The engineering of heterologous host for natural products overproduction

Dissertation
zur Erlangung des Grades
des Doktors der Naturwissenschaften
der Naturwissenschaftlich-Technischen Fakultät
der Universität des Saarlandes

von
Mariia Lopatniuk

Saarbrücken
2019

Tag des Kolloquiums: 23.01.2020

Dekan: Prof. Dr. Guido Kickelbick

Vorsitzender: Prof. Dr. Uli Kazmaier

Berichterstatter: Prof. Dr. Andriy Luzhetskyy

Prof. Dr. Rolf Müller

Akad. Mitarbeiter: Dr.-Ing. Michael Kohlstedt

Diese Arbeit entstand unter der Anleitung von Prof. Dr. Andriy Luzhetskyy in der Fachrichtung 8.2, Pharmazeutische Biotechnologie der Naturwissenschaftlich-Technischen Fakultät III der Universität des Saarlandes von December 2014 bis September 2019.

Acknowledgements

First of all, I would like to express my deepest gratitude to Prof. Andriy Luzhetskyy for his supervision during my PhD. I want to thank him, for believing in me and for giving me the opportunity to work in his group. I am very grateful for exciting and challenging projects, which helped me to gain a lot of experience and knowledge.

I want to thank Prof. Rolf Müller, for being my second supervisor. I am grateful for his critical questions and suggestions during my committee meetings.

Special gratitude goes to Dr. Maksym Myronovskyi for his patience during all these years. I thank him for leading me on my PhD journey and sharing his skills with me. I would also like to thank him for his critical reading of all my manuscripts. I know how much time and effort it took, and I am very grateful for this.

I would like to express many thanks to Birgit Rosenkränzer for teaching me a lot during the first few months of my stay in AMEG. I would also like to thank Dr. Yuriy Rebets for his ideas and support during my PhD. I owe my thanks to Dr. Constanze Paulus and Marc Stierhof for answering all my organic chemistry and purification questions. And my special gratitude goes to Dr. Yousra Ahmed, for being there whenever I needed her support and advice. And, of course, this work would not be possible without my students, Alessia Nottebrock, Stephanie Schreiner, Julia Wildfeuer, and Florian Riedel. I want to thank all members of the group, former and present, for a friendly working environment and your help with my projects.

Last but not the least I want to express my gratitude to Prof. Jesko Köhnke for collaboration on thioholgamide project. I would also like to thank Asfandyar Sikandar, for sharing a thioholgamide project with me. I'm very grateful for your support in professional and private life.

Також хочу подякувати своїй сім'ї за підтримку і розуміння протягом всього мого життя, і особливо протягом останніх років, коли я так далеко. Я безмежно вдячна своїм батькам за те, що подарували мені життя, навчили приймати самостійні рішення і бути відповідальною за них. Дякую вам за все!

List of publications

Lopatniuk M., Ostash B., Makitrynskyy R., Walker S., Luzhetskyy A., Fedorenko V. Testing the utility of site-specific recombinases for manipulations of genome of moenomycin producer Streptomyces ghanaensis ATCC14672, J Appl Genet. **2015**, 56(4):547-550.

Lopatniuk M., Myronovskyi M., Luzhetskyy A. *Streptomyces albus*: A New Cell Factory for Non-Canonical Amino Acids Incorporation into Ribosomally Synthesized Natural Products, ACS Chem Biol, **2017**, 12, 2362−2370.

Koshla O., **Lopatniuk M.,** Rokytskyy I., Yushchuk O., Dacyuk Y., Fedorenko V., Luzhetskyy A., Ostash B. Properties of *Streptomyces albus* J1074 mutant deficient in $tRNA^{Leu}_{UAA}$ gene *bldA,* Arch Microbiol, **2017**, 199:1175–1183.

Busche T., Tsolis K.C., Koepff J., Rebets Y, Rückert C., Hamed M.B., Bleidt A., Wiechert W., **Lopatniuk M.**, Ahmed Y., Anné J., Karamanou S., Oldiges M., Kalinowski J., Luzhetskyy A., Economou A., Multi-Omics and Targeted Approaches to Determine the Role of Cellular Proteases in Streptomyces Protein Secretion, Front Microbiol, **2018**, 4;9:1174.

Kuzhyk Y., **Lopatniuk M.**, Luzhetskyy A., Fedorenko V., Ostash B. Genome Engineering Approaches to Improve Nosokomycin A Production by Streptomyces ghanaensis B38.3. Indian J Microbiol, **2019,** 59(1):109-111.

Lopatniuk M., Myronovskyi M., Nottebrock A., Busche T., Kalinowski J., Ostash B., Fedorenko V., Luzhetskyy A. Effect of “ribosome engineering” on the transcription level and production of *S. albus* indigenous secondary metabolites, Appl Microb Biot, **2019**, 103(17):7097-7110.

Lopatniuk M., Myronovskyi M., Nottebrock A., Busche T., Kalinowski J., Ostash B., Fedorenko V., Luzhetskyy A. Rational ribosome engineering to improve secondary metabolite production, *to be submitted*

Lopatniuk M., Wildfeuer J., Riedel F, Dahlem C., Kiemer A., Luzhetskyy A., The platform for biosynthetic engineering of RiPPs**,** *to be submitted*

Conference contributions

Lopatniuk M., Myronovskyi M., Luzhetskyy A., The new strategy for studying the effect of *rpsL* mutations on antibiotic production, *6th HIPS Symposium, Saarbrücken, Germany,* **2015** (Poster)

Lopatniuk M., Myronovskyi M., Luzhetskyy A., Development of the new strategy for studying the effect of point-mutations in *rpsL* gene on antibiotic production level, *VAAM Workshop Biology of Natural Products-Producing Microorganisms, Frankfurt, Germany,* **2015** (Talk)

Lopatniuk M., Myronovskyi M., Luzhetskyy A., Cloning of the cinnamycin biosynthesis gene cluster and incorporation of unnatural amino acids into the structure of lantibiotic, *7th HIPS Symposium, Saarbrücken, Germany,* **2016** (Poster)

Lopatniuk M., Myronovskyi M., Luzhetskyy A., Site-directed incorporation of unnatural amino acids into lantibiotic cinnamycin in *Streptomyces albus*, *VAAM Workshop Biology of Natural Products-Producing Microorganisms, Freiburg, Germany,* **2016** (Talk)

Lopatniuk M., Myronovskyi M., Luzhetskyy A., Site-directed incorporation of unnatural amino acids into lantibiotic cinnamycin in *Streptomyces albus*, *18th International Symposium on the Biology of Actinomycetes (ISBA), Jeju, Korea,* **2017** (Poster)

Wildfeuer J., Riedel F, **Lopatniuk M.,** Dahlem C., Kiemer A., Luzhetskyy A., The platform for biosynthetic engineering of RiPPs**,** *10th HIPS Symposium, Saarbrücken, Germany,* **2019** (Poster)

Lopatniuk M., Wildfeuer J., Riedel F, Dahlem C., Kiemer A., Luzhetskyy A., The platform for biosynthetic engineering of RiPPs**,** *VAAM Workshop Biology of Natural Products-Producing Microorganisms, Jena, Germany,* **2019** (Talk)

Abstract

Streptomyces are a prolific source of diverse bioactive natural products. However, most of the corresponding biosynthetic gene clusters are expressed poorly under laboratory conditions. Therefore, the optimized host strains for heterologous expression of biosynthetic gene clusters and the production of natural products in sufficient yields are in great need.

The first part of this thesis is dedicated to the optimization of the *S. albus* host strain to increase the natural product production titers through ribosomal engineering. For the first time, we showed that ribosomal engineering has a significant impact on the whole transcriptome of the cell, what, in turn, has a strong effect on the expression level of indigenous and heterologously expressed biosynthetic gene clusters.

The second part is dedicated to RiPPs natural products derivatization in *Streptomyces* hosts via two approaches: the stop-codon suppression and rational codon randomization. By using the first approach, we succeed in incorporating unnatural amino acids into a potent lantibiotic cinnamycin and have obtained five new derivatives. The platform for rational codon randomization was developed, enabling the fast and efficient derivatization of an anticancer RiPP thioholgamide A. These two approaches proved to be efficient for the RiPPs derivatization and might find its application in bio-orthogonal chemistry, target fishing, and improvement of the pharmacokinetics of the RiPP lead compounds.

Zusammenfassung

Streptomyceten sind eine ergiebige Quelle diverser, bioaktiver Naturstoffe. Jedoch werden die meisten dazugehörigen biosynthetischen Gencluster nur geringfügig unter Laborbedingungen exprimiert. Daher sind optimierte Wirtstämme für die heterologe Expression von Genclustern und Produktion der Naturstoffe in ausreichender Menge von großem Bedarf.

Der erste Teil dieser Arbeit beschäftigt sich mit der Optimierung des Wirtstammes *S. albus*, um die Produktion mittels Veränderungen auf ribosomaler Ebene zu erhöhen. Zum ersten Mal zeigen wir, dass Veränderungen dieser Art einen signifikanten Einfluss auf das gesamte Transkriptom der Zelle haben, was wiederum einen starken Effekt auf das Expressionslevel von eigenen und heterolog exprimierten Clustern ausübt.

Der zweite Teil beschäftigt sich mit der Derivatisierung von RiPP-Naturstoffen in *Streptomyceten* über zwei Ansätze: Stopp-Codon-Unterdrückung und rationale Codon-Randomisierung. Unter Nutzung des ersten Ansatzes konnten wir erfolgreich unnatürliche Aminosäuren in das wirksame Lantibiotikum Cinnamycin einbauen und erhielten fünf neue Derivate. Die rationale Codon-Randomisierung wurde entwickelt und ermöglicht die schnelle und effiziente Derivatisierung des antikanzerogenen RiPP Naturstoffes Thioholgamid A. Beide Ansätze sind effiziente Methoden zur Derivatisierung von RiPP-Naturstoffen und könnten Anwendung in der bio-orthogonalen Chemie, „target fishing“ und bei der Verbesserung der Pharmakokinetik der RiPP-Leitstrukturen finden.

Table of Contents

1. Introduction

1.1 Natural products discovery from Actinobacteria

Microbes have been an important source of natural products ever since the first antibiotic penicillin was discovered in 1928. During almost one century, natural products discovery has contributed a large number of active compounds to the market [1, 2]. Due to the great variety of biological activities, NPs found their niche in veterinary, pharmaceutical, and agricultural industries [1-3]. In medicine, natural products are prevalently used as antibacterial and anticancer agents [1, 4], immunosuppressants [1] or antiparasitic compounds [5, 6], with cephalosporins, vancomycin, teicoplanin, erythromycin, rapamycin, actinomycin D, doxorubicin being the most important examples [1, 7-9]. The phylum Actinobacteria produces over 70 % of all known antibiotics of natural origin, with a single genus *Streptomyces* producing about 55 % [2].

Natural products (NPs) discovery rate was very high until 1985; then it dropped significantly at the end of the 20th century [10] due to several reasons. First, the rate of rediscovery of already known compounds was increasing year by year, and this made screening programs less efficient and more expensive [11]. On the other hand, strict requirements for drug toxicity, time and costs required for preclinical and clinical development, and short lifespan of patents made drug development from natural products less profitable [1]. As a result of this big pharmaceutical companies cut most of their natural product discovery programs in the past three decades [12, 13] and their focus shifted from NPs drug development towards high-throughput screening (HTS) of synthetic compounds libraries [12] and direct combinatorial chemistry [14]. However, this synthetic approach turned out to be not as effective as it was expected. According to United States Food and Drug Administration (FDA) among all drugs approved from 1981 to 2014 only 27 % are purely synthetic compounds, while the remaining 73 % are of natural origin (Figure 1) [15]. Exclusively synthetic compounds cover only 20 % of all antibacterial drugs, 13 % of anticancer, 31 % of antiparasitic compounds, and 10 % of antiviral compounds; the rest are NP-derived [15]. These statistical data imply that NPs remain the primary source of lead compounds covering anti-infective and anticancer approved drugs [16, 17].

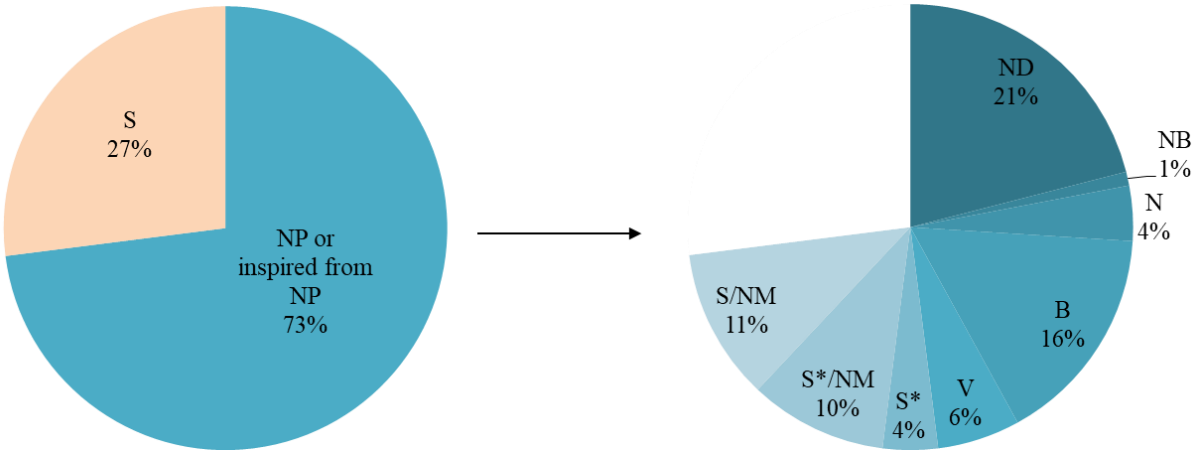


Figure 1. Categorization of drugs approved by FDA from 1981 till 2014. Altogether 1562 drugs were analyzed [15]. S – synthetic compounds; ND – natural product derivatives; NB – botanical drugs; N – unaltered natural products; B – biological macromolecules; V – vaccines; S* – synthetic drugs with natural product pharmacophore; S*/NM – synthetic drugs with natural product pharmacophore / mimic of natural product; S/NM – synthetic drugs / mimic of natural product.

NPs are more fruitful in drug discovery than synthetic compounds and are still in great need due to many reasons. In contrast to synthetic compounds, NPs often possess a more complex chemical structure and higher molecular weight than synthetic compounds. NPs contain numerous stereocenters, what makes it difficult to synthesize such molecules via chemical synthesis [17]. NPs usually have better penetration through the cell membrane than synthetic compounds, what allows them to reach the target molecules efficiently; that's why they usually have better *in vivo* activity. Compare to synthetic compounds, semisynthetic already exhibit better pharmacological characteristics and the reason behind this is that they are typically based on NP scaffold [17]. Such advantage of NPs over libraries of synthetic compounds can be explained by a long process of natural evolution and selection [11]. In the course of evolution, bacteria that gained the ability to produce biologically active compounds had a preference for survival. Thus, NPs evolved as active and functional compounds able to penetrate microbes membranes and inhibit the target enzymes and interfere with macromolecules [11].

Today, when we face a problem of growing resistance of pathogens to available bioactive compounds, the most promising way to confront it is to look for new drugs in NPs. But this is only possible if we solve the main problems in drug discovery: rediscovery of already known compounds and unsustainable supply of compounds in sufficient amounts for bioactivity testing. Resolving these problems, together with the government support of NPs

development, can restore the interest of the pharmaceutical industry in NPs as a source of new drugs.

The rediscovery problem can be solved with the recent development of sequencing and bioinformatics technologies. Genome sequencing and bioinformatic analysis revealed the hidden potential of bacteria to produce more NPs than it was thought before [18-23]. For instance, Actinomycetes sp. were shown to encode more than 20 NPs biosynthetic pathways, while under laboratory conditions, they produce around 2-3 compounds [13]. According to the bioinformatic analysis, only 2 % of antibiotics have been discovered so far from the *Streptomyces* source [10]. Implementation of genome sequencing, along with genome mining, enables the discovery of encoded biosynthetic gene clusters that may code for potentially new NPs with new activities. Targeted expression of these new BGCs allows solving the problem of rediscovery. However, this cluster centered approach requires the development of hosts for efficient heterologous expression of new BGCs.

Most NPs are produced in tiny amounts preventing their discovery due to weak expression of their BGCs [24]. Development of host strains for effective expression of interesting BGCs would solve the problem of unsustainable supply of NPs in amount sufficient for their biological testing. Such hosts are now being developed based on *S. albus, S. lividans, S. coelicolor*, etc. Already developed intermediate strains showed promising features of BGCs expression, compounds detection, and production [13] [25].

Today a strain-centered approach is substituted by a cluster-centered approach. The turn from the single strain as a source of NP to bioinformatically preselected unique BGCs may fill the NPs discovery pipeline with new pharmacophores and help us to stand against spreading multidrug-resistant pathogens.

1.2 Heterologous hosts in NPs discovery.

In the 21st century discovery of NPs often relies on heterologous expression of new uncharacterized BGCs in suitable host strains [26]. Host strains are widely used for expression, refactoring of BGCs, and derivatization of the NPs [27]. Genetic engineering of BGCs is usually performed in *E. coli* using well-established protocols, and final recombinant constructs are expressed in suitable hosts. This allows performing biosynthetic studies, yields improvement and derivatization of the compounds in a time-efficient way.

A model host strain should have the following characteristics:

1. amenability to genetic manipulations,
2. fast growth rate,
3. low production level of native secondary metabolites (to facilitate NPs identification and purification),
4. rich pool of metabolic precursors and biosynthetic machinery that can support diverse biosynthetic pathways,
5. high success rate of heterologous expression of foreign BGCs,
6. high production yields of heterologously expressed BGCs [28].

Today a lot of resources are dedicated to the development of the panel of host strains to facilitate the discovery of NPs and to increase the production titers of the compounds. Since *Streptomyces* are one of the most prolific producers of NPs, the focus of this chapter will be on the *Streptomyces* hosts. The key feature of *Streptomyces* is their metabolic capacity to support the biosynthesis of different NPs. *Streptomyces* possesses a lot of intrinsic BGCs, which can compete with heterologously expressed BGCs for precursors. To reduce the metabolic burden and to redirect the precursor flux towards the biosynthesis of heterologously expressed BGCs, the intrinsic clusters need to be removed. Therefore, since the 2000s, a lot of efforts have been directed towards the development of cluster-free host strains, or chassis [29]. Such chassis strains not only ensure the flow of precursor molecules into the biosynthetic pathway of desired NP but also greatly simplify the NP purification process, due to significantly reduced secondary metabolites background.

Today there is a number of optimized host strains developed, which derive from *S. coelicolor, S. lividans, S. avermitilis,* and *S. albus* [30].

***S. coelicolor* host strains**

S. coelicolor A3(2) is the best-studied model *Streptomyces* strain that was among the first hosts used for heterologous expression. It was the first well-characterized *Streptomyces* strain, with available protocols for genetic manipulations, protoplast transformation, and conjugation [31]. *S. coelicolor* A3(2) can naturally produce polyketide antibiotic actinorhodin (act), PKS-NRPS hybrid undecylprodigiosin (red), NRPS-derived calcium-dependent antibiotic

(cda) and type I polyketide CPK (cpk) [32]. Nowadays, there are several of *S. coelicolor* A3(2) -derived host strains available.

S. coelicolor CH999 host strain was developed for expression of polyketide BGCs. It is deficient in actinorhodin and undecylprodigiosin production, due to deletion of act cluster and mutation in red cluster [33]. It was used for heterologous expression of polyketide neocarcillin from *S. carcinostaticus*, non-ribosomal peptide epothilone from *Sorangium cellulosum*, etc. (Table 3).

S. coelicolor M512 derived from *S. coelicolor* A3(2). The genes of pathway-specific activators for actinorhodin and undecylprodigiosin biosynthesis, *actII*-ORF4 and *redD,* respectively, were deleted in strain M512 [34]. This strain was constructed to study the transcriptional regulation of actinorhodin and undecylprodigiosin biosynthesis, but it was also successfully used as a host for heterologous expression. Aminocoumarins clorobiocin and novobiocin from *Streptomyces sp.*, liponucleoside caprasamycin from *Streptomyces sp.*, etc. were expressed in this strain (Table 3).

S. coelicolor M1146 (ΔactΔredΔcpkΔcda) is another host strain derived from *S. coelicolor* A3(2) [35]. In this strain, actinorhodin, undecylprodigiosin, calcium-dependent antibiotic, and CPK BGCs were deleted. The strain showed a 3-fold increase in the production of shikimate pathway derived chloramphenicol and a 5-fold increase in the production of non-ribosomal peptide congocidine, compared to *S. coelicolor* A3(2) [35]. It was also successfully used for heterologous expression of polyketide FK506 from *S. tsukubaensis*, nucleoside-peptide nikkomycine from *S. tendae*, etc. (Table 3). The strains *S. coelicolor* M1152 and M1154 derived from M1146 by subsequent introduction of *rpoB* and *rpsL* mutations (details in sections 1.3 The effect of mutations in *rpoB* gene on the production yields of NPs and 1.4 The effect of mutations in *rpsL* and *rsmG* genes on the production yields of NPs) [35]. The introduction of these mutations further improved production yield of heterologously expressed BGCs, compared to *S. coelicolor* A3(2). 20-40-folds increased production yields of heterologously expressed BGCs were observed [35]. The new hosts were successfully used for expression of lanthipeptide streptocollin from *S. collinus*, non-ribosomal peptide holomycin from *S. clavuligerus*, and many others (Table 3).

Table 3. Heterologous expression of NPs BGCs in host strains.

An original strain	Derived host	Heterologously expressed NP	Original producer	NP class	Reference
S. coelicolor A3(2)	CH999	medermycin	*Streptomyces sp.* AM-7161	polyketide	[36]
		neocarzilin	*S. carzinostaticus*	polyketide	[37]
		epothilone	*Sorangium cellulosum*	non-ribosomal peptide	[38]
	M512	clorobiocin and novobiocin	*Streptomyces sp.*	aminocoumarins	[39] [40]
		fostriecin	*S.pulveraceus*	polyketide	[41]
		caprazamycin	*Streptomyces sp.* MK730-62F2	liponucleoside	[42]
	M1146	nikkomycin	*S. tendae* Tü 901	nucleoside-peptide	[43]
		gougerotin	*S.graminearus*	nucleoside-peptide	[44]
		GE2270	*Planobispora rosea*	thiopeptide	[45]
		cephamycin C	*S. clavuligerus* ATCC 27064	beta-lactam	[46]
		FK506	*S. tsukubaensis*	polyketide	[47]
		clorobiocin	*Streptomyces sp.*	aminocoumarin	[48]
		chuangxinmycin	*Actinoplanes tsinanensis*	indole	[49]
		violapyrones	*S. violascens*	polyketide	[50]
	M1152	indolocarbazole	*S. sanyensis* FMA	indolocarbazole	[51]
		desotamides	*S. scopuliridis* SCSIO ZJ46	non-ribosomal peptide	[52]
		streptocollin	*S. collinus* Tü365	lanthipeptide	[53]
		chaxamycins A to D	*S. leeuwenhoekii*	polyketide	[54]
		roseoflavin	*S. davawensis* JCM 4913	riboflavin analogue	[55]
	M1154	caprazamycin	*Streptomyces sp.* MK730-62F2	liponucleoside	[48]
		napsamycin	*Streptomyces sp.* DSM5940	uridylpeptide	[56]
		holomycin	*S. clavuligerus*	non-ribosomal peptide	[57]
S. lividans 66	TK21	xylanase	*Streptomyces* Sp-No-36a	enzyme	[58]
		pikromycin	*S. venezuelae*	polyketide	[59]
	TK23	thioviridamide	*S. olivoviridis*	thiopeptide	[60]
		nikkomycin	*S. tendae* Tü 901	Nucleoside-peptide	[61]
		daunorubicin	*S. peucetius*	polyketide	[62]
		daptomycin	*S. roseosporus* NRRL 11379	cyclic lipopeptide	[63]
	TK24	labyrinthopeptins	*Actinomadura sp.*	class III lantibiotic	[64]
		berninamycin	*S. bernensis* UC 5144	thiopeptide	[65]
		syringolin	*Pseudomonas sp.*	NRPS/PKS hybrid product	[66]
		spinosyn	*Saccharopolyspora spinosa*	polyketide	[67]
	K4-114 and K4-155	deoxyoleandolide	*S. antibioticus*	polyketide	[68]
		platensimycin and platencin	*S. platensis*	terpene	[69]
		enterocin	*S. maritimus*	aromatic polyketide	[70]
		fredericamycin	*S. griseus* ATCC 49344	polyketide	[71]
		R1128	*Streptomyces* sp. R1128	anthraquinone	[72]
		6-deoxyerythronolide B	*Saccharopolyspora erythraea*	polyketide	[73]
	RedStrep 1.1-1.8	mithramycin A	*S. argillaceus* ATCC12956	glycosylated polyketide	[74]

Table 3. (Continued).

An original strain	Derived host	Heterologously expressed NP	Original producer	NP class	Reference
S. avermitillis	MA-4680	milbemycin	*S. hygroscopicus subsp. aureolacrimosus*	glycosylated macrolide	[75]
		thiolactomycin	*S. thiolactonus* NRRL 15439	NRPS/PKS hybrid product	[76]
	SUKA5	pladienolide	*S. platensis*	polyketide	[77]
		streptomycin	*S. griseus*	aminoglycoside	[77]
	SUKA17 and SUKA22	pladienolide	*S. platensis*	polyketide	[78]
		aureothin	*S. thioluteus* HKI-227	polyketide	[78]
		nemadectin	*S. microflavus* neau3	polyketide	[78]
		asukamycin	*S.nodosus* subsp. *asukaensis*	polyketide	[78]
		resistomycin	*S. resistomycificus*	polyketide	
		cephamycin	*S. clavuligerus* ATCC 27064	beta-lactam	[77]
		chloramphenicol	*S. venezuelae*	shikimate	[78]
		rebeccamycin	*Saccharothrix aerocolonigenes* ATCC39243	indolocarbazole	[79]
		holomycin	*S. clavuligerus*	non-ribosomal peptide	[78]
		bafilomycin	*Kitasatospora sp*.	polyketide	[78]
		sangivamycin	*S. rimosus*	nucleoside	[78]
S. albus	J1074	fredericamycin	*S. griseus* ATCC 49344	polyketide	[80]
		lysolipin	*S. tendae* Tü 4042	polyketide	[81]
		moenomycin	*S. ghanaensis*	glycophospholipid	[82]
		trichostatin A	*S. platensis*	polyketide	[83]
		thaxtomin	*S. scabies*	non-ribosomal peptide	[84]
		epoxomycin	*S, hygroscopicus* ATCC 53709	NRPS/PKS hybrid product	[85]
		thiocoraline	*Micromonospora sp*.	non-ribosomal peptide	[86]
		rebeccamycin	*Saccharotrix sp*.	indolocarbazole	[79]
		holomycin	*S. clavuligerus*	non-ribosomal peptide	
		chromopyrrolic acid	*Nonomuraea sp*.	indolocarbazole	[87]
		benastatins	*Actinomadura* sp.	polyketide	[88]
		steffimycin	*S. steffisburgensis* NRRL 3193	aromatic polyketide	[89]
	Del14	mensacarcin	*S. bottropensis*	polyketide	[28]
		cinnamycin	*S. cinnamoneus*	lantibiotic	[28]
		nybomycin	*S.albus* subsp. *chlorinus* NRRL B-24108	not reported	[90]
		tunicamycin	*S. lysosuperificus*	nucleoside	[28]

S. coelicolor M1317 (*ΔactΔredΔcpkΔcda rpoB*[C1298T] *ΔgcsΔsrsAΔrppA*) contains deletions of 7 indigenous BGCs [91]. It derived from M1152 by deleting three type III

polyketide synthase BGCs. This strain was constructed as a chassis for expression and study of type III polyketide synthases and their biosynthetic products.

***S. lividans* host strains**

S. lividans is another widely used strain for heterologous expression of BGCs. *S. lividans* is genetically closely related to *S. coelicolor*. Both strains contain act, red and cda BGCs. Unlike *S. coelicolor*, which can produce these compounds in laboratory conditions, in *S. lividans,* the corresponding clusters are silent [92]. The unique feature of *S. lividans* is a low level of internal proteases activity, and therefore it is widely used as a host for expression of antimicrobial peptides [93] and recombinant proteins [94]. Several *S. lividans* host strains derived from the wild type strain *S. lividans* 66 (or 1326).

S. lividans TK21 (SLP2$^-$, SLP3$^-$) derived from *S. lividans* 66. It was constructed by eliminating two endogenous plasmids SLP2 and SLP3 [95, 96]. The enzyme xylanase and type I PK pikromycin was successfully expressed in this host (Table 3).

S. lividans TK23 (SLP2$^-$, SLP3$^-$) is another plasmid-free strain derived from *S. lividans* 66 [97]. The strain was obtained due to genetic instability of the parental strain *S. lividans* 66; therefore, it is genetically different from TK21. Polyketide daunorubicin, cyclic lipopeptide daptomycin, and lots more NPs were expressed in this strain (Table 3).

S. lividans TK24 (SLP2$^-$, SLP3$^-$, *rpsL* [K88E]) derived from *S. lividans* TK21, by introducing a point mutation in the *rpsL* gene [98]. Of all *S. lividans* strains, TK24 is the most commonly used host. For instance, class III lanthipeptide labyrinthopeptin from *Actinomadura sp*., thiopeptide berninamycin from *S. bernensis* and macrolide spinosyn from *Saccharopolyspora spinosa* were expressed in this host (Table 3).

S. lividans K4-114 and K4-155 (Δ*act* SLP2$^-$, SLP3$^-$, *rpsL* [K88E]) are further improved host strains for overexpression of polyketide BGCs. The strains derived from TK24 by deletion of the actinorhodin biosynthetic pathway [73]. K4-114 and K4-155 are genotypically identical, with the only difference being that K4-114 was obtained as a result of substitution of act cluster with erythromycin resistance gene on a temperature-sensitive vector, and K4-155 with a suicide vector [73]. These strains were used for the expression of polyketides deoxyoleandolide, enterocin, and fredericamycin, etc. (Table 3).

Recently, based on TK24 eight genome-minimized *S. lividans* RedStrep host strains were developed [74]: RedStrep1 (Δ*act*); RedStrep1.1 (Δ*act*Δ*red*); RedStrep1.3 (Δ*act*Δ*red*Δ*cda*); RedStrep1.4 (Δ*act*Δ*red*Δ*cda*Δ*cpk*) with additionally deleted coelimycin P1 BGC and genes encoding gamma-butyrolactone; RedStrep1.5 (Δ*act*Δ*red*Δ*cda*Δ*hrdD*) derived from RedStrep1.3 with deletion of the transcriptional regulator gene *hrdD*; RedStrep1.6 (Δ*act*Δ*red*Δ*cda*Δ*mel*) derived from RedStrep1.3, additionally deleted melanin BGC; RedStrep1.7 (Δ*act*Δ*red*Δ*cda*ΔcpkΔ*mel*) derived from RedStrep1.4, additionally deleted melanin BGC; RedStrep1.8 (Δ*act*Δ*red*Δ*cda*ΔhrdDΔ*mel*) derived from RedStrep1.5, additionally deleted melanin BGC [74]. *S. lividans* RedStrep hosts were successfully used for the expression of a mithramycin BGC (Table 3). The highest production level was observed in RedStrep1.3 strain (Δ*act*Δ*red*Δ*cda*), 3-fold increase, compared to TK24 [74].

***S. avermitilis* host strains**

S. avermitilis MA-4680 (ATCC 31267) is a producer of an important antiparasitic drug avermectin. This strain was isolated from a soil sample in Japan, during the screening program for the discovery of antiparasitic compounds in 1978 [99]. *S. avermitilis* is also used as a host strain, as it is well studied and genetically amenable (Table 3). Number of optimized host strains was developed from *S. avermitilis* MA-4680 [77].

S. avermitilis SUKA5 (Δ*avr*Δ*flp*Δ*olm*) was the first cluster-free host, with deleted avermectin, filipin, and oligomycin BGCs [77]. *S. avermitilis* SUKA17 and SUKA22 (Δ*avr*Δ*flp*Δ*olm*Δ*terpene* BGCs) – two isogenic strains derived from SUKA5 with an additionally deleted BGCs for terpenes geosmin and neopentalenolactone, and carotenoid BGC [77, 78]. Heterologous expression of numerous NPs in the original *S. avermitilis* strain and *S. avermitilis*-derived hosts were reported: beta-lactam cephamycin C from *S. clavuligerus*, bafilomycin from *Kitasatospora sp*., rebeccamycin from *Saccharotrix sp*., asukamycin and lots more (Table 3).

***S. albus* host strains**

S. albus G is a wild type strain isolated from soil sample. It was widely used in 1970th-1980th for studying the plaque formation by bacteriophages and restriction endonucleases in Streptomyces [100, 101]. *S. albus* J1074 is an *S. albus* G derived strain, deficient in *Sal*I restriction-modification system [102]. Therefore, *S. albus* J1074 has higher conjugation

efficiency compared to the parental strain. *S. albus* J1074 is widely used as a host strain due to the high success rate of heterologous expression of various BGCs. This strain was successfully used for expression of polyketides fredericamycin from *S. griseus* and benastatin from *Actinomadura* sp., non-ribosomal peptides holomycin from *S. clavuligerus* and thiocoraline from *Micromonospora* sp., indolocarbazole rebeccamycin from *Saccharotrix* sp., etc. (Table 3).

In a year of 2018 hosts derived from J1074 were constructed by overexpression of the *crp* gene encoding a cAMP receptor protein (CRP) and deletion of *pfk* gene encoding a phosphofructokinase [103]. CRP is a pleiotropic DNA-binding regulator that was shown to have a positive effect on the antibiotic production. Phosphofructokinase encoded by the gene *pfk* catalyzes the rate-limiting step in glycolysis. The deletion of the *pfk* gene redirects the carbon flux towards the pentose phosphate pathway, which in turn increases a supply of a reducing agent NADPH, which is used in the process of secondary metabolites biosynthesis [104, 105]. The developed strains, *S. albus+crp* and *S. albus Δpfk+crp* were used for heterologous expression of actinorhodin BGC. The production of analyzed actinorhodin increased by 60 % for *S. albus +crp* and 80 % for *S. albus Δpfk +crp*, compared to J1074 [103].

Recently the construction of a first cluster-free *S. albus* Del14 chassis strain was reported [28]. In this strain, 15 endogenous BGCs were consequently deleted from the genome of *S. albus* J1074. The deletion of indigenous BGCs increases production of heterologously expressed polyketides dismethylmensacarcin by 2,5-fold and pyridinopyrone A by 2-fold, nucleoside tunicamycin by 2-fold, compared to J1074 strain [28]. *S. albus* Del14 was used successful heterologous expression of nybomycin BGC from *S. albus* subsp. *chlorinus* (Table 3). *S. albus* Del14 was further improved by introducing of additional *phi*C31 integration sites, providing the two new Del14-derived hosts: *S. albus* B2P1 and B4. Addition of extra *phi*C31 integration sites increases the copy number of heterologously expressed BGCs and often leads to higher production. New host strains showed further increase in production yields of dismethylmensacarcin by 4-fold and pyridinopyrone A by 2,5-fold, compared to J1074 [28].

Other *Streptomyces* host strains

S. grisoefuscus is another host strain used for heterologous expression of BGCs [92]. It has a very short doubling time of about 1 hour, while for other *Streptomyces,* it is in a range of 4-6 hours [106]. The efficient protoplast transformation protocol with high regeneration efficiency is developed for this strain. An aminonucleoside antibiotic puromycin was successfully expressed in *S. grisoefuscus*. However, the production titer was lower compared to another host strain *S. lividans* 66 [92].

S. ambofaciens BES2074, derived from the wild type spiramycin producer *S. ambofaciens* BES1260, is used as a host for heterologous production of NPs [107]. This strain is deficient in spiramycin production, due to mutation in the corresponding cluster. BES2074 was used for heterologous expression of lipopeptide A54145, with an increased yield of 285 % compared to native producer *S. fradiae* [108].

S. cinnamonensis C730.1 and C730.7 are industrial strains for the production of polyketide monensin, with moderate and high yields respectively. They were used as well for the heterologous production of polyketide tetracenomycine from *S. glaucescens* [109]. The high production yields of 3,13 g/L and 4,95 g/L were obtained for C730.1 and C730.7, respectively. These production yields are about 15-20-folds higher than in the native producer strain (0,26 g/L) and another host *S. lividans* TK24 (0,23 g/L) [109, 110].

BGCs are often expressed differently in various host strains. For example, aminocoumarins novobiocin and clorobiocin were expressed in higher yields in *S. coelicolor* hosts, then in *S. lividans* or *S. avermitilis* [111] [78]. Another example is the successful heterologous production of terpene platensin in *S. lividans*, whereas other host strains of *S. albus* and *S. coelicolor* did not produce any detectable amount of the compound [69]. The glycophospholipid moenomycin was expressed in high amounts in *S. albus* J1074 strain, and comparatively less in *S. lividans* hosts, but in *S. coelicolor* hosts the production was barely detectable [82]. The nybomycin BGC was successfully expressed in *S. albus* Del14 host but failed to be expressed in another host strain, *S. lividans* TK24 [90].

The reasons for differences in expression of BGCs in different hosts are not entirely understood. Therefore, it is unlikely that a single “super host” for efficient expression of all

BGCs can be constructed in the near future. The hosts from different strains possess differences in regulatory network, pool of precursors, and resistance might influence the expression efficiency. In the last decades, a broad panel of host strains was constructed to choose from depending on the BGC of interest. The panel was proved to be sufficient for the expression of BGCs of different source and nature. The further improvement of the constructed panel of hosts is the most promising way to achieve high expression rates and yields of interesting NPs.

1.3 The effect of mutations in *rpoB* gene on the production yields of NPs

In the last century, it was shown that rifampicin resistance mutations are often associated with overproduction of NPs by Actinobacteria [112]. Rifampicin is a polyketide antibiotic that targets DNA-dependent RNA-polymerase and inhibits the transcription (Figure 2a and 2b). Mutations that confer resistance to rifampicin are located in the *rpoB* gene that encodes the β-subunit of RNA-polymerase [113]. Such mutations decrease the affinity of rifampicin to RNA-polymerase [114]. All known rifampicin resistance mutations can be assigned to three conserved regions of RNA-polymerase: region I – 165 – 175aa, region II – 420 – 450aa and region III – 591 – 605aa [115]. Among these mutations, only those appearing in region II were shown to have an effect on the NPs production level. Often a single *rpoB* mutation has a different impact on the production of different NPs. Besides, one mutation can have a different degree of positive effect on the production level of various natural compounds.

The exact mechanism of how *rpoB* mutations influence the production level of NPs in *Streptomycetes* is not entirely understood. The RNA-polymerase with certain *rpoB* mutations was reported to mimic the stringent response [98, 116]. Stringent response is an adaptation mechanism that allows bacteria to survive under the nutrient depletion by readjusting the expression of numerous genes, including the activation of the secondary metabolism [98, 117]. Stringent response is triggered by increased concentration of the signal molecule guanosine tetraphosphate (ppGpp) in bacterial cells [117]. However, the role of *rpoB* mutations in mimicking the stringent response is still unknown. Furthermore, it was shown that RNA-polymerase with certain *rpoB* mutations could mimic the stringent response even in the absence of ppGpp [118]. Therefore, *rpoB* mutations decouple the expression of BGCs

from ppGpp regulation, which allows earlier start of production and often overproduction of NPs [117].

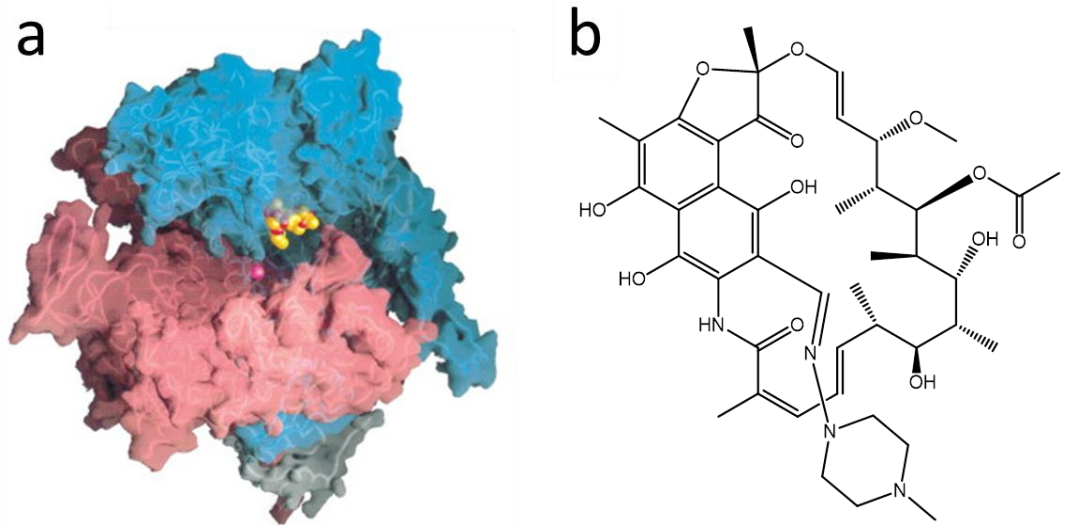


Figure 2. (a) Three-dimensional structure of RNA polymerase from *Thermus aquaticus* in complex with rifampicin [119]. Subunits of RNA polymerase are shown in colors: beta, cyan; beta', pink. Mg^{2+} ion at the active site is shown as a magenta sphere. The rifampicin is shown at the binding pocket (carbon, orange; oxygen, red; nitrogen, blue); (b) The chemical structure of the antibiotic rifampicin.

Numerous *rpoB* mutations increasing antibiotic production have been described. The most prominent among them are H437Y, H437R, S442Y, S442F, S433L, and Q424K (Table 1) [120, 121]. For instance, mutation S433L leads to a 50-fold overproduction of actinorhodin in *S. coelicolor* native producer strain [120]. Mutation H437R was reported to increase the actinomycin production by 7-fold in *S. antibioticus* strain [120].

Due to the pronounced effect of *rpoB* mutations on the NPs production yields, they are used in the construction of host strains for NPs overexpression. For instance, S433L mutation was introduced in *S. coelicolor* M1146 derived host, M1152. The new host exhibits a higher production level of indigenous NP actinorhodin by 10-folds and heterologously expressed chloramphenicol BGC by 20-fold, compared to M1146 [35]. Therefore, the introduction of *rpoB* mutations is a valuable tool for the construction of highly productive host strains for heterologous expression of NPs biosynthetic pathways.

Table 1. The examples of *rpoB* mutations and their effect on the NPs production level in the native producer strain [120].

Amino acid substitution	Microorganism	Natural product	Increase in production
Q424K	*S. griseus*	Streptomycin	4-fold
S433L	*S. coelicolor*	Actinorhodin	50-fold
H437Y	*S. antibioticus*	Actinomycin	7-fold
	Amicolatopsis orientalis	Vancomycin	2-fold
	Saccharopolyspora erythraea	Erythromycin	2-fold
H437R	*S. antibioticus*	Actinomycin	7-fold
	Saccharopolyspora erythraea	Erythromycin	4-fold
S442Y	*Amicolatopsis orientalis*	Vancomycin	3-fold

1.4 The effect of mutations in *rpsL* and *rsmG* genes on the production yields of NPs

Similarly to rif^R mutations, streptomycin resistance mutations (str^R) were shown to increase the production of NPs in Actinobacteria [122, 123]. Streptomycin belongs to aminoglycoside antibiotics that target the ribosome by binding to small subunit and inhibit the protein synthesis (Figure 3a-c). Streptomycin binding pocket is formed by 16S rRNA together with S12 protein (Figure 3b). This pocket lies in the decoding center of the ribosome that is responsible for the accuracy of the protein synthesis on the ribosome. Binding of streptomycin to the pocket affects the proof-reading activity of the ribosome during the elongation step and, therefore, induces miscoding of the mRNA information [124, 125].

