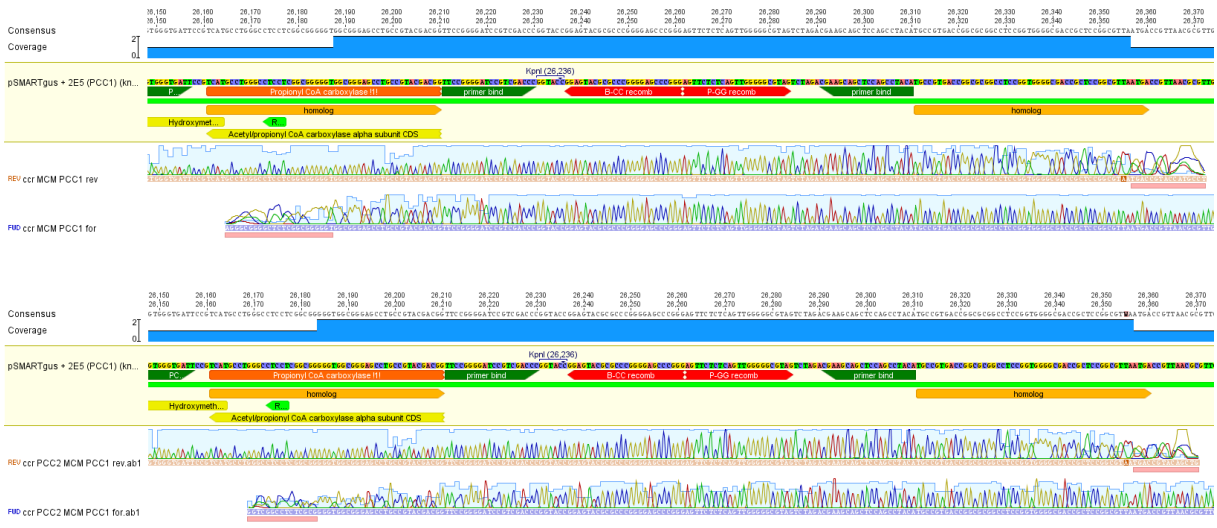


Figure IV.S7: Alignment of the Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 sequencing results to the corresponding genome sequence.

Gel picture for Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 ΔVDH and *S. albus* J1074 ΔVDH

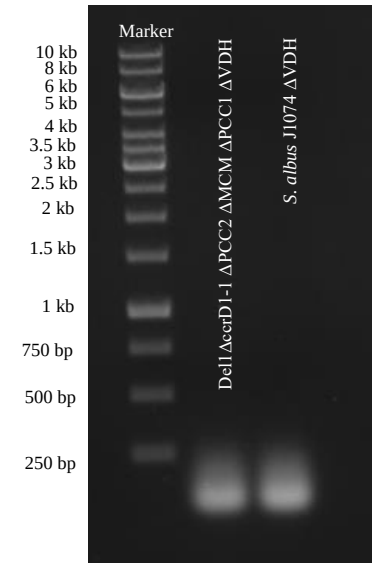


Figure IV.S8: Gel picture of the PCR products for the VDH (212 bp) knockout in the Del1ΔccrD1-1 ΔPCC2 ΔMCM ΔPCC1 and S. albus J1074strain after marker excision.

4. An influence of the copy number of biosynthetic gene clusters on the production level of antibiotics in a heterologous host

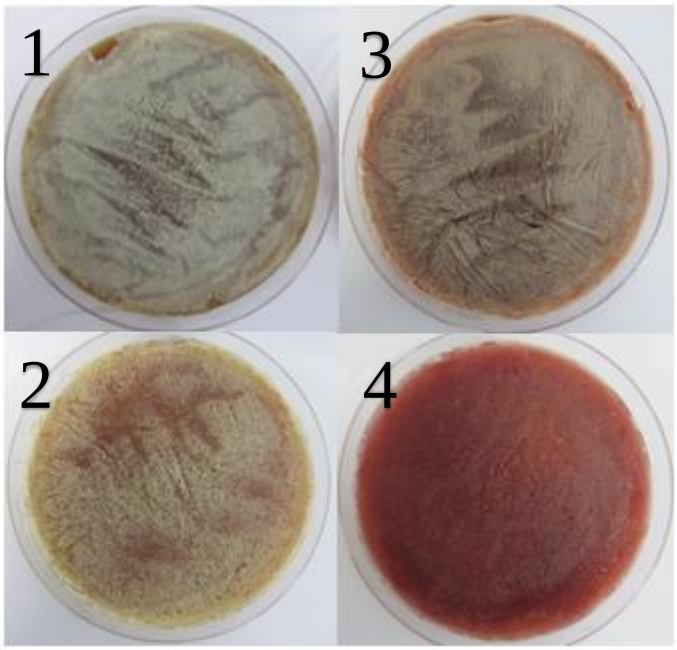


Figure 4.1: Aerial mycelium and spore formation in S. albus ΔpseB4::41-2C-06 (1), S. albus J1074::41-2C-06 (2), S. albus T1::41-2C-06 (3) and S. albus T11::41-2C-06 (4). Intensity of the red color, which originates from the production of demethoxyaranciamycinone and derivatives, increases from 1 to 4. In 4 there is no visible spore formation and the strain is growing weaker in comparison to 1, 2 and 3.

Table 4.1: NMR data of 2-(1-hydroxyethyl)-1,8-dihydroxy-3-methylanthraquinone (600 / 150MHz, $CDCl_3$, T = 25°C, solvent as internal reference)

pos.	δ_C ppm	δ_H ppm (*J* Hz)	COSY[a]	HMBC[a]
1	160.6			1-OH, 13-H_3
1-OH		12.88 s		
2	137.8			1-OH, 4-H, 12-H_3, 13-H_3
3	145.3			13-H_3
4	122.9	7.65 s	(13-H_3)	13-H_3
4a	131.4			
5	120.0	7.82 d (7.6)	6-H	6-H, 7-H
6	137.3	7.68 t (8.0)	5-H, 7-H	5-H, 7-H, (8-OH)
7	124.7	7.29 d (8.4)	6-H	5-H, 8-OH
8	162.5			6-H, 7-H, 8-OH
8-OH		11.96		
8a	115.8			5-H, 7-H, 8-OH
9	193.1			(4-H), (5-H)
9a	114.3			1-OH, 4-H
10	181.6			4-H, 5-H
10a	133.6			6-H
11	66.9	5.16 br t (6.8)	12-H_3	(4-H), 12-H_3
11-OH		3.86 br s		
12	22.2	1.62 d (6.8)	11-H	^{1}J
13	20.6	2.47 s	(4-H)	^{1}J, 4-H

[a]weak signals in brackets