Mutations leading to streptomycin resistance are often located in the *rpsL* gene that encodes S12 protein of the 30S ribosomal subunit [126, 127]. Str^R mutations in *rpsL* gene occur at two regions of S12 protein: region I, covering amino acids from 41 to 47 (TPKKPNS) and region II, covering amino acids from 83 to 94 (RGGRVKDLPGVR) [126]. These two regions of S12 protein form two loops that lie near the decoding center of the ribosome. Mutations in regions I and II change the three-dimensional conformation of the streptomycin binding

pocket and therefore prevent streptomycin from binding. Moreover, these conformational changes increase the translation accuracy on the ribosome [125]. The *rpsL* mutations were also reported to increase the stability of the ribosome in the late growth phase and elevate the overall protein synthesis [128, 129]. The latter can be explained by increased expression of a ribosomal recycling factor (RRF) observed in *rpsL* mutants [130].

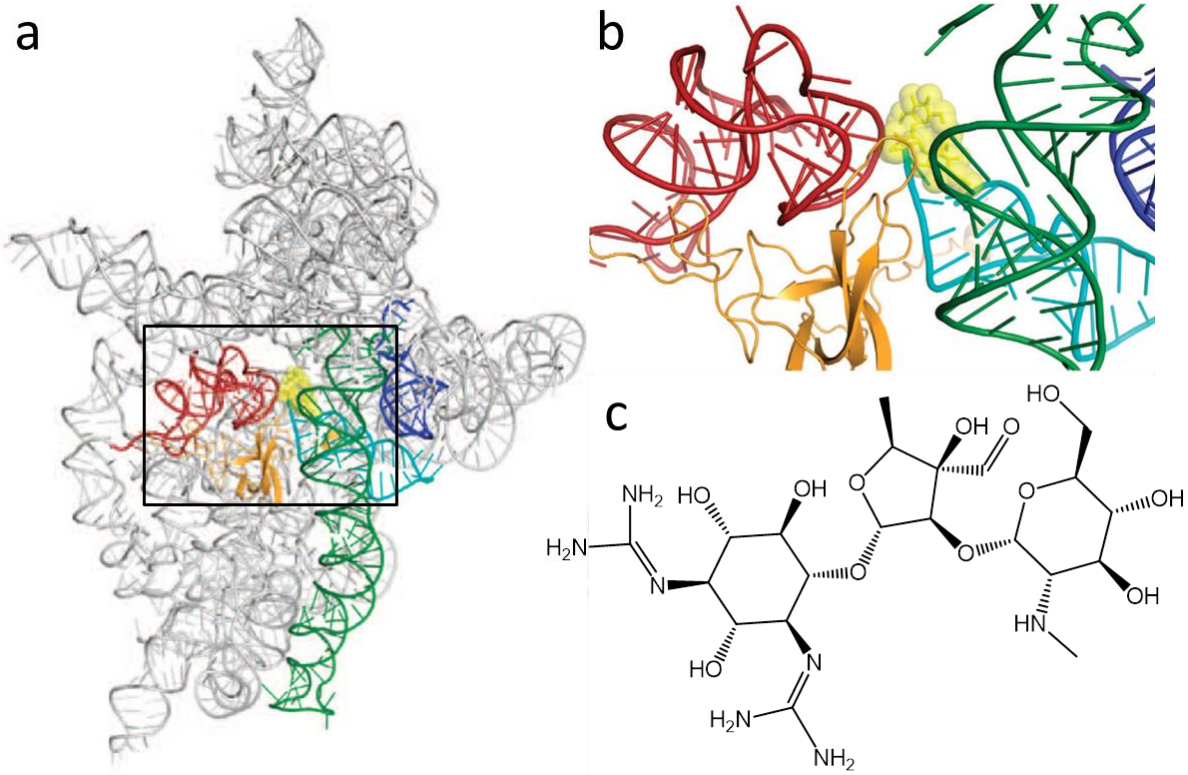


Figure 3. (a) Three-dimensional structure of the 30S ribosomal subunit in complex with antibiotic streptomycin. Streptomycin binding region is marked with black rectangle [124]; (b) The magnified image of the streptomycin binding pocket. Streptomycin is shown in yellow spheres, S12 protein in orange, 16S rRNA helixes are colored in grey, red, blue, green and cyan; (c) The chemical structure of antibiotic streptomycin.

The connection between the *rpsL* mutations and increased antibiotic production in Actinomycetes was an object of numerous studies. Only *rpsL* mutations in the region II of S12 protein have been reported to increase the antibiotic production [131]. It is believed that higher accuracy of translation and enhanced protein synthesis in the late growth phase when antibiotics are synthesized are the cause of this effect [128, 129]. Evidences exist that *rpsL* mutations influence antibiotic production not only on the translational level but also on the transcriptional level [132]. For instance, it was reported that K88E *rpsL* mutation

increases the expression of actinorhodin BGC in *S. coelicolor* by inducing the transcription of pathway-specific regulatory gene *act*II-ORF4 [132]. The molecular mechanism of how *rpsL* mutations influence the transcription, for now, is elusive.

Different *rpsL* mutations have been reported to increase the production level of various NPs (Table 3). For instance, mutation K88E increases actinorhodin production in *S. coelicolor* by 35-fold and oligomycin in *S. avermitilis* by 70-fold [131]. Mutations in the *rpsL* gene have also been reported to improve the production yields of heterologously expressed NPs. For example, K88E mutation was introduced in *S. coelicolor* M1154 host [35] and *S. lividans* TK24 [98], and this increased the production level of heterologously expressed BGCs (see chapter 1.2 Heterologous hosts in NPs discovery).

Another type of mutations that lead to streptomycin resistance and NPs overproduction is located in *rsmG* gene. The r*smG* gene encodes for a methyltransferase G that is responsible for methylation of guanine in position 527 (G527) of bacterial 16S rRNA. Methylated G527 is present on the conserved 530 loop of bacterial 16S rRNA, which, together with regions I and II of S12 protein, form the streptomycin binding pocket [127]. Disruption of the *rsmG* gene function leads to non-methylated G527 what decreases the affinity of streptomycin to its binding pocket and results in streptomycin resistance phenotype [126, 127, 133]. Similarly to *rpsL* mutations, deletion of the *rsmG* gene increases the protein biosynthesis level in the late growth phase [133]. However, unlike *rpsL* mutations, deletion of *rsmG* gene does not influence the ribosome stability or expression of RRF and therefore, is responsible for increased translational activity via an unknown mechanism [133].

Disruption of *rsmG* gene function was reported to increases the antibiotic production level in Actinomycetes (Table 2). As an examples, *rsmG* gene dysfunction enhances actinomycin production in *S. antibioticus* by 6-fold and actinorhodin production in *S. coelicolor* by 25-fold [131]. The deletion of the *rsmG* gene was also used to activate silent BGCs in *Streptomyces* sp. [134]. Unfortunately, it is still not clear how disruption of *rsmG* gene influences antibiotic production. Similarly to *rpsL* mutations, for *rsmG* deletion, the increase in transcription level of BGCs of overexpressed NPs was reported [132, 133]. This indicates that both, *rpsL* mutations and *rsmG* deletion, have an effect on the antibiotic

production by making changes of the BGC expression on the transcriptional and translational level.

Mutations in *rpoB*, *rpsL,* and *rsmG* genes all increase the production of NPs via different mechanisms. Therefore, they can be used for the development of the host strains with improved yields of heterologously expressed clusters, and a combination of these mutations in one strain can have a synergistic effect [135]. For instance, a combination of eight different antibiotic-resistance mutations was reported to increase the actinorhodin production in *S. coelicolor* by 180-fold, compared to the wild type [132]. Therefore, *rpoB*, *rpsL,* and *rsmG* mutations are used in the development of a panel of NPs overproducing strains.

Table 3. The examples of str^R mutations and their effect on the NPs production level in the native producer strain [131].

Mutation	Microorganism	Natural product	Increase in production
K88E *rpsL*	*S. coelicolor*	Actinorhodin	35-fold
	S. avermitilis	Oligomycin	70-fold
	S. antibioticus	Actinomycin	5-fold
K88R *rpsL*	*S. antibioticus*	Actinomycin	4-fold
	S. parvulus	Actinomycin	5-fold
	S. albus industrial strain	Salinomycin	1.5-fold [135]
Deletion of *rsmG*	*S. griseus*	Streptomycin	3-fold [134]
	S. antibioticus	Actinomycin	6-fold
	S. parvulus	Actinomycin	3-fold
	S. coelicolor	Actinorhodin	25-fold

1.5 Ribosomally synthesized and post-translationally modified peptides. Lanthipeptides

Natural products, isolated from bacteria, possess a high structural diversity. Most of them belong to one of the big classes of NPs, based on the biosynthetic origin and chemical structure [136]. The most abundant classes of bacterial NPs are terpenes, alkaloids, polyketides, and non-ribosomal peptides [137]. The significant part of NPs that made to the pharmaceutical industry belongs to the two biggest classes, polyketides and non-ribosomal peptides. The compounds erythromycin, avermectin, spinosyn, rifamycin, rapamycin, daptomycin, vancomycin, teixobactin, actinomycin, etc. belongs to these two classes [138, 139].

Recently another big class of ribosomally synthesized and post-translationally modified peptide (RiPP) NPs was discovered [140]. Development of the sequencing technologies and genome mining tools in 21st century revealed that RiPPs BGCs are more abundant than it was thought before [141]. Despite the great variety of RiPPs structures, they all share the common mechanism of biosynthesis (Figure 4a). Biosynthetic gene clusters of RiPPs contain a gene that encodes a precursor peptide and genes encoding for a set of modification enzymes [141, 142]. The precursor peptide usually consists of the following parts: the N-terminal leader peptide, the core peptide, and, in some cases, the C-terminal follower peptide. The core peptide is transformed into the final product after undergoing all modifications, including proteolytic cleavage of the leader and the follower peptides (Figure 4a) [143]. The Leader and the follower peptides serve as recognition elements for the modification enzymes and are essential for the maturation of the core peptide [141, 144]. The tailoring enzymes catalyze different post-translational modifications of RiPPs including heterocyclization, methylation, hydroxylation, dehydration to di- and mono-sulfide bond formation, etc. (Figure 4b) [142, 143]. High structural diversity of RiPPs endow them with a wide range of biological activities, such as antimicrobial [145], antifungal [146, 147], anticancer [148], and antiviral [149, 150]. Based on key features in their structures and post-translational modifications, RiPPs are divided into subfamilies (Table 4) [140, 142, 143]. The most widely spread RiPPs subfamilies in bacteria are lanthipeptides, thiopeptides, linear azole-containing peptides (LAPs) and lasso peptides [151].

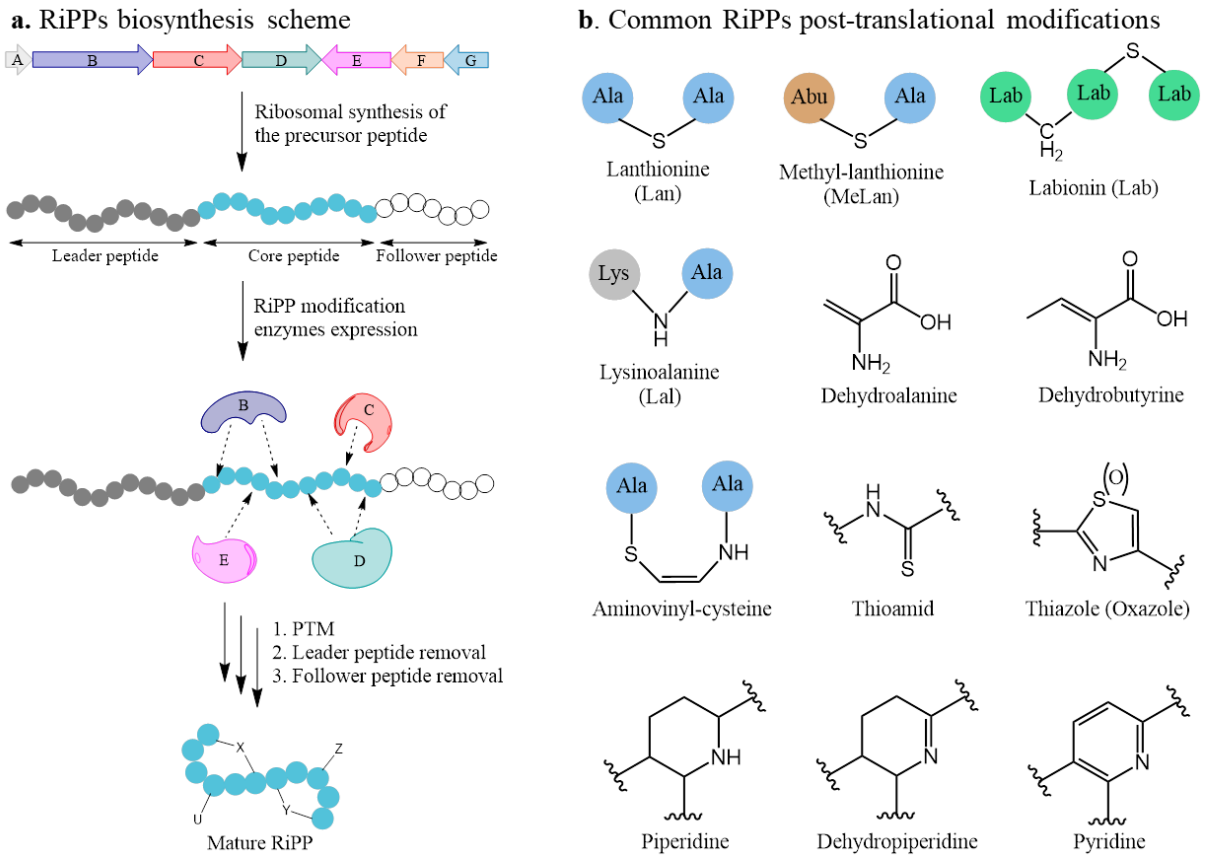


Figure 4. Key features of RiPPs. (a) Biosynthesis of RiPPs; (b) the most common post-translational modifications found in RiPPs.

Lanthipeptides

Subfamily of lanthipeptides is the largest group of RiPPs, and the first one that was described [152]. The distinct feature of this group is the presence of unusual amino acids such as lanthionine (Lan), methyl-lantionine (MeLan) or labionine (Lab) that are formed through post-translational modifications of the core peptide (Figure 4b) [142]. These unusual amino acids are formed through dehydration of Ser/Thr to dehydroalanine (Dha)/ dehydrobutyrine (Dhb) respectively. The subsequent cyclization of Dha/Dhb with Cys residue results in the formation of Lan/MeLan (Figure 5a) [153]. Lanthipeptides are subdivided into four classes (I-IV), depending on the mechanism of Lan and MeLan formation. In class I of lanthipeptides, the formation of Lan/MeLan is catalyzed by two distinct enzymes. In contrast to class I, in classes II, III, and IV formation of Lan/MeLan residues is catalyzed by one multifunctional enzyme (Figure 5b). In class I of lanthipeptides dehydration of Ser/Thr is catalysed by dehydratase LanB, and subsequent cyclization with cysteine by cyclase LanC. Evidences exist

that LanB and LanC work in a multienzyme complex to perform their functions [140, 143, 154, 155]. In class II of lanthipeptides, a single enzyme LanM is responsible for dehydration and cyclization (Figure 5b). It consists of two domains: the N-terminal dehydratase domain and the C-terminal cyclase domain. The cyclase domain shows high sequence homology to LanC of class I lanthipeptides. The lanthionine formation in class III and IV is catalyzed by multifunctional enzymes LanKC and LanL, respectively. LanKC and LanL consist of three domains: the N-terminal lyase domain, the central kinase domain, and the C-terminal cyclase domain (Figure 5b). The kinase domain of both, LanKC and LanL, activates Ser/Thr via phosphorylation and then the lyase domain eliminates the phosphate group what results in dehydrated amino acids Dha/Dhb (Figure 5a). The cyclase domain of LanL (class IV) shows homology to the LanC cyclase enzyme (class I). The cyclase domain of LanKS (class III) doesn't show such homology (Figure 5b).

Table 4. RiPPs subfamilies from microbes [142].

RiPP subfamily	**Defining key features**
Lanthipeptides	Lan, MeLan or Lab thioether amino acids
Linaridins	dehydro amino acids
Cyanobactins	N-to-C macrocyclized peptides from *Cyanobacteria*
Thiopeptides	central six-membered nitrogen-containing ring, dehydration and cyclodehydration
Lasso peptides	N-terminal macrolactam with C-terminal tail threaded through the ring
Proteusins	D-amino acids and C- methylations
Linear azole-containing peptides (LAPs)	oxazole or thiazole rings
Bottromycins	N-terminal macrocyclic amidine
Microcins	include lasso peptide and LAPs, produced by members of the *Enterobacteriaceae* family,
Microviridins	lactones made from Glu/Asp and Ser/Thr side chains and lactams made from Lys and Glu/Asp residues
Sactipeptides	intramolecular thioether linkage between Cys side chain and alpha-carbons of other amino acid
Bacterial head-to-head cyclized peptides	N-to-C cyclized peptides differing from cyanobactins in the biosynthetic machinery employed for macrocyclization
Autoinducing peptides	peptides containing a cyclic ester or a thioester
Methanobactin	peptidic chelator, produced by methanotrophic bacteria
Streptide	a Trp-to-Lys carbon-carbon crosslink
Thioamides	peptides containing thioamide linkages installed post-translationally

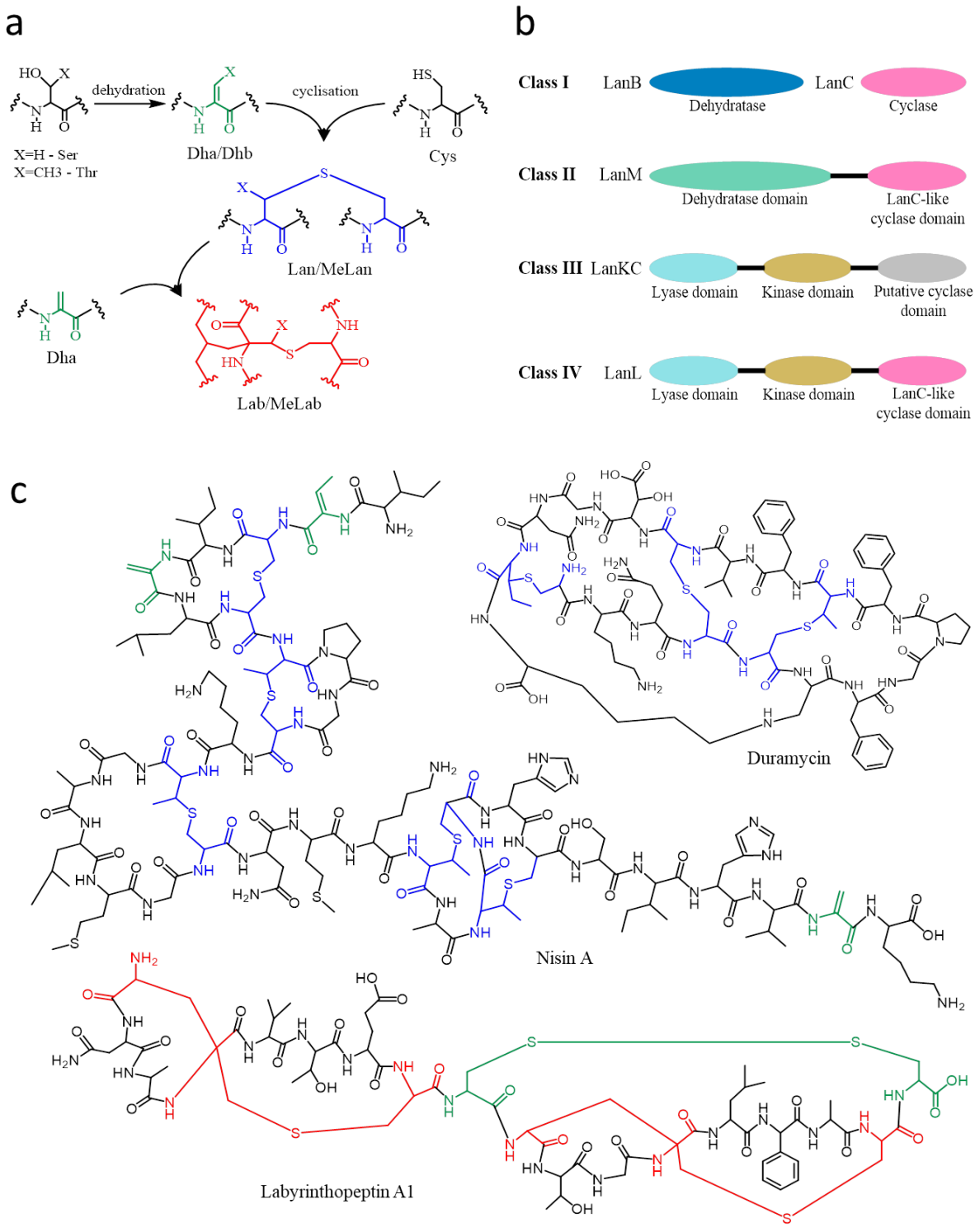


Figure 5. (a) Formation of lanthionine (Lan), methyl-lanthionine (MeLan) and labionine (Lab) bridges; (b) Structures of lanthionine synthetase from four different classes of lanthipeptides; (c) Examples of lanthipeptide NPs [143].

Representatives of a lanthipeptides subfamily exhibit a wide range of activities. Nisin A (Figure 5c) is widely used in the food industry as a preservative [156]. Several RiPP-derived antibiotics are now undergoing clinical trials. For instance, duramycin (Figure 5c) is in clinical trials for cystic fibrosis [157], microbisporicin is in the final stages of pre-clinical studies as a very potent compound against Gram-positive pathogens [158], and actagardine currently is in development against *Clostridium difficile* [159]. Antiviral compound labyrinthopeptin A1 (Figure 5c) proved to be a very potent agent with activity against human immunodeficiency virus (HIV) and herpes simplex virus (HSV) [158].

Linear azole-containing peptides (LAPs)

The linear azole-containing peptides (LAPs) are a group of RiPPs characterized by the presence of oxazole and/or thiazole heterocycles that are important for biological activity. Oxazole rings are formed via cyclodehydration of serine or threonine residues of the core peptide, while thiazole rings are formed via cyclodehydration of cysteine (Figure 6a and 6b) [140, 143]. Oxazole/thiazole heterocycles can be further reduced to oxazoline/thiazoline. Beside heterocycles formation LAPs usually undergo other post-translational modifications, such as dehydration, methylation, and acetylation. The dehydroalanine residues found in LAPs are incorporated in the same way as in the class I lanthipeptides, by the protein that possesses high homology to LanB [143]. To the subfamily of LAPs belong DNA gyrase inhibitor microcin B17, antibacterials goadsporin with the activity against plant pathogen *Streptomyces scabies* and plantazolicin with selective activity against *Bacillus antracis* [143].

Thiopeptides

Thiopeptides are another subfamily of highly modified RiPPs. They are characterized by the presence of six-membered N-containing ring (azacycle) (Figure 7) [143]. The azacycle is formed through the condensation of two dehydroalanine residues followed by oxidations and reductions (Figure 7b). The azacycle can be present in different oxidation states: piperidine, dehydropiperidine, imidazopiperidine, pyridine, and hydroxypyridine (Figure 7a) [160]. Besides azacycle, dehydrated amino acids, oxazole- or thiazole-rings and indoles are usually present in thiopeptides (Figure 7c). The mechanism of oxazole- or thiazole-ring formation in thiopeptides is the same as in LAPs.

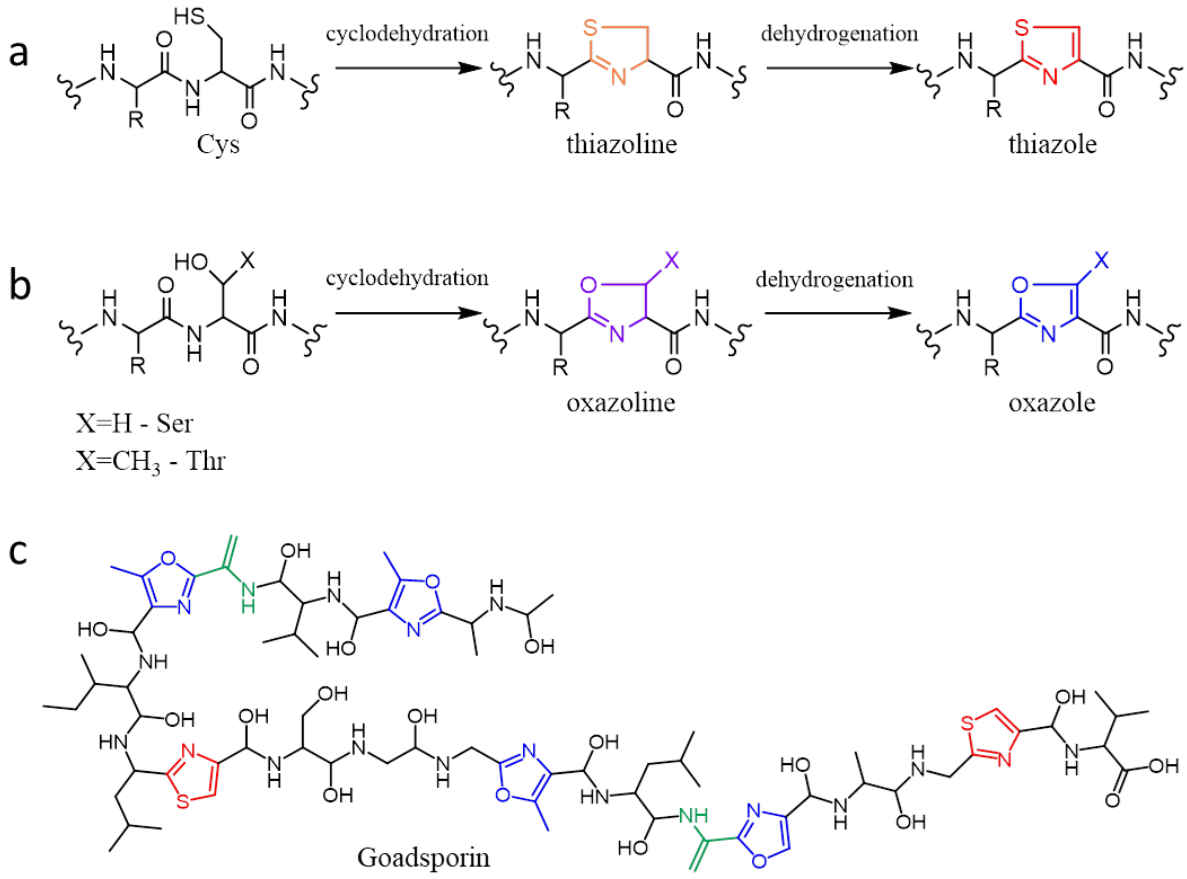


Figure 6. (a) Formation of thiazole and thiazoline heterocycles; (b) Formation of oxazole and oxazoline heterocycles; (c) Structure of LAP goadsporin with PTM represented in colors.

Thiopeptides are active against Gram-positive bacteria. Most of thiopeptides inhibit protein biosynthesis by binding either to 50S ribosomal subunit or to translational elongation factor Tu (EF-Tu) [160]. Thiostrepton and micrococcin (Figure 7c) exhibit strong activity against malaria parasite – *Plasmodium falciparum*. Thiopeptide GE2270A is now in clinical trials for treatment of infections caused by *Clostridium difficile*. Some representatives of this subfamily also show anticancer activities [143].

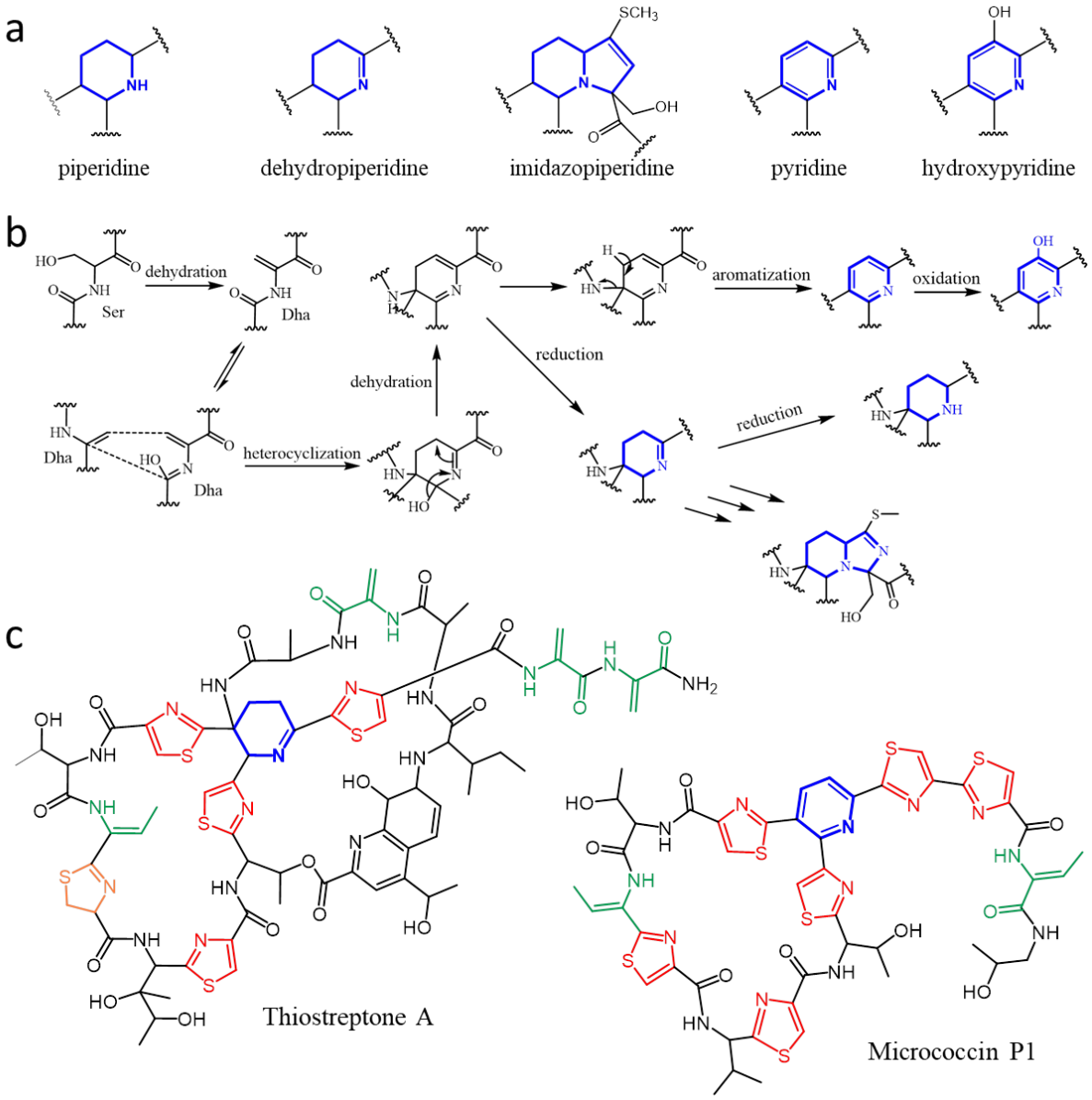


Figure 7. (a) Different kinds of azoline heterocycles; (b) Formation of azoline heterocycles; (c) Structures thiopeptides thiostrepton A and micrococcin P1. Post-translational modifications are represented in different colours.

Lassso peptides

The subfamily lasso peptides are characterized by the presence of a specific lasso knotted structure (Figure 8) [143]. The core peptide usually consists of about 20 residues. After cleavage of the leader peptide, the N-terminal amino group of the core peptide forms an isopeptide bond with side chain a carboxylic group on glutamate or aspartate in position 7-9 of the core peptide, forming the macrocycle (Figure 8). During ring closure, the C-terminal tail of the core peptide is trapped within the ring, and the structure is stabilized by

steric interactions between the amino acid residues on the tail and the ring [143, 161]. Lasso peptides are the growing group of NPs with potent activities, usually as antimicrobials, enzyme inhibitors and receptor antagonists [143]. The relevant examples of lasso peptides are microcin J25 [162] and capistruin [163] with antibacterial activity, and siamycin I and II as anti-HIV agents [143].

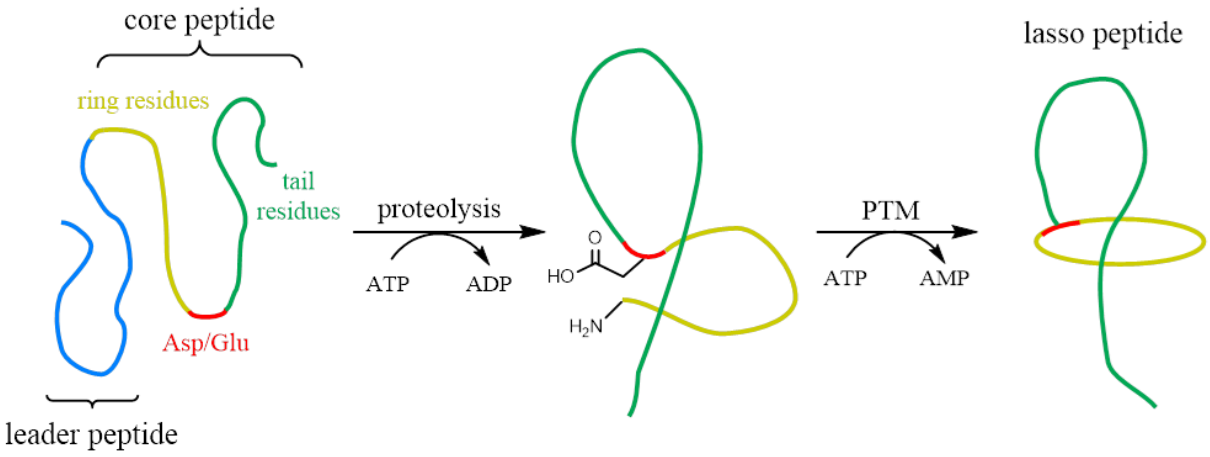


Figure 8. Formation of the lasso peptides structure [161].

The growing interest to RiPPs as a group of NPs is observed in the last decades [164]. The biosynthesis of RiPPs through precursor peptide and physical separation of the leader peptide containing recognition sites for modification enzymes from the core peptide allow generation of RiPPs derivatives [165]. Through a codon substitution in the core region of the precursor peptide gene, the derivatives with corresponding amino acids substitutions can be obtained. Due to the presence of recognition elements for modification enzymes in the leader peptide combinatorial biosynthetic approach can be used for diversification of RiPPs. The modification enzymes from other RiPPs pathways can be recruited via integration of their recognition sequences into the leader peptide sequence [164]. This biosynthetic potential of RiPPs can be exploited to create a range of new natural compounds for biotechnological or medical use.

1.6 Outline of the work

This thesis can be divided into two parts. The first part is dedicated to optimization of the *S. albus* J1074 via ribosome engineering, to increase the production yield of heterologously expressed BGCs. A list of *rpsL* mutations alone and in combination with *rsmG* deletion was studied in details to understand their effect on the production level of different NPs (Appendix I. page 121). Besides, the significant impact of ribosomal engineering on the transcription level of more than half genes in the genome was observed (Chapter 2. Publication I, page 45).

The second part of the thesis is dedicated to RiPPs derivatization. The publication II (page 78) describes our work on the introduction of unnatural amino acids into the structure of cinnamycin lanthipeptide in *S. albus* J1074 through utilizing an orthogonal pyrrolysyl-tRNA synthase /$tRNA^{Pyl}$ pair. Five new derivatives of cinnamycin were obtained via incorporation of three distinct pyrrolysine analogues into two different positions in the cinnamycin structure. The production yields of new derivatives were sufficient for structure elucidations and bio-activity assays.

The codon randomization, as a second approach for RiPPs derivatization, was implemented to generate a range of thioholgamide A derivatives (Appendix II, page 131). Here we present a platform for fast and efficient codon randomization of thioholgamide precursor peptide in the *S. lividans* host strain. We have obtained 58 new thioholgamide derivatives by randomizing only five amino acid residues.

References

1. Demain, A.L., *Importance of microbial natural products and the need to revitalize their discovery.* J Ind Microbiol Biotechnol, 2014. **41**(2): p. 185-201.
2. Demain, A.L. and J.L. Adrio, *Contributions of microorganisms to industrial biology.* Mol Biotechnol, 2008. **38**(1): p. 41-55.
3. Dayan, F.E., C.L. Cantrell, and S.O. Duke, *Natural products in crop protection.* Bioorganic & Medicinal Chemistry, 2009. **17**(12): p. 4022-4034.
4. Baker, D.D., et al., *The value of natural products to future pharmaceutical discovery.* Natural Product Reports, 2007. **24**(6): p. 1225-1244.
5. Omura, S. and A. Crump, *Ivermectin: panacea for resource-poor communities?* Trends Parasitol, 2014. **30**(9): p. 445-55.
6. Takahashi, Y. and T. Nakashima, *Actinomycetes, an Inexhaustible Source of Naturally Occurring Antibiotics.* Antibiotics (Basel), 2018. **7**(2).
7. Butler, M.S., et al., *Glycopeptide antibiotics: back to the future.* J Antibiot (Tokyo), 2014. **67**(9): p. 631-44.

8. Van Bambeke, F., *Glycopeptides and glycodepsipeptides in clinical development: a comparative review of their antibacterial spectrum, pharmacokinetics and clinical efficacy.* Curr Opin Investig Drugs, 2006. **7**(8): p. 740-9.
9. Nelson, M.L. and S.B. Levy, *The history of the tetracyclines.* Ann N Y Acad Sci, 2011. **1241**: p. 17-32.
10. Watve, M.G., et al., *How many antibiotics are produced by the genus Streptomyces?* Archives of Microbiology, 2001. **176**(5): p. 386-390.
11. Baltz, R.H., *Renaissance in antibacterial discovery from actinomycetes.* Current Opinion in Pharmacology, 2008. **8**(5): p. 557-563.
12. Koehn, F.E. and G.T. Carter, *The evolving role of natural products in drug discovery.* Nature Reviews Drug Discovery, 2005. **4**(3): p. 206-220.
13. Katz, L. and R.H. Baltz, *Natural product discovery: past, present, and future.* J Ind Microbiol Biotechnol, 2016. **43**(2-3): p. 155-176.
14. Sun, H.H., et al., *Recent advances in combinatorial biosynthesis for drug discovery.* Drug Design Development and Therapy, 2015. **9**: p. 823-833.
15. Newman, D.J. and G.M. Cragg, *Natural Products as Sources of New Drugs from 1981 to 2014.* J Nat Prod, 2016. **79**(3): p. 629-661.
16. Butler, M.S. and A.D. Buss, *Natural products - The future scaffolds for novel antibiotics?* Biochemical Pharmacology, 2006. **71**(7): p. 919-929.
17. Stratton, C.F., D.J. Newman, and D.S. Tan, *Cheminformatic comparison of approved drugs from natural product versus synthetic origins.* Bioorganic & Medicinal Chemistry Letters, 2015. **25**(21): p. 4802-4807.
18. Blin, K., et al., *antiSMASH 4.0-improvements in chemistry prediction and gene cluster boundary identification.* Nucleic Acids Research, 2017. **45**(W1): p. W36-W41.
19. Skinnider, M.A., et al., *PRISM 3: expanded prediction of natural product chemical structures from microbial genomes.* Nucleic Acids Research, 2017. **45**(W1): p. W49-W54.
20. Kim, J. and G.S. Yi, *PKMiner: a database for exploring type II polyketide synthases.* Bmc Microbiology, 2012. **12**.
21. van Heel, A.J., et al., *BAGEL3: automated identification of genes encoding bacteriocins and (non-)bactericidal posttranslationally modified peptides.* Nucleic Acids Research, 2013. **41**(W1): p. W448-W453.
22. Weber, T., *In silico tools for the analysis of antibiotic biosynthetic pathways.* International Journal of Medical Microbiology, 2014. **304**(3-4): p. 230-235.
23. Weber, T. and H.U. Kim, *The secondary metabolite bioinformatics portal: Computational tools to facilitate synthetic biology of secondary metabolite production.* Synthetic and Systems Biotechnology, 2016. **1**(2): p. 69-79.
24. Baltz, R.H., *Gifted microbes for genome mining and natural product discovery.* J Ind Microbiol Biotechnol, 2017. **44**(4-5): p. 573-588.
25. Baltz, R.H., *Genetic manipulation of secondary metabolite biosynthesis for improved production in Streptomyces and other actinomycetes.* J Ind Microbiol Biotechnol, 2016. **43**(2-3): p. 343-370.
26. Monciardini, P., et al., *Discovering new bioactive molecules from microbial sources.* Microbial Biotechnology, 2014. **7**(3): p. 209-220.
27. Gross, H., *Strategies to unravel the function of orphan biosynthesis pathways: recent examples and future prospects.* Applied Microbiology and Biotechnology, 2007. **75**(2): p. 267-277.
28. Myronovskyi, M., et al., *Generation of a cluster-free Streptomyces albus chassis strains for improved heterologous expression of secondary metabolite clusters.* Metab Eng, 2018. **49**: p. 316-324.

29. Gao, H., et al., *Engineering of a genome-reduced host: practical application of synthetic biology in the overproduction of desired secondary metabolites.* Protein & Cell, 2010. **1**(7): p. 621-626.
30. Nah, H.J., et al., *Cloning and Heterologous Expression of a Large-sized Natural Product Biosynthetic Gene Cluster in Streptomyces Species.* Frontiers in Microbiology, 2017. **8**.
31. Hopwood, D.A., *Forty years of genetics with Streptomyces: from in vivo through in vitro to in silico.* Microbiology-Uk, 1999. **145**: p. 2183-2202.
32. Zhang, H.R., et al., *Methods and options for the heterologous production of complex natural products.* Natural Product Reports, 2011. **28**(1): p. 125-151.
33. Mcdaniel, R., et al., *Engineered Biosynthesis of Novel Polyketides.* Science, 1993. **262**(5139): p. 1546-1550.
34. Floriano, B. and M. Bibb, *afsR is a pleiotropic but conditionally required regulatory gene for antibiotic production in Streptomyces coelicolor A3(2).* Molecular Microbiology, 1996. **21**(2): p. 385-396.
35. Gomez-Escribano, J.P. and M.J. Bibb, *Engineering Streptomyces coelicolor for heterologous expression of secondary metabolite gene clusters.* Microbial Biotechnology, 2011. **4**(2): p. 207-215.
36. Ichinose, K., et al., *Cloning, sequencing and heterologous expression of the medermycin biosynthetic gene cluster of Streptomyces sp. AM-7161: towards comparative analysis of the benzoisochromanequinone gene clusters.* Microbiology-Sgm, 2003. **149**: p. 1633-1645.
37. Otsuka, M., et al., *Cloning, sequencing, and functional analysis of an iterative type I polyketide synthase gene cluster for biosynthesis of the antitumor chlorinated polyenone neocarzilin in "Streptomyces carzinostaticus".* Antimicrobial Agents and Chemotherapy, 2004. **48**(9): p. 3468-3476.
38. Tang, L., et al., *Cloning and heterologous expression of the epothilone gene cluster.* Science, 2000. **287**(5453): p. 640-642.
39. Freitag, A., et al., *Metabolic engineering of the heterologous production of clorobiocin derivatives and elloramycin in Streptomyces coelicolor M512.* Metab Eng, 2006. **8**(6): p. 653-661.
40. Eustaquio, A.S., et al., *Heterologous expression of novobiocin and clorobiocin biosynthetic gene clusters.* Applied and Environmental Microbiology, 2005. **71**(5): p. 2452-2459.
41. Su, C., et al., *Construction of the co-expression plasmids of fostriecin polyketide synthases and heterologous expression in Streptomyces.* Pharmaceutical Biology, 2015. **53**(2): p. 269-274.
42. Kaysser, L., et al., *Identification and Manipulation of the Caprazamycin Gene Cluster Lead to New Simplified Liponucleoside Antibiotics and Give Insights into the Biosynthetic Pathway.* Journal of Biological Chemistry, 2009. **284**(22): p. 14987-14996.
43. Wang, L., et al., *[Engineering and heterologous expression of a nikkomycin biosynthetic gene cluster].* Wei Sheng Wu Xue Bao, 2015. **55**(6): p. 707-18.
44. Du, D., et al., *Improvement of gougerotin and nikkomycin production by engineering their biosynthetic gene clusters.* Applied Microbiology and Biotechnology, 2013. **97**(14): p. 6383-6396.
45. Flinspach, K., et al., *Heterologous expression of the thiopeptide antibiotic GE2270 from Planobispora rosea ATCC 53733 in Streptomyces coelicolor requires deletion of ribosomal genes from the expression construct.* Plos One, 2014. **9**(3): p. e90499.
46. Martinez-Burgo, Y., et al., *Heterologous expression of Streptomyces clavuligerus ATCC 27064 cephamycin C gene cluster.* J Biotechnol, 2014. **186**: p. 21-9.
47. Jones, A.C., et al., *Phage p1-derived artificial chromosomes facilitate heterologous expression of the FK506 gene cluster.* Plos One, 2013. **8**(7): p. e69319.
48. Flinspach, K., et al., *Heterologous Expression of the Biosynthetic Gene Clusters of Coumermycin A(1), Clorobiocin and Caprazamycins in Genetically Modified Streptomyces coelicolor Strains.* Biopolymers, 2010. **93**(9): p. 823-832.

49. Shi, Y.Y., et al., *Biosynthesis of antibiotic chuangxinmycin from Actinoplanes tsinanensis.* Acta Pharmaceutica Sinica B, 2018. **8**(2): p. 283-294.
50. Hou, L.K., et al., *Overexpression of a type III PKS gene affording novel violapyrones with enhanced anti-influenza A virus activity.* Microbial Cell Factories, 2018. **17**.
51. Li, T., et al., *Cloning, Characterization and Heterologous Expression of the Indolocarbazole Biosynthetic Gene Cluster from Marine-Derived Streptomyces sanyensis FMA.* Mar Drugs, 2013. **11**(2): p. 466-488.
52. Li, Q.L., et al., *Identification of the Biosynthetic Gene Cluster for the Anti-infective Desotamides and Production of a New Analogue in a Heterologous Host.* J Nat Prod, 2015. **78**(4): p. 944-948.
53. Iftime, D., et al., *Streptocollin, a Type IV Lanthipeptide Produced by Streptomyces collinus Tu 365.* Chembiochem, 2015. **16**(18): p. 2615-2623.
54. Castro, J.F., et al., *Identification and Heterologous Expression of the Chaxamycin Biosynthesis Gene Cluster from Streptomyces leeuwenhoekii.* Applied and Environmental Microbiology, 2015. **81**(17): p. 5820-5831.
55. Jankowitsch, F., et al., *Genome Sequence of the Bacterium Streptomyces davawensis JCM 4913 and Heterologous Production of the Unique Antibiotic Roseoflavin.* Journal of Bacteriology, 2012. **194**(24): p. 6818-6827.
56. Kaysser, L., et al., *Identification of a Napsamycin Biosynthesis Gene Cluster by Genome Mining.* Chembiochem, 2011. **12**(3): p. 477-487.
57. Robles-Reglero, V., et al., *Transcriptional analysis and proteomics of the holomycin gene cluster in overproducer mutants of Streptomyces clavuligerus.* J Biotechnol, 2013. **163**(1): p. 69-76.
58. Iwasaki, A., H. Kishida, and M. Okanishi, *Molecular-Cloning of a Xylanase Gene from Streptomyces Sp-No-36a and Its Expression in Streptomycetes.* Journal of Antibiotics, 1986. **39**(7): p. 985-993.
59. Pyeon, H.R., et al., *Heterologous expression of pikromycin biosynthetic gene cluster using Streptomyces artificial chromosome system.* Microbial Cell Factories, 2017. **16**.
60. Izawa, M., T. Kawasaki, and Y. Hayakawa, *Cloning and Heterologous Expression of the Thioviridamide Biosynthesis Gene Cluster from Streptomyces olivoviridis.* Applied and Environmental Microbiology, 2013. **79**(22): p. 7110-7113.
61. Bormann, C., V. Mohrle, and C. Bruntner, *Cloning and heterologous expression of the entire set of structural genes for nikkomycin synthesis from Streptomyces tendae Tu901 in Streptomyces lividans.* Journal of Bacteriology, 1996. **178**(4): p. 1216-1218.
62. Filippini, S., et al., *Streptomyces Peucetius Daunorubicin Biosynthesis Gene, Dnrf - Sequence and Heterologous Expression.* Microbiology-Uk, 1995. **141**: p. 1007-1016.
63. Penn, J., et al., *Heterologous production of daptomycin in Streptomyces lividans.* J Ind Microbiol Biotechnol, 2006. **33**(2): p. 121-8.
64. Krawczyk, J.M., et al., *Heterologous Expression and Engineering Studies of Labyrinthopeptins, Class III Lantibiotics from Actinomadura namibiensis.* Chemistry & Biology, 2013. **20**(1): p. 111-122.
65. Malcolmson, S.J., et al., *The posttranslational modification cascade to the thiopeptide berninamycin generates linear forms and altered macrocyclic scaffolds.* Proc Natl Acad Sci U S A, 2013. **110**(21): p. 8483-8.
66. Huang, F., et al., *Heterologous expression and antitumor activity analysis of syringolin from Pseudomonas syringae pv. syringae B728a.* Microbial Cell Factories, 2018. **17**(1): p. 31.
67. Zhao, C., et al., *Heterologous Expression of Spinosyn Biosynthetic Gene Cluster in Streptomyces Species Is Dependent on the Expression of Rhamnose Biosynthesis Genes.* Journal of Molecular Microbiology and Biotechnology, 2017. **27**(3): p. 190-198.

68. Shah, S., et al., *Cloning, characterization and heterologous expression of a polyketide synthase and P-450 oxidase involved in the biosynthesis of the antibiotic oleandomycin.* Journal of Antibiotics, 2000. **53**(5): p. 502-508.
69. Smanski, M.J., et al., *Expression of the Platencin Biosynthetic Gene Cluster in Heterologous Hosts Yielding New Platencin Congeners.* J Nat Prod, 2012. **75**(12): p. 2158-2167.
70. Piel, J., et al., *Cloning, sequencing and analysis of the enterocin biosynthesis gene cluster from the marine isolate 'Streptomyces maritimus': evidence for the derailment of an aromatic polyketide synthase.* Chemistry & Biology, 2000. **7**(12): p. 943-955.
71. Chen, Y., E. Wendt-Pienkowski, and B. Shen, *Identification and utility of FdmR1 as a Streptomyces antibiotic regulatory protein activator for fredericamycin production in Streptomyces griseus ATCC 49344 and heterologous hosts.* Journal of Bacteriology, 2008. **190**(16): p. 5587-5596.
72. Marti, T., et al., *Cloning, nucleotide sequence, and heterologous expression of the biosynthetic gene cluster for R1128, a non-steroidal estrogen receptor antagonist - Insights into an unusual priming mechanism.* Journal of Biological Chemistry, 2000. **275**(43): p. 33443-33448.
73. Ziermann, R. and M.C. Betlach, *Recombinant polyketide synthesis in Streptomyces: Engineering of improved host strains.* Biotechniques, 1999. **26**(1): p. 106-+.
74. Novakova, R., et al., *Increased heterologous production of the antitumoral polyketide mithramycin A by engineered Streptomyces lividans TK24 strains.* Applied Microbiology and Biotechnology, 2018. **102**(2): p. 857-869.
75. Kim, M.S., et al., *Engineered biosynthesis of milbemycins in the avermectin high-producing strain Streptomyces avermitilis.* Microbial Cell Factories, 2017. **16**.
76. Tao, W., et al., *A genomics-led approach to deciphering the mechanism of thiotetronate antibiotic biosynthesis.* Chemical Science, 2016. **7**(1): p. 376-385.
77. Komatsua, M., et al., *Genome-minimized Streptomyces host for the heterologous expression of secondary metabolism.* Proc Natl Acad Sci U S A, 2010. **107**(6): p. 2646-2651.
78. Komatsu, M., et al., *Engineered Streptomyces avermitilis Host for Heterologous Expression of Biosynthetic Gene Cluster for Secondary Metabolites.* Acs Synthetic Biology, 2013. **2**(7): p. 384-396.
79. Sanchez, C., et al., *The biosynthetic gene cluster for the antitumor rebeccamycin: characterization and generation of indolocarbazole derivatives.* Chemistry & Biology, 2002. **9**(4): p. 519-31.
80. Wendt-Pienkowski, E., et al., *Cloning, sequencing, analysis, and heterologous expression of the fredericamycin biosynthetic gene cluster from Streptomyces griseus.* Journal of the American Chemical Society, 2005. **127**(47): p. 16442-16452.
81. Lopez, P., et al., *Isolation of the lysolipin gene cluster of Streptomyces tendae Tu 4042.* Gene, 2010. **461**(1-2): p. 5-14.
82. Makitrynskyy, R., et al., *Genetic factors that influence moenomycin production in streptomycetes.* J Ind Microbiol Biotechnol, 2010. **37**(6): p. 559-566.
83. Kudo, K., et al., *Biosynthetic Origin of the Hydroxamic Acid Moiety of Trichostatin A: Identification of Unprecedented Enzymatic Machinery Involved in Hydroxylamine Transfer.* Journal of the American Chemical Society, 2017. **139**(20): p. 6799-6802.
84. Jiang, G.D., et al., *High-Yield Production of Herbicidal Thaxtomins and Thaxtomin Analogs in a Nonpathogenic Streptomyces Strain.* Applied and Environmental Microbiology, 2018. **84**(11).
85. Schorn, M., et al., *Genetic basis for the biosynthesis of the pharmaceutically important class of epoxyketone proteasome inhibitors.* Acs Chemical Biology, 2014. **9**(1): p. 301-9.
86. Lombo, F., et al., *Deciphering the biosynthesis pathway of the antitumor thiocoraline from a marine actinomycete and its expression in two Streptomyces species.* Chembiochem, 2006. **7**(2): p. 366-376.

87. Chae, C.S., et al., *Production of chromopyrrolic acid by coexpression of inkOD in a heterologous host Streptomyces albus.* Bioorganic & Medicinal Chemistry Letters, 2009. **19**(6): p. 1581-1583.
88. Xu, Z., A. Schenk, and C. Hertweck, *Molecular analysis of the benastatin biosynthetic pathway and genetic engineering of altered fatty acid-polyketide hybrids.* Journal of the American Chemical Society, 2007. **129**(18): p. 6022-30.
89. Gullon, S., et al., *Isolation, characterization, and heterologous expression of the biosynthesis gene cluster for the antitumor anthracycline steffimycin.* Applied and Environmental Microbiology, 2006. **72**(6): p. 4172-4183.
90. Rodriguez Estevez, M., et al., *Heterologous Expression of the Nybomycin Gene Cluster from the Marine Strain Streptomyces albus subsp. chlorinus NRRL B-24108.* Mar Drugs, 2018. **16**(11).
91. Thanapipatsiri, A., et al., *A Streptomyces coelicolor host for the heterologous expression of Type III polyketide synthase genes.* Microbial Cell Factories, 2015. **14**.
92. Baltz, R.H., *Streptomyces and Saccharopolyspora hosts for heterologous expression of secondary metabolite gene clusters.* J Ind Microbiol Biotechnol, 2010. **37**(8): p. 759-72.
93. Roldan-Tapia, M., et al., *Streptomyces as Overexpression System for Heterologous Production of an Antimicrobial Peptide.* Protein and Peptide Letters, 2017. **24**(6): p. 483-488.
94. Anne, J., et al., *Recombinant protein production and streptomycetes.* J Biotechnol, 2012. **158**(4): p. 159-167.
95. Valverde, J.R., S. Gullon, and R.P. Mellado, *Modelling the metabolism of protein secretion through the Tat route in Streptomyces lividans.* Bmc Microbiology, 2018. **18**.
96. Hopwood, D.A., et al., *Plasmids, Recombination and Chromosome Mapping in Streptomyces-Lividans-66.* Journal of General Microbiology, 1983. **129**(Jul): p. 2257-2269.
97. Dyson, P. and H. Schrempf, *Genetic Instability and DNA Amplification in Streptomyces-Lividans 66.* Journal of Bacteriology, 1987. **169**(10): p. 4796-4803.
98. Ochi, K., *From microbial differentiation to ribosome engineering.* Bioscience Biotechnology and Biochemistry, 2007. **71**(6): p. 1373-1386.
99. Ikeda, H., K. Shin-ya, and S. Omura, *Genome mining of the Streptomyces avermitilis genome and development of genome-minimized hosts for heterologous expression of biosynthetic gene clusters.* J Ind Microbiol Biotechnol, 2014. **41**(2): p. 233-250.
100. Chater, K.F. and L.C. Wilde, *Restriction of a Bacteriophage of Streptomyces-Albus G Involving Endonuclease Sali.* Journal of Bacteriology, 1976. **128**(2): p. 644-650.
101. Cox, K.L. and R.H. Baltz, *Restriction of Bacteriophage Plaque-Formation in Streptomyces Spp.* Journal of Bacteriology, 1984. **159**(2): p. 499-504.
102. Chater, K.F. and L.C. Wilde, *Streptomyces-Albus-G Mutants Defective in the Salgi Restriction-Modification System.* Journal of General Microbiology, 1980. **116**(Feb): p. 323-334.
103. Kallifidas, D., et al., *Rational engineering of Streptomyces albus J1074 for the overexpression of secondary metabolite gene clusters.* Microbial Cell Factories, 2018. **17**(1): p. 25.
104. Gunnarsson, N., A. Eliasson, and J. Nielsen, *Control of fluxes towards antibiotics and the role of primary metabolism in production of antibiotics.* Molecular Biotechnology of Fungal Beta-Lactam Antibiotics and Related Peptide Synthetases, 2004. **88**: p. 137-178.
105. Borodina, I., et al., *Antibiotic overproduction in Streptomyces coelicolor A3(2) mediated by phosphofructokinase deletion.* Journal of Biological Chemistry, 2008. **283**(37): p. 25186-25199.
106. Flowers, T.H. and S.T. Williams, *Measurement of Growth-Rates of Streptomycetes - Comparison of Turbidimetric and Gravimetric Techniques.* Journal of General Microbiology, 1977. **98**(Jan): p. 285-289.
107. Richardson, M.A., et al., *Cloning of Spiramycin Biosynthetic Genes and Their Use in Constructing Streptomyces-Ambofaciens Mutants Defective in Spiramycin Biosynthesis.* Journal of Bacteriology, 1990. **172**(7): p. 3790-3798.

108. Alexander, D.C., et al., *Development of a genetic system for combinatorial biosynthesis of lipopeptides in Streptomyces fradiae and heterologous expression of the A54145 biosynthesis gene cluster.* Appl Environ Microbiol, 2010. **76**(20): p. 6877-87.
109. Li, C., et al., *High titer production of tetracenomycins by heterologous expression of the pathway in a Streptomyces cinnamonensis industrial monensin producer strain.* Metab Eng, 2009. **11**(6): p. 319-27.
110. Baltz, R.H., *Strain improvement in actinomycetes in the postgenomic era.* J Ind Microbiol Biotechnol, 2011. **38**(6): p. 657-666.
111. Eustaquio, A.S., et al., *Heterologous expression of novobiocin and clorobiocin biosynthetic gene clusters.* Appl Environ Microbiol, 2005. **71**(5): p. 2452-9.
112. Hu, H.F. and K. Ochi, *Novel approach for improving the productivity of antibiotic-producing strains by inducing combined resistant mutations.* Applied and Environmental Microbiology, 2001. **67**(4): p. 1885-1892.
113. Cai, X.C., et al., *Rifampicin-Resistance Mutations in the rpoB Gene in Bacillus velezensis CC09 have Pleiotropic Effects.* Frontiers in Microbiology, 2017. **8**.
114. Xu, M., et al., *Cross-resistance of Escherichia coli RNA polymerases conferring rifampin resistance to different antibiotics.* Journal of Bacteriology, 2005. **187**(8): p. 2783-92.
115. Hu, H., Q. Zhang, and K. Ochi, *Activation of antibiotic biosynthesis by specified mutations in the rpoB gene (encoding the RNA polymerase beta subunit) of Streptomyces lividans.* Journal of Bacteriology, 2002. **184**(14): p. 3984-91.
116. Hu, H.F., Q. Zhang, and K. Ochi, *Activation of antibiotic biosynthesis by specified mutations in the rpoB gene (encoding the RNA polymerase beta subunit) of Streptomyces lividans.* Journal of Bacteriology, 2002. **184**(14): p. 3984-3991.
117. Hesketh, A., et al., *The global role of ppGpp synthesis in morphological differentiation and antibiotic production in Streptomyces coelicolor A3(2).* Genome Biol, 2007. **8**(8).
118. Xu, J., et al., *A rifampicin resistance mutation in the rpoB gene confers ppGpp-independent antibiotic production in Streptomyces coelicolor A3(2).* Molecular Genetics and Genomics, 2002. **268**(2): p. 179-189.
119. Campbell, E.A., et al., *Structural mechanism for rifampicin inhibition of bacterial RNA polymerase.* Cell, 2001. **104**(6): p. 901-912.
120. Tanaka, Y., et al., *Activation and products of the cryptic secondary metabolite biosynthetic gene clusters by rifampin resistance (rpoB) mutations in actinomycetes.* Journal of Bacteriology, 2013. **195**(13): p. 2959-70.
121. Tanaka, Y., et al., *Activation and Products of the Cryptic Secondary Metabolite Biosynthetic Gene Clusters by Rifampin Resistance (rpoB) Mutations in Actinomycetes.* Journal of Bacteriology, 2013. **195**(13): p. 2959-2970.
122. Shima, J., et al., *Induction of actinorhodin production by rpsL (encoding ribosomal protein S12) mutations that confer streptomycin resistance in Streptomyces lividans and Streptomyces coelicolor A3(2).* Journal of Bacteriology, 1996. **178**(24): p. 7276-7284.
123. Hosoya, Y., et al., *Acquisition of certain streptomycin-resistant (str) mutations enhances antibiotic production in bacteria.* Antimicrobial Agents and Chemotherapy, 1998. **42**(8): p. 2041-2047.
124. Demirci, H., et al., *A structural basis for streptomycin-induced misreading of the genetic code.* Nature Communications, 2013. **4**.
125. Ramakrishnan, V., *Ribosome structure and the mechanism of translation.* Cell, 2002. **108**(4): p. 557-572.
126. Holberger, L.E. and C.S. Hayes, *Ribosomal Protein S12 and Aminoglycoside Antibiotics Modulate A-site mRNA Cleavage and Transfer-Messenger RNA Activity in Escherichia coli.* Journal of Biological Chemistry, 2009. **284**(46): p. 32188-32200.
127. Benitez-Paez, A., et al., *Impairing methylations at ribosome RNA, a point mutation-dependent strategy for aminoglycoside resistance: The rsmG case.* Biomedica, 2014. **34**: p. 41-49.

128. Hosaka, T., et al., *The novel mutation K87E in ribosomal protein S12 enhances protein synthesis activity during the late growth phase in Escherichia coli.* Molecular Genetics and Genomics, 2004. **271**(3): p. 317-324.
129. Okamoto-Hosoya, Y., T. Hosaka, and K. Ochi, *An aberrant protein synthesis activity is linked with antibiotic overproduction in rpsL mutants of Streptomyces coelicolor A3(2).* Microbiology-Sgm, 2003. **149**: p. 3299-3309.
130. Hosaka, T., J. Xu, and K. Ochi, *Increased expression of ribosome recycling factor is responsible for the enhanced protein synthesis during the late growth phase in an antibiotic-overproducing Streptomyces coelicolor ribosomal rpsL mutant.* Molecular Microbiology, 2006. **61**(4): p. 883-897.
131. Tanaka, Y., et al., *Antibiotic Overproduction by rpsL and rsmG Mutants of Various Actinomycetes.* Applied and Environmental Microbiology, 2009. **75**(14): p. 4919-4922.
132. Wang, G.J., T. Hosaka, and K. Ochi, *Dramatic activation of antibiotic production in Streptomyces coelicolor by cumulative drug resistance mutations.* Applied and Environmental Microbiology, 2008. **74**(9): p. 2834-2840.
133. Nishimura, K., et al., *Mutations in rsmG, encoding a 16S rRNA methyltransferase, result in low-level streptomycin resistance and antibiotic overproduction in Streptomyces coelicolor A3(2).* Journal of Bacteriology, 2007. **189**(10): p. 3876-3883.
134. Tanaka, Y., S. Tokuyama, and K. Ochi, *Activation of secondary metabolite-biosynthetic gene clusters by generating rsmG mutations in Streptomyces griseus.* Journal of Antibiotics, 2009. **62**(12): p. 669-673.
135. Tamehiro, N., et al., *Innovative approach for improvement of an antibiotic- overproducing industrial strain of Streptomyces albus.* Applied and Environmental Microbiology, 2003. **69**(11): p. 6412-6417.
136. doi:10.1039/9781847551535-00001
137. Choudoir, M.J., C. Pepe-Ranney, and D.H. Buckley, *Diversification of Secondary Metabolite Biosynthetic Gene Clusters Coincides with Lineage Divergence in Streptomyces.* Antibiotics-Basel, 2018. **7**(1).
138. Hill, A.M., *The biosynthesis, molecular genetics and enzymology of the polyketide-derived metabolites.* Natural Product Reports, 2006. **23**(2): p. 256-320.
139. Meier, J.L. and M.D. Burkart, *The chemical biology of modular biosynthetic enzymes.* Chem Soc Rev, 2009. **38**(7): p. 2012-2045.
140. Sikandar, A. and J. Koehnke, *The role of protein-protein interactions in the biosynthesis of ribosomally synthesized and post-translationally modified peptides.* Natural Product Reports, 2019.
141. Yang, X. and W.A. van der Donk, *Ribosomally Synthesized and Post-Translationally Modified Peptide Natural Products: New Insights into the Role of Leader and Core Peptides during Biosynthesis.* Chemistry-a European Journal, 2013. **19**(24): p. 7662-7677.
142. Ortega, M.A. and W.A. van der Donk, *New Insights into the Biosynthetic Logic of Ribosomally Synthesized and Post-translationally Modified Peptide Natural Products.* Cell Chemical Biology, 2016. **23**(1): p. 31-44.
143. Arnison, P.G., et al., *Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature (vol 30, pg 108, 2013).* Natural Product Reports, 2013. **30**(12): p. 1568-1568.
144. Dunbar, K.L. and D.A. Mitchell, *Revealing Nature's Synthetic Potential through the Study of Ribosomal Natural Product Biosynthesis (vol 8, pg 473, 2013).* Acs Chemical Biology, 2013. **8**(5): p. 1083-1083.
145. Zheng, Q.F., et al., *Thiopeptide Antibiotics Exhibit a Dual Mode of Action against Intracellular Pathogens by Affecting Both Host and Microbe.* Chemistry & Biology, 2015. **22**(8): p. 1002-1007.

146. Mohr, K.I., et al., *Pinensins: The First Antifungal Lantibiotics.* Angewandte Chemie-International Edition, 2015. **54**(38): p. 11254-11258.
147. Essig, A., et al., *Copsin, a Novel Peptide-based Fungal Antibiotic Interfering with the Peptidoglycan Synthesis.* Journal of Biological Chemistry, 2014. **289**(50): p. 34953-34964.
148. Hayakawa, Y., et al., *Thioviridamide, a novel apoptosis inducer in transformed cells from Streptomyces olivoviridis.* Journal of Antibiotics, 2006. **59**(1): p. 1-5.
149. Ferir, G., et al., *The Lantibiotic Peptide Labyrinthopeptin A1 Demonstrates Broad Anti-HIV and Anti-HSV Activity with Potential for Microbicidal Applications.* Plos One, 2013. **8**(5).
150. Richard, A.S., et al., *Virion-associated phosphatidylethanolamine promotes TIM1-mediated infection by Ebola, dengue, and West Nile viruses.* Proc Natl Acad Sci U S A, 2015. **112**(47): p. 14682-14687.
151. Letzel, A.C., S.J. Pidot, and C. Hertweck, *Genome mining for ribosomally synthesized and post-translationally modified peptides (RiPPs) in anaerobic bacteria.* BMC Genomics, 2014. **15**.
152. Gross, E. and J.L. Morell, *The structure of nisin.* Journal of the American Chemical Society, 1971. **93**(18): p. 4634-5.
153. van der Donk, W.A. and S.K. Nair, *Structure and mechanism of lanthipeptide biosynthetic enzymes.* Curr Opin Struct Biol, 2014. **29**: p. 58-66.
154. Li, B., et al., *Structure and mechanism of the lantibiotic cyclase involved in nisin biosynthesis.* Science, 2006. **311**(5766): p. 1464-1467.
155. Helfrich, M., K.D. Entian, and T. Stein, *Structure-function relationships of the lanthionine cyclase SpaC involved in biosynthesis of the Bacillus subtilis peptide antibiotic subtilin.* Biochemistry, 2007. **46**(11): p. 3224-3233.
156. Lubelski, J., et al., *Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin.* Cellular and Molecular Life Sciences, 2008. **65**(3): p. 455-476.
157. Jones, A.M. and J.M. Helm, *Emerging Treatments in Cystic Fibrosis.* Drugs, 2009. **69**(14): p. 1903-1910.
158. Gomes, K.M., R.S. Duarte, and M.D.D. Bastos, *Lantibiotics produced by Actinobacteria and their potential applications (a review).* Microbiology-Sgm, 2017. **163**(2): p. 109-121.
159. Boakes, S., et al., *Organization of the genes encoding the biosynthesis of actagardine and engineering of a variant generation system.* Molecular Microbiology, 2009. **72**(5): p. 1126-1136.
160. Zheng, Q.F., H. Fang, and W. Liu, *Post-translational modifications involved in the biosynthesis of thiopeptide antibiotics.* Organic & Biomolecular Chemistry, 2017. **15**(16): p. 3376-3390.
161. Hegemann, J.D., et al., *Lasso Peptides: An Intriguing Class of Bacterial Natural Products.* Accounts of Chemical Research, 2015. **48**(7): p. 1909-1919.
162. Rebuffat, S., et al., *Microcin J25, from the macrocyclic to the lasso structure: Implications for biosynthetic, evolutionary and biotechnological perspectives.* Current Protein & Peptide Science, 2004. **5**(5): p. 383-391.
163. Knappe, T.A., et al., *Isolation and structural characterization of capistruin, a lasso peptide predicted from the genome sequence of Burkholderia thailandensis E264.* Journal of the American Chemical Society, 2008. **130**(34): p. 11446-11454.
164. Sardar, D. and E.W. Schmidt, *Combinatorial biosynthesis of RiPPs: docking with marine life.* Current Opinion in Chemical Biology, 2016. **31**: p. 15-21.
165. Hudson, G.A. and D.A. Mitchell, *RiPP antibiotics: biosynthesis and engineering potential.* Current Opinion in Microbiology, 2018. **45**: p. 61-69.

2. Publications

I

Effect of "ribosome engineering" on the transcription level and production of *S. albus* indigenous secondary metabolites

Lopatniuk M., Myronovskyi M., Nottebrock A., Busche T., Kalinowski J., Ostash B., Fedorenko V., Luzhetskyy A.

Appl Microb Biot, **2019**, 103(17):7097-7110. https://doi.org/10.1007/s00253-019-10005-y.
Published online 19th July 2019

Lopatniuk M. has conducted experiments on construction the BAC-library with mutated *rpsL*-variants, construction of the platform strain, metabolic profile of the strains, and writing of the manuscript. **Myronovskyy M**. supervised the work, participated in planning of the experiments and manuscript evaluation. **Nottebrock A**. conducted experiments on metabolic profiling of mutant strains with *rsmG*-deletion. **Busche T**. has performed RNA-isolation from the mutant strains, RNA-sequencing, differential analysis of the data, and manuscript evaluation. **Kalinowski J.** has provided the RNA-sequencing facility and participated in manuscript evaluation. **Ostash B**. participated in planning of the experiments and manuscript correction. **Fedorenko V.** participated in planning of the experiments. **Luzhetskyy A.** supervised the work, participated in planning of the experiments and critical manuscript reading and correction.

Author's name	Contribution to the work, %
Lopatniuk M.	65
Myronovskyi M.	5
Nottebrock A.	5
Busche T.	5
Kalinowski J.	5
Ostash B.	5
Fedorenko V.	5
Luzhetskyy A.	5

Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-019-10005-y

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Effect of "ribosome engineering" on the transcription level and production of *S. albus* indigenous secondary metabolites

Maria Lopatniuk[1] · Maksym Myronovskyi[1] · Alessia Nottebrock[1] · Tobias Busche[2] · Jörn Kalinowski[2] · Bohdan Ostash[3] · Viktor Fedorenko[3] · Andriy Luzhetskyy[1,4]

Received: 18 January 2019 / Revised: 11 June 2019 / Accepted: 30 June 2019

Abstract

Significant resources are invested into efforts to improve the production yields of natural products from *Actinobacteria*, a well-recognized source of leads for several industries, most notably pharmaceutical one. Introduction of changes into genes for ribosomal protein S12 (*rpsL*) and/or 16S rRNA methylation (*rsmG*) is one of traditional approaches (referred to as ribosomal engineering) towards actinobacterial strain improvement. Yet, true potential of ribosome engineering remains unknown as it is currently coupled to empirical selection for aminoglycoside-resistance; *rpsL* mutations without such phenotypic expression could not be isolated. Here, we report a systematic and rational ribosome engineering approach to study the effect of a range of *rpsL* mutations on the production level of different biosynthetic gene clusters (BGC). The severe effect of diverse *rpsL* mutations together with deletion of *rsmG* engineered in *Streptomyces albus* has been revealed on the transcription level of several indigenous BGCs. The aforementioned mutations strongly impacted the transcription of indigenous BGCs, possibly because they alter the transcription of BGC-situated and global regulatory genes. The *rsmG* deletion with certain *rpsL* mutations can have a synergistic effect on the transcription level of indigenous BGCs. Our work thus provides the first streptomycete platform for rational engineering and study of virtually any nonlethal *rpsL* mutation. The tremendous effect of ribosome engineering on the transcription profile of the strains was reported for the first time. A library of described *S. albus rpsL**/Δ*rsmG* strains represents a useful tool for overproducing known secondary metabolites and activating silent biosynthetic gene clusters in *Actinobacteria*.

Keywords Rational ribosome engineering · Natural products · Transcription profile · *Streptomyces*

Introduction

Members of the phylum *Actinobacteria* produce the majority of "specialized metabolites" that are used in clinical practice. Among this group, *Streptomyces* species are considered the most valuable because they produce over two-thirds of clinically used drugs of natural origin, and they remain the main source of new antimicrobial, antiparasitic, antitumoral, and antiviral compounds (Genilloud 2017). The rapid development of genome sequencing technologies along with bioinformatics tools for mining biosynthetic gene clusters (BGCs), such as antiSMASH (Weber et al. 2015) and PRISM (Skinnider et al. 2015), has allowed the hidden potential of *Actinobacteria* to produce a huge variety of natural compounds to be revealed (Baltz 2017). However, most of the BGCs in *actinomycetes* have been found to be either silent under standard laboratory conditions or to encode compounds that are detectable in crude extracts as only microgram quantities, which is far below the amount required for further

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-019-10005-y) contains supplementary material, which is available to authorized users.

✉ Andriy Luzhetskyy
Andriy.Luzhetskyy@helmholtz-hips.de

[1] Department Pharmacy, Actinobacteria Metabolic Engineering Group, Saarland University, Uni Campus C2.3, 66123 Saarbrücken, Germany

[2] Centre for Biotechnology (CiBiTec), Bielefeld University, Universitätsstraße 27, 33615 Bielefeld, Germany

[3] Department of Genetics and Biotechnology, Ivan Franko National University of Lviv, Hrushevskyy Street., 4, Lviv 79005, Ukraine

[4] Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Uni Campus E8.1, 66123 Saarbrücken, Germany

Published online: 19 July 2019

exploration (Ren et al. 2017). In addition, metabolic engineering of the relevant biosynthetic pathways is often hindered because it is difficult to perform genetic manipulations in the native producers (Bekiesch et al. 2016). To overcome these major obstacles during drug discovery and development, and successfully acquire these invaluable multiple, potentially bioactive molecules, we need a robust production platform that supplies natural products in addition to their derivatives, analogs, and mimetics in sufficient quantities. Thus, BGCs, which are responsible for the production of bioactive molecules, are often expressed heterologous in well-studied and/or engineered host strains, such as *Streptomyces coelicolor* (Gomez-Escribano and Bibb 2011), *S. venezuelae* (Kim et al. 2015), *S. avermitilis* (Komatsu et al. 2013), and *S. albus* (Myronovskyi et al. 2018; Zaburannyi et al. 2014). However, BGCs are usually expressed at low levels in host strains, and this slows the drug development process (Bekiesch et al. 2016).

It has been reported that certain streptomycin resistance mutations often result in increases in the production yields of natural products in *Actinobacteria*, although the specific mechanism underlying this phenomenon is not well understood (Tanaka et al. 2009a). These mutations occur in the *rpsL* gene, which encodes the S12 protein of the ribosomal small subunit. The S12 protein is located in the ribosomal A-site and plays a crucial role in the decoding process (Holberger and Hayes 2009). Streptomycin binds to the ribosome A-site and induces decoding errors. Mutated S12 variants stabilize the open A-site conformation and prevent streptomycin from binding (Holberger and Hayes 2009). Many streptomycin resistance mutations have been demonstrated to be useful tools for improving antibiotic production levels (Ochi 2007). Most of these mutations are located near Asp-89 in the conserved loop region of the S12 protein (Arg-83–Arg-94) (Okamoto-Hosoya et al. 2003b). This loop is located at the ribosomal active site proximal to the A-site and is believed to be important for translation accuracy because mutations in this region lead to hyper-accurate ribosome phenotypes (Agarwal et al. 2011). The K88E, L90K, and R94G substitutions activated undecylprodigiosin production in *S. lividans* (Okamoto-Hosoya et al. 2003b); the K88R mutation increased salinomycin production by 1.5-fold in an industrial strain of *S. albus (Tamehiro* et al. 2003*)*; in *S. coelicolor*, strains with the P91S substitution produced an up to 10-fold more actinorhodin than was produced by the original strain (Okamoto-Hosoya et al. 2003a); and *S. coelicolor* strains with both the K88E mutation and an insertion mutation at GI92 produced substantially higher levels of actinorhodin (Wang et al. 2009). Mutations in the *rpsL* gene are widely used in industry to improve the level of production in a variety of antibiotic-producing strains (Beltrametti et al. 2006; Tamehiro et al. 2003). However, some reports have shown that identical mutations can have the opposite effect on the production yields of different antibiotics (Baltz 2011). For example, the K88E mutations abolished avermectin production in *S. avermitilis,* while the K88R mutation decreased erythromycin production by 4-fold in *S. erythraea* (Tanaka et al. 2009a). Thus, mutations in the *rpsL* gene influence antibiotic production levels in different ways. The reasons for these differences remain poorly understood.

Another method of "ribosome engineering" involves the inactivation of the *rsmG* gene, which encodes an *S*-adenosylmethionine (SAM)-dependent 16S rRNA methyltransferase. The deletion of the *rsmG* gene resulted in the overproduction of actinorhodin in *S. coelicolor* (Nishimura et al. 2007). Mutations in this gene have also been shown to activate the expression of silent or poorly expressed BGCs in *S. griseus* (Tanaka et al. 2009b).

One significant obstacle to studying the effects of *rpsL* and *rsmG* mutations on natural products biosynthesis is the random nature of their origin and the fact that the effect of other unidentified mutations within the actinobacterial genome cannot be excluded. Furthermore, some mutations were studied in merodiploid state (wild-type allele on the chromosome and a mutated one on multicopy plasmid), blurring the conclusions of the study. Finally, the data were obtained in different actinomycetes strains, producing biogenetically different antibiotics.

The aim of this study was bipartite. First, we set out to develop a platform for precise and straightforward engineering of any viable *rpsL* mutation into the genome of *Streptomyces albus* J1074, one of the most popular strains for heterologous expression purposes. Our approach allowed us to introduce mutations in an essential gene without leaving any extra DNA sequences within the genome. Second, we recreated in J1074 a subset of most useful *rpsL* mutations and systemically studied their effects on the transcription level and production level of native BGCs. We further studied the effects of *rsmG* gene deletion on secondary metabolism in the context of *rpsL* mutations. Comprehensive metabolomic and transcriptomic analysis of the generated *rpsL/rsmG* mutants portrayed their deep impact on natural product biosynthesis and, unexpectedly, changes in the transcriptional activity of a number of genes for regulatory proteins and sigma factors. Besides novel prospects for exploration of ribosome engineering, our work provides, in a single and convenient streptomycete species, perhaps the most diverse library of *rpsL* mutants for practical needs of natural product research.

Materials and methods

Strains, plasmids, and medium conditions

The *S. albus* delPse strain was used as the original strain to introduce mutations and deletions. All strains derived during this study are listed in Table 1. *Escherichia coli* XL1-Blue was used

Table 1 Descriptions of the strains used in this study

Name of the strain	Description
Streptomyces albus ΔpseB4 strain	Initial strain used for our research (Bilyk and Luzhetskyy 2014)
S. albus ΔrsmG	*S. albus ΔpseB4* strain with knocked out *rsmG* gene
S. albus K88E	*S. albus ΔpseB4* strain with K88E mutation in *rpsL* gene
S. albus K88E ΔrsmG	*S. albus ΔpseB4* strain with K88E mutation in *rpsL* gene and knocked out *rsmG* gene
S. albus GI92	*S. albus ΔpseB4* strain with GI92 mutation in *rpsL* gene
S. albus GI92 ΔrsmG	*S. albus ΔpseB4* strain with GI92 mutation in *rpsL* gene and knocked out *rsmG* gene
S. albus K88E-GI92	*S. albus ΔpseB4* strain with K88E and GI92 mutations in *rpsL* gene
S. albus K88E-GI92 ΔrsmG	*S. albus ΔpseB4* strain with K88E and GI92 mutations in *rpsL* gene and knocked out *rsmG* gene
S. albus P91S	*S. albus ΔpseB4* strain with P91S mutation in *rpsL* gene
S. albus P91S ΔrsmG	*S. albus ΔpseB4* strain with P91S mutation in *rpsL* gene and knocked out *rsmG* gene
S. albus K88E-P91S	*S. albus ΔpseB4* strain with K88E and P91S mutations in *rpsL* gene
S. albus K88E-P91S ΔrsmG	*S. albus ΔpseB4* strain with K88E and P91S mutations in *rpsL* gene and knocked out *rsmG* gene
S. albus R86P	*S. albus ΔpseB4* strain with R86P mutation in *rpsL* gene
S. albus R86P ΔrsmG	*S. albus ΔpseB4* strain with R86P mutation in *rpsL* gene and knocked out *rsmG* gene
S. albus R94G	*S. albus ΔpseB4* strain with R94G mutation in *rpsL* gene
S. albus R94G ΔrsmG	*S. albus ΔpseB4* strain with R94G mutation in *rpsL* gene and knocked out *rsmG* gene

for cloning experiments, and ET12567 pUZ8002 was used for intergeneric conjugation. The following antibiotics were used at the indicated concentrations: apramycin, 50 μg/ml; kanamycin, 50 μg/ml; hygromycin, 50 μg/ml; phosphomycin, 200 μg/ml, and nalidixic acid, 50 μg/ml. The strains were cultivated on standard solid media as follows: *S. albus* on mannitol soya flour (MS) medium (20 g of soya powder, 20 g of mannitol, 16 g of agar, 1 l of water) and *E. coli* on LB agar (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 16 g of agar, 1 l of water).

DNA manipulation

All DNA manipulations were performed according to standard protocols (Sambrook and Russell 2006). The *S. albus* BAC library was used to introduce mutations into the *rpsL* gene and the deletion of *rsmG*. All of the primers used in this study are listed in Table 1.

Construction of a BAC library with different rpsL gene variants

Two-step PCR mutagenesis was performed to construct *rpsL* gene variants with point mutations. A pair of primers specific to a certain mutation was used in combination with rpsL-chk-for (-rev) primers to amplify the *rpsL* gene in two steps (Fig. 1, Table S1). Moreover, RedET technology was used to replace the native *rpsL* gene in BAC 1A11 with a hygromycin resistance gene (*hph*) flanked by MssI restriction sites (Zhang et al. 2000). Then, the resistance gene was cut out with MssI endonuclease, and the resulting linearized BAC was used for recombination with mutated *rpsL* variants. The nine BACs obtained in these experiments were sequenced to verify the presence of mutations.

Construction of the S. albus platform strain

The apramycin resistance gene (*am*) was amplified with the am-rpsL-for and am-rpsL-rev primers. The resulting cassette was introduced upstream of the *rpsL* gene on BAC 1F1 via RedET. The resulting BAC, 1F1-am-rpsL, was transferred conjugally into the *S. albus delPse* strain. The first/second crossover was selected by the presence/absence of GusA beta-glucuronidase activity (Myronovskyi et al. 2011). The generated strain, *S. albus/am-rpsL,* was used for further modifications. The hygromycin resistance gene (*hyg*) was amplified with the hyg-rpsL-for and hyg-rpsL-rev primers. The resulting cassette was introduced downstream of the *rpsL* gene on BAC 1F1 via RedET. The resulting BAC, 1F1-rpsL-hyg, was conjugated into the *S. albus/am-rpsL* strain. The new strain, *S. albus/am-rpsL-hyg* (*S. albus DM*), contained an *rpsL* gene flanked with resistance markers on each side.

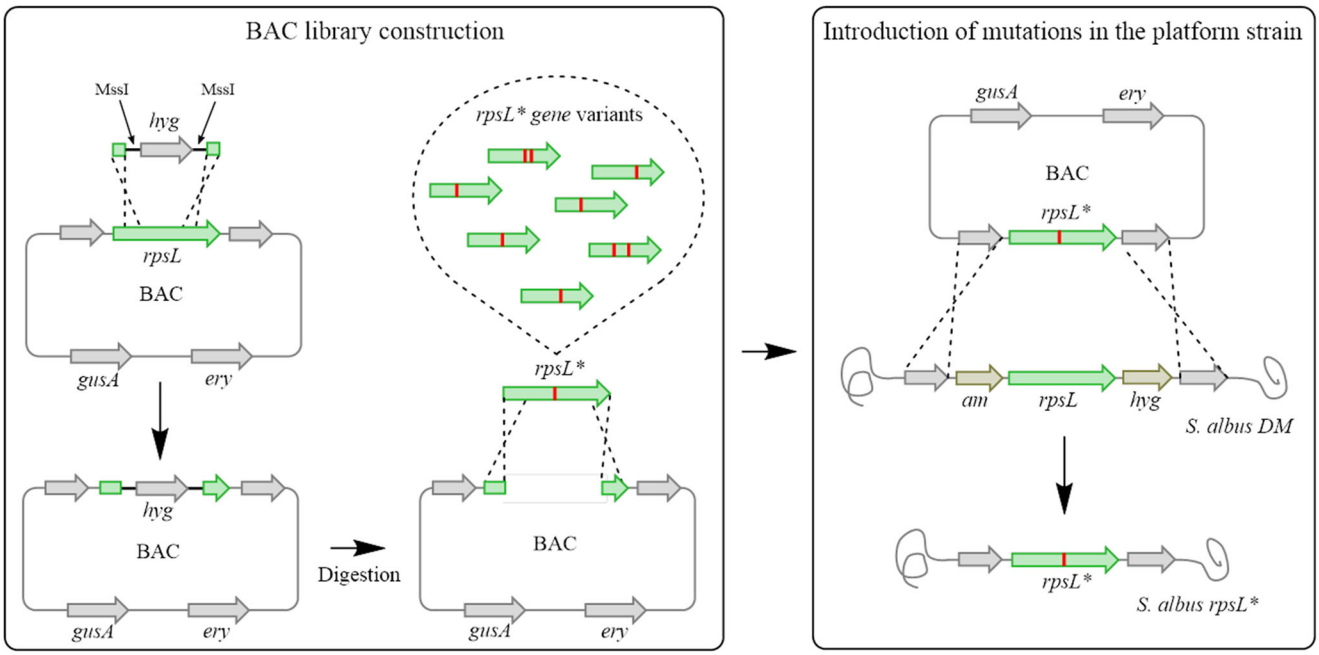


Fig. 1 Experimental workflow of the construction of a BAC library of diverse *rpsL* gene variants and the introduction of mutations into the *S. albus DM* platform strain

Knock-out of the rsmG methyltransferase gene

The function of the *rsmG* gene was abolished by an in-frame deletion. First, the apramycin resistance cassette (Iterative Marker Excision System, IMES) in the patt-saac-oriT plasmid (Myronovskyi et al. 2014) was amplified with the del-rsmG-for and del-rsmG-rev primers. Then, the amplified fragment was used to replace the *rsmG* gene in BAC 1 L5 using RedET recombination. The resulting recombinant BAC 1L5del*rsmG*:*Am* was introduced via conjugation into *S. albus* strains possessing *rpsL* mutations. Screening for double-crossover mutants was performed on MS medium supplemented with 50 μg/ml of apramycin and 70 μg/ml of X-gluc. The excision of the marker was performed by expressing phiC31 integrase in the pKHint31 plasmid in double-crossover mutants (Myronovskyi et al. 2014). The gene deletion and marker excision were confirmed via PCR using an appropriate primer set (rsmG-chk-for and rsmG-chk-rev).

Determination of MIC

The MICs were determined by incubation of spore suspension in liquid TSB media with different concentrations of streptomycin, paromomycin, and gentamycin, starting with 200 μg/ml and then diluted twice in each next well. The strains were incubated in 96-well plates for 48 h at 28 °C 900 rpm platform. Then, the mycelium was stained with thiazolyl blue tetrazolium bromide, to visualize the growth of the strains.

Antibiotic extraction

To extract the antibiotics, the strains were grown in tryptic soy broth (TSB) medium for 48 h at 28 °C and 180 rpm, and 1 ml of the seed culture was then transferred to 50 ml of production medium NL19 (20 g of soya powder, 20 g of mannitol, 1 l of water). After the cultures were grown for 6 days on a rotary shaker at 28 °C and 180 rpm, the cells were harvested by centrifugation. The supernatant was extracted with equal amounts of ethyl acetate. To evaluate the levels of production in the cultures, extracts were dried and dissolved in methanol.

RNA isolation, library preparation, cDNA sequencing, and bioinformatics

For total RNA isolation, *S. albus* cells were grown in NL19 medium (for indigenous BGC expression). Then, 2 ml of 2-day and 3-day cultures were spun down for 20 s at 14,000× rpm, and the pellets were immediately frozen in liquid nitrogen and stored at − 80 °C. Total RNA extraction was performed using an RNeasy Kit (Qiagen, Hilden, Germany) as previously described (Huser et al. 2003). An RNase-Free DNase set (Qiagen) was used two times for on-column DNA digestion, and an additional DNase treatment was then performed with a DNase I kit (Roche Diagnostics, Mannheim, Germany) to ensure that all DNA was completely removed. To check the RNA samples for DNA contamination, PCR was performed using oligonucleotides designed to create two different products approximately 150 bp and 500 bp in size. Initially, RNA quality was checked with Trinean Xpose

(Gentbrugge, Belgium) and Agilent RNA Nano 6000 kits on an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). A Ribo-Zero rRNA Removal Kit for bacteria was obtained from Illumina (San Diego, CA, USA) and used to remove ribosomal RNA molecules from isolated total RNA. rRNA removal was checked using an Agilent RNA Pico 6000 kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). A TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) was used to prepare cDNA libraries (Koepff et al. 2017). The resulting cDNAs were pair-end sequenced on an Illumina HiSeq 1500 system (San Diego, CA, USA) using a 70 bp read length. RNA-seq raw data files are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under E-MTAB-7715. The cDNA reads were mapped to the *Streptomyces albus* J1074 (NC_020990.1) using bowtie2 v2. 2.7(Langmead and Salzberg 2012) with default settings for paired-end read mapping. All mapped sequence data were converted from SAM to BAM format with SAMtools v1.3 (Li et al. 2009) and imported to the software ReadXplorer v. 2.2. Short read alignments and differential gene expression were illustrated using ReadXplorer 2.2.0 (Hilker et al. 2016) and DEseq (Anders and Huber 2010), respectively.

HPLC-MS analyses

The extracts were analyzed on a Dionex Ultimate 3000 HPLC coupled with a Bruker AmaZon mass spectrometer with electrospray ionization. The Nucleodur BEH C18 column (1.7 μm, 100 mm × 2.1 mm) was used with water + 0.1 % formic acid and acetonitrile + 0.1 % formic acid as the mobile phase. The 18-min linear gradient was used (5 to 95%) with a flow rate of 0.6 ml/min.

Results

Construction of a BAC library with diverse rpsL gene variants

To investigate the effects of different mutations in the *rpsL* gene on the expression levels of diverse BGCs in *S. albus* J1074, a BAC library containing mutated *rpsL* genes was constructed. Seven mutations, including K88R, K88E, GI92, P91S, R86P, L90K, R94G, K88E-GI92, and K88E-P91S, and combinations of these mutations were chosen for this study. All of the selected mutations and combinations have previously been shown to cause antibiotic overproduction and/or cryptic cluster activation (Okamoto-Hosoya et al. 2003b; Okamoto-Hosoya et al. 2000; Shima et al. 1996; Tanaka et al. 2009a; Wang et al. 2009).

Site-directed mutagenesis of the *rpsL* gene was performed using two-step PCR, with an appropriate pair of primers used for each different mutation (Table S1). We obtained nine amplified *rpsL* genes, each of which contained a mutation in a desired location. The BAC vector containing the *rpsL* gene was modified to allow the site-specific introduction of mutated *rpsL* genes. To perform these experiments, a cassette containing the *hph* gene flanked by MssI endonuclease recognition sites was constructed by PCR (Fig. 1). The cassette was next introduced into the native *rpsL* gene on the BAC via RedET (Zhang et al. 2000) by replacing a few nucleotides at the same time. Digesting the modified BAC with the MssI endonuclease restriction enzyme provided us with a linearized vector that was disrupted in the middle of the *rpsL* gene. Then, the linearized BAC vector and the mutated *rpsL* gene variants were used to obtain a BAC library containing a set of vectors with mutations inserted in the *rpsL* gene via RedET in *E. coli* (Fig. 1). All nine BACs were sequenced to prove that the mutations were present (data not shown).

Construction of a platform strain for the direct introduction of mutated rpsL variants into the chromosome

The *S. albus ΔpseB4* strain (Bilyk and Luzhetskyy 2014) was used to construct a platform strain to enable the convenient and efficient introduction of point mutations directly into the chosen gene on the chromosome. The aim was to flank the *rpsL* locus with two selective markers. Because an erythromycin resistance gene (*ery*) was already present in the BAC backbone, we decided to use *am* and *hyg* resistance genes for the platform strain. Two BACs, each containing a resistance gene (*am* or *hyg*) flanking the *rpsL* gene, were constructed and used to insert the corresponding resistance gene into the *S. albus* chromosome. The final strain, *S. albus* DM, carried the *rpsL* gene flanked by two resistance markers (Fig. 1).

This platform strain allowed us to substitute the native *rpsL* gene, now flanked by markers, with the mutated *rpsL* allele in the BAC via double crossover. Positive clones then are selected based on their sensitivity to apramycin and hygromycin. After substitution, no scars or additional nucleotide sequences are left on the chromosome (Fig. 1). This strategy can be used to introduce viable mutations into any essential gene.

Introduction of mutated rpsL variants into the platform strain

All BACs with mutated *rpsL* genes were transferred into the platform strain via conjugation with *E. coli*, and clones were selected after double crossover based on the loss of GusA activity (Fig. 1) (Myronovskyi et al. 2011). Two of the mutations, K88R and L90K, could not be introduced into the platform strain. The L90K mutation increased the production of undecylprodigiosin by *S. lividans* when the mutated L90K-*rpsL* variant was on the replicative plasmid (Okamoto-Hosoya

et al. 2003b). The K88R mutation was previously described as being associated with a low level of resistance to streptomycin and an increase in the production of salinomycin in *Streptomyces* sp. (Tamehiro et al. 2003). In our experiments, the mutated genes would be located directly on the chromosomes and present as one copy, and the resulting genomes would lack native *rpsL* gene as a second copy. Our data suggest that L90K and K88R *rpsL* substitutions are viable only under permissive conditions, such as the presence of wild type *rpsL* gene or other, as-yet-unknown mutations.

Finally, from the clones obtained after double crossover, we selected those that were sensitive to apramycin and hygromycin because they were expected to carry a mutated *rpsL* gene. All new strains possessing mutations (Table 1) were verified by amplification of the *rpsL* gene from the chromosome of the derived strains following by sequencing of the amplified product (data not shown).

Knock out of the 16S rRNA methyltransferase G gene in rpsL mutant strains

Another "ribosome engineering" approach that can be used to influence natural product biosynthesis is to inactivate the 16S rRNA methyltransferase G (*rsmG*) gene by mutating its active site or deleting the whole gene (Tanaka et al. 2009a, b). Similar to the *rpsL* mutants, strains with a mutated (or deleted) *rsmG* gene possessed resistance to streptomycin. An in-frame deletion was introduced in the *rsmG* gene in an array of aforementioned *rpsL* mutants. To achieve a markerless knock-out, the IMES was used (Myronovskyi et al. 2014). The *rsmG* gene was replaced with an apramycin resistance gene, which was also excised by PhiC31 recombinase leaving a 48 nucleotide-long fragment. We succeeded in obtaining eight strains with deleted *rsmG* genes, including one strain with an unmodified *rpsL* gene and seven strains with mutations in *rpsL* (Table 1). There were no obvious differences in the growth or formation of aerial mycelium and spores between the *S. albus rsmG* mutant and the *rpsL-rsmG* double mutants.

Some *rpsL* mutations were reported to confer different level of resistance to aminoglycoside antibiotics (Okamoto-Hosoya et al. 2000; Tanaka et al. 2009a; Wang et al. 2009, 2008). We have analyzed the minimal inhibitory concentration of all constructed strains on three antibiotics: streptomycin, paromomycin, and gentamicin (Table 2). Mutation K88E increased the resistance of the strain to streptomycin with MIC 200 μg/ml, alone and in combination with GI92 and P91S. Deletion of *rsmG* gene was reported to give a low level of resistance to streptomycin (Tanaka et al. 2009a); however, we observed the same MIC of 200 μg/ml in *S. albus* del *rsmG* strain as in K88E mutants. Mutations GI92 and R86P resulted in MIC of 25 μg/ml for both, P91S—12.5 μg/ml, and the *S. albus* strain with R94G mutation was almost the same sensitive to streptomycin as the original *S. albus ΔpseB4*, MIC of 3.13 μg/ml and 1.56 μg/ml, respectively. Additionally combination of *rsmG* deletion with the *rpsL* mutations elevated the MIC to 200 μg/ml almost in all of them, with the only exception of R94G, here it was increased to 50 μg/ml. We observed a slight increase of MIC for paromomycin in mutants with GI92, K88E-GI92, P91S, and K88E-P91S, but in combination with *rsmG* deletion, this effect was diminished. With gentamycin we did not observe any changes, all strains are highly sensitive to gentamicin. This analysis showed that with our methodology, we can obtain mutation that does not confer the high level of resistance to streptomycin and can be simply lost in the usual screening for *rpsL* mutations with selection on high concentrations of antibiotic.

Effect of "ribosome engineering" on the transcription level and production of S. albus indigenous secondary metabolites

S. albus ΔpseB4 strain derives from J1074 that in turn is a *Sal*I system-deficient strain of *S. albus* G (Zaburannyi et al. 2014). Different genome mining tools such as AntiSMASH and PRISM reveal from 22 to 26 NP BGCs in *S. albus ΔpseB4* genome (Table S2). Most of the clusters are silent or expressed at the low level under the standard laboratory conditions (Olano et al. 2014).

Some of the mutations that we used in our experiments reported to activate cryptic BGCs in different *Streptomyces* strains (Shima et al. 1996). To explore how *rpsL* and double *rpsL-rsmG* mutations influence the expression level of internal BGCs and the production of the corresponding secondary metabolites, we performed secondary metabolite extraction and transcriptome analysis in the *S. albus ΔpseB4* strain and the mutant strains carrying the following mutations: K88E, GI92, K88E-GI92, K88E-GI92 *ΔrsmG*, P91S, K88E-P91S, and K88E-P91S *ΔrsmG* (Table 1).

The production level of antimycins in *S. albus* strains was calculated by comparison of the areas of peaks for five ions of *m/z* 519.34, 533.36, 547.37, 561.39, 575.42 $[M-H]^-$, that correspond to antimycin A3, A2, A1, and two more derivatives, respectively (Fig. S4). Regarding candicidins, the UV absorption at wavelength 404 nm was used for comparison of the production level in *S. albus* strains (Fig. S5). There was a clear correlation between the production level of antimycins and candicidins in all examined strains (Fig. 2a, c). Interestingly, the levels of transcription of both BGCs were also strongly correlated, indicating that these BGCs are jointly regulated. The transcriptional levels of the pathway-specific regulatory genes FscRI-FscRIV of the candicidin BGC were correlated with the transcriptional levels of candicidin biosynthetic structural genes (Fig. 2b).

With regard to antimycins, we identified a correlation between the transcription of the antimycin sigma factor gene and the BGC (Fig. 2c). Considering that both clusters are regulated

Table 2 Level of resistance of *S. albus* delPse mutants to streptomycin, paromomycin, and gentamycin antibiotics

Strain ID	MIC, μg/ml		
	Streptomycin	Paromomycin	Gentamicin
S. albus delPse	1.56	0.39	< 0.39
S. albus K88E	*200*	0.39	< 0.39
S. albus GI92	25	0.78	< 0.39
S. albus K88E-GI92	*200*	1.56	< 0.39
S. albus P91S	12.5	0.78	< 0.39
S. albus K88E-P91S	*200*	0.78	< 0.39
S. albus R86P	25	0.39	< 0.39
S. albus R94G	3.13	0.39	< 0.39
S. albus delPse:del *rsmG*	*200*	< 0.39	< 0.39
S. albus K88E:del *rsmG*	*200*	0.39	< 0.39
S. albus GI92:del *rsmG*	*200*	0.39	< 0.39
S. albus K88E-GI92:del *rsmG*	*200*	0.39	< 0.39
S. albus P91S:del *rsmG*	*200*	0.39	< 0.39
S. albus K88E-P91S:del *rsmG*	*200*	< 0.39	< 0.39
S. albus R86P:del *rsmG*	*200*	0.39	< 0.39
S. albus R94G:del *rsmG*	50	0.39	< 0.39

MICs were determined in liquid TSB media. Strains were inoculated from spore cultures into 96-well plates with different concentrations of antibiotics and incubated at 28 °C 900 rpm for 48 h. The highest level of resistance is marked in italics

by the same LuxR family cluster-situated regulator, FscRI (McLean et al. 2016) and that the transcription levels of both BGCs observed among different *rpsL* mutants were almost indistinguishable, our data indicate that *rpsL* mutations influence transcription at a higher regulatory level and that the signal is then transmitted to a cluster-situated regulator, FscRI, which in turn influences the transcription of candicidin via the regulatory proteins FscRII-FscRIV (Fig. 2b) and that of antimycin via AntA sigma factor (Fig. 2c). However, the mutations do not necessarily affect production in the same way. The increased transcription induced in the two clusters in the *ΔrsmG* and KE-PS *ΔrsmG* strains did not result in the enhanced production of both compounds; instead, production was nearly abolished.

The accumulation of the antimycin derivative N-formyl Me-ester antimycic acid (Seo et al. 2001) was detected in some of the strains with *rpsL* mutations (Fig. 2d and Fig. S6). In the *S. albus K88E* strain, we detected the presence of a compound using a UV-absorption specific for antimycic acid that had a mass corresponding to that of N-formyl Me-ester antimycic acid of *m/z* 295 $[M–H]^-$ (Fig. 3 and Fig. S6). The *S. albus GI92* strain produced 4-fold more of the compound. The KE-GI alone or combined with the subsequent deletion of *rsmG*-boosted production 8-fold and 13-fold, respectively (Fig 2d and Fig. S6). The antimycin BGC in *S. albus* lacks two of the genes that are involved in the biosynthesis of the antimycin dilactone core: kynurenidase and phosphopantetheinyl transferase (PPT). Presumably, *S. albus* uses kynurenidase in tryptophan catabolism and another phosphopantetheinyl transferase for the biosynthesis of antimycin (Seipke and Hutchings 2013). The transcription of the kynurenidase gene was not different among the mutant strains. The *S. albus* genome encodes two phosphopantetheinyl transferase genes, XNR_RS02845 and XNR_RS28340. The transcription of the latter of these two genes was not affected by *rpsL* mutations or *rsmG* deletion. However, there were some changes in the transcription of the former gene, XNR_RS02845, which is part of the paulomycin BGC. An increase in the transcription of XNR_RS02845 was detected in mutant strains with higher N-formyl Me-ester antimycic acid production (Fig. 2d), suggesting that XNR_RS02845 is involved in antimycin biosynthesis. Thus, the decrease in the production of antimycin observed in the *ΔrsmG* and KE-PS *ΔrsmG* strains may have been because the transcription of the paulomycin BGC was nearly completely abolished in these strains. Hence, in these strains, the level of production of antimycin does not match the transcription level of the cluster. Such cross-talk between antimycin and paulomycin BGCs was not reported before and requires further investigation.

Paulomycins production was calculated by comparison of mass peak areas of four paulomycins: Paulomycin A and B, and Paulomenol A and B, with *m/z* 785.33, 771.32, 660.36, and 646.32 $[M–H]^-$, respectively (Fig. S7a). These compounds were as well characterized by UV absorption at wavelength 246 and 322 nm, that is specific for paulomycins (Fig. S7.B). The same mutations had a completely opposite effect on paulomycin production. Mutations that had a positive

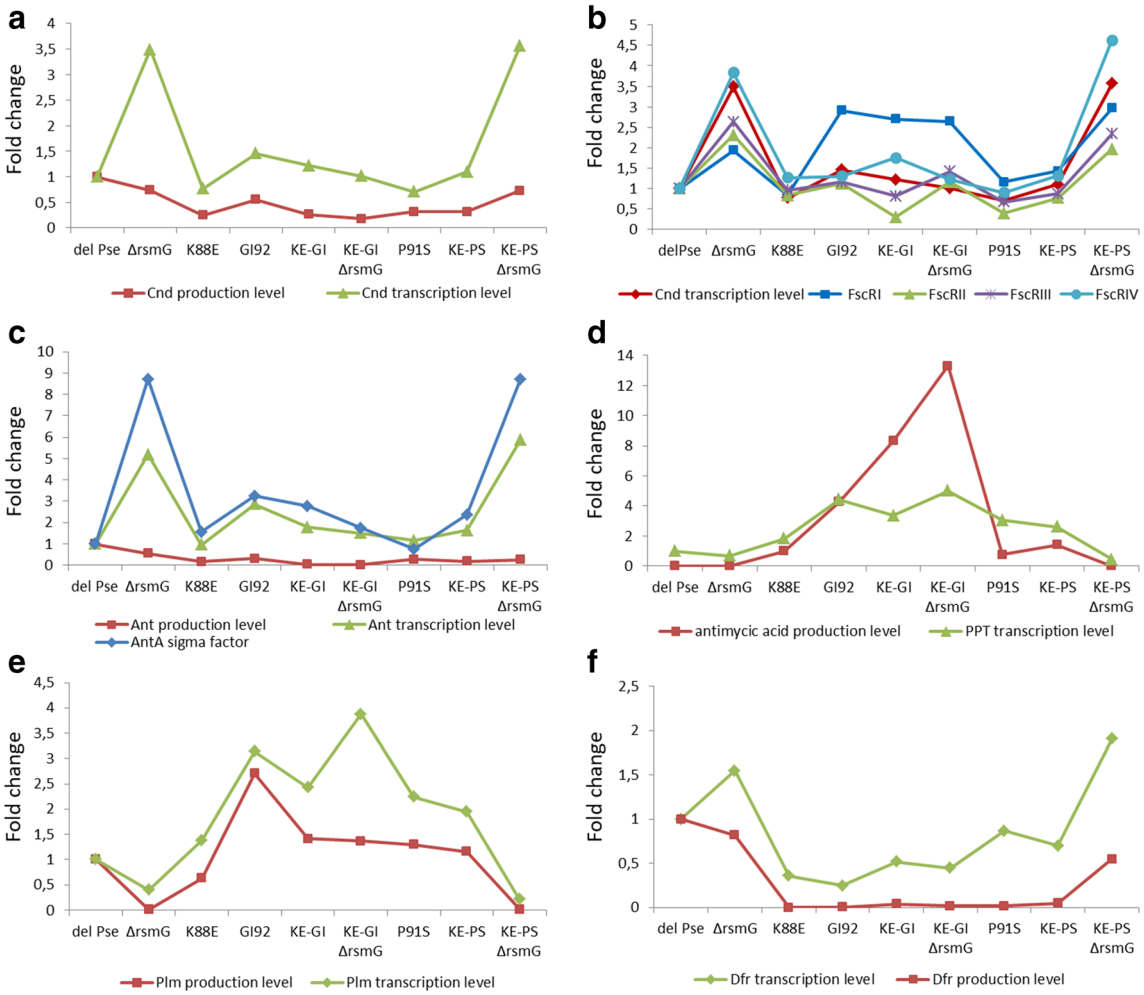


Fig. 2 Comparison of the production levels of candicidins (**a**), candicidins BGC regulatory proteins (**b**), antimycins (**c**), antimycic acid (**d**), paulomycins (**e**), and desferrioxamine (**f**) at the transcription level in strains with *rpsL* mutations combined with the deletion of the *rsmG* gene. The transcription and production levels observed in the original strain were defined as 1. Ant stands for antimycin, Cnd—candicidin, Plm—paulomycin, Dfr—desferrioxamine

effect on the transcription of antimycin and candicidin BGCs were found to abolish the transcription of the cluster for paulomycin and vice versa (Fig. 2a, c, e). We observed an almost 3-fold higher level of production of paulomycins in the *S. albus GI92* strain and a slightly higher level of its production in the K88E-GI92 combination strain as well as the K88E-GI92 *ΔrsmG* and P91S strains than was observed in the *S. albus ΔpseB4* strain (Fig. 2e). In strains with mutations, the production level of paulomycin matched the transcription level of the cluster. *RsmG* deletion alone decreased transcription of the cluster, whereas it was increased by 2-fold in the KE-GI combination and surprisingly increased even further in combination with *rsmG* deletion. Despite the negative effect *rsmG* deletion exerted on paulomycin BGC transcription, when combined with KE-GI, it had a synergistic positive effect. The *rsmG* deletion had a completely opposite effect on the transcription/production level in the strain with the KE-PS *rpsL* combination. While the transcription of the paulomycin BGC was almost twofold higher in the KE-PS mutant strain, it was nearly entirely abolished when combined with *rsmG* deletion. Additionally, we observed a correlation between transcription of the paulomycin BGC and the level of transcription of two LuxR-family transcriptional regulatory genes, *Plm2* and *Plm30* (Gonzalez et al. 2016) (Fig. S8). Two other transcriptional regulatory genes, *Plm1* (TetR-family transcriptional regulator) and *Plm10* (SARP-family transcriptional regulator), had lower transcription levels. Therefore, the transcription of the paulomycin BGC was correlated with the level of production of paulomycins in all mutants.

Additionally we observed changes in the production of siderophore desferrioxamine B and E, with ions of *m*/*z* 559.46 and 599.44 $[M–H]^-$, respectively (Fig. S4). The production of desferrioxamines was significantly lower in all strains with *rpsL* mutations as well as in the strain with KE-GI mutations combined with the deletion of the *rsmG* gene. The transcription level of siderophore desferrioxamine was

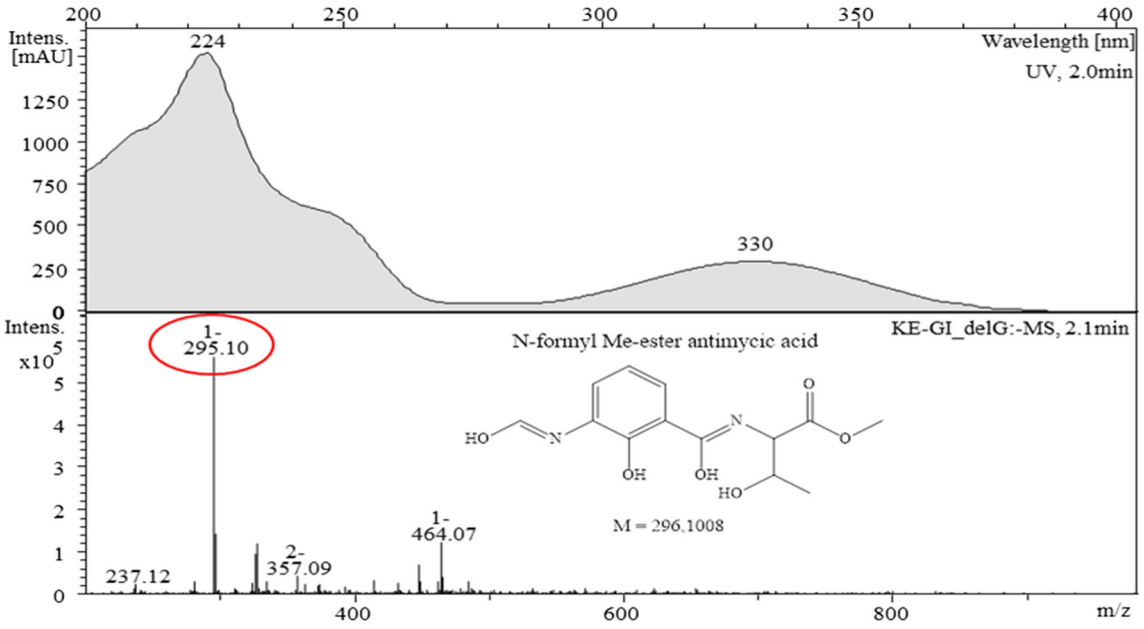


Fig. 3 The mass and UV spectrum of N-formyl Me-ester antimycic acid in *S. albus* KE-GI:del *rsmG* mutant strain

also changed in strains with *rpsL* mutations and *rsmG* gene deletion (Fig. 2f). The *S. albus ΔrsmG* strain produced slightly less than was produced by the original strain, whereas the transcription level of the desferrioxamine BGC was 1.6-fold higher. We further found that compared with the KE-PS combination strain, the *S. albus K88E-P91S ΔrsmG* strain restored the production of desferrioxamine. Transcription of the desferrioxamine BGC was also increased in this strain.

We noticed that mutations that increased paulomycin production/transcription in *S. albus* simultaneously exhibited a decrease in the production/transcription of antimycins, candicidins and desferrioxamine (Fig. 2a, c e, f). Consequently, the signal that elevates the expression of the paulomycin BGC must have a negative effect on the transcription of the antimycin, candicidin, and desferrioxamine BGCs.

The number of indigenous BGCs possessed by *S. albus* is much higher than the number of natural compounds this species produces (Seipke 2015). Thus, we analysed the transcription level of the remaining BGCs in the *S. albus* mutant strains (Fig. 4, Table S3–S4) even though we were unable to detect corresponding natural products. In 23 of the 26 BGCs, transcription was altered by more than 2-fold in at least one of the mutant strains. We also observed that for some clusters, certain mutations increased transcription, while others decreased transcriptions (BGC 2, 3, 5, 7, and 17). All mutations had a strong negative effect on the transcription of the bacteriocin cluster (BGC 13). The most dramatic changes in BGC transcription were detected in the strains with the KE-GI mutation and *rsmG* deletion and those with KE-GI*ΔrsmG* and KE-PS*ΔrsmG* combinations.

We observed changes in the transcription levels of known global transcriptional regulatory proteins (Table 3). The differences observed in the transcription levels of *bldD* (Al-Bassam et al. 2014; Elliot et al. 1998), *afsQ1/2* (Daniel-Ivad et al. 2017; Rodriguez et al. 2013; Wang et al. 2013), *relA* (Chakraburtty and Bibb 1997; Rodriguez et al. 2013) and *afsR* (Santos-Beneit et al. 2011) were smaller than twofold; thus, we cannot affirm that they are responsible for the alterations observed in the transcription of indigenous BGCs. However, we found that the transcription of the two-component signal transduction system *phoP/R* (Rodriguez et al. 2013) was abolished in *S. albus* strains with P91S mutations and the KE-PS combined mutant. Interestingly, in GI92 and KE-GI mutants, the transcription level of the *phoR* gene was 3- and 2-folds higher, respectively, whereas the transcription level of *phoP* in the GI92 mutant strain was nearly twofold lower. The transcription of *afsK* (Rajkarnikar et al. 2006), encoding a serine-threonine protein kinase, was almost 3-fold higher in the KE-GI mutant and 2.4-fold higher in the KE-GI *Δ rsmG* combined mutant (Table 3). Interestingly, the transcription of the TetR-family regulatory protein gene *atrA* (Hirano et al. 2008; Li et al. 2015; Wang et al. 2015) increased in the GI92, KE-GI, and KE-GI *Δ rsmG* strains in a gradual manner. We observed no noticeable changes in the transcription of the GntR-like repressor gene *dasR* or the global regulator gene *adpA* (Higo et al. 2012; Ohnishi et al. 2005; Rabyk et al. 2018).

Furthermore, we observed considerable differences in the transcription level of sigma factors among the mutant strains (Fig. S9 and Table S5), with 23 out of the 33 reviewed sigma factors exhibiting more than 2-fold changes in the level of transcription in at least one mutant strain when compared to the level observed in the original strain.

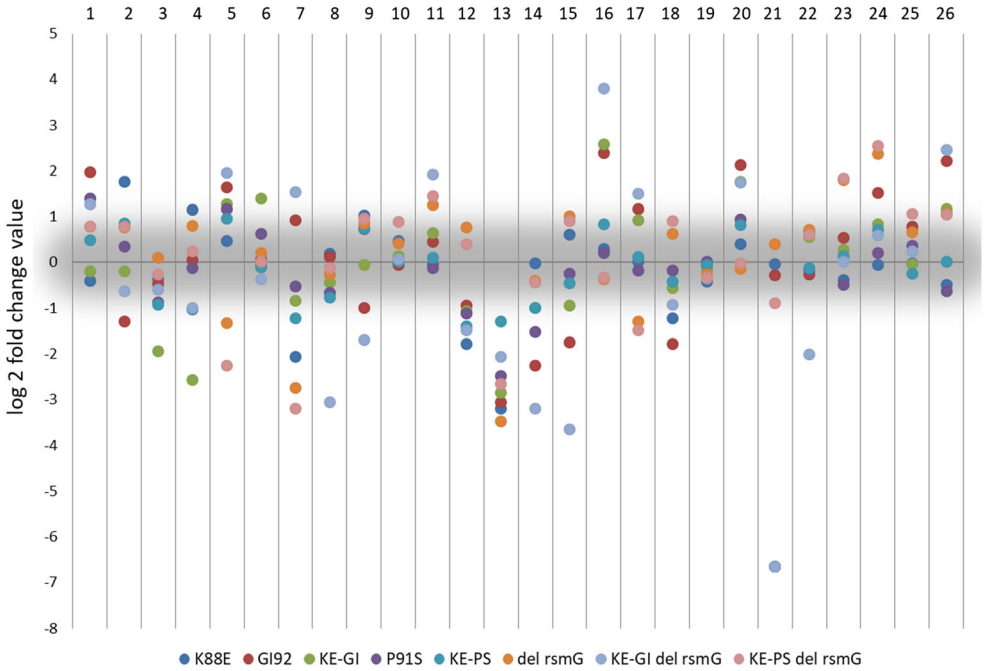


Fig. 4 Transcription levels of 26 BGCs of *S. albus* in strains with *rpsL* mutations and *rsmG* deletion in comparison to that of the original strain. Data represent log2-fold change values. Clusters analysed: 1 hybrid NRPS/PKS, 2 alteramide/frontalamide, 3 terpene/hopene, 4 bacteriocin, 5 paulomycin, 6 bacteriocin, 7 NRPS, 8 siderophore/aerobactin-like, 9 terpene/geosmin, 10 echosides/oxasolomycin, 11 terpene/albaflavenone, 12 lanthipeptide, 13 bacteriocin, 14 labirynthopeptide/SapB, 15 NRPS, 16 NRPS/gramicidin-like, 17 NRPS, 18 siderophore/desferrioxamine, 19 ectoine, 20 NRPS/indigoidin, 21 polyprenyl synthase/carotenoids, 22 type III PKS/tetrahydroxynaphtalene, 23 type I PKS/candicidins, 24 hybrid NRPS/PKS/antimycin, 25 hybrid NRPS/PKS, and 26 type I PKS. Values shaded in gray represent smaller than twofold changes in transcription and are considered not significantly different from the value of the original strain.

We also detected a dramatic increase in the transcription of hypothetical protein genes (XNR_RS13105-13110), with a 100-fold change observed in the GI92 mutant and a 71-fold change observed in the KE-GI Δ *rsmG* strain (Table S6). The transcription levels of members of a cluster of unknown genes (XNR_RS12890-12915) consisting of SAM-dependent methyl-transferase genes, hypothetical genes, and amine oxidase genes were enhanced by up to 18-fold in the KE-PS Δ *rsmG* mutant strain. Surprisingly, the transcription of the sulfur metabolism genes operon was increased by 10- to 20-fold in all of the evaluated mutants. We also noted that the transcription of an unknown ATP-binding response regulator receiver gene (XNR_RS06415) was abolished.

From these data, we conclude that mutations in the *rpsL* gene and *rsmG* deletion exert a tremendous effect on the transcription of indigenous BGCs, probably by altering the transcription of pleiotropic regulatory genes and sigma factors, which in turn influence the transcription of cluster-situated regulatory genes.

Discussion

A major driver behind this study was the development of rational ribosome engineering platform for *Streptomyces*. The latter was designed on basis of *S. albus* J1074, and its utility was confirmed via rapid and reliable introduction of a set of *rpsL* point mutations into J1074 genome, alone and in combination with *rsmG* deletion. We chose a set of different *rpsL* mutations that have been reported to elevate the production level of various antibiotics in different strains (see details in the Introduction section). The most important drawback of previously obtained data is that origin of *rpsL* and *rsmG* mutations generated via selection for spontaneous streptomycin-resistance mutants is indeed random. Thus, one cannot exclude a possibility that other mutations could have occurred on the chromosome that may also have influenced antibiotic production. In addition, several mutations have been obtained in merozygote strains (Okamoto-Hosoya et al. 2003b). In the latter, ribosome population would be heterogeneous with regard to S12 protein, as some ribosomes would possess wild-type S12 protein, while the other would carry a mutant S12. The quantity of mutant ribosomes would likely be fluctuating in response to growth phase, nutrition status, etc. and hence difficult to control and associate with phenotypic outcomes. We wanted to introduce point mutations directly into the *rpsL* gene on the *S. albus* chromosome without causing any other mutations and without maintaining the native *rpsL* gene background.

Many different techniques based on homologous recombination (i.e., site-specific recombination techniques (Herrmann et al. 2012; Myronovskyi and Luzhetskyy 2013), iterative

Table 3 Comparison of the transcription level of pleiotropic regulatory genes in mutant strains to the original *S. albus* strain. Data represent fold-changes. The transcription level in the original strain was defined as 1

	Δ rsmG	*K88E*	*GI92*	*KE-GI*	*KE-GI Δ rsmG*	*P91S*	*KE-PS*	*KE-PS Δ rsmG*
bldD	1.24	0.82	0.88	0.62	0.64	0.75	0.64	1.14
afsQ1/2	0.84	1.04	0.99	0.74	0.84	1.06	1.03	0.79
afsK	1.49	1.53	1.39	2.89	2.41	1.36	1.35	1.5
afsR	1.14	1.25	0.81	0.66	1.01	0.61	0.75	1.08
phoP	1.2	1.11	0.54	1.09	0.66	0	0	0.82
phoR	1.54	1.18	3.11	2.11	0.65	0	0	1.57
relA	1.36	1.35	0.9	1.02	0.83	1.31	1.5	1.73
atrA	1.04	1.35	2.75	3.44	4.18	1.82	1.74	0.9
dasR	0.82	0.82	0.86	0.74	0.96	1.41	0.86	1.01
adpA	1.23	0.96	0.83	0.76	1.48	0.78	0.85	1.19

0 to 0.25	0.25 to 0.5	0.5 to 0.75	0.75 to 1.5	1.5 to 2	2 to 4	4 and more

marker-free excision systems (Myronovskyi et al. 2014)) and programmed endonucleases (Cas9) (Tao et al. 2018; Tong et al. 2015)) are available for *Streptomyces* genome editing. These are very useful for obtaining gene/cluster deletions but are not as useful for inserting point mutations into essential genes in the genome. Recently, the CRISPR/Cas system was reported to introduce the K88E point mutation into the *rpsL* gene of *S. lividans* (Cobb et al. 2015). However, in that study, streptomycin resistance was used to select positive clones. Thus, that system cannot be used to generate point mutations into random essential genes because such mutations usually do not introduce any resistance phenotype, and not all *rpsL* mutations lead to resistance as well. Another disadvantage of the CRISPR/Cas system is its off-target effects, which can lead to unwanted alterations in the genome; these may directly or indirectly interfere with the effects of the introduced point mutation. In consideration of these drawbacks, we established a system that allows the point mutations to be introduced directly into the chromosome of a strain in a robust, rapid, and site-specific manner. This system requires the construction of a platform strain in which the gene of interest is flanked by two resistance markers and the construction of a BAC library (any genome library can be used) containing clones with point mutations in the desired positions. Homologous recombination between the BAC backbone and the chromosome will result in the whole region of the chromosome located between the resistance genes being removed and replaced by the gene of interest in the homologous region of the BAC containing the mutation. After the mutation is introduced into the chromosome, no scar or resistance genes are left, and this allows resistance genes to be re-used in the same strain. This approach can be useful for the precise introduction of mutations into essential gene without requiring the use of direct antibiotic selection of mutant strains. In addition, this system can be used to construct industrial strains because it leaves no genetic material from an unrelated organism.

The generated library of J1074-borne *rpsL/rsmG* mutations opens the door for systematic scrutiny of their effects and mechanisms, particularly on secondary metabolome; below we comment on our most important findings. The mutations exerted significant effects on the transcription profiles of the strains. In all, 88 % of the BGCs (23 out of 26) displayed significant changes in transcription levels. The differences observed in the transcription of regulatory (Table 2) and sigma factors genes (Fig. S2) indicate that the mutations triggered transcriptional regulatory mechanisms, which in turn altered the expression of the BGCs. Most likely, these pleiotropic regulators and sigma factors can influence the activity of

cluster-specific regulators and thereby modulate the transcription of BGCs. An interesting hypothesis has been proposed in which *rpsL* mutations are predicted to enhance the activity of guanosine-3′,5′-tetraphosphate synthase RelA, which is located close to a S12 protein on the ribosome. This effect, in turn, could increase the amount of ppGpp in cells. In *E. coli,* ppGpp binds to RNA polymerase and can change its affinity for different promoters (Ochi 2007). Nevertheless, this does not explain how ppGpp influences transcription in *Streptomyces* species because as gram-positive bacteria, they possess no ppGpp-binding site on their RNA-polymerase. A recent study found that increasing the amount of ppGpp did not directly influence RNA polymerase but did decrease the pool of GTP that was converted to ppGpp by the RelA protein (Krasny and Gourse 2004). Those authors proposed that decreasing the pool of GTP depressed transcription at promoters with an initiating guanine nucleotide. ppGpp could thereby influence transcription indirectly by affecting the GTP pool in cells.

The same *rpsL* mutations exerted opposite effects on the transcription of antimycin and candicidin versus paulomycin BGCs. This difference might be due to alterations in the expression of versatile transcriptional regulators that act as activators of one cluster and repressors of another. This type of versatile regulatory effect has been described for AdpA regulatory protein in *S. ansochromogenes* (Zhang et al. 2017) and for arginine biosynthesis in *S. coelicolor* (Perez-Redondo et al. 2012) and *Bacillus subtilis* (Miller et al. 1997).

Interestingly, *rsmG* deletion in some cases abolished and in other cases enhanced the effect of the *rpsL* mutations. Deleting *rsmG* in *S. albus* strains in combination with the KE-GI mutation increased the effect of the mutation, whereas deleting it in combination with the KE-PS mutation completely abolished the effect of the mutation (Figs. 2a–f). We therefore conclude that deleting the *rsmG* gene abolishes the effect of the KE-PS combination, regardless of whether it has a positive or negative effect on transcription.

The *rpsL* mutations and *rsmG* deletion appear to affect both transcription and translation. At the transcriptional level, they change the expression of genes for pleiotropic regulators and sigma factors, leading to major repercussions within entire transcription regulatory network. However, they were also shown previously to increase ribosome stability and overall protein synthesis (Hosaka et al. 2006), and our data indicate that altered transcriptional regulation alone cannot explain the effects of *rpsL* mutations on secondary metabolome. Together, these changes in transcriptional regulation, and protein synthesis significantly alter BGC expression and very often increase yields of natural products. Therefore, developed technology of the rational introduction of *rpsL* and *rsmG* mutations can be widely applied to improve expression of BGCs and subsequently production of corresponding natural products. The optimized production of natural products will facilitate their supply for pharmacodynamics and pharmacokinetic studies and thus positively influence the entire drug discovery and development chain.

Funding information This work was supported by the ERC starting grant EXPLOGEN No. 281623, BMBF grant „MyBio“ 031B0344B to AL and DAAD scholarship Referat: 322 PZK: A/12/84540 to ML.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain studies with human participants or animals performed by any of authors.

References

Agarwal D, Gregory ST, O'Connor M (2011) Error-prone and error-restrictive mutations affecting ribosomal protein S12. J Mol Biol 410(1):1–9. https://doi.org/10.1016/j.jmb.2011.04.068

Al-Bassam MM, Bibb MJ, Bush MJ, Chandra G, Buttner MJ (2014) Response regulator heterodimer formation controls a key stage in *Streptomyces* development. PLoS Genet 10(8):e1004554. https://doi.org/10.1371/journal.pgen.1004554

Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11(10):R106. https://doi.org/10.1186/Gb-2010-11-10-R106

Baltz RH (2011) Strain improvement in actinomycetes in the postgenomic era. J Ind Microbiol Biotechnol 38(6):657–666. https://doi.org/10.1007/s10295-010-0934-z

Baltz RH (2017) Gifted microbes for genome mining and natural product discovery. J Ind Microbiol Biotechnol 44(4-5):573–588. https://doi.org/10.1007/s10295-016-1815-x

Bekiesch P, Basitta P, Apel AK (2016) Challenges in the heterologous production of antibiotics in *Streptomyces*. Arch Pharm 349(8):594–601. https://doi.org/10.1002/ardp.201600058

Beltrametti F, Rossi R, Selva E, Marinelli F (2006) Antibiotic production improvement in the rare actinomycete *Planobispora rosea* by selection of mutants resistant to the aminoglycosides streptomycin and gentamycin and to rifamycin. J Ind Microbiol Biotechnol 33(4):283–288. https://doi.org/10.1007/s10295-005-0061-4

Bilyk B, Luzhetskyy A (2014) Unusual site-specific DNA integration into the highly active pseudo-attB of the *Streptomyces albus J1074* genome. Appl Microbiol Biotechnol 98(11):5095–5104. https://doi.org/10.1007/s00253-014-5605-y

Chakraburtty R, Bibb M (1997) The ppGpp synthetase gene (relA) of *Streptomyces coelicolor* A3(2) play's a conditional role in antibiotic production and morphological differentiation. J Bacteriol 179(18):5854–5861. https://doi.org/10.1128/jb.179.18.5854-5861.1997

Cobb RE, Wang YJ, Zhao HM (2015) High-efficiency multiplex genome editing of *streptomyces* species using an engineered CRISPR/Cas System. ACS Synth Biol 4(6):723–728. https://doi.org/10.1021/sb500351f

Daniel-Ivad M, Hameed N, Tan S, Dhanjal R, Socko D, Pak P, Gverzdys T, Elliot MA, Nodwell JR (2017) An engineered allele of afsQ1 facilitates the discovery and investigation of cryptic natural products. ACS Chem Biol 12(3):628–634. https://doi.org/10.1021/acschembio.6b01002

Elliot M, Damji F, Passantino R, Chater K, Leskiw B (1998) The bldD gene of *Streptomyces coelicolor* A3(2): a regulatory gene involved in morphogenesis and antibiotic production. J Bacteriol 180(6):1549–1555

Genilloud O (2017) *Actinomycetes*: still a source of novel antibiotics. Nat Prod Rep 34(10):1203–1232. https://doi.org/10.1039/c7np00026j

Gomez-Escribano JP, Bibb MJ (2011) Engineering *Streptomyces coelicolor* for heterologous expression of secondary metabolite gene clusters. Microb Biotechnol 4(2):207–215. https://doi.org/10.1111/j.1751-7915.2010.00219.x

Gonzalez A, Rodriguez M, Brana AF, Mendez C, Salas JA, Olano C (2016) New insights into paulomycin biosynthesis pathway in *Streptomyces albus* J1074 and generation of novel derivatives by combinatorial biosynthesis. Microb Cell Factories 15:56. https://doi.org/10.1186/s12934-016-0452-4

Herrmann S, Siegl T, Luzhetska M, Petzke L, Jilg C, Welle E, Erb A, Leadlay PF, Bechthold A, Luzhetskyy A (2012) Site-specific recombination strategies for engineering *Actinomycete* genomes. Appl Environ Microbiol 78(6):1804–1812. https://doi.org/10.1128/Aem.06054-11

Higo A, Hara H, Horinouchi S, Ohnishi Y (2012) Genome-wide distribution of AdpA, a global regulator for secondary metabolism and morphological differentiation in *Streptomyces*, revealed the extent and complexity of the AdpA regulatory network. DNA Res 19(3):259–273. https://doi.org/10.1093/dnares/dss010

Hilker R, Stadermann KB, Schwengers O, Anisiforov E, Jaenicke S, Weisshaar B, Zimmermann T, Goesmann A (2016) ReadXplorer 2-detailed read mapping analysis and visualization from one single source. Bioinformatics 32(24):3702–3708. https://doi.org/10.1093/bioinformatics/btw541

Hirano S, Tanaka K, Ohnishi Y, Horinouchi S (2008) Conditionally positive effect of the TetR-family transcriptional regulator AtrA on streptomycin production by *Streptomyces griseus*. Microbiol-Sgm 154:905–914. https://doi.org/10.1099/mic.0.2007/014381-0

Holberger LE, Hayes CS (2009) Ribosomal protein S12 and aminoglycoside antibiotics modulate A-site mRNA cleavage and transfer-messenger RNA activity in *Escherichia coli*. J Biol Chem 284(46):32188–32200. https://doi.org/10.1074/jbc.M109.062745

Hosaka T, Xu J, Ochi K (2006) Increased expression of ribosome recycling factor is responsible for the enhanced protein synthesis during the late growth phase in an antibiotic-overproducing *Streptomyces coelicolor* ribosomal rpsL mutant. Mol Microbiol 61(4):883–897. https://doi.org/10.1111/j.1365-2958.2006.05285.x

Huser AT, Becker A, Brune I, Dondrup M, Kalinowski J, Plassmeier J, Puhler A, Wiegrabe I, Tauch A (2003) Development of a *Corynebacterium glutamicum* DNA microarray and validation by genome-wide expression profiling during growth with propionate as carbon source. J Biotechnol 106(2-3):269–286. https://doi.org/10.1016/j.jbiotec.2003.08.006

Kim EJ, Yang I, Yoon YJ (2015) Developing *Streptomyces venezuelae* as a cell factory for the production of small molecules used in drug discovery. Arch Pharm Res 38(9):1606–1616. https://doi.org/10.1007/s12272-015-0638-z

Koepff J, Keller M, Tsolis KC, Busche T, Ruckert C, Hamed MB, Anne J, Kalinowski J, Wiechert W, Economou A, Oldiges M (2017) Fast and reliable strain characterization of *Streptomyces lividans* through micro-scale cultivation. Biotechnol Bioeng 114(9):2011–2022. https://doi.org/10.1002/bit.26321

Komatsu M, Komatsu K, Koiwai H, Yamada Y, Kozone I, Izumikawa M, Hashimoto J, Takagi M, Omura S, Shin-ya K, Cane DE, Ikeda H (2013) Engineered *Streptomyces avermitilis* host for heterologous expression of biosynthetic gene cluster for secondary metabolites. ACS Synth Biol 2(7):384–396. https://doi.org/10.1021/sb3001003

Krasny L, Gourse RL (2004) An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. EMBO J 23(22):4473–4483. https://doi.org/10.1038/sj.emboj.7600423

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9(4):357–359. https://doi.org/10.1038/nmeth.1923

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Proc GPD (2009) The sequence alignment/map format and SAMtools. Bioinformatics 25(16):2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Li XX, Yu TF, He Q, McDowall KJ, Jiang BY, Jiang ZB, Wu LZ, Li GW, Li QL, Wang SM, Shi YY, Wang LF, Hong B (2015) Binding of a biosynthetic intermediate to AtrA modulates the production of lidamycin by *Streptomyces globisporus*. Mol Microbiol 96(6):1257–1271. https://doi.org/10.1111/mmi.13004

McLean TC, Hoskisson PA, Seipke RF (2016) Coordinate regulation of antimycin and candicidin biosynthesis. Msphere 1(6). https://doi.org/10.1128/mSphere.00305-16

Miller CM, Baumberg S, Stockley PG (1997) Operator interactions by the *Bacillus subtilis* arginine repressor/activator, AhrC: novel positioning and DNA-mediated assembly of a transcriptional activator at catabolic sites. Mol Microbiol 26(1):37–48. https://doi.org/10.1046/j.1365-2958.1997.5441907.x

Myronovskyi M, Luzhetskyy A (2013) Genome engineering in actinomycetes using site-specific recombinases. Appl Microbiol Biotechnol 97(11):4701–4712. https://doi.org/10.1007/s00253-013-4866-1

Myronovskyi M, Welle E, Fedorenko V, Luzhetskyy A (2011) Beta-glucuronidase as a sensitive and versatile reporter in *actinomycetes*. Appl Environ Microbiol 77(15):5370–5383. https://doi.org/10.1128/AEM.00434-11

Myronovskyi M, Rosenkranzer B, Luzhetskyy A (2014) Iterative marker excision system. Appl Microbiol Biotechnol 98(10):4557–4570. https://doi.org/10.1007/s00253-014-5523-z

Myronovskyi M, Rosenkranzer B, Nadmid S, Pujic P, Normand P, Luzhetskyy A (2018) Generation of a cluster-free *Streptomyces albus* chassis strains for improved heterologous expression of secondary metabolite clusters. Metab Eng 49:316–324. https://doi.org/10.1016/j.ymben.2018.09.004

Nishimura K, Hosaka T, Tokuyama S, Okamoto S, Ochi K (2007) Mutations in rsmG, encoding a 16S rRNA methyltransferase, result in low-level streptomycin resistance and antibiotic overproduction in *Streptomyces coelicolor* A3(2). J Bacteriol 189(10):3876–3883. https://doi.org/10.1128/Jb.01776-06

Ochi K (2007) From microbial differentiation to ribosome engineering. Biosci Biotechnol Biochem 71(6):1373–1386. https://doi.org/10.1271/bbb.70007

Ohnishi Y, Yamazaki H, Kato JY, Tomono A, Horinouchi S (2005) AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. Biosci Biotechnol Biochem 69(3):431–439. https://doi.org/10.1271/Bbb.69.431

Okamoto-Hosoya Y, Sato TA, Ochi K (2000) Resistance to paromomycin is conferred by rpsL mutations, accompanied by an enhanced antibiotic production in *Streptomyces coelicolor* A3(2). J Antibiot 53(12):1424–1427

Okamoto-Hosoya Y, Hosaka T, Ochi K (2003a) An aberrant protein synthesis activity is linked with antibiotic overproduction in rpsL mutants of *Streptomyces coelicolor* A3(2). Microbiol-Sgm 149:3299–3309. https://doi.org/10.1099/mic.0.26490-0

Okamoto-Hosoya Y, Okamoto S, Ochi K (2003b) Development of antibiotic-overproducing strains by site-directed mutagenesis of the rpsL gene in *Streptomyces lividans*. Appl Environ Microbiol 69(7):4256–4259. https://doi.org/10.1128/Aem.69.7.4256-4259.2003

Olano C, Garcia I, Gonzalez A, Rodriguez M, Rozas D, Rubio J, Sanchez-Hidalgo M, Brana AF, Mendez C, Salas JA (2014) Activation and identification of five clusters for secondary metabolites in *Streptomyces albus* J1074. Microb Biotechnol 7(3):242–256. https://doi.org/10.1111/1751-7915.12116

Perez-Redondo R, Rodriguez-Garcia A, Botas A, Santamarta I, Martin JF, Liras P (2012) ArgR of *Streptomyces coelicolor* is a versatile

regulator. PLoS One 7(3):e32697. https://doi.org/10.1371/journal.pone.0032697

Rabyk M, Yushchuk O, Rokytskyy I, Anisimova M, Ostash B (2018) Genomic insights into evolution of AdpA family master regulators of morphological differentiation and secondary metabolism in *Streptomyces*. J Mol Evol 86(3-4):204–215. https://doi.org/10.1007/s00239-018-9834-z

Rajkarnikar A, Kwon HJ, Ryu YW, Suh JW (2006) Catalytic domain of AfsKav modulates both secondary metabolism and morphologic differentiation in *Streptomyces avermitilis* ATCC 31272. Curr Microbiol 53(3):204–208. https://doi.org/10.1007/s00284-006-0062-1

Ren HQ, Wang B, Zhao HM (2017) Breaking the silence: new strategies for discovering novel natural products. Curr Opin Biotechnol 48: 21–27. https://doi.org/10.1016/j.copbio.2017.02.008

Rodriguez H, Rico S, Diaz M, Santamaria RI (2013) Two-component systems in *Streptomyces*: key regulators of antibiotic complex pathways. Microb Cell Factories 12:127. https://doi.org/10.1186/1475-2859-12-127

Sambrook J, Russell DW (2006) The condensed protocols from molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Santos-Beneit F, Rodriguez-Garcia A, Martin JF (2011) Complex transcriptional control of the antibiotic regulator afsS in *Streptomyces*: PhoP and AfsR are overlapping, competitive activators. J Bacteriol 193(9):2242–2251. https://doi.org/10.1128/Jb.01462-10

Seipke RF (2015) Strain-level diversity of secondary metabolism in *Streptomyces albus*. PLoS One 10(1):e0116457. https://doi.org/10.1371/journal.pone.0116457

Seipke RF, Hutchings MI (2013) The regulation and biosynthesis of antimycins. Beilstein J Org Chem 9:2556–2563. https://doi.org/10.3762/bjoc.9.290

Seo Y, Cho KW, Rho JR, Mo SJ, Shin J (2001) A new analog of antimycin from *Streptomyces* sp M03033. J Microbiol Biotechnol 11(4):663–667

Shima J, Hesketh A, Okamoto S, Kawamoto S, Ochi K (1996) Induction of actinorhodin production by rpsL (encoding ribosomal protein S12) mutations that confer streptomycin resistance in *Streptomyces lividans* and *Streptomyces coelicolor* A3(2). J Bacteriol 178(24): 7276–7284

Skinnider MA, Dejong CA, Rees PN, Johnston CW, Li HX, Webster ALH, Wyatt MA, Magarvey NA (2015) Genomes to natural products PRediction Informatics for Secondary Metabolomes (PRISM). Nucleic Acids Res 43(20):9645–9662. https://doi.org/10.1093/nar/gkv1012

Tamehiro N, Hosaka T, Xu J, Hu HF, Otake N, Ochi K (2003) Innovative approach for improvement of an antibiotic- overproducing industrial strain of *Streptomyces albus*. Appl Environ Microbiol 69(11):6412–6417. https://doi.org/10.1128/Aem.69.11.6412.6417.2003

Tanaka Y, Komatsu M, Okamoto S, Tokuyama S, Kaji A, Ikeda H, Ochi K (2009a) Antibiotic overproduction by rpsL and rsmG mutants of various *Actinomycetes*. Appl Environ Microbiol 75(14):4919–4922. https://doi.org/10.1128/Aem.00681-09

Tanaka Y, Tokuyama S, Ochi K (2009b) Activation of secondary metabolite-biosynthetic gene clusters by generating rsmG mutations in *Streptomyces griseus*. J Antibiot 62(12):669–673. https://doi.org/10.1038/ja.2009.97

Tao WX, Yang AN, Deng ZX, Sun YH (2018) CRISPR/Cas9-based editing of *Streptomyces* for discovery, characterization, and production of natural products. Front Microbiol 9:1660. https://doi.org/10.3389/Fmicb.2018.01660

Tong YJ, Charusanti P, Zhang LX, Weber T, Lee SY (2015) CRISPR-Cas9 based engineering of actinomycetal genomes. ACS Synth Biol 4(9):1020–1029. https://doi.org/10.1021/acssynbio.5b00038

Wang GJ, Hosaka T, Ochi K (2008) Dramatic activation of antibiotic production in *Streptomyces coelicolor* by cumulative drug resistance mutations. Appl Environ Microbiol 74(9):2834–2840. https://doi.org/10.1128/Aem.02800-07

Wang G, Inaoka T, Okamoto S, Ochi K (2009) A novel insertion mutation in *Streptomyces coelicolor* ribosomal S12 protein results in paromomycin resistance and antibiotic overproduction. Antimicrob Agents Ch 53(3):1019–1026. https://doi.org/10.1128/Aac.00388-08

Wang R, Mast Y, Wang J, Zhang WW, Zhao GP, Wohlleben W, Lu YH, Jiang WH (2013) Identification of two-component system AfsQ1/Q2 regulon and its cross-regulation with GlnR in *Streptomyces coelicolor*. Mol Microbiol 87(1):30–48. https://doi.org/10.1111/mmi.12080

Wang WF, Tian JZ, Li L, Ge M, Zhu H, Zheng GS, Huang H, Ruan LJ, Jiang WH, Lu YH (2015) Identification of two novel regulatory genes involved in pristinamycin biosynthesis and elucidation of the mechanism for AtrA-p-mediated regulation in *Streptomyces pristinaespiralis*. Appl Microbiol Biotechnol 99(17):7151–7164. https://doi.org/10.1007/s00253-015-6638-6

Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Muller R, Wohlleben W, Breitling R, Takano E, Medema MH (2015) antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43(W1):W237–W243. https://doi.org/10.1093/nar/gkv437

Xu J, Zhang J, Zhuo J, Li Y, Tian Y, Tan H (2017) Activation and mechanism of a cryptic oviedomycin gene cluster via the disruption of a global regulatory gene, adpA, in *Streptomyces ansochromogenes*. J Biol Chem 292(48):19708–19720. https://doi.org/10.1074/jbc.M117.809145

Zaburannyi N, Rabyk M, Ostash B, Fedorenko V, Luzhetskyy A (2014) Insights into naturally minimised *Streptomyces albus* J1074 genome. BMC Genomics 15:97. https://doi.org/10.1186/1471-2164-15-97

Zhang YM, Muyrers JPP, Testa G, Stewart AF (2000) DNA cloning by homologous recombination in *Escherichia coli*. Nat Biotechnol 18(12):1314–1317

Applied Microbiology and Biotechnology

Supplementary material

Rational ribosome engineering to improve secondary metabolite production

Maria Lopatniuk[1], Maksym Myronovskyi[1], Alessia Nottebrock[1], Tobias Busche[2], Jörn Kalinowski[2], Bohdan Ostash[3], Viktor Fedorenko[3], Andriy Luzhetskyy[1,4]*

[1]Department Pharmacy, Actinobacteria Metabolic Engineering Group, Saarland University, Uni Campus C2.3, 66123, Saarbrücken, Germany

[2]Bielefeld University, Centre for Biotechnology (CiBiTec), Universitätsstraße 27, 33615, Bielefeld, Germany

[3]Department of Genetics and Biotechnology, Ivan Franko National University of Lviv, Hrushevskyy str., 4, 79005, Lviv, Ukraine.

[4]Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Uni Campus E8.1, 66123, Saarbrücken, Germany

*Corresponding author: Prof. Andriy Luzhetskyy

E-mail: Andriy.Luzhetskyy@helmholtz-hips.de

Tel: +49 681 302 70200

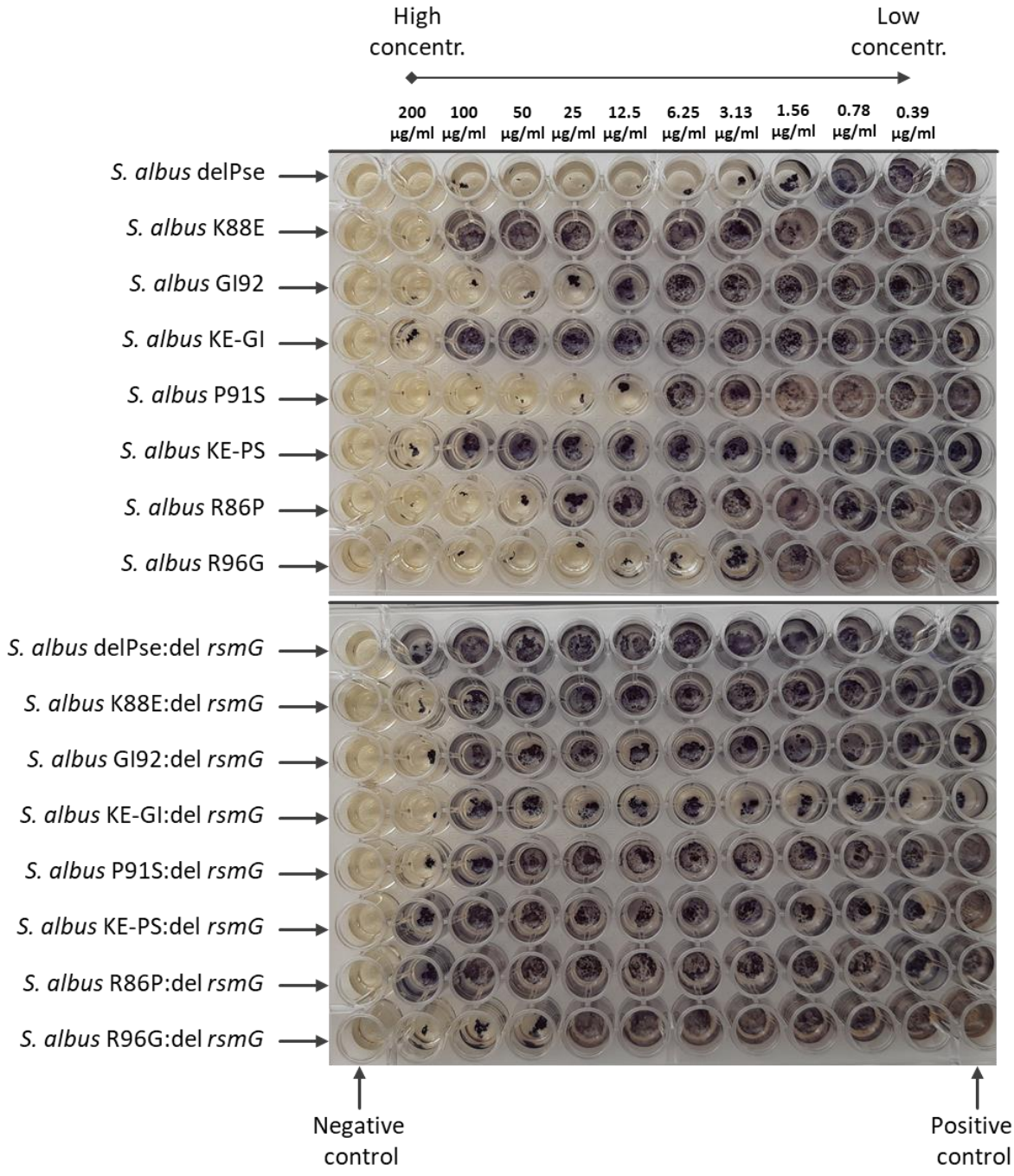


Figure S1. MIC of *S. albus* delPse and mutants strains on streptomycin. Negative control represents the well without antibiotic and cell culture. Positive control represents the well without antibiotic and with cell culture (as a reference for normal growing culture).

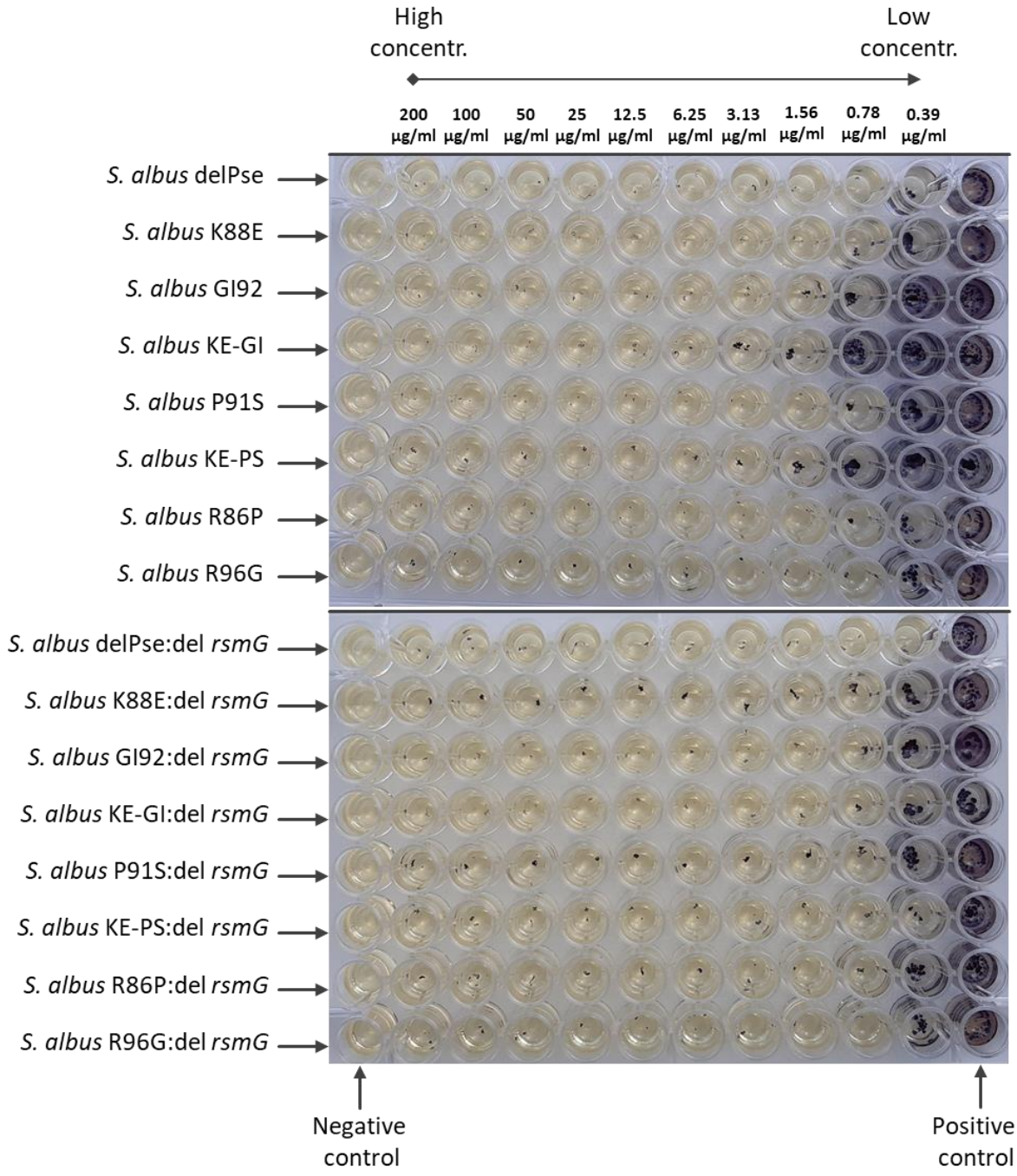


Figure S2. MIC of *S. albus* delPse and mutants strains on paromomycin. Negative control represents the well without antibiotic and cell culture. Positive control represents the well without antibiotic and with cell culture (as a reference for normal growing culture).

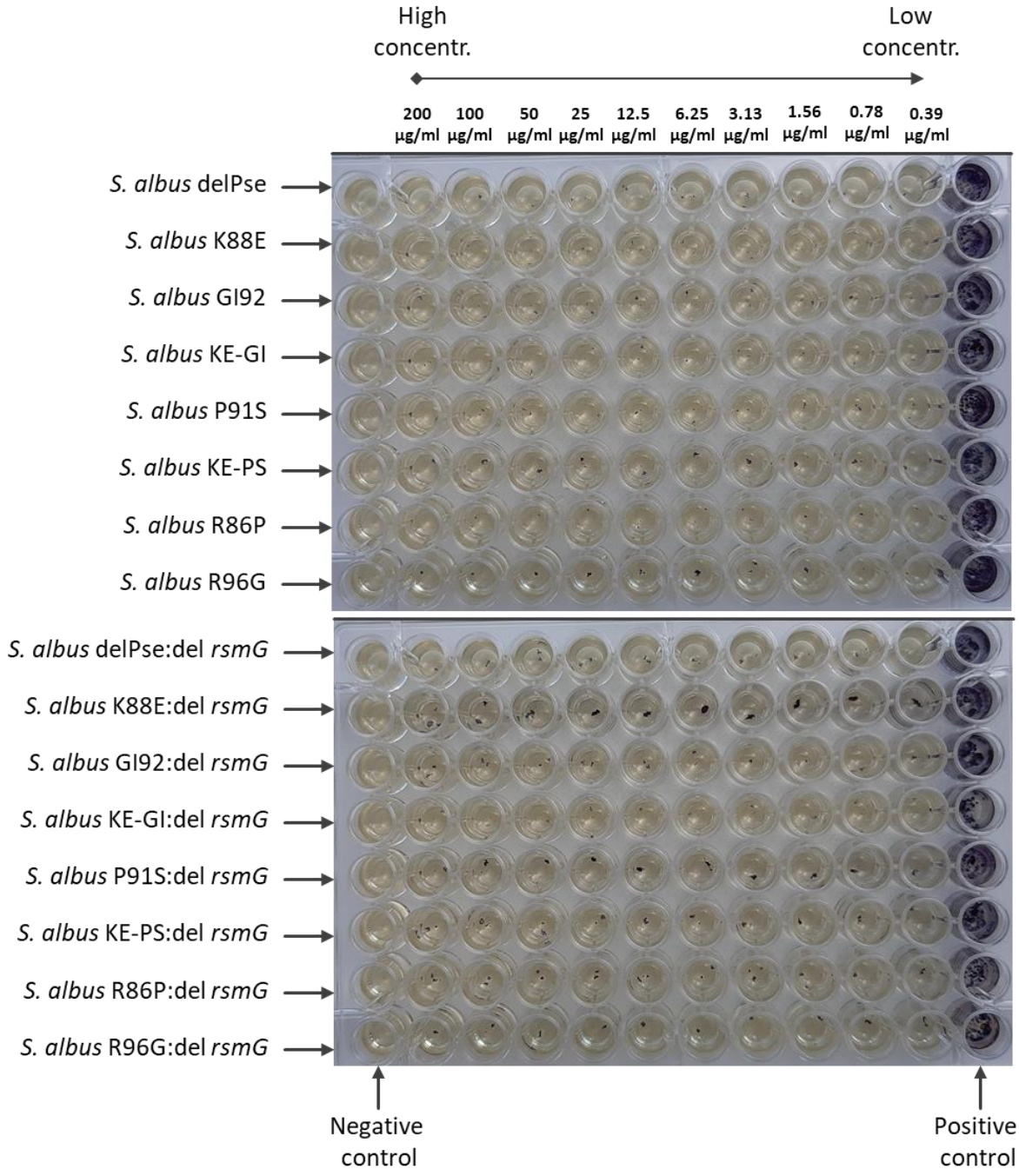


Figure S3. MIC of *S. albus* delPse and mutants strains on gentamicin. Negative control represents the well without antibiotic and cell culture. Positive control represents the well without antibiotic and with cell culture (as a reference for normal growing culture).

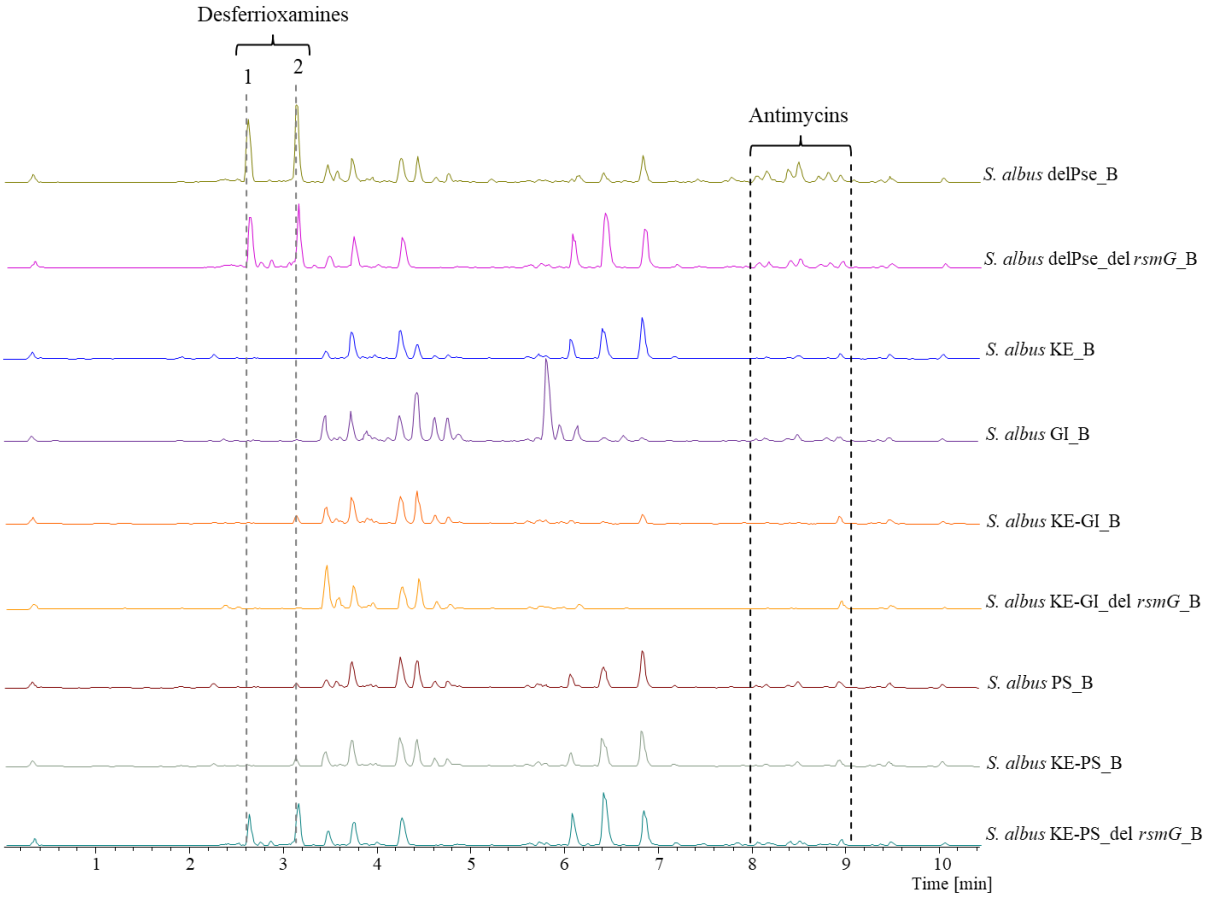


Figure S4. Comparison of base peak chromatograms in negative mode of butanol extracts from *S. albus* delPse and mutant strains. **1** – Desferrioxamine B, *m/z* 559.46 $[M - H]^-$; **2** – Desferrioxamine E, *m/z* 599.44 $[M - H]^-$.Among compounds found in the retention time from 8 to 9 minutes antimycins were observed with ions of *m/z* 519.34, 533.36, 547.37, 561.39, 575.42 $[M - H]^-$.

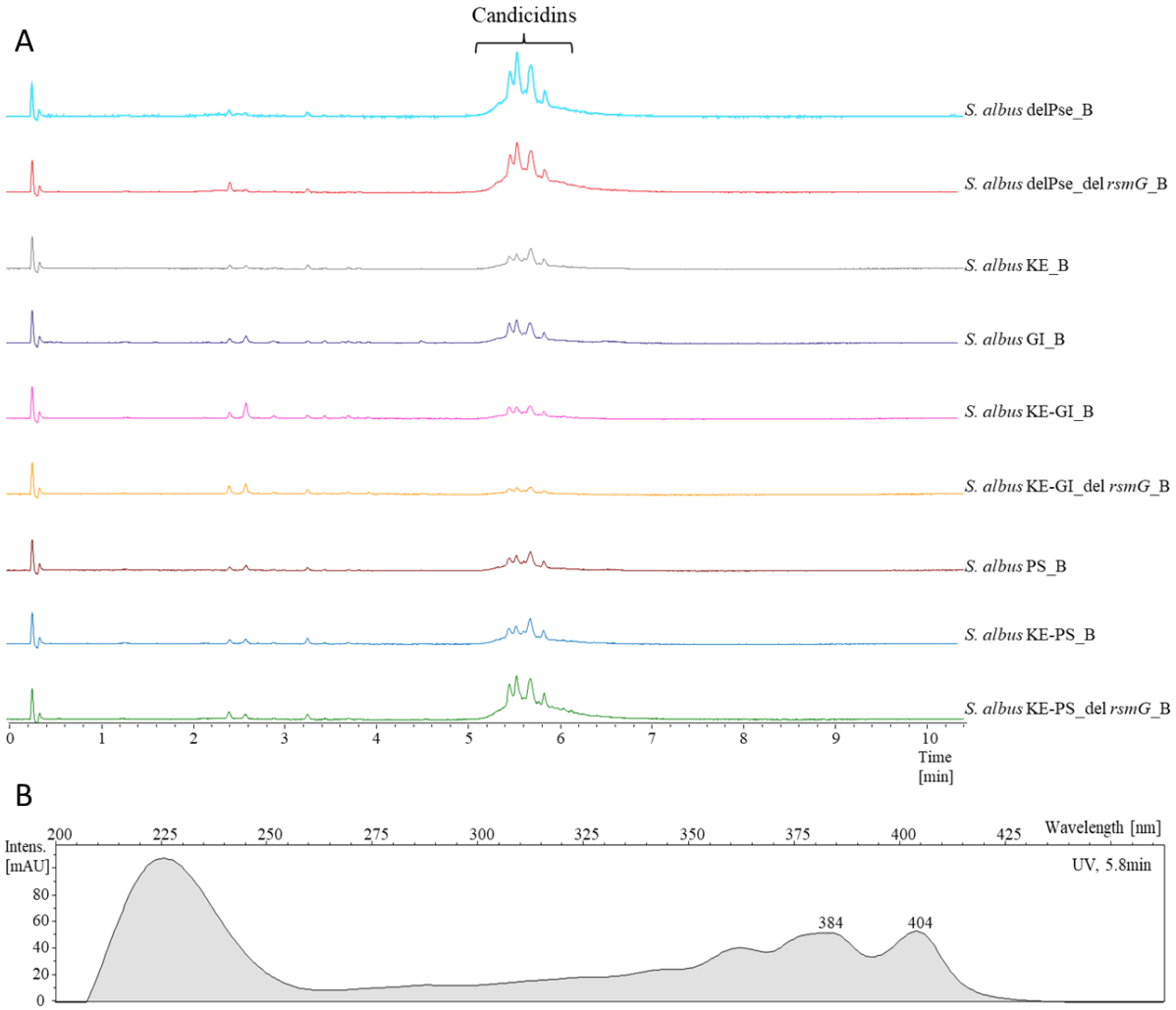


Figure S5. **A**. Comparison of UV chromatograms of butanol extracts from *S. albus* delPse and mutant strains at wavelength 404nm. The production level was evaluated by comparison of the UV 404nm peak areas from 5.4 until 6.0 min. **B**. The UV spectrum of candicidins in the butanol extract of *S. albus* delPse at the retention time 5.8 min.

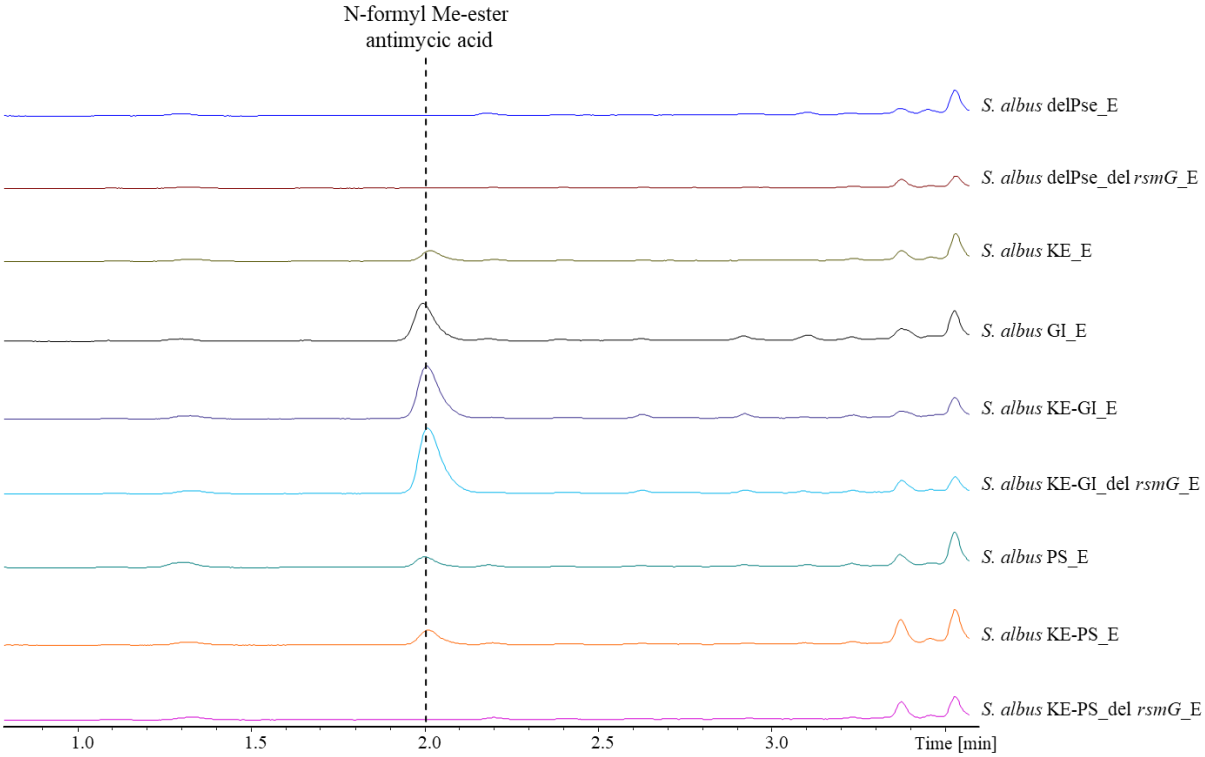


Figure S6. Comparison of the production level of N-formyl Me-ester antimycic acid in *S. albus* delPse and mutants strains.

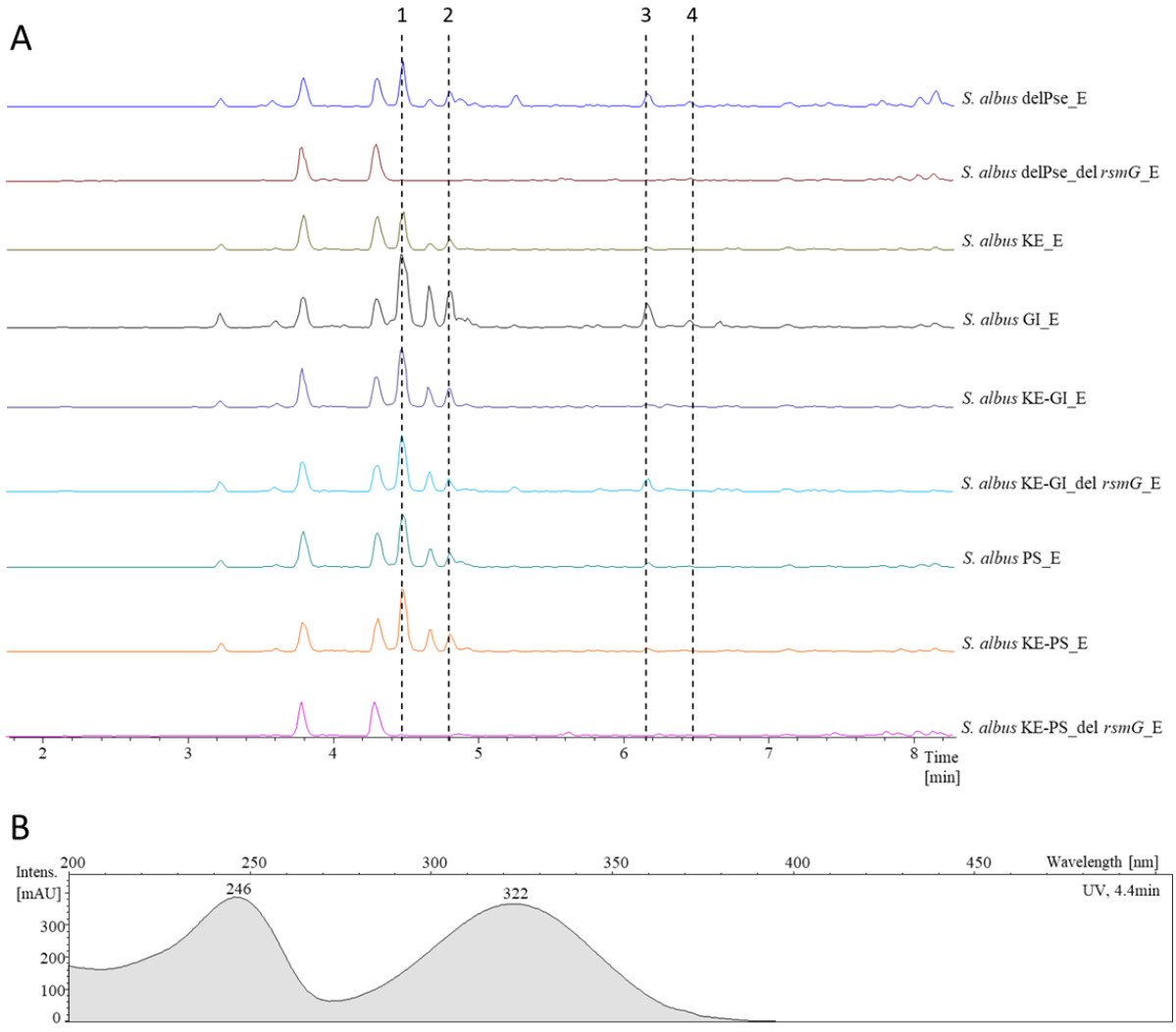


Figure S7. A. Comparison of base peak chromatograms in negative mode of butanol extracts from *S. albus* delPse and mutant strains. **1** – Paulomenol B, *m/z* 646.32 $[M - H]^-$; **2** – Paulomenol A , *m/z* 660.36 $[M - H]^-$; **3** – Paulomycin B , *m/z* 771.32 $[M - H]^-$; **4** – Paulomycin A , *m/z* 785.33 $[M - H]^-$. **B**. The UV spectrum of paulomycins in the butanol extract of *S. albus* delPse at the retention time 4.4 min.

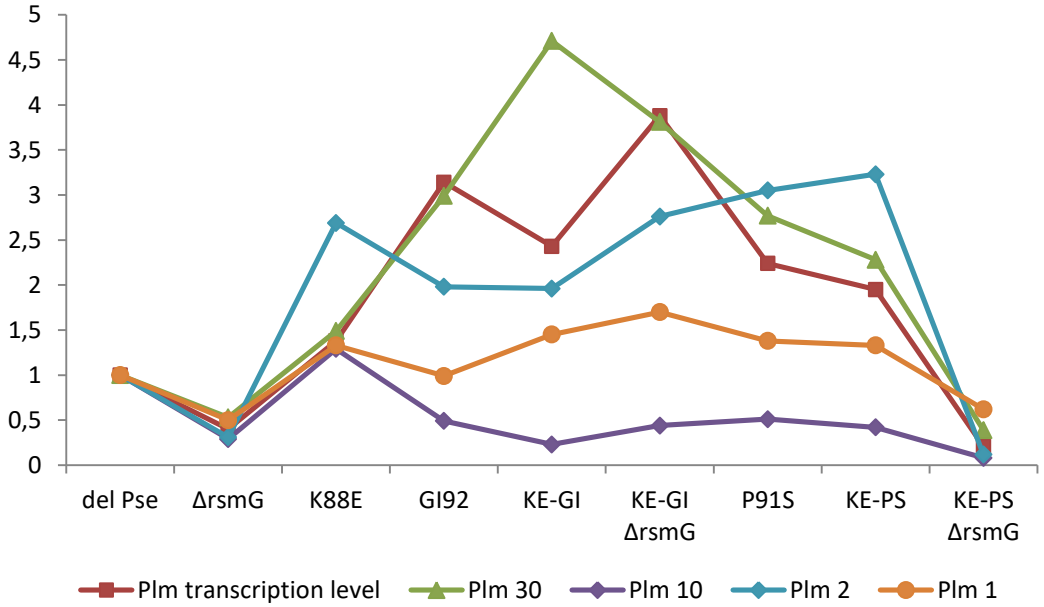


Figure S8. Comparison of the transcription level from paulomycin BGC with the transcription of regulatory genes from the cluster. Plm 1 correspond to TetR-family transcriptional regulator, Plm 2 – LuxR-family transcriptional regulator, Plm 10 – SARP-family transcriptional regulator and Plm 30 – LuxR-family transcriptional regulator.

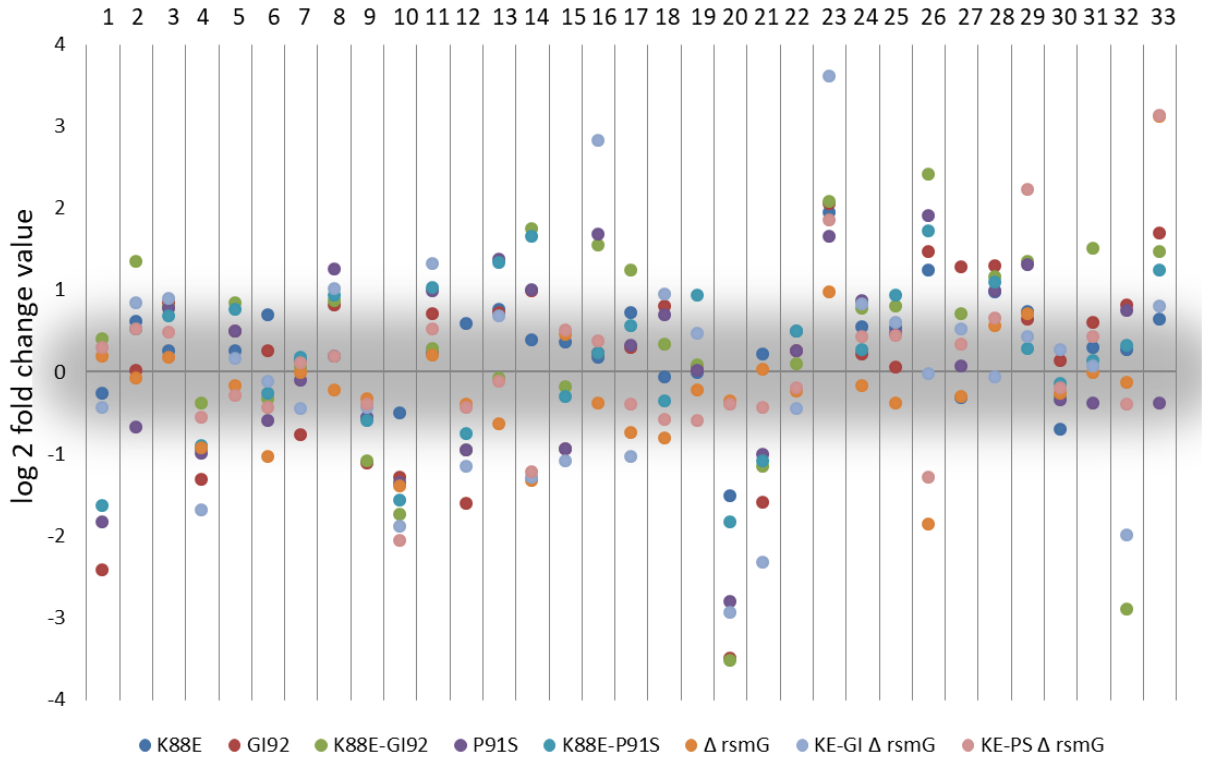


Figure S9. Transcription level of 33 sigma factors of *S. albus* in strains with *rpsL* mutations and *rsmG* deletion, compared to original strain. Data represents the log2 fold change values. 1 - sigma24; 2 - sigma24; 3 - sigma factor; 4 - sigma factor WhiG; 5 - SigB/SigF/SigG family; 6 - sigma factor; 7 - SigB/SigF/SigG family; 8 - rpoE sigma factor; 9 – sigE; 10 - SigB/SigF/SigG family; 11 - sigma factor HrdD; 12 - sigma -70 family; 13 - sigE family; 14 - SigB/SigF/SigG family; 15 - SigB/SigF/SigG family; 16 - sigE family; 17 - sig M; 18 - sigE family; 19 - sigma24; 20 - sigE family; 21 - sigma24; 22 - sigma factor ShbA; 23 - sigma factor; 24 - ECF sigma factor; 25 - sigma-70 family; 26 - ECF family; 27 - sigma-70 family; 28 - sigma-70 family; 29 - sig K; 30 - ECF family; 31 - ECF family; 32 – SigL; 33 - antA sigma factor.

Table S1. Sequences of primers; positions of mutations are underlined.

Primers name	Sequence (5´- 3´)	Application
K88E-for	GCAGGAGCACTCCATCGTGCTCGTGCGTGGTG GCCGTGTGGAGGACCTGCCGGGTGTTCGCTAC AAGATCATCCGCGGCTCCCTCGACA	Amplification of *rpsL* gene with K88E mutation
K88E-rev	TGTCGAGGGAGCCGCGGATGATCTTGTAGCGA ACACCCGGCAGGTCCTCCACACGGCCACCACG CACGAGCACGATGGAGTGCTCCTGC	
L90K-for	GCACTCCATCGTGCTCGTGCGTGGTGGCCGTG TGAAGGACAAGCCGGGTGTTCGCTACAAGATC ATCCGCGGCTCCCTCGACA	Amplification of *rpsL* gene with L90K mutation
L90K-rev	TGTCGAGGGAGCCGCGGATGATCTTGTAGCGA ACACCCGGCTTGTCCTTCACACGGCCACCACG CACGAGCACGATGGAGTGC	
P91S-for	CTCCATCGTGCTCGTGCGTGGTGGCCGTGTGA AGGACCTGTCGGGTGTTCGCTACAAGATCATC CGCGGCTCCCTCGACAC	Amplification of *rpsL* gene with P91S mutation
P91S-rev	GTGTCGAGGGAGCCGCGGATGATCTTGTAGCG AACACCCGACAGGTCCTTCACACGGCCACCAC GCACGAGCACGATGGAG	
R94G-for	CTCCATCGTGCTCGTGCGTGGTGGCCGTGTGA AGGACCTGCCGGGTGTTGGCTACAAGATCATC CGCGGCTCCCTCGACACCCAGGGTGT	Amplification of *rpsL* gene with R94G mutation
R94G-rev	ACACCCTGGGTGTCGAGGGAGCCGCGGATGAT CTTGTAGCCAACACCCGGCAGGTCCTTCACAC GGCCACCACGCACGAGCACGATGGAG	
GI92-for	GCCGTGTGAAGGACCTGCCGGGGGGTGTTCGC TACAAGATCAT	Amplification of *rpsL* gene with GI92 insertion
GI92-rev	ATGATCTTGTAGCGAACACCCCCCGGCAGGTC CTTCACACGGC	
K88E-GI92-for	TCGTGCGTGGTGGCCGTGTGGAGGACCTGCCG GGGGGTGTTCGCTACAAGATCAT	Amplification of *rpsL* gene with K88E-GI92 combination
K88E-GI92-rev	ATGATCTTGTAGCGAACACCCCCCGGCAGGTC CTCCACACGGCCACCACGCACGA	
K88E-P91S-	TCGTGCGTGGTGGCCGTGTGGAGGACCTGTCG	Amplification of *rpsL*

for	GGTGTTCGCTACAAGATC	gene with K88E-P91S combination
K88E-P91S-rev	GATCTTGTAGCGAACACCCGACAGGTCCTCCACACGGCCACCACGCACGA	
K88R-for	CGTGCGTGGTGGCCGTGTGAGGGACCTGCCGGGTGTTCGCT	Amplification of *rpsL* gene with K88R mutation
K88R-rev	AGCGAACACCCGGCAGGTCCCTCACACGGCCACCACGCACG	
R86P-for	CGTGCTCGTGCGTGGTGGCCCTGTGAAGGACCTGCCGGGTG	Amplification of *rpsL* gene with R86P mutation
R86P-rev	CACCCGGCAGGTCCTTCACAGGGCCACCACGCACGAGCACG	
rpsL-Red-for	CCTGCAGGAGCACTCCATCGTGCTCGTGCGTGGTGGCCGTGTGAAGGACCGTTTAAACAATACTTGACATATCACTGT	Amplification of a *hyg* gene for an introduction into *rpsL* gene on the BAC
rpsL-Red-rev	ACACCCTGGGTGTCGAGGGAGCCGCGGATGATCTTGTAGCGAACACCCGGGTTTAAACTCAGGCGCCGGGGGCGGTGT	
rpsL-chk-for	GGCACACAGAAACCGGAGA	Amplification of *rpsL* gene with mutations and sequencing of the *rpsL* gene from modified BACs
rpsL-chk-rev	TGAGGGAGGTCACCAGAGG	
am-rpsL-for	TCTGTGGCCGAAAACACACAGAGGGTGGGAGGGGGCGTGATTCCGGGGATCCGTCGACC	Amplification of *am* resistance gene for its introduction into BAC 1F1
am-rpsL-rev	CCCCGTACCCCGGGGCGGACCGGCGCCCTCCGCTGCATGTGTAGGCTGGAGCTGCTTC	
hyg-rpsL-for	TCCGAGCTGCTTGAGAAGGGCCCGTACGACTCCGGTCGTATATAGGAACTTCGAAGTTCC	Amplification of a *hyg* resistance gene for its introduction into BAC 1F1
hyg-rpsL-rev	CAGGGACTCCGGGTGACGCGCCCCGCGAAAACCCCACCCGATAACTTCGTATAGCATACATTATACGAAGTTATGGAACTTCGGAATAGGAACT	
del-rsmG-for	GGCCGGCCCCTGTGACGGGACCGGCCCCTCGGTAAAAAGACGCGGGTGTCTGTAGGCTGGAGCTGCTTCG	Amplification of a *am* resistance gene for deletion of the *rsmG* gene on the BAC 1L5
del-rsmG-rev	TTCGTCAGACATCAGTACGTTCGAGTTCGCGG	

	TACGGAAGGACGGTCCCCTTCCGGGGATCCGTCGACCC	
rsmG-chk-for	GGATTCACTCTCCTCGACTT	Sequencing of the *rsmG* gene fragment
rsmG-chk-rev	CCTCGGGACGCCCGTGGGCG	

Table S2. Natural product BGCs identified in *S. albus* J1074 by genome mining and computational analysis.

Cluster	Type	Location	Product
1	Hybrid PKS-NRPS	462-61269	Unknown
2	Alteramide	239019-258716	Frontalamide
3	Terpene	308546-335040	Hopene
4	Bacteriocin	417358-434605	Unknown
5	Deoxysugars	674471-722273	Paulomycin
6	Bacteriocin	879983-891448	Unknown
7	NRPS	1136314-1199510	Lipopeptide
8	Siderophore	1268479-1282477	Aerobactin-like
9	Terpene	1541823-1555798	Geosmin
10	Echosides	1818600-1833697	Oxasolomycin
11	Terpene	1865969-1883984	Albaflavenone
12	Lanthipeptide	2386240-2403633	Unknown
13	Bacteriocin	2561155-2566257	Lactococcin 972-family
14	Labirynthopeptide	2717782-2726087	SapB
15	NRPS	3553899-3603948	Unknown
16	NRPS	3892879-3983189	Gramicidin-like
17	NRPS	4469268-4514112	Unknown
18	Siderophore	4745388-4752013	Desferrioxamine
19	Ectoine synthase	5639338-5643151	Ectoine
20	NRPS	6350051-6381031	Indigoidin
21	Polyprenyl synthase	6400999-6430494	Carotenoids
22	Type III PKS	6520372-6561837	Tetrahydroxynaphtalene, flaviolin
23	Type I PKS	6566245-6721884	Candicidins
24	Hybrid NRPS/PKS	6730528-6755217	Antimycin
25	Hybrid NRPS/PKS	6755265-6776627	Unknown
26	Type I PKS	6776705-6838358	Unknown

Table S3. Transcription level of indigenous clusters in mutant strains compared to *S.albus delPse* strain. Data represent values in fold change.

fold change								
	Δ rsmG	K88E	GI92	KE-GI	KE-GI Δ rsmG	P91S	KE-PS	KE-PS Δ rsmG
PKS-NRPS hybrid	1,73	0,76	3,92	0,88	2,42	2,65	1,41	1,73
Alteramide/Frontalamide	1,7	3,38	0,41	0,88	0,65	1,27	1,81	1,72
Terpene/ Hopanoid	1,07	0,78	0,72	0,26	0,66	0,55	0,53	0,83
Bacteriocin 1	1,74	2,23	1,04	0,17	0,5	0,92	0,49	1,18
Paulomycin	0,4	1,38	3,14	2,43	3,88	2,24	1,95	0,21
Bacteriocin 2	1,16	0,96	1,05	2,62	0,78	1,54	0,93	1,01
NRPS	0,15	0,24	1,9	0,56	2,89	0,7	0,43	0,11
Siderophore	0,83	1,14	1,09	0,74	0,12	0,63	0,59	0,92
Terpene / Geosmin	1,82	2,04	0,5	0,96	0,31	1,67	1,69	1,95
Echosides / Oxasolomycin	1,34	1,38	0,97	1,1	1,05	1,01	1,01	1,85
Terpene/ Albaflavenone	2,39	0,96	1,37	1,56	3,78	0,92	1,07	2,72
Lanthipeptide	1,71	0,29	0,52	0,48	0,36	0,46	0,38	1,33
Bacteriocin 3	0,09	0,11	0,12	0,14	0,24	0,18	0,41	0,16
Labirynthopeptide / SapB	0,76	0,99	0,21	0,5	0,11	0,35	0,5	0,74
NRPS	2,02	1,52	0,3	0,52	0,08	0,85	0,73	1,88
NRPS / Gramicidin like	0,78	1,23	5,26	6,0	14,06	1,16	1,78	0,8
NRPS	0,41	1,01	2,24	1,9	2,85	0,89	1,09	0,36
Siderophore/ Desferrioxamine	1,55	0,43	0,29	0,68	0,53	0,89	0,75	1,88
Ectoine	0,83	0,75	0,95	0,89	0,79	1,01	0,97	0,8
NRPS/ Indigoidin	0,91	1,32	4,37	3,4	3,36	1,92	1,76	0,99
Polyprenyl synthase / Carotenoids	1,32	0,98	0,82	0,01	0,01	0,01	0,01	0,54
Type III PKS / Tetrahydronaphtelene	1,65	1,6	0,83	1,48	0,25	0,9	0,92	1,53
Type I PKS / Candicidins	3,49	0,77	1,46	1,22	1,01	0,71	1,1	3,56
Hybrid NRPS/PKS / Antimycin	5,19	0,96	2,86	1,78	1,51	1,16	1,64	5,86
Hybrid NRPS/PKS	1,59	0,99	1,73	0,98	1,19	1,29	0,84	2,1
PKS I	2,09	0,71	4,68	2,26	5,54	0,65	1,01	2,07

Table S4. Transcription level of indigenous clusters in mutant strains compared to *S.albus delPse* strain. Data represent values in log2 fold change.

log2 fold change								
	Δ *rsmG*	K88E	GI92	KE-GI	KE-GI Δ r*smG*	P91S	KE-PS	KE-PS Δ *rsmG*
PKS-NRPS hybrid	0,791	-0,396	1,971	-0,184	1,275	1,406	0,496	0,790
Alteramide/Frontalamide	0,766	1,757	-1,286	-0,184	-0,621	0,345	0,856	0,782
Terpene/ Hopanoid	0,098	-0,358	-0,474	-1,943	-0,599	-0,862	-0,916	-0,268
Bacteriocin 1	0,799	1,157	0,057	-2,556	-1	-0,120	-1,029	0,238
Paulomycin	-1,322	0,465	1,651	1,281	1,956	1,163	0,963	-2,251
Bacteriocin 2	0,214	-0,058	0,070	1,389	-0,358	0,622	-0,104	0,014
NRPS	-2,736	-2,058	0,925	-0,836	1,531	-0,514	-1,217	-3,184
Siderophore	-0,268	0,189	0,124	-0,434	-3,058	-0,666	-0,761	-0,120
Terpene / Geosmin	0,863	1,028	-1	-0,058	-1,689	0,739	0,757	0,963
Echosides / Oxasolomycin	0,422	0,464	-0,043	0,137	0,070	0,014	0,014	0,887
Terpene/ Albaflavenone	1,257	-0,058	0,454	0,641	1,918	-0,120	0,097	1,443
Lanthipeptide	0,773	-1,785	-0,943	-1,058	-1,473	-1,120	-1,395	0,411
Bacteriocin 3	-3,473	-3,184	-3,058	-2,836	-2,058	-2,473	-1,286	-2,643
Labirynthopeptide / SapB	-0,395	-0,014	-2,251	-1	-3,184	-1,514	-1	-0,434
NRPS	1,014	0,604	-1,736	-0,943	-3,643	-0,234	-0,454	0,910
NRPS / Gramicidin like	-0,358	0,298	2,395	2,584	3,813	0,214	0,831	-0,321
NRPS	-1,286	0,014	1,163	0,925	1,510	-0,168	0,124	-1,473
Siderophore/ Desferrioxamine	0,632	-1,217	-1,785	-0,556	-0,915	-0,168	-0,415	0,910
Ectoine	-0,268	-0,415	-0,074	-0,168	-0,340	0,014	-0,043	-0,321
NRPS/ Indigoidin	-0,136	0,400	2,127	1,765	1,748	0,941	0,815	-0,014
Polyprenyl synthase / Carotenoids	0,400	-0,029	-0,286	-6,643	-6,643	-6,643	-6,643	-0,888
Type III PKS / Tetrahydroxynaphtelene	0,722	0,678	-0,268	0,565	-2	-0,152	-0,120	0,613
Type I PKS / Candicidins	1,803	-0,377	0,545	0,286	0,014	-0,494	0,137	1,831
Hybrid NRPS/PKS / Antimycin	2,375	-0,058	1,516	0,831	0,594	0,214	0,713	2,550
Hybrid NRPS/PKS	0,669	-0,014	0,790	-0,029	0,250	0,367	-0,251	1,070
PKS I	1,063	-0,494	2,226	1,176	2,469	-0,621	0,014	1,049

Table S5. Transcription level of sigma factors in mutant strains compared to *S. albus delPse* strain. Data represent values in log2 fold change.

Gene's ID XNR_RS_	family of sigma factor	Δ rsmG	K88E	GI92	KE-GI	KE-GI Δ rsmG	P91S	KE-PS	KE-PS Δ rsmG
03030	sigma24	0,19	-0,26	-2,42	0,4	-0,43	-1,83	-1,63	0,29
03690	sigma24	-0,07	0,62	0,01	1,34	0,83	-0,67	0,52	0,52
05170	sigma factor	0,18	0,25	0,84	0,79	0,89	0,77	0,68	0,48
05970	sigma factor WhiG	-0,93	-0,94	-1,32	-0,39	-1,68	-0,99	-0,9	-0,55
07380	SigB/SigF/SigG family	-0,16	0,25	0,49	0,84	0,16	0,49	0,76	-0,28
07675	sigma factor	-1,02	0,69	0,25	-0,33	-0,12	-0,59	-0,26	-0,43
07760	SigB/SigF/SigG family	-0,01	0,14	-0,77	0,06	-0,45	-0,11	0,18	0,11
07865	rpoE sigma factor	-0,21	0,19	0,81	0,87	1,01	1,26	0,94	0,18
08220	sigE	-0,33	-0,56	-1,12	-1,09	-0,43	-0,57	-0,6	-0,39
09755	SigB/SigF/SigG family	-1,39	-0,51	-1,29	-1,74	-1,88	-1,36	-1,57	-2,05
10635	sigma factor HrdD	0,20	0,23	0,71	0,28	1,32	0,99	1,03	0,52
11175	sigma -70 family	-0,39	0,59	-1,61	-0,96	-1,15	-0,95	-0,76	-0,43
13665	sigE family	-0,64	0,76	0,72	-0,08	0,68	1,37	1,33	-0,12
14315	SigB/SigF/SigG family	-1,32	0,39	0,99	1,75	-1,28	1	1,65	-1,21
14320	SigB/SigF/SigG family	0,45	0,36	-0,94	-0,19	-1,08	-0,94	-0,31	0,50
14400	sigE family	-0,39	0,17	0,23	1,55	2,81	1,68	0,23	0,37
14850	sig M	-0,73	0,72	0,3	1,24	-1,02	0,32	0,56	-0,39
16255	sigE family	-0,81	-0,06	0,8	0,33	0,94	0,7	-0,36	-0,57
16370	sigma 24	-0,21	-0,01	0,05	0,08	0,46	0,01	0,94	-0,59
17300	sigE family	-0,36	-1,52	-3,5	-3,52	-2,94	-2,8	-1,83	-0,39
17485	sigma 24	0,02	0,21	-1,59	-1,15	-2,32	-1,01	-1,09	-0,43
18855	sigma factor ShbA	-0,23	0,5	0,26	0,1	-0,45	0,26	0,49	-0,20
19155	sigma factor	0,97	1,94	2,05	2,08	3,60	1,65		1,85
19540	ECF sigma factor	-0,16	0,55	0,22	0,77	0,83	0,87	0,27	0,43

19730	sigma-70 family	-0,37	0,54	0,06	0,8	0,60	0,47	0,94	0,44
19800	ECF family	-1,86	1,24	1,46	2,41	-0,02	1,9	1,72	-1,28
19995	sigma-70 family	-0,30	-0,32	1,28	0,71	0,51	0,07		0,33
22170	sigma-70 family	0,56	0,97	1,29	1,16	-0,05	0,99	1,1	0,65
25815	sig K	0,71	0,74	0,64	1,34	0,42	1,31	0,28	2,22
26190	ECF family	-0,26	-0,7	0,13	-0,31	0,26	-0,35	-0,14	-0,20
27905	ECF family	-0,01	0,29	0,6	1,5	0,07	-0,39	0,13	0,43
28030	sigL	-0,13	0,27	0,82	-2,9	-2	0,75	0,32	-0,39
29215	antA sigma factor	3,12	0,64	1,69	1,47	0,79	-0,39	1,24	3,13

Table S6. Transcription level of genes that undergo the most changes in mutant strains, compared to *S. albus delPse* strain. Data represent values in fold change.

XNR_RS12890-12915 – an unknown cluster of genes consisting of SAM-dependant methyl-transferase genes, hypothetical genes and amine oxidase gene.

XNR_RS13105-13110 – hypothetical genes.

XNR_RS03500-03550 – sulfur metabolism genes.

XNR_RS06415 – unknown ATP-binding response regulator receiver.

	Δ rsmG	K88E	GI92	KE-GI	KE-GI Δ rsmG	P91S	KE-PS	KE-PS Δ rsmG
XNR_RS12890-12915	5,5	7,07	4,1	4,47	3,02	9,41	5,41	18,87
XNR_RS13105-13110	0,51	16,5	103,35	1,29	71,15	1,63	12,89	0,5
XNR_RS03500-03550	8,96	9,07	15,37	14,73	18,37	18,84	11,32	17,56
XNR_RS06415	0,004	0,002	0,001	0,001	0,001	0,014	0,001	0,001

II

Streptomyces albus: A New Cell Factory for Non-Canonical Amino Acids Incorporation into Ribosomally Synthesized Natural Products

Lopatniuk M., Myronovskyi M., Luzhetskyy A.

ACS Chem Biol, **2017**, 12, 2362−2370. DOI: 10.1021/acschembio.7b00359

Published online 31st July 2017

Lopatniuk M. has done cloning of the cinnamycin biosynthetic gene cluster, expression of the last, incorporation of unnatural amino acids, structures elucidation and manuscript writing. **Myronovskyy M**. supervised the work, provided the system for pyrrolysine analogues incorporation, and participated in planning of the experiments and manuscript writing. **Luzhetskyy A.** supervised the work, participated in planning of the experiments and critical manuscript reading and correction.

Author's name	Contribution to the work, %
Lopatniuk M.	70
Myronovskyi M.	15
Luzhetskyy A.	15

ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

Streptomyces albus: A New Cell Factory for Non-Canonical Amino Acids Incorporation into Ribosomally Synthesized Natural Products

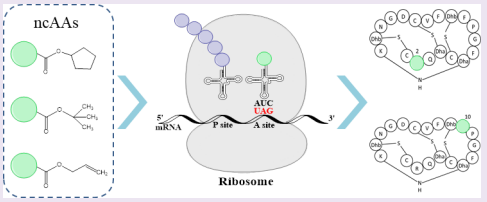
Mariia Lopatniuk,[†] Maksym Myronovskyi,[†] and Andriy Luzhetskyy*[,†,‡]

[†]Department of Pharmaceutical Biotechnology, Saarland University, 66123 Saarbrücken, Germany

[‡]Helmholtz-Institute for Pharmaceutical Research, Saarland Campus, Building C2.3, 66123 Saarbrücken, Germany

Supporting Information

ABSTRACT: The incorporation of noncanonical amino acids (ncAAs) with different side chains into a peptide is a promising technique for changing the functional properties of that peptide. Of particular interest is the incorporation of ncAAs into peptide-derived natural products to optimize their biophysical properties for medical and industrial applications. Here, we present the first instance of ncAA incorporation into the natural product cinnamycin in streptomycetes using the orthogonal pyrrolysyl-tRNA synthetase/tRNA^{Pyl} pair from *Methanosarcina barkeri*. This approach allows site-specific incorporation of ncAAs *via* the read-through of a stop codon by the suppressor tRNA^{Pyl}, which can carry different pyrrolysine analogues. Five new deoxycinnamycin derivatives were obtained with three distinct pyrrolysine analogues incorporated into diverse positions of the antibiotic. The combination of partial hydrolysis and MS/MS fragmentation analysis was used to verify the exact position of the incorporation events. The introduction of ncAAs into different positions of the peptide had opposite effects on the peptide's biological activity.



Ribosomally synthesized and post-translationally modified peptides (RiPPs) represent a large class of structurally divergent natural products with potent biological activities. Despite their wide diversity, they share a common mechanism of biosynthesis. The ribosomally synthesized precursor peptide consists of an N-terminal leader peptide and C-terminal core peptide. The core peptide further undergoes a variety of modifications, followed by proteolytic removal of the leader peptide.[1] The large complexity of RiPP structures is achieved by different modification enzymes, the activities of which vary over a huge range, from cyclization to dehydration and di- and monosulfide bond formation.[2] Because this class of natural products exhibits a variety of structures, they possess a wide range of biological activities, such as antimicrobial,[3] antifungal,[4,5] and antiviral[6,7] activities.

The biosynthesis of RiPPs through precursor peptides provides an opportunity to alter the amino acids in the final structure. Each single codon in a prepeptide gene corresponds to a certain amino acid in the peptide backbone, and site-specific codon substitution enables controlled replacement of amino acids, which in turn can alter the structure and functions of the new peptide. Recently, it became possible to further expand the set of proteinogenic amino acids[8] that can be incorporated into peptides with unnatural amino acids.[9] The most interesting of these are noncanonical amino acids (ncAAs) with new reactive groups that can endow a protein with new physicochemical properties or enable bioorthogonal protein conjugation, also known as "click chemistry."[10−12]

Two methods of incorporating ncAAs into the peptide structure have been described. The "genetic code engineering" strategy requires the construction of a mutant strain, auxotrophic for a specific amino acid. When cultivated in the absence of this amino acid, the mutant strain, as a substitute, is able to incorporate structurally related ncAAs into the structure of the protein. Using this approach, ncAAs have been incorporated into the lantibiotic lichenicidin[13] and the lasso peptide capistruin[14] using *Escherichia coli*. This method has the limitations that only isostructural ncAAs can be incorporated and that the strategy has been implemented only in *E. coli* and not in streptomycetes, one of the natural producers of RiPPs. The "genetic code expansion" method represents the second strategy for incorporating ncAAs into the structure of peptides. In this approach, an orthogonal aminoacyl-tRNA synthetase/suppressor tRNA (aaRS/tRNA) pair is utilized to introduce a wide variety of ncAAs into the protein structure, using stop-codon read-through.[15] Pyrrolysyl-tRNA synthetase/tRNA^{Pyl} (pylRS/tRNA^{Pyl}) from *Methanosarcina barkeri*/*mazei*[16−18] and tyrosyl-tRNA synthetase/tRNA^{Tyr} from *Methanococcus jannaschii*[19,20] are the most widely used aaRS/tRNA pairs for stop-codon suppression. They have been successfully implemented in *E. coli*, *Mycobacterium*, and eukaryotes. In particular, the pyrrolysyl-tRNA synthetase/tRNA^{Pyl} pair was widely used for incorporation of ncAAs into RiPPs in *E. coli*. Very efficient examples are the incorporation of ncAAs into lasso peptide MccJ25[21] and different lantipeptides.[22,23] There is also an example of utilizing the pyrrolysyl-tRNA synthetase/tRNA^{Pyl} pair for incorporation of ncAAs into the thiopeptide antibiotic in the producer strain, *Bacillus cereus*.[24] However, there have

Received: May 3, 2017
Accepted: July 31, 2017
Published: July 31, 2017

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

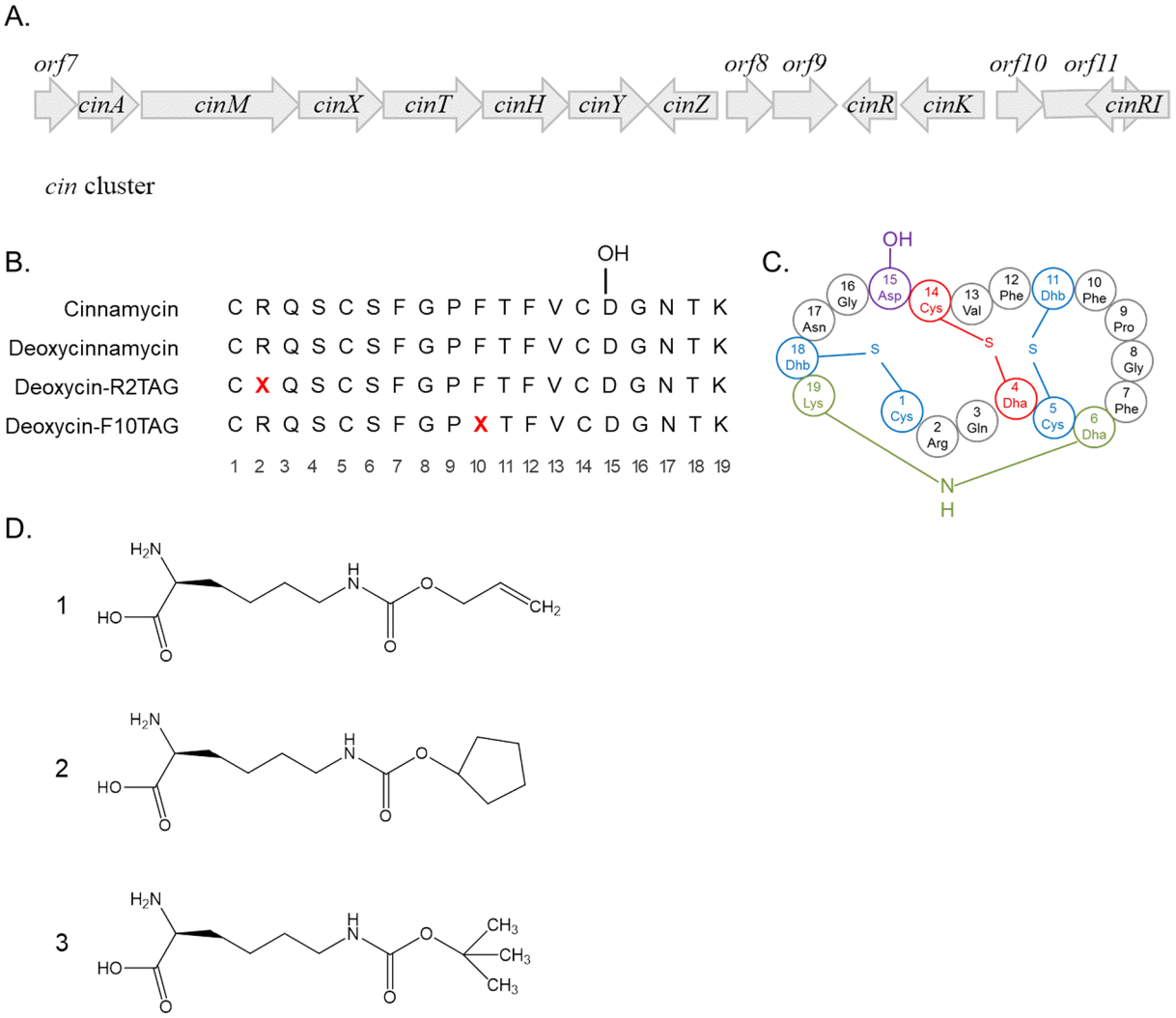


Figure 1. (A) Structure of the cinnamycin biosynthetic gene cluster. (B) The amino acid composition of the cinnamycin core-peptide, with incorporation positions indicated by the red X. (C) Structure of the cinnamycin lantibiotic. (D) Chemical structure of the noncanonical amino acids used in feeding experiments, with 1, H-Lys-Alloc−OH; 2, H-Lys-Cyc−OH; and 3, H-Lys-Boc−OH.

only been two reports demonstrating the exploitation of this type of ncAA incorporation system in streptomycetes, where ncAAs were incorporated into the reporter proteins GFP and GusA.[25,26] There has been no example of the introduction of ncAAs into the structure of secondary metabolites in streptomycetes. In our work, we aimed to incorporate ncAAs into a natural product in streptomycetes. Our group previously optimized the pylRS/tRNA^{Pyl} pair from *Methanosarcina barkeri* for the inducible incorporation of the pyrrolysine analogue Cyc into the GusA reporter in *Streptomyces albus* J1074.[25] In the present work, we chose to use the cinnamycin biosynthetic gene cluster to study the incorporation of ncAAs into natural products using the pylRS/tRNA^{Pyl} system (Figure 1, panel A). Cinnamycin belongs to a group of lantibiotics that are characterized by the presence of unique lanthionine (thioether) bridges (Figure 1, panel C).[27] Cinnamycin is a structural analogue of duramycin, which is currently undergoing clinical studies for cystic fibrosis treatment.[28,29] By directly binding to phosphatidylethanolamine (PE) in cell membranes,[30,31] cinnamycin can be exploited as an apoptosis imaging agent in cancer therapy;[32] it also acts as an antitumor agent[33] and inhibits the entry of many enveloped viruses including Ebola, Zika, dengue, and West Nile viruses by blocking their attachment to T-cells.[34]

Here, we describe the first instance of the utilization of the pylRS/tRNA^{Pyl} pair to incorporate ncAAs into a natural product in its natural production environment—streptomycetes. We obtained five cinnamycin derivatives with the ncAAs H-Lys-Alloc−OH (Alk), H-Lys-Cyc−OH (Cyc), and H-Lys-Boc−OH (Boc) incorporated into two different positions of lanthipeptide (Figure 1, panel D). The structures of the cinnamycin derivatives were verified by high-resolution mass spectrometry (HRMS) as well as partial hydrolysis followed by MS/MS fragmentation analysis. The influence of ncAA incorporation on the antibiotic activity was also described.

RESULTS AND DISCUSSION

Cloning of the Cinnamycin Biosynthetic Gene Cluster and its Expression in *S. albus*. In this work, we aimed to introduce ncAAs into the structure of the lanthipeptide antibiotic cinnamycin using the pyrrolysine system. To implement this strategy, a stop codon must be introduced into the desired position of the prepeptide gene and then suppressed by the pylRS/tRNA^{Pyl} pair. Upon pyrrolysine

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

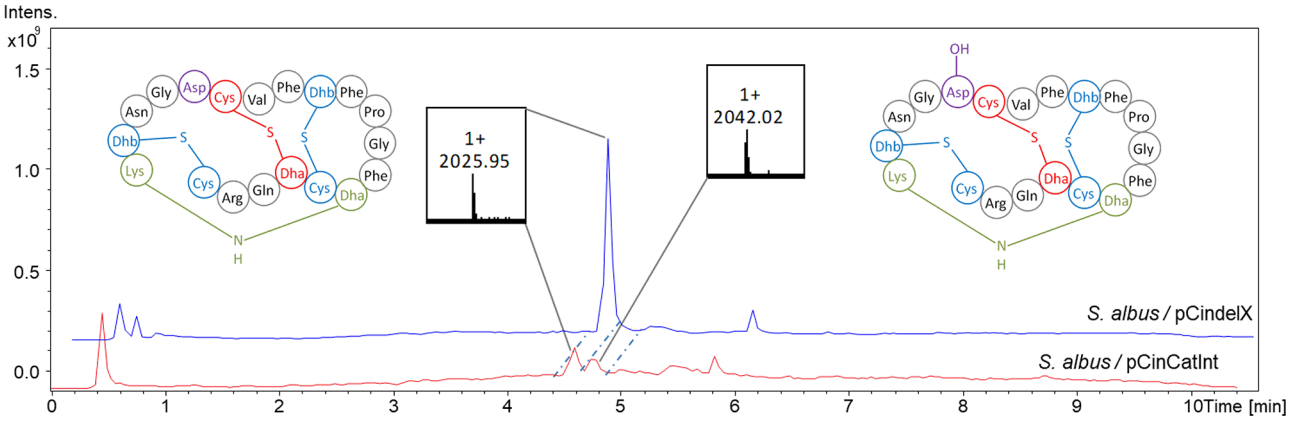


Figure 2. Comparison of the production of cinnamycin and deoxycinnamycin by *S. albus* strains. Extracted mass chromatogram in the range of 2000−3000 Da. The chromatogram of a sample from *S. albus* pCinCatInt is represented in red, and one from *S. albus* pCindelX is represented in blue.

analogue supplementation, the stop codon will be translated with the incorporation of an ncAA at the determined position. Such manipulations of the chromosome of the producing strain are challenging and time-consuming. Therefore, we decided to clone the *cin* cluster from the chromosome of *Streptomyces cinnamoneus* DSM 40005 to perform all genetic manipulations in *E. coli* and then to express new constructs in the heterologous host *Streptomyces albus*.[35−37] The sequence of the cinnamycin biosynthetic gene cluster (*cin*) from *S. cinnamoneus* has been previously reported.[38,39] Transformation associated recombination (TAR) in *Saccharomyces cerevisiae* BY4742 was used to directly clone the cinnamycin biosynthetic gene cluster from the chromosome of *S. cinnamoneus*.[40] The 600 bp homologous regions from the right and left side of the cluster were cloned into the *S. cerevisiae*/*E. coli*/*Streptomyces* shuttle vector pCLY10.[41] Yeast spheroplasts were transformed with a mixture of linearized capture vector and *S. cinnamoneus* chromosomal DNA. The clones containing the cloned *cin* cluster were identified by PCR. The plasmid with an intact *cin* cluster was named pCLY10cin. The sequence of the plasmid pCLY10cin was confirmed by sequencing and restriction mapping.

The vectors pCLY10cin, with the intact cluster, and pSOKTpyr-tsr, with the pylRS/tRNA^{Pyl} genes, are integrative in streptomycetes and contain the same VWB integrative system. Therefore, they cannot be maintained simultaneously in *Streptomyces* cells. To keep both plasmids in the same *Streptomyces* strain, the region with the VWB integrase gene and its attP site on the plasmid pCLY10cin was replaced by the region with the φC31 integrase gene and the cognate attP site, yielding pCinCatInt. *S. albus* pCinCatInt, harboring the *cin* cluster, was grown in soya-mannitol (SM) productive medium, and then the lanthipeptide was extracted from a culture broth with 1-butanol. Cinnamycin production was verified by HPLC-MS (Figure 2) and a bioassay against *Bacillus subtilis*. HRMS analysis revealed the presence of two compounds, with masses corresponding to cinnamycin (accurate mass −2039.844 Da) and deoxycinnamycin without a hydroxyl group on the aspartic acid residue in position 15 (Asp15-OH; accurate mass −2023.849 Da).

The presence of the nonhydroxylated cinnamycin derivative can be explained by insufficient activity of the hydroxylase CinX. The *cinX* gene in the cinnamycin biosynthetic gene cluster encodes an α-ketoglutarate/iron(II)-dependent hydroxylase that is involved in this decoration.[42] To simplify the profile of produced cinnamycins and further purification experiments, we decided to delete the *cinX* gene. The central part of the *cinX* gene was deleted in frame in the plasmid pCinCatInt, yielding pCindelX. pCindelX was expressed in *S. albus* J1074. HPLC-MS analysis revealed that *S. albus* pCindelX produces only deoxycinnamycin without the hydroxyl group in position Asp15. Surprisingly, the production level of deoxycinnamycin was approximately 5 times higher than the total cinnamycins produced by the *S. albus* pCinCatInt strain with the original cluster (Figure 2). Despite this higher production level, *S. albus* pCindelX exhibits lower bioactivity against *B. subtilis* than *S. albus* pCinCatInt (Figure S.1). It has been reported that Asp15-OH is important for the activity of cinnamycin.[43] The decreased biological activity of deoxycinnamycin likely allows the higher production level in the host strain.

Incorporation of Non-Canonical Amino Acids into the Structure of Cinnamycin. Two different positions, Arg2 and Phe10, in the cinnamycin prepeptide gene were chosen for ncAA incorporation. Neither of them is involved in the formation of lanthionine, methyl-lanthionine, or Lys-Dha bridges (Figure 1, panel B), and their substitution should not cause critical structural changes. However, these amino acids have different impacts on the recognition of the target phospholipid by cinnamycin. Phe10 is one of the amino acids involved in the formation of a hydrophobic cleft in the cinnamycin molecule that directly interacts with the glycerol moiety and fatty acid chain of PE.[44] Arg2 is localized in the hydrophilic part of the lantibiotic structure and is not relevant for target recognition. To incorporate ncAAs into positions Arg2 and Phe10, their codons in the prepeptide gene must be replaced with a TAG amber stop codon. Two plasmids, pCinR2TAG and pCinF10TAG, were constructed with TAG codons in positions 2 and 10, respectively. The in-frame insertion of TAG codons was achieved by overlap extension PCR amplification of the prepeptide gene followed by RedET.[45] The plasmids were transformed into *S. albus* to generate two strains: *S. albus* pCinF10TAG and *S. albus* pCinR2TAG. The resulting strains did not produce any cinnamycins, which indicates that the TAG stop codon is not suppressed by the *S. albus* strain without the plasmid pSOKTpyr-tsr.

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

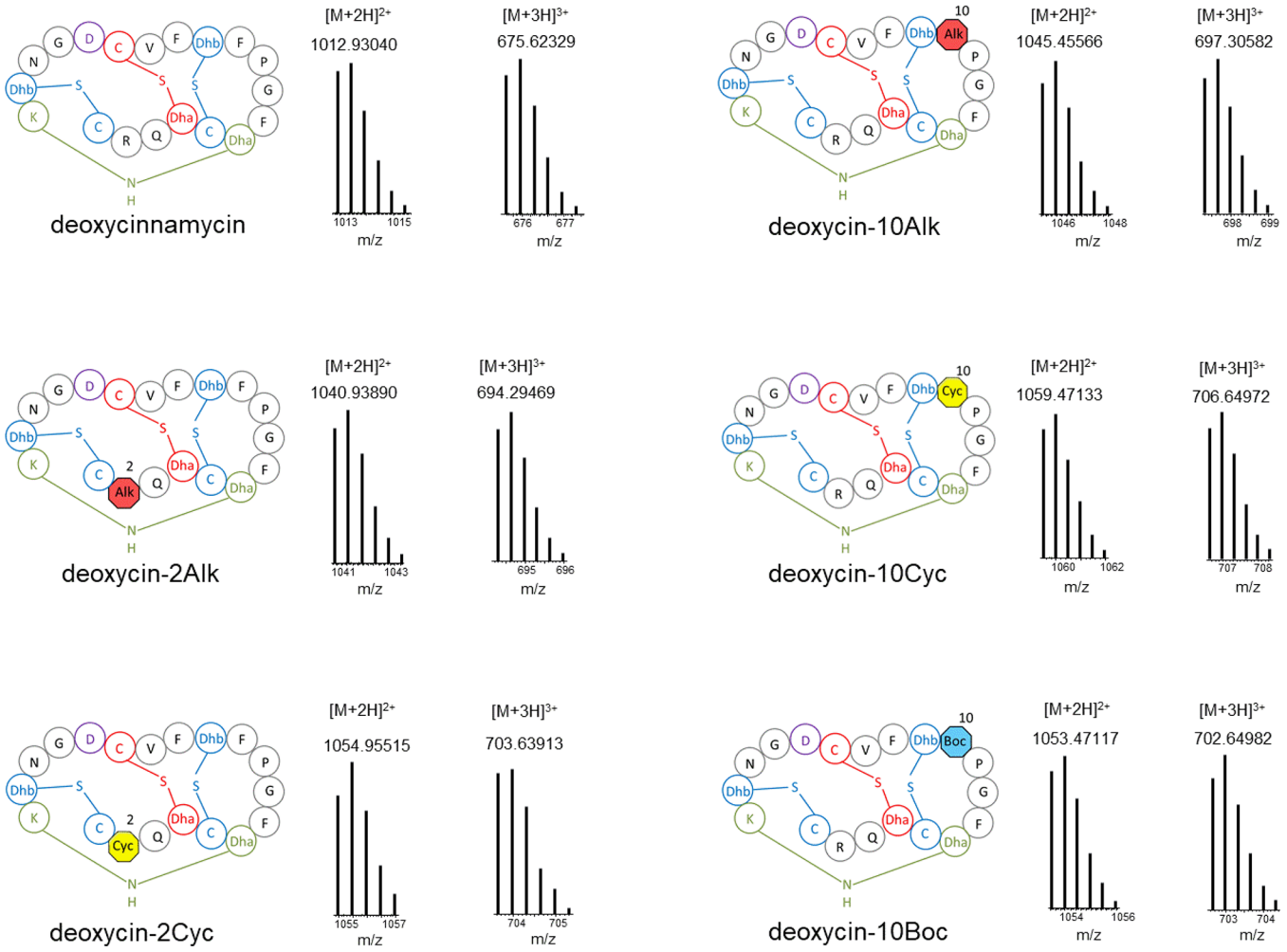


Figure 3. Structures of the new cinnamycins with incorporated noncanonical amino acids. Incorporation positions are marked with numbers. Distinct amino acids are shown in different colors: H-Lys-Alloc−OH (Alk), red; H-Lys-Cyc−OH (Cyc), yellow; and H-Lys-Boc−OH (Boc), blue.

Next, the plasmid pSOKTpyr-tsr, containing two genes, *pylS* and *pylT*, that encode pyrrolysyl-tRNA synthetase and $tRNA^{Pyl}$ from *Methanosarcina barkeri*, respectively, was transformed into *S. albus* pCinR2TAG and *S. albus* pCinF10TAG strains. In the presence of pyrrolysine or its analogues, products of these genes can suppress the stop codon by the incorporation of these amino acids. The unnatural amino acids Alk, Cyc, and Boc were used for incorporation. The strains *S. albus* pCinR2TAG pSOKTpyr-tsr and *S. albus* pCinF10TAG pSOKTpyr-tsr were grown in the presence of thiostrepton to induce the expression of the *pylS* and *pylT* genes. Then, 1 mL of the preculture was inoculated into a new medium, supplemented with thiostrepton and 20 mM of ncAA, and cultivated for another 5 days. After the compounds were extracted from the culture, the crude extract was purified on a cross-linked dextran polymer bead LH20 column and analyzed on an HPLC coupled with HRMS. Five new deoxycinnamycins with incorporated ncAAs were detected (Figure 3). Three of them contained the amino acids Alk, Cyc, and Boc incorporated into position 10 of the deoxycinnamycin structure: deoxycin-10Alk (accurate mass −2088.897 Da), deoxycin-10Cyc (accurate mass −2116.928 Da), and deoxycin-10Boc (accurate mass −2104.928 Da; Figure 3).

The production yield of new deoxycinnamycins with substitution in position 2 was much lower. Only two derivatives with incorporation of Alk and Cyc were detected: deoxycin-2Alk (accurate mass −2079.864 Da) and deoxycin-2Cyc (accurate mass −2107.895 Da; Figure 3). Among the ncAAs used, Alk was incorporated at the highest rate, yielding 8 mg/L of the cinnamycin derivative in position 10 and 0.75 mg/L in position 2. The incorporation efficiency of the amino acids Cyc and Boc was lower; the yields of deoxycin-10Cyc, deoxycin-10Boc, and deoxycin-2Cyc were 1.75 mg/L, 0.31 mg/L, and 0.032 mg/L, respectively. The diversity in the incorporation rates of different ncAAs can be explained by the varied binding affinity of the pyrrolysyl-tRNA synthetase to these amino acids.[46] However, the large difference in the incorporation efficiency of Alk into positions 2 and 10 can be explained only by the position effect. The low yield of new deoxycinnamycins with incorporation in position 2 may be due to the very close proximity of this amino acid to the leader peptide and to the recognition sequence for the protease that removes the leader peptide. Therefore, substitution in this position may interfere with the proteolysis reaction that releases the active cinnamycin.

Structure Verification of the New Cinnamycin Derivatives. To verify the exact position of the ncAAs in the structure of the new deoxycinnamycins, the combination of partial hydrolysis and MS/MS fragmentation of deoxycinnamycin and the most abundant derivative deoxycin-10Alk was

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

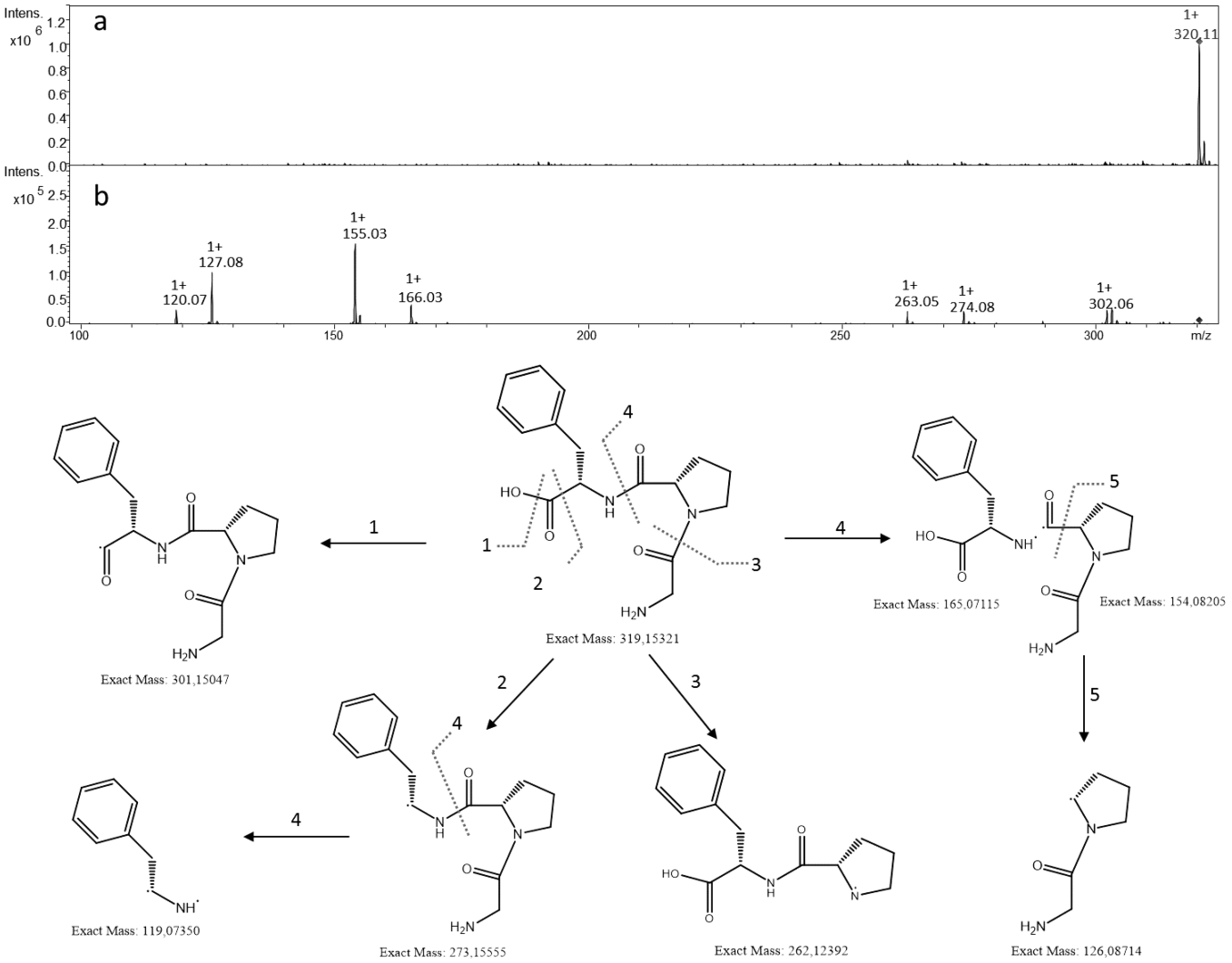


Figure 4. MS/MS fragmentation pattern of the deoxycinnamycin ion with a mass of 320.11^{+} after hydrolysis; positive mode ionization. (a) Mass spectrum of the sample after hydrolysis with a fragment corresponding to the amino acids Gly-Pro-Phe in positions 8−9−10. (b) MS/MS fragmentation analysis of the ion with a mass of 320.11^{+}. Fragmentation positions of the parental ion are demonstrated with numbers from 1 to 5.

used. Deoxycinnamycin and deoxycin-10Alk were purified using size-exclusion chromatography on a dextran polymer bead LH20 column and using reverse-phase chromatography on a preparative C18 column. Then, 0.5 mg of deoxycinnamycin and deoxycin-10Alk were partially hydrolyzed in 1 M hydrochloric acid. Tripeptides containing amino acids 8−9−10 from deoxycinnamycin and deoxycin-10Alk were identified using HPLC-HRMS. A fragment with an accurate mass of 319.153 Da, corresponding to the tripeptide Gly-Pro-Phe, was detected in the hydrolysate of deoxycinnamycin. Another fragment with an accurate mass of 384.201 Da that corresponds to the tripeptide Gly-Pro-Alk was identified in the hydrolysate of deoxycin-10Alk. HPLC-MS/MS analysis of these tripeptides further confirmed their structure (Figures 4 and 5). Daughter dipeptides with amino acids 8−9 and 9−10, respectively, as well as other fragmentation events were detected (a loss of $[H_2O]$ = 18 in mass, [CO] = 28 in mass and $[CO + H_2O]$ = 46 in mass). The data obtained from the partial hydrolysis of deoxycinnamycin and deoxycin-10Alk with subsequent MS/MS fragmentation provided clear evidence that the amino acid Alk was incorporated into position 10 of the structure of deoxycin-10Alk.

The native, not hydrolyzed, compounds deoxycinnamycin, deoxycin-10Alk, deoxycin-2Alk, and deoxycin-10Cyc were examined by MS/MS fragmentation analysis. In the case of deoxycinnamycin, deoxycin-10Alk, and deoxycin-2Alk, the losses of Phe12 (147.069 Da), Val13 (99.067 Da), and the fragment with both amino acid residues together, Phe12-Val13 (246.136 Da), were detected (Figure S.2). Deoxycin-10Cyc was unstable during ionization. In addition to its full-size ion $[2116.925 + 3H]^{3+}$ = 706.649 Da and butanol adduct $[2116.925 + But +3H]^{3+}$ = 730.328 Da, a nonselective fragment $[2004.875 + 3H]^{3+}$ = 669.299 Da and butanol adduct $[2004.875 + But +3H]^{3+}$ = 692.978 Da were detected (Figure S.3). These fragments appear due to the loss of the cyclopentyl formate group of the Cyc residue (112.050 Da) during ionization and correspond to masses of the deoxycin-10Cyc and its adduct after the loss of this group. The MS/MS fragmentation analysis of the daughter fragment lacking the cyclopentyl formate group revealed fragments lacking the next amino acids and amino acid combinations: Cyc10 lacking cyclopentyl formate (127.746 Da), Pro9-Cyc10 lacking cyclopentyl formate (225.771 Da), Phe12 (147.309 Da), Val13 (99.769 Da), and Phe12-Val13 (245.717; Figure S.4).

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

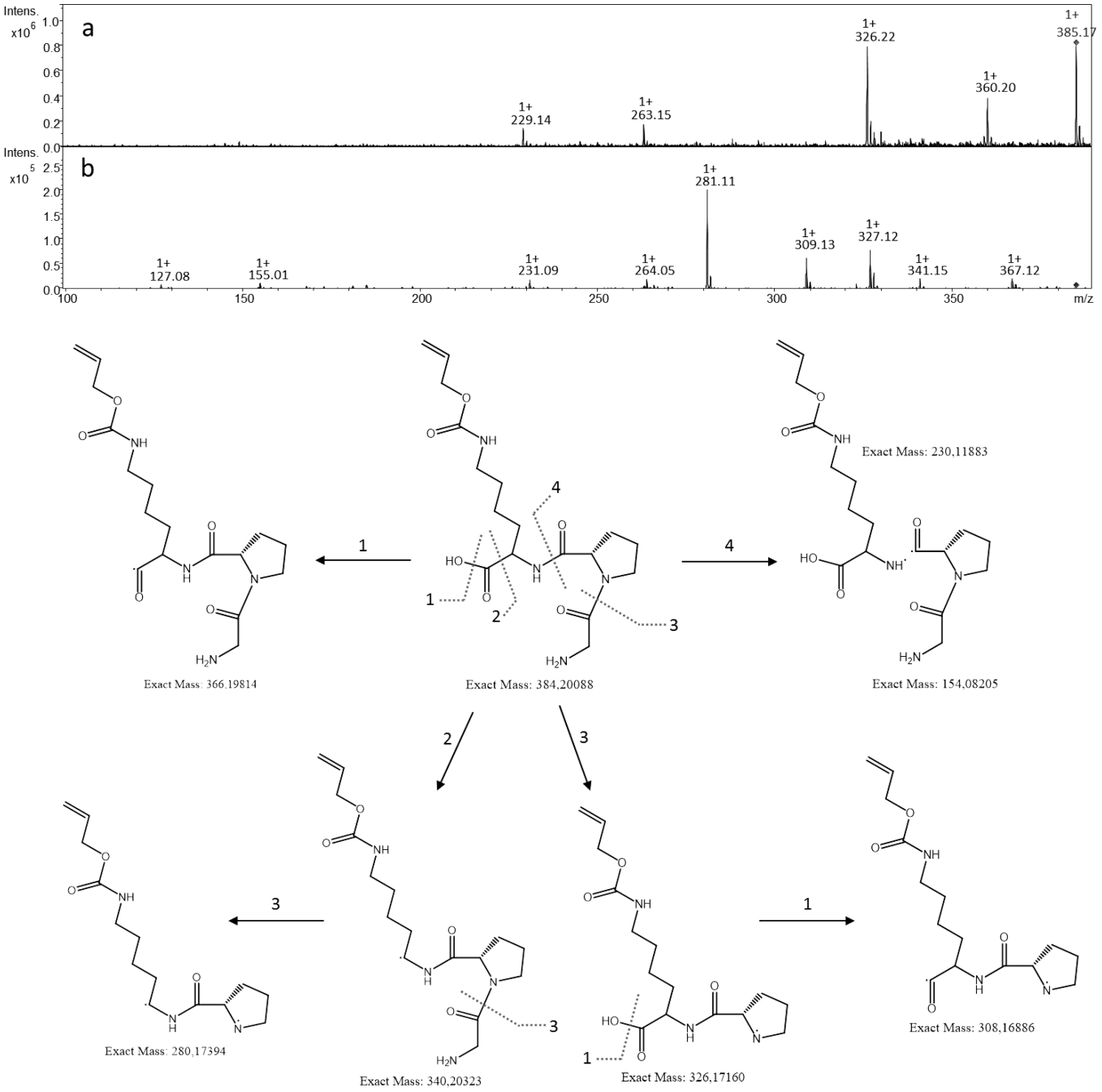


Figure 5. MS/MS fragmentation pattern of a deoxycin-10Alk ion with a mass of 385.17^{+} after hydrolysis; positive mode ionization. (a) Mass spectrum of the sample after hydrolysis with a fragment corresponding to the amino acids Gly-Pro-Alk in positions 8−9−10. (b) MS/MS fragmentation analysis of the ion with a mass 385.17^{+}. Fragmentation positions of the parental ion are indicated with numbers from 1 to 4.

Due to the low production level of the rest of the compounds, the obtained amounts of these compounds were insufficient for MS/MS analysis. Thus, deoxycin-2Cyc and deoxycin-10Boc were only analyzed by HPLC-HRMS. Like deoxycin-10Cyc, deoxycin-10Boc is unstable during ionization. In addition to its full-size ion $[2104.926 + 3H]^{3+}$ = 702.649 Da and butanol adduct $[2104.926 + But + 3H]^{3+}$ = 726.328 Da, two nonselective fragments, $[2004.873 + 3H]^{3+}$ = 669.298 Da and $[2048.864 + 3H]^{3+}$ = 683.962 Da, were detected (Figure S.5). According to the mass loss, they could correspond to the cleavage of *tert*-butyl formate and isobutane residues from the Boc amino acid (Figure S.5).

Altogether, the fragmentation patterns and partial hydrolysis confirm the site-specific incorporation of ncAAs in the selected positions of the lanthipeptide cinnamycin. Nonspecific incorporation events were not detected.

Activity Assay of the New Cinnamycins. The two chosen positions for unnatural amino acid incorporation are in distinct regions of cinnamycin that contribute differently to its antibiotic function. To explore the effect of ncAA incorporation, deoxycinnamycin, deoxycin-10Alk, and deoxycin-2Alk were purified. Pure cinnamycin together with the purified new deoxycinnamycins were used in a minimal inhibitory concentration (MIC) assay against *B. subtilis* (Table S.1). The MIC assay revealed that the activity of the new

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

deoxycinnamycin, without the hydroxyl group at the Asp15 position, was 64 times lower than that of original cinnamycin with the hydroxyl group. The importance of Asp15-OH for target recognition has previously been speculated;[43] however, it was not known to what extent the absence of Asp15-OH affects the activity. An opposite effect was observed when Arg2 and Phe10 were substituted with Alk. The replacement of the hydrophobic aromatic Phe with Alk decreased the anti-*Bacillus* activity of deoxycin-10Alk 2-fold compared with deoxycinnamycin. Phe10 is part of a hydrophobic cleft in the cinnamycin molecule that directly interacts with the glycerol moiety and fatty acid chain of PE;[44] therefore, its substitution may affect target recognition and the overall antibiotic activity. By contrast, the replacement of positively charged Arg in position 2 with hydrophobic Alk increased the activity of the new cinnamycin 2-fold. The reason behind this enhanced biological activity is unclear.

CONCLUSIONS

We have demonstrated for the first time the use of the pylRS/tRNA^{Pyl} system to incorporate ncAAs into RiPPs in streptomycetes. Three different ncAAs were introduced into two distinct positions of the lantibiotic cinnamycin. The structures of the new deoxycinnamycin derivatives were verified by the combination of partial hydrolysis and MS/MS fragmentation analysis. The incorporation rate remains low but is in the range of 8−0.032 mg/L, depending on the ncAA and the position of incorporation. To improve the incorporation efficiency, further investigation is needed. In addition, recent studies have described the possibility of changing the substrate specificity of the pyrrolysyl-tRNA synthetase, which would further expand the library of ncAAs that can be incorporated into a peptide by pyrrolysyl-tRNA synthetase.[47,48] These results confirm the possibility of incorporating unnatural amino acids with diverse side chains into the cinnamycin structure, and this strategy may be implemented for other ribosomally synthesized peptides. In addition, side chains with highly reactive groups can further be used for click chemistry and peptide labeling to identify targets of other RiPPs.[49]

METHODS

Strains, Plasmids, Medium, and Culture Conditions. *Streptomyces albus* J1074 was used as a host for the expression of the cinnamycin gene cluster. *Escherichia coli* XL1-Blue was used for cloning experiments, and ET12567 pUZ8002 was used for intergeneric conjugation. The yeast strain *Saccharomyces cerevisiae* BY4742 was used to clone the cinnamycin gene cluster. The following antibiotics were used at the indicated concentration: apramycin 50 μg/mL, kanamycin 50 μg/mL, thiostrepton 25 μg/mL (in liquid medium), phosphomycin 200 μg/mL, and nalidixic acid 50 μg/mL. The strains were cultivated on standard media: *S. albus* on SM medium (20 g of soya powder, 20 g of mannitol, 16 g of agar), *E. coli* on LB agar (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 16 g of agar), and *S. cerevisiae* on YPD (10 g of yeast extract, 20 g of peptone, 20 g of dextrose, 16 g of agar). For the extraction of cinnamycin, *S. albus* was grown in a tryptic soy broth (TSB) medium for 5 days at 28 °C.

DNA Manipulation. All DNA manipulations were performed according to standard protocols.[50]

Cloning of the Cinnamycin Biosynthesis Gene Cluster by Exploiting the Transformation-Associated Recombination (TAR) technique. TAR cloning was performed using the standard protocol.[51,52] To capture the *cin* cluster from the chromosome of *Streptomyces cinnamoneus cinnamoneus* DSM40005, the capture vector pCLY10cinAB was constructed. This vector is based on the pCLY10 *E. coli*/yeast/*Streptomyces* shuttle vector[41] and carries the yeast CEN6-ARS4, Leu auxotrophic marker, origin of DNA transfer, VWB integrase gene and appropriate attP site, origin of replication in *E. coli* ori15A, and apramycin resistance gene aac(3)IV; in addition, the plasmid carries the left and right homologous regions, flanking the *cin* cluster, with an endonuclease *Bgl*II restriction site in between. The homologous arms were constructed by two-step PCR. First, the left arm was created by amplifying the 600 bp flanking region on the left side of the cluster with the primers CinL-Hind-for and CinL-Bgl-rev (Table S.2). The right homologous arm was created in the same manner with the primers CinR-Bgl-for and CinR-Not-rev (Table S.2). Then, the two PCR products were mixed together and amplified with the primers CinL-Hind-for and CinR-Not-rev. Subsequently, we obtained the left and right homologous arms linked by a *Bgl*II restriction site. The resulting construct was cloned into the plasmid pCLY10 between *Hind*III and *Not*I restriction sites. This plasmid was used to capture the gene cluster. The chromosomal DNA was isolated using a standard protocol.[50] The plasmid with the captured *cin* cluster was named pCLY10cin.

Replacement of the VWB Integrase Gene and Its attP Site by the Region with the φC31 Integrase Gene and Required attP Site. The chloramphenicol resistance gene *cat* was amplified from the plasmid pUCCat with the primers CinCat-for and CinCat-rev. The resulting PCR product was used to replace a 9732 bp fragment in pCLY10cin containing the apramycin resistance gene *aac(3)IV*, yeast CEN6-ARS4, *Leu* auxotrophic marker, origin of DNA transfer, and VWB integrase gene with the appropriate attP site. The plasmid with the *cat* gene was named pCinCat. Subsequently, a 5508 bp fragment from the plasmid pUCCatInt containing the *aac(3)IV* gene, origin of transfer and φC31 integrase gene, and required attP site was integrated into the *cat* gene on the plasmid pCinCat. The resulting plasmid was named pCinCatInt.

Knockout of the cinX Hydroxylase Gene. The function of the *cinX* gene was abolished by replacing the main part of the *cinX* gene with hygromycin resistance gene *hyg*. The hygromycin resistance gene from the plasmid pHygOK was amplified with the primers CinHyg-for and CinHyg-rev. The PCR product was used for RedET to replace the main part of the *cinX* gene. Subsequently, the *hyg* gene was removed by digestion of the plasmid with *Kpn*I restriction enzyme and self-ligation.

Feeding of the *S. albus* Strains with ncAAs and Extraction of Cinnamycins from the Culture Medium. Streptomyces strains for the production of cinnamycins were grown in TSB medium for 1 day, to reach the stationary phase. Subsequently, 1 mL of culture was inoculated in 50 mL of TSB medium supplemented with the amino acids Alk, Boc, or Cyc at a concentration of 20 mM when needed. After 5 days of growing on a rotary shaker, the culture was harvested by centrifugation. Extraction from biomass was performed with a 1:1 (v/v) methanol−acetone mixture. The supernatant was extracted first with ethyl acetate, and then the water phase was extracted with 1-butanol. The upper butanol phase was evaporated, and the dry extract was dissolved in methanol and used for further purification and measurements. As a control, a culture without supplementation was used.

Purification of the New Cinnamycins. The methanol extract obtained after butanol extraction was used for purification of the compound on a 1-m-long column packed with cross-linked dextran polymer beads. Methanol was used as a solvent for elution. Fractions were collected every 10 min with a flow rate of 1 mL/min. The fractions containing cinnamycin were pooled together, evaporated, and dissolved in methanol. Afterward, the prepurified methanol extract was further purified on a Dionex Ultimate 3000 preparative HPLC using a C18 column (linear gradient from 5% to 95% acetonitrile). The fractions with cinnamycin were again evaporated and dissolved in methanol. After these two purification steps, the cinnamycins were used for HPLC-HRMS analysis as well as for hydrolysis.

HPLC-HRMS and HPLC-MS/MS Analyses. HPLC-HRMS analyses were performed on a Dionex Ultimate 3000 HPLC A coupled with an LTQ Orbitrap high-resolution mass spectrometer using a C18 column (100 × 2.1 mm). Cinnamycins were analyzed using a linear 9 min gradient from 5% to 95% acetonitrile and a 0.5

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

mL/min flow rate. HPLC-MS/MS analyses were performed on a Dionex Ultimate 3000 HPLC coupled with a Bruker AmaZon mass spectrometer using a C18 column (100 × 2.1 mm) and a linear 18 min gradient from 5% to 95% acetonitrile, 0.6 mL/min flow rate.

Partial Hydrolysis. Purified deoxycinnamycins (0.5 mg) were evaporated and dissolved in 1 mL of 1 N hydrochloric acid (HCl). Then, 1 mL of dissolved cinnamycin was divided into 10 samples of 100 μL each. All samples were placed simultaneously on a thermoblock heated to 95 °C. The reaction was stopped at different time points (15, 30 min and 1, 3, 6, 9, 12, 15, 18 h) by fast evaporation of the acid under nitrogen. Dry samples were later dissolved in methanol and used for HPLC-MS/MS analyses.

Bioassay. A sterile 96-well microtiter plate was used for the experiment. An amount of 25 μg of cinnamycin and its derivatives in methanol solution were transferred into the second column of the plate and evaporated. Then, 100 μL of LB medium was dispensed into all wells, and 100 μL of additional LB was added to the wells in the second column containing the antibiotic and mixed. Two-fold dilutions of the antibiotics were made starting from the second column. Afterward, an overnight culture of *Bacillus subtilis* was diluted 100 times, and 100 μL of the diluted culture was added to each well except the first control column (the volume in the wells of the first column was adjusted up to 200 μL with LB medium). The plate was incubated at 37 °C overnight and then scanned with a POLARstar Omega plate reader.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00359.

Supplementary figures, details of unhydrolyzed cinnamycin MS/MS fragmentation, and primer sequences (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: a.luzhetskyy@mx.uni-saarland.de.

ORCID

Andriy Luzhetskyy: 0000-0001-6497-0047

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The research was supported by a Collaborative Research Project, StrepSynth, funded by the Programme: Cooperation (FP7-KBBE-2013-7).

■ REFERENCES

(1) Dunbar, K. L., and Mitchell, D. A. (2013) Revealing Nature's Synthetic Potential through the Study of Ribosomal Natural Product Biosynthesis (vol 8, pg 473, 2013). *ACS Chem. Biol. 8*, 1083−1083.

(2) Ortega, M. A., and van der Donk, W. A. (2016) New Insights into the Biosynthetic Logic of Ribosomally Synthesized and Post-translationally Modified Peptide Natural Products. *Cell Chem. Biol. 23*, 31−44.

(3) Zheng, Q. F., Wang, Q. L., Wang, S. F., Wu, J. Q., Gao, Q., and Liu, W. (2015) Thiopeptide Antibiotics Exhibit a Dual Mode of Action against Intracellular Pathogens by Affecting Both Host and Microbe. *Chem. Biol. 22*, 1002−1007.

(4) Mohr, K. I., Volz, C., Jansen, R., Wray, V., Hoffmann, J., Bernecker, S., Wink, J., Gerth, K., Stadler, M., and Muller, R. (2015) Pinensins: The First Antifungal Lantibiotics. *Angew. Chem., Int. Ed. 54*, 11254−11258.

(5) Essig, A., Hofmann, D., Munch, D., Gayathri, S., Kunzler, M., Kallio, P. T., Sahl, H. G., Wider, G., Schneider, T., and Aebi, M. (2014) Copsin, a Novel Peptide-based Fungal Antibiotic Interfering with the Peptidoglycan Synthesis. *J. Biol. Chem. 289*, 34953−34964.

(6) Ferir, G., Petrova, M. I., Andrei, G., Huskens, D., Hoorelbeke, B., Snoeck, R., Vanderleyden, J., Balzarini, J., Bartoschek, S., Bronstrup, M., Sussmuth, R. D., and Schols, D. (2013) The Lantibiotic Peptide Labyrinthopeptin A1 Demonstrates Broad Anti-HIV and Anti-HSV Activity with Potential for Microbicidal Applications. *PLoS One 8*, e64010.

(7) Richard, A. S., Zhang, A., Park, S. J., Farzan, M., Zong, M., and Choe, H. (2015) Virion-associated phosphatidylethanolamine promotes TIM1-mediated infection by Ebola, dengue, and West Nile viruses. *Proc. Natl. Acad. Sci. U. S. A. 112*, 14682−14687.

(8) Zhang, Y., Baranov, P. V., Atkins, J. F., and Gladyshev, V. N. (2005) Pyrrolysine and selenocysteine use dissimilar decoding strategies. *J. Biol. Chem. 280*, 20740−20751.

(9) Liu, C. C., and Schultz, P. G. (2010) Adding New Chemistries to the Genetic Code. *Annu. Rev. Biochem. 79*, 413−444.

(10) Lang, K., and Chin, J. W. (2014) Cellular Incorporation of Unnatural Amino Acids and Bioorthogonal Labeling of Proteins. *Chem. Rev. 114*, 4764−4806.

(11) Hao, Z. Y., Hong, S. L., Chen, X., and Chen, P. R. (2011) Introducing Bioorthogonal Functionalities into Proteins in Living Cells. *Acc. Chem. Res. 44*, 742−751.

(12) Peng, T., and Hang, H. C. (2016) Site-Specific Bioorthogonal Labeling for Fluorescence Imaging of Intracellular Proteins in Living Cells. *J. Am. Chem. Soc. 138*, 14423−14433.

(13) Oldach, F., Al Toma, R., Kuthning, A., Caetano, T., Mendo, S., Budisa, N., and Sussmuth, R. D. (2012) Congeneric Lantibiotics from Ribosomal In Vivo Peptide Synthesis with Noncanonical Amino Acids. *Angew. Chem., Int. Ed. 51*, 415−418.

(14) Al Toma, R. S., Kuthning, A., Exner, M. P., Denisiuk, A., Ziegler, J., Budisa, N., and Sussmuth, R. D. (2015) Site-directed and global incorporation of orthogonal and isostructural noncanonical amino acids into the ribosomal lasso peptide capistruin. *ChemBioChem 16*, 503−509.

(15) Young, T. S., and Schultz, P. G. (2010) Beyond the Canonical 20 Amino Acids: Expanding the Genetic Lexicon. *J. Biol. Chem. 285*, 11039−11044.

(16) Hancock, S. M., Uprety, R., Deiters, A., and Chin, J. W. (2010) Expanding the Genetic Code of Yeast for Incorporation of Diverse Unnatural Amino Acids via a Pyrrolysyl-tRNA Synthetase/tRNA Pair. *J. Am. Chem. Soc. 132*, 14819−14824.

(17) Al Toma, R. S., Kuthning, A., Exner, M. P., Denisiuk, A., Ziegler, J., Budisa, N., and Sussmuth, R. D. (2015) Site-Directed and Global Incorporation of Orthogonal and Isostructural Noncanonical Amino Acids into the Ribosomal Lasso Peptide Capistruin. *ChemBioChem 16*, 503−509.

(18) Polycarpo, C. R., Herring, S., Berube, A., Wood, J. L., Soll, D., and Ambrogelly, A. (2006) Pyrrolysine analogues as substrates for pyrrolysyl-tRNA synthetase. *FEBS Lett. 580*, 6695−6700.

(19) Chin, J. W., Martin, A. B., King, D. S., Wang, L., and Schultz, P. G. (2002) Addition of a photocrosslinking amino acid to the genetic code of Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A. 99*, 11020−11024.

(20) Wang, F., Robbins, S., Guo, J. T., Shen, W. J., and Schultz, P. G. (2010) Genetic Incorporation of Unnatural Amino Acids into Proteins in Mycobacterium tuberculosis. *PLoS One 5*, e9354.

(21) Piscotta, F. J., Tharp, J. M., Liu, W. R., and Link, A. J. (2015) Expanding the chemical diversity of lasso peptide MccJ25 with genetically encoded noncanonical amino acids. *Chem. Commun. 51*, 409−412.

(22) Shi, Y. X., Yang, X. A., Garg, N., and van der Donk, W. A. (2011) Production of Lantipeptides in Escherichia coli. *J. Am. Chem. Soc. 133*, 2338−2341.

(23) Bindman, N. A., Bobeica, S. C., Liu, W. S. R., and van der Donk, W. A. (2015) Facile Removal of Leader Peptides from Lanthipeptides by Incorporation of a Hydroxy Acid. *J. Am. Chem. Soc. 137*, 6975−6978.

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

(24) Luo, X. Z., Zambaldo, C., Liu, T., Zhang, Y. H., Xuan, W. M., Wang, C., Reed, S. A., Yang, P. Y., Wang, R. E., Javahishvili, T., Schultz, P. G., and Young, T. S. (2016) Recombinant thiopeptides containing noncanonical amino acids. *Proc. Natl. Acad. Sci. U. S. A. 113*, 3615−3620.
(25) Myronovskyi, M., Welle, E., Fedorenko, V., and Luzhetskyy, A. (2011) beta-Glucuronidase as a Sensitive and Versatile Reporter in Actinomycetes. *Appl. Environ. Microbiol. 77*, 5370−5383.
(26) He, J., Van Treeck, B., Nguyen, H. B., and Melancon, C. E., 3rd (2016) Development of an Unnatural Amino Acid Incorporation System in the Actinobacterial Natural Product Producer Streptomyces venezuelae ATCC 15439. *ACS Synth. Biol. 5*, 125−132.
(27) Jung, G. (1991) Lantibiotics - Ribosomally Synthesized Biologically-Active Polypeptides Containing Sulfide Bridges and Alpha-Beta-Didehydroamino Acids. *Angew. Chem., Int. Ed. Engl. 30*, 1051−1068.
(28) Jones, A. M., and Helm, J. M. (2009) Emerging Treatments in Cystic Fibrosis. *Drugs 69*, 1903−1910.
(29) Oliynyk, I., Varelogianni, G., Roomans, G. M., and Johannesson, M. (2010) Effect of duramycin on chloride transport and intracellular calcium concentration in cystic fibrosis and non-cystic fibrosis epithelia. *Apmis 118*, 982−990.
(30) Chatterjee, C., Paul, M., Xie, L. L., and van der Donk, W. A. (2005) Biosynthesis and mode of action of lantibiotics. *Chem. Rev. 105*, 633−683.
(31) Sahl, H. G., and Bierbaum, G. (1998) Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annu. Rev. Microbiol. 52*, 41−79.
(32) Ogawa, K., and Aoki, M. (2014) Radiolabeled apoptosis imaging agents for early detection of response to therapy. *Sci. World J. 2014*, 732603.
(33) Stafford, J. H., and Thorpe, P. E. (2011) Increased Exposure of Phosphatidylethanolamine on the Surface of Tumor Vascular Endothelium. *Neoplasia 13*, 299−U125.
(34) Tabata, T., Petitt, M., Puerta-Guardo, H., Michlmayr, D., Wang, C., Fang-Hoover, J., Harris, E., and Pereira, L. (2016) Zika Virus Targets Different Primary Human Placental Cells, Suggesting Two Routes for Vertical Transmission. *Cell Host Microbe 20*, 155−166.
(35) Baltz, R. H. (2010) Streptomyces and Saccharopolyspora hosts for heterologous expression of secondary metabolite gene clusters. *J. Ind. Microbiol. Biotechnol. 37*, 759−772.
(36) Lopatniuk, M. M., Ostash, B. E., Luzhetskyy, A. N., Walker, S., and Fedorenko, V. A. (2014) Generation and study of the strains of streptomycetes–heterologous hosts for the production of moenomycin. *Russ. J. Genet. 50*, 360−365.
(37) Myronovskyi, M., Tokovenko, B., Brotz, E., Ruckert, C., Kalinowski, J., and Luzhetskyy, A. (2014) Genome rearrangements of Streptomyces albus J1074 lead to the carotenoid gene cluster activation. *Appl. Microbiol. Biotechnol. 98*, 795−806.
(38) Widdick, D. A., Dodd, H. M., Barraille, P., White, J., Stein, T. H., Chater, K. F., Gasson, M. J., and Bibb, M. J. (2003) Cloning and engineering of the cinnamycin biosynthetic gene cluster from Streptomyces cinnamoneus cinnamoneus DSM 40005. *Proc. Natl. Acad. Sci. U. S. A. 100*, 4316−4321.
(39) O'Rourke, S., Widdick, D., and Bibb, M. (2017) A novel mechanism of immunity controls the onset of cinnamycin biosynthesis in Streptomyces cinnamoneus DSM 40646. *J. Ind. Microbiol. Biotechnol. 44*, 563−572.
(40) Kouprina, N., and Larionov, V. (2016) Transformation-associated recombination (TAR) cloning for genomics studies and synthetic biology. *Chromosoma 125*, 621−632.
(41) Bilyk, O., Sekurova, O. N., Zotchev, S. B., and Luzhetskyy, A. (2016) Cloning and Heterologous Expression of the Grecocycline Biosynthetic Gene Cluster. *PLoS One 11*, e0158682.
(42) Okesli, A., Cooper, L. E., Fogle, E. J., and van der Donk, W. A. (2011) Nine Post-translational Modifications during the Biosynthesis of Cinnamycin. *J. Am. Chem. Soc. 133*, 13753−13760.
(43) Zhao, M., Li, Z. X., and Bugenhagen, S. (2008) Tc-99m-labeled duramycin as a novel phosphatidylethanolamine-binding molecular probe. *J. Nucl. Med. 49*, 1345−1352.
(44) Rzeznicka, II, Sovago, M., Backus, E. H., Bonn, M., Yamada, T., Kobayashi, T., and Kawai, M. (2010) Duramycin-induced destabilization of a phosphatidylethanolamine monolayer at the air-water interface observed by vibrational sum-frequency generation spectroscopy. *Langmuir 26*, 16055−16062.
(45) Muyrers, J. P. P., Zhang, Y. M., Testa, G., and Stewart, A. F. (1999) Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res. 27*, 1555−1557.
(46) Katayama, H., Nozawa, K., Nureki, O., Nakahara, Y., and Hojo, H. (2012) Pyrrolysine Analogs as Substrates for Bacterial Pyrrolysyl-tRNA Synthetase in Vitro and in Vivo. *Biosci., Biotechnol., Biochem. 76*, 205−208.
(47) Takimoto, J. K., Dellas, N., Noel, J. P., and Wang, L. (2011) Stereochemical Basis for Engineered Pyrrolysyl-tRNA Synthetase and the Efficient in Vivo Incorporation of Structurally Divergent Non-native Amino Acids. *ACS Chem. Biol. 6*, 733−743.
(48) Lacey, V. K., Louie, G. V., Noel, J. P., and Wang, L. (2013) Expanding the Library and Substrate Diversity of the Pyrrolysyl-tRNA Synthetase to Incorporate Unnatural Amino Acids Containing Conjugated Rings. *ChemBioChem 14*, 2100−2105.
(49) McKay, C. S., and Finn, M. G. (2014) Click Chemistry in Complex Mixtures: Bioorthogonal Bioconjugation. *Chem. Biol. 21*, 1075−1101.
(50) Sambrook, J., and Russell, D. W. (2006) *The Condensed Protocols from Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
(51) Kouprina, N., and Larionov, V. (2008) Selective isolation of genomic loci from complex genomes by transformation-associated recombination cloning in the yeast Saccharomyces cerevisiae. *Nat. Protoc. 3*, 371−377.
(52) Noskov, V. N., Koriabine, M., Solomon, G., Randolph, M., Barrett, J. C., Leem, S. H., Stubbs, L., Kouprina, N., and Larionov, V. (2001) Defining the minimal length of sequence homology required for selective gene isolation by TAR cloning. *Nucleic Acids Res. 29*, 32e.

DOI: 10.1021/acschembio.7b00359
ACS Chem. Biol. 2017, 12, 2362−2370

Supporting information

Streptomyces albus: a New Cell Factory for Non-Canonical Amino Acids Incorporation into Ribosomally Synthetized Natural Products

Mariia Lopatniuk[1], Maksym Myronovskyi[1], and Andriy Luzhetskyy[1,2]*

1. Department of Pharmaceutical Biotechnology, Saarland University, 66123 Saarbrücken, Germany
2. Helmholtz-Institute for Pharmaceutical Research Saarland, Campus, Building C2.3, 66123 Saarbrücken, Germany

Antibiotic conc. µg/ml / Sample name	0	62.5	31.3	15.6	7.81	3.91	1.95	0.98	0.49	0.24	0.12	0
cinnamycin	0.04	0.042	0.038	0.062	0.04	0.052	0.063	0.049	0.053	**1.071**	**1.247**	**1.153**
deoxycinnamycin	0.033	0.044	0.049	**1.305**	**0.994**	**1.037**	**0.876**	**1.318**	**1.131**	**1.323**	**1.253**	**1.349**
deoxycin-10Alk	0.04	0.064	**0.841**	**1.259**	**1.179**	**1.229**	**1.19**	**1.19**	**1.258**	**1.295**	**0.894**	**1.057**
deoxycin-2Alk	0.038	0.068	0.088	0.051	**1.4**	**1.137**	**1.065**	**1.255**	**1.281**	**1.094**	**1.416**	**1.029**

Table S1. MIC analysis of the new cinnamycins compared to cinnamycin and deoxycinnamycin against the test-culture *Bacillus subtilis*. The viability of the strain was detected by optical density of the culture. Values lower 0.1 were considered as not viable, and values higher than 0.8 correspond to a growing culture, indicating resistance.

Primer name	Primers sequences, direction 5´-3´
CinL-Hind-for	AAAAAAGCTTCTTGTAGAAGGCGAGCATCG
CinL-Bgl-rev	ATGGGACCCGACCACGCCCAGATCTGCTCCTCGGGCACGAGCCG
CinR-Bgl-for	CGGCTCGTGCCCGAGGAGCAGATCTGGGCGTGGTCGGGTCCCAT
CinR-Not-rev	AAAAGCGGCCGCCGTCGGAGGTGGTGAAGAG
FTAG-for	GAGCTGCAGCTTCGGCCCGTAGACCTTCGTGTGCGACGGCA
FTAG-rev	TGCCGTCGCACACGAAGGTCTACGGGCCGAAGCTGCAGCTC
RTAG-for	CACGGAAGCCTTCGCCTGCTAGCAGAGCTGCAGCTTCGGCC
RTAG-rev	GGCCGAAGCTGCAGCTCTGCTAGCAGGCGAAGGCTTCCGTG
Cin1-for	TGGCAGAACAACAGCGACA
Cin2-rev	GTGGAACTTGTCGGCCAGT
CinXdel-for	CTCGACCCGGCCCGGTTCGGGCGCGAGATGAAGGCGGTCACGGA GGTACCAATACTTGACATATCACTGT
CinXdel-rev	GGTTGGCGATGCAGTGCCAGAAGAGCTTCTCCGGGTCCTGCTCCA CCTTGGGTACCTCAGGCGCCGGGGGCGGTG
cinXchk-for	TGATCGGCATCTCCGGGTTC
cinXchk-rev	CGGTCCTGCTCCGAACATC
CinCat-for	GGTTCATGTGCAGCTCCATCAGCAAAAGGGGATGATAAGTTTATC ACCACTGTGGTGTGACCGGAACAG
CinCat-rev	CCGACGCCCGTACAACCGTCGGCCTGCCTCGCGGGTCTAACGAAA ATGGAAGTGTAAAGCCTGGGGTGC
CinHyg-for	CGTCCCTTGACTTCTGGACCAAGGACATCGCCGCCACGGAAGCCT TCGCCGTTTAAACAATACTTGACATATCACTGT
CinHyg-rev	CGCGTCATCGCACCGGGCGAGCATTACCGCCTAGAGGCAGCAGC CACTTAGTTTAAACTCAGGCGCCGGGGGCGGTGT

Table S2. Oligonucleotides sequences used for amplification reaction

Figure S.1. Bioassay of two *S. albus* strains, harboring the plasmids pCinCatInt and pCindelX, against *Bacillus subtilis*.

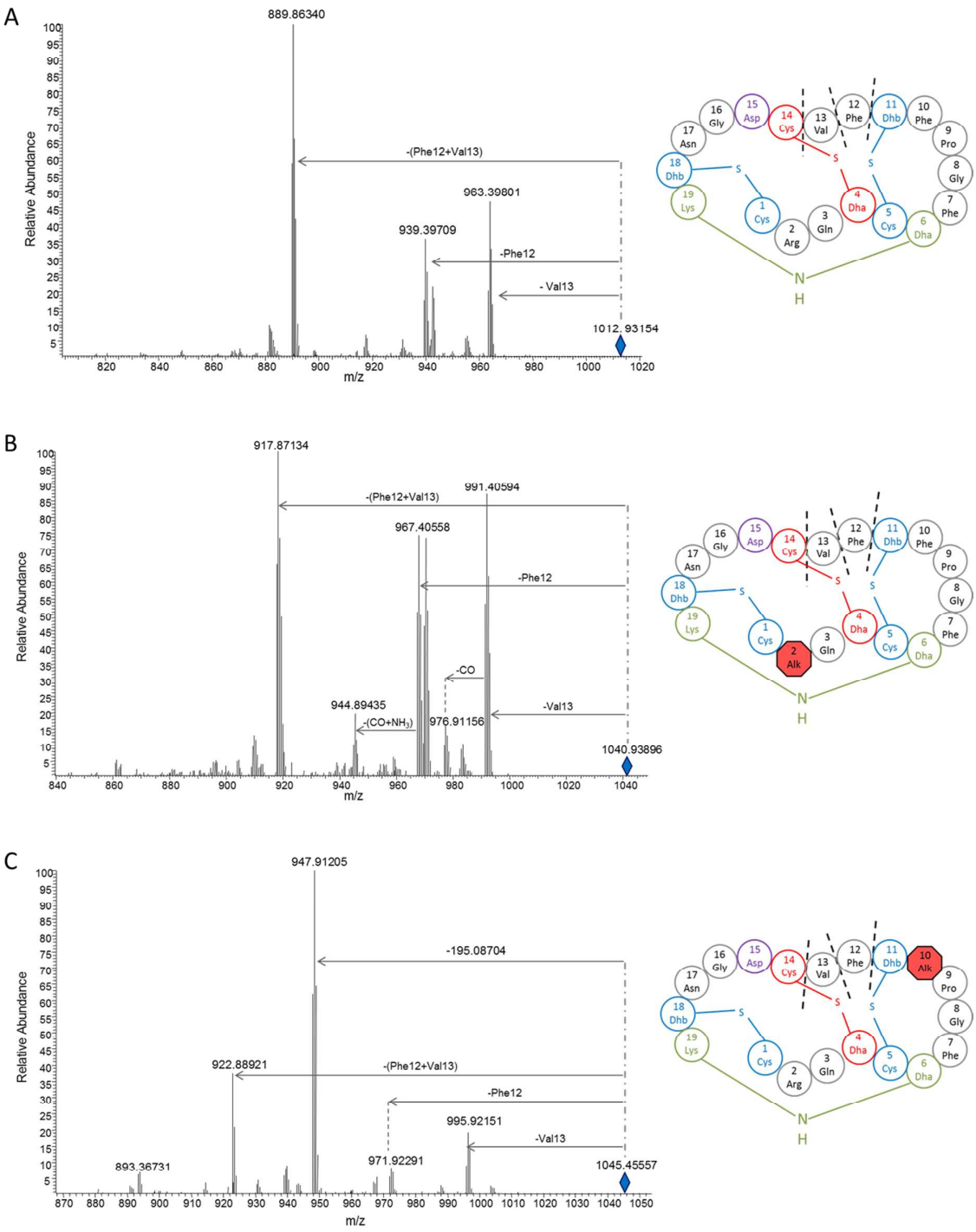


Figure S.2. MS/MS fragmentation analysis of deoxycinnamycin, deoxycin-10Alk and deoxycin-2Alk; positive mode ionization. Fragmentation positions are indicated with dashed lines. (A) Fragmentation of the deoxycinnamycin triple-charged ion with a mass $[2023.848 + 3H]^{3+} = 675.623$ Da. (B) Fragmentation of the deoxycin-10Alk triple-charged ion with a mass $[2088.896 + 3H]^{3+} = 697.306$ Da. (C) Fragmentation of the deoxycin-2Alk triple-charged ion with a mass $[2079.862 + 3H]^{3+} = 694.295$.

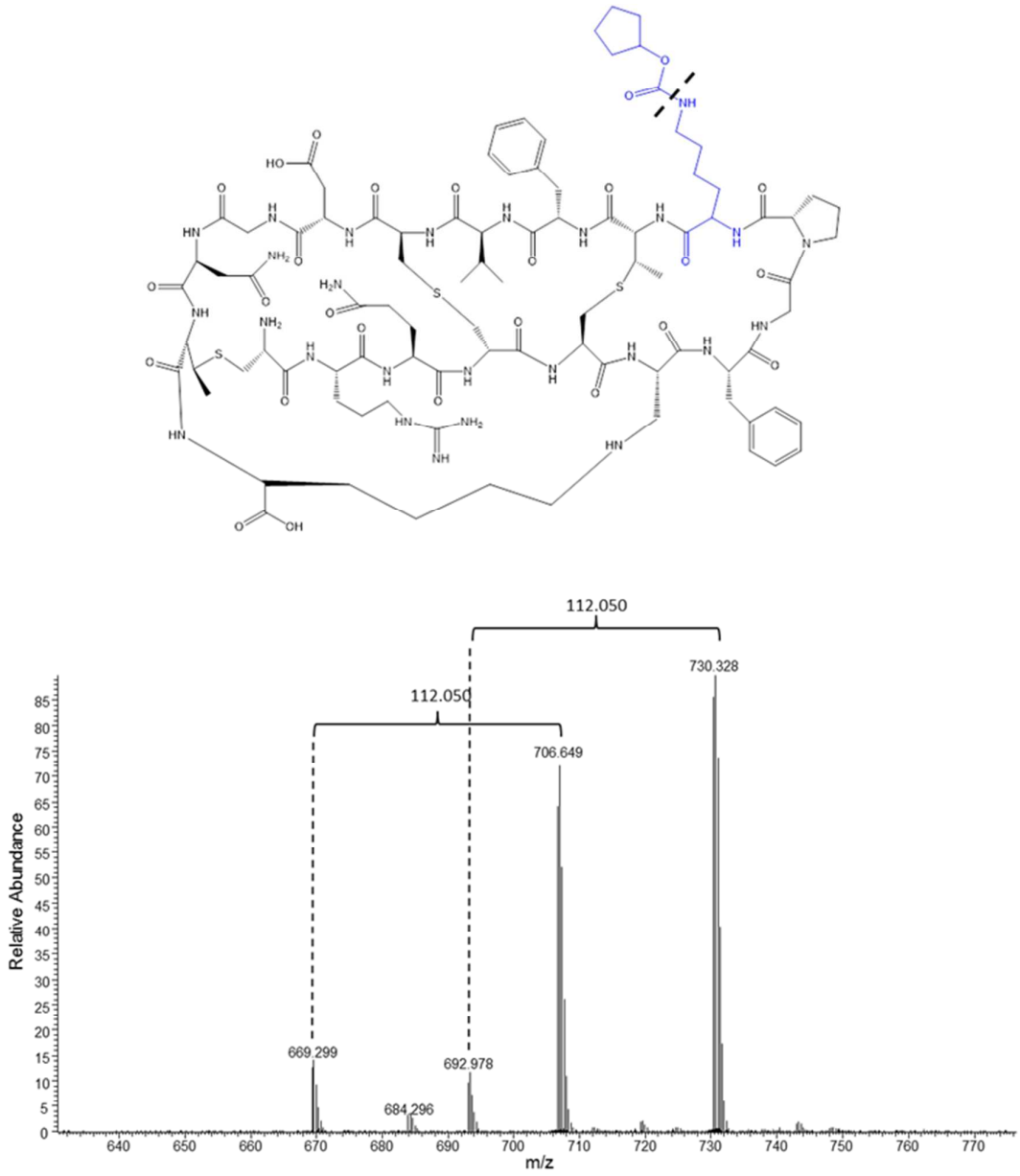


Figure S.3. Non-selective fragmentation of deoxycin-10Cyc and its butanol adduct during ionization. Loss of a mass of 112,050 Da from the triple-charged ion $[2116.925 + 3H]^{3+}$ = 706.649 Da and its butanol adduct $[2116.925 + But + 3H]^{3+}$ = 730.328 Da correspond to the loss of the cyclopentyl formate group of the Cyc residue. The cleavage position is indicated with dashed line.

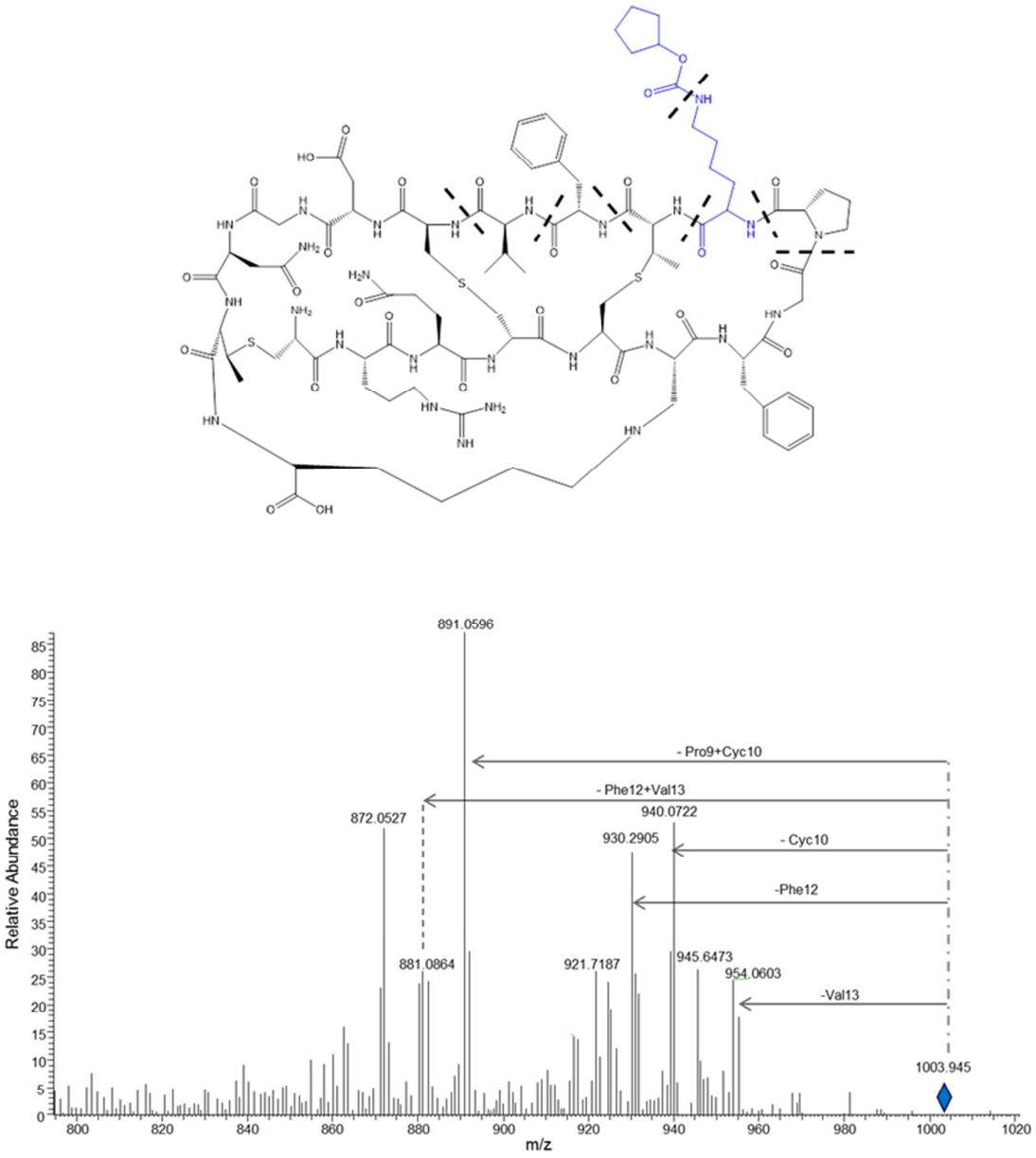


Figure S.4. MS/MS fragmentation analysis of the triple-charged ion of deoxycin-10Cyc, with a mass $[2004.875 + 3H]^{3+} = 669.299$, lacking the cyclopentyl formate group; positive mode ionization. Fragmentation positions are indicated with dashed lines.

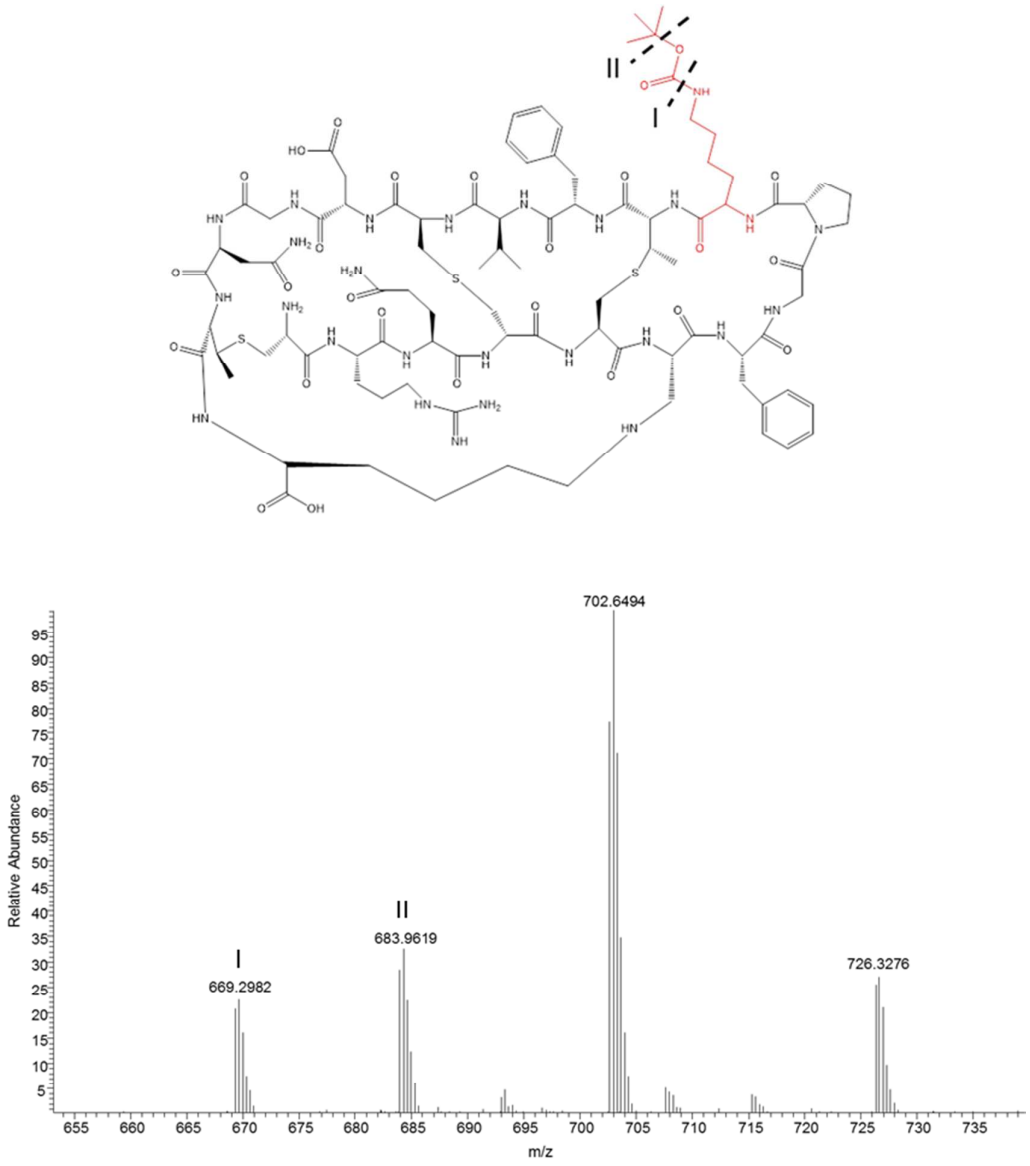


Figure S.5. Non-selective fragmentation of deoxycin-10Boc during ionization. Cleavage positions are indicated with dashed lines.

III

Properties of *Streptomyces albus* J1074 mutant deficient in $tRNA^{Leu}_{UAA}$ gene *bldA*

Koshla O., Lopatniuk M., Rokytskyy I., Yushchuk O., Dacyuk Y., Fedorenko V., Luzhetskyy A., Ostash B.

Arch Microbiol, **2017**, 199:1175–1183. DOI 10.1007/s00203-017-1389-7

Published online 20th May 2017

Koshla O. generated *S. albus* strains, used in this study, conducted experiments on strains growth, secondary metabolites production, participated in writing the manuscript. **Lopatniuk M.** constructed BAC for *bldA* deletion and performed analysis of metabolic extracts. **Rokytskyy I.** constructed plasmids for β-galactosidase reporter system. **Yushchuk O.** conducted experiments on strains morphology. **Dacyuk Y**. performed SEM. **Fedorenko V.** participated in planning of the experiments. **Luzhetskyy A.** participated in planning of the experiments and manuscript correction. **Ostash B.** supervised the work, participated in planning of the experiments and wrote the manuscript.

Author's name	Contribution to the work, %
Koshla O	60
Lopatniuk M.	5
Rokytskyy I.	5
Yushchuk O.	5
Dacyuk Y.	5
Fedorenko V.	5
Luzhetskyy A.	5
Ostash B.	10

Arch Microbiol (2017) 199:1175–1183
DOI 10.1007/s00203-017-1389-7

ORIGINAL PAPER

Properties of *Streptomyces albus* J1074 mutant deficient in $tRNA^{Leu}_{UAA}$ gene *bldA*

Oksana Koshla[1] · Maria Lopatniuk[1,2] · Ihor Rokytskyy[1] · Oleksandr Yushchuk[1] · Yuriy Dacyuk[3] · Victor Fedorenko[1] · Andriy Luzhetskyy[2] · Bohdan Ostash[1]

Received: 30 March 2017 / Revised: 6 May 2017 / Accepted: 16 May 2017 / Published online: 20 May 2017

Abstract *Streptomyces albus* J1074 is one of the most popular and convenient hosts for heterologous expression of gene clusters directing the biosynthesis of various natural metabolic products, such as antibiotics. This fuels interest in elucidation of genetic mechanisms that may limit secondary metabolism in J1074. Here, we report the generation and initial study of J1074 mutant, deficient in gene *bldA* for $tRNA^{Leu}_{UAA}$, the only tRNA capable of decoding rare leucyl TTA codon in *Streptomyces*. The *bldA* deletion in J1074 resulted in a highly conditional Bld phenotype, with depleted formation of aerial hyphae on certain solid media. In addition, *bldA* mutant of J1074 was unable to produce endogenous antibacterial compounds and two heterologous antibiotics, moenomycin and aranciamycin, whose biosynthesis is directed by TTA-containing genes. We have employed a new TTA codon-specific β-galactosidase reporter system to provide genetic evidence that J1074 *bldA* mutant is impaired in translation of TTA. In addition, we have discussed the possible reasons for differences in the phenotypes of *bldA* mutants described here and in previous studies, providing knowledge to study *bldA*-based regulation of antibiotic biosynthesis.

Keywords *Streptomyces albus* · tRNA · Antibiotics · β-galactosidase

Introduction

The actinobacterium *Streptomyces albus* J1074 was isolated in 1980 as a *S. albus* G derivative deficient in the *Sal*GI restriction-modification system (Chater and Wilde 1980). Later, J1074 served as a model to study phage restriction phenomena in streptomycetes, and in 1990s it has gained prominence as a host for heterologous expression of antibiotic biosynthesis genes (Rodriguez et al. 1993, 2000). Fast, highly dispersed growth and genetic amenability make J1074 a strain of first choice for expression of heterologous gene clusters from actinobacteria (Baltz 2010). Previous studies (Lopatniuk et al. 2014; Iqbal et al. 2016) portray J1074 as an optimal starting point to develop an efficient platform for heterologous expression (metagenomic) purposes. It is expected that such a platform would greatly increase our ability to discover bioactive natural products encrypted in environmental DNA and silent gene clusters of actinobacteria. In this context, it is essential to elucidate genetic mechanisms that control secondary metabolism in J1074 to find limiting factors and the ways to circumvent them. It has to be mentioned that regulation of antibiotic production has been relatively well studied in few models of streptomycetes (Liu et al. 2013), although it is not yet understood to what extent this knowledge can be extrapolated onto new strains, such as J1074.

Communicated by Erko Stackebrandt.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-017-1389-7) contains supplementary material, which is available to authorized users.

✉ Bohdan Ostash
b.ostash@lnu.edu.ua

[1] Department of Genetics and Biotechnology, Ivan Franko National University of Lviv, 4 Hrushevskoho st., Lviv 79005, Ukraine

[2] Actinobacteria Metabolic Engineering Group, Department of Pharmaceutical Biotechnology, Helmholtz Institute for Pharmaceutical Research Saarland, Saarland University, UdS Campus C2 3, 66123 Saarbrucken, Germany

[3] Department of Physics of Earth, Ivan Franko National University of Lviv, 4 Hrushevskoho st., Lviv 79005, Ukraine

Transcriptional control of gene expression is arguably the most important regulatory layer in bacteria, and antibiotic production is no exception. In addition, streptomycetes possess a highly unusual regulatory switch based on leucyl (TTA) codon and its cognate $tRNA^{Leu}_{UAA}$, encoded by gene *bldA*. TTA codons are located exclusively in genes for auxiliary functions such as morphogenesis and antibiotic production, and *bldA* deficiency is not lethal. There is a considerable temporal gap between occurrence of UUA-containing transcripts and their subsequent translation (Gramajo et al. 1993; Pope et al. 1996; Rebets et al. 2006). Failure to develop aerial mycelium and spores on top of substrate mycelium is a hallmark of all *bldA*-mutants (the "Bald" phenotype) studied to date. The latter is thought to be the result of blocked translation of TTA-containing gene for master regulator AdpA (Takano et al. 2003; Nguyen et al. 2003). The influence of *bldA* deficiency on antibiotic biosynthesis remains more controversial, as well as the exact chain of events linking *bldA* to delayed antibiotic production (Guthrie and Chater 1990; Gramajo et al. 1993; Chater and Chandra 2008; Makitrynskyy et al. 2013).

The effects of *bldA* deletion on J1074 have not been studied so far, and it remained unknown to what extent colony development and antibiotic production will be affected by the former. Here, we show that on certain (minimal) solid media *bldA*-deficient mutant J1074 produces aerial hyphae and spores less densely than the parental strain; in the same time, under many culture conditions parental and mutant strains are phenotypically indistinguishable. In line with many other reports, our *bldA* mutant's ability to express TTA codon-containing antibiotic biosynthesis gene clusters is strictly dependent on $tRNA^{Leu}_{UAA}$. Finally, we describe a novel reporter system that was used to study TTA codon-based regulatory mechanisms in *S. albus*.

Materials and methods

Microorganisms, vectors, and culture conditions

Streptomyces albus SAM2, a derivative of J1074 carrying single $attB^{\varphi C31}$ site (Bilyk and Luzhetskyy 2014), was used throughout the study. *Escherichia coli* DH5α was used for routine subcloning. *E. coli* ET12567 (pUB307) was used to perform intergeneric conjugation from *E. coli* to *S. albus*. *Escherichia coli* strains were grown under standard conditions (Sambrook and Russell 2001). *Bacillus cereus* ATCC19637 and *Debaryomyces hansenii* VKM Y-9 were used as antibiotic-sensitive test cultures (growth temperature −30 °C). RedET-mediated gene replacements (Datsenko and Wanner 2000) were carried out with the help of REDIRECT system (Gust et al. 2003). All vectors, cosmids, plasmids, and BAC DNAs are listed in Table 1. For intergeneric matings, *S. albus* strains were grown on soy-flour mannitol (SFM) agar (Kieser et al. 2000) at 30 °C. To reveal endogenous antibacterial and antifungal activities, *S. albus* strains were grown on SG2 agar (in g/L: glucose 20, yeast extract 5, soytone 10, pH 7.2 prior to autoclaving) and other solid media as detailed in Electronic Supplementary Materials (ESM). For submerged production of moenomycin and aranciamycin *S. albus* strains were grown in liquid medium SG1 (SG2 supplemented with 2 g/L $CaCO_3$) at 28 °C for 5 days. For phenotypic examinations, *S. albus* were grown on SFM and SMMS agar (Kieser et al. 2000), as well as other media as described in ESM. Wherever needed, strains were maintained in the presence of apramycin (25 μg/mL); chromogenic substrate X-Gal was added to the media to a final concentration of 50 μg/mL; cumate (50 mM) was used to induce *SCO3479* expression from plasmids pRV4 and pRV3.

Plasmids and BACs construction

Bacterial artificial chromosome (BAC) BAC_1N12 was used to prepare *bldA* (*XNR_1995*) knockout BAC. For this purpose, apramycin resistance cassette *aac(3)IV-oriT* flanked with P-GG and B-CC sites was amplified from plasmid patt-saac-oriT (Myronovskyi et al. 2014) with primers Am-bldAdelF and Am-bldAdelR (all primer sequences are given in Table s1, ESM). The resulting 1378-bp PCR product was used to replace *bldA* gene in BAC_1N12 using recombineering. The resulting BAC was named 1N12d_bldA. This and other constructs generated in course of the work were verified by sequencing. Plasmid pTOSbldA (complementation of the *bldA* deletion). The *bldA* gene together with putative promoter region was amplified with primers bldA-Hind-for and bldA-Xba-rev yielding a product 675 bp in length. The PCR product was digested with enzymes *Hin*dIII–*Xba*I and cloned into respective sites of vector pTOS to give pTOSbldA. Plasmid pTESsco3479 (expression of β-galactosidase gene *SCO3479* under promoter *ermEp**). Gene *SCO3479* was amplified with primers Sco3479upXbaI and Sco3479rpMfeIBgl from the chromosome of *S. coelicolor*. The PCR product, 3016 bp in length, was digested with restriction endonucleases *Xba*I and *Bgl*II, and then ligated to *Xba*I–*Bgl*II sites of vector pTES. Plasmids pOOB109 and pOOB110. The 3.3-kb fragment of *S. coelicolor* M145 genome that encompassed the entire coding sequence of *SCO3479* plus 0.3-kb putative promoter region was amplified with primers Sco3479upBglII and 3479rpMfeIBglII. The resulting amplicon was treated with restriction endonuclease *Bgl*II and cloned into *Bam*HI + *Bgl*II-digested vector pTES. Two clones were selected, referred to as pOOB109 and pOOB110, which contained *SCO3479* in two orientations within vector pTES. In pOOB109, *SCO3479* is under control of its own

Table 1 Plasmids, cosmids, and BACs used in this work

Name	Description	Source
BAC_1N12	Cm^r; pSMART-based bacterial artificial chromosome (BAC) carrying a segment of *S. albus* genome with gene *bldA*	This work
1N12_ bldA-I	BAC 1N12 derivative, replacement of *bldA* with IMES-flanked cassette *aac(3)IV-oriT*	This work
moeno38	SuperCos1-based, carries entire *moe* cluster 1 except *moeR5moeS5*, Km^rAp^r	Ostash et al. (2013)
moeno38-6	moeno38, replacement of *neo* with $int^{\varphi C31}$-$oriT^{RK2}$-*hyg* cassette (Hy^r), Δ*moeH5* (Km^r)	Ostash et al. (2013)
pOJ436ara	pOJ436-based cosmid, carries entire aranciamycin biosynthesis gene (*ara*) cluster	Luzhetskyy et al. (2007)
pTES	Am^r; phiC31-based *Streptomyces* integrative vector; expression of cloned gene from *ermEp**	Herrmann et al. (2012)
pTOS	Am^r; VWB-based *Streptomyces* integrative vector	Herrmann et al. (2012)
pIJ773	Source of FRT-flanked *aac(3)IV-oriT* cassette	Gust et al. (2003)
patt-saac-oriT	Source of IMES-flanked *aac(3)IV-oriT* cassette	Myronovskyi et al. (2014)
pALFLP	pSG5-based plasmid expressing recombinase Flp; Hy^r	Fedoryshyn et al. (2008)
pUWLint31	pIJ101-based plasmid expressing Int-phiC31; Hy^r	Myronovskyi et al. (2014)
pUWLDre	pIJ101-based, expresses SSR Dre to evict pTOS vector sequences	Herrmann et al. (2012)
pGCymRP21	Am^rSp^r; phiC31-based *Streptomyces* integrative vector; expression of a cloned gene from cumate-inducible promoter *cmt*-P21	Horbal et al. (2014)
pTESsco3479	pTES carrying *SCO3479* under control of *ermEp**	This work
pOOB109	pTES carrying *SCO3479* under control of tandem promoter—truncated *ermEp* + full-length *SCO3479p*	This work
pOOB110	pTES carrying *SCO3479* under control of full-length *SCO3479p*	This work
pOOB114	pTES carrying "dead" version of *SCO3479* (stop codon in 19th position) under control of *ermEp**	This work
pRV3	pGCymRP21 carrying mutant (TTA codon in 19th position) version of *SCO3479* under control of cmt-P21	This work
pRV4	pGCymRP21 carrying native version of SCO3479 under control of *cmt*-P21	This work
pTOSbldA	pTOS derivative carrying *bldA* gene along with upstream 0.6-kb region	This work

promoter as well as truncated ermEp*; and in pOOB110, *SCO3479* is under control of its own promoter. Plasmid pOOB114. 3017-bp fragment containing entire coding sequence of *SCO3479* plus 29-bp putative promoter was amplified from plasmid pOOB109 with primers lacZ_XbaI_TGA2 and 3479rpMfeIBglII. In this way, leucyl codon CTG in 19th position of *SCO3479* was replaced with stop codon TGA. The resulting amplicon was treated with XbaI and MfeI and cloned into XbaI–EcoRI sites of pTES to give pOOB114. Plasmid pRV3. 3017-bp fragment containing entire coding sequence of *SCO3479* plus 29-bp putative promoter was amplified from plasmid pOOB109 with primers lacZ_XbaI_TTA1 and Sco3479_6Hisrp. In this way, leucyl codon CTG in 8th position of *SCO3479* was replaced with synonymous codon TTA and six histidine codons CAC were appended upstream of stop codon. The resulting amplicon was treated with *Xba*I and *Mfe*I and cloned into *Spe*I–*Eco*RI sites of pGCymRP21 to give pRV3. In the latter, *SCO3479* replaced *gusA* gene. Plasmid pRV4. 3017-bp fragment containing entire coding sequence of *SCO3479* plus 29-bp putative promoter was amplified from plasmid pOOB109 with primers Sco3479upXbaI and Sco3479_6Hisrp. In this way, native sequence of *SCO3479* was appended upstream of hexahistidine-stop codon sequence $[(CAC)_6$-TGA]. The resulting amplicon was treated with *Xba*I and *Mfe*I and cloned into *Spe*I–*Eco*RI sites of pGCymRP21 to give pRV4.

Generation and verification of the *S. albus* recombinant strains

All constructs were transferred into *S. albus* conjugally, as described elsewhere; gene replacements in *S. albus* were generated as described in Myronovskyi et al. (2014). Apramycin resistance marker flanked with B-GG and P-GG sites was evicted from the genome of *bldA* mutant with recombinase Int-phiC31 (plasmid pUWLint31). pTOS vector sequences from *bldA* complementation strain were evicted with recombinase Dre (plasmid pUWLDre). PCR was employed to confirm the presence of the plasmids, cosmids, and expected gene replacements in the chromosomes of streptomycetes.

Determination of antibiotic production

Moenomycin production was analyzed in *S. albus* strains carrying cosmid moeno38-6. The latter leads to accumulation of two moenomycins in *S. albus*—nosokomycin

A_2 and its precursor lacking terminal galacturonic residue (Ostash et al. 2013; Lopatniuk et al. 2014). Strains were grown in SG1. Quantity of moenomycins was determined by antibiotic disc (Ø 5 mm, Whatman) diffusion assay against spores (10^7 per plate) of moenomycin-sensitive *Bacillus cereus* ATCC19637 as described in Makitrynskyy et al. (2010). Productivity index (PI) was calculated according to the equation: PI = (Ø growth inhibition zone, mm/Ø disc, mm) − 1. PI = 0 is the lowest possible value and corresponds to antibiotic production below detection limit of the bioassay (~2% of production level of the control strain). The PIs were referred back to equal amounts of dry biomass (10 mg) in different strains.

For aranciamycin production, control and pOJ436ara-carrying strains were grown for 24 h in preculture medium (tryptic soy broth, TSB; Himedia) at 30 °C and 1 mL of the preculture was used to inoculate 50 mL (in 300-mL flasks with glass beads) of main medium SG1. After 5 days of growth, biomass was separated from supernatant via centrifugation. Spent medium (10–20 mL) was extracted with equal volume of ethylacetate. Ethylacetate extract was dried in vacuo, then reconstituted in methanol, and subjected to TLC (immobile phase: silicagel plates F60, Merck; mobile phase: chloroform:methanol = 95:5) and disc diffusion assay against *B. cereus* ATCC19637 spores (10^7 per plate) on modified minimal agar (g/L: KH_2PO_4 3, K_2HPO_4 7, sodium citrate × $4H_2O$ 0.5, $MgSO_4$ × $7H_2O$ 0.1, $(NH_4)_2SO_4$ 1, glucose 2, bacto peptone 0.3, agar 16) (Anagnostopoulos and Spizizen 1961).

Aranciamycin production and native antibiotic activity of *S. albus* strains were also monitored using agar plug antibiotic diffusion assay. Briefly, strains were grown on SG1 agar for 5 days. Then, agar plugs (Ø 5 mm) were cut off the lawn and stacked on top of TSB agar plates with test culture *D. hansenii* spread immediately prior to the experiments or *B. cereus* spores as it is described above. Halos of growth inhibition around the plugs were measured after 18 h of incubation.

Scanning electron microscopy (SEM)

Small pieces of 3-day-old lawns were cut off the OM or SFM agar plate samples, vacuum-dried, and directly analyzed on a Jeol JSM-T220A scanning microscope.

Results

Generation and growth characteristics of *S. albus* strains carrying *bldA* knockout

For *S. albus* J1074 *bldA* gene knockout, we prepared BAC 1N12_bldA-I, where *bldA* was replaced with *aac(3)IV-oriT* cassette flanked with B-GG and P-GG sites for recombinase PhiC31. With this BAC, we successfully generated respective *S. albus* mutant (ΔbldA-I) and then its markerless derivative (upon expression of PhiC31 from plasmid pUWLInt31). The markerless *bldA* mutant was referred to as OK3. OK3 genotype was confirmed by PCR (see Fig. S1, Electronic Supplementary Materials, ESM). We also introduced *S. albus* SAM2 *bldA* gene on vector pTOS back into OK3, and, after pTOS sequences eviction, generated markerless complemented strain OK3 + bldA.

In submerged culture, OK3 and the parent (SAM2) strains exhibited similar dynamics of growth and biomass accumulation (Fig. S2). The strains were also incubated on a number of solid media and analyzed after 3 and 5 days. On most media, OK3 was macroscopically and microscopically indistinguishable from the parent (Figs. S3, S4). Nevertheless, we have found that certain minimal media did result in visible changes in lawn morphology of OK3 (see Fig. S4). The most pronounced morphological differences were seen on SFM and SMMS (Figs. 1 and S5). On these media, OK3 produced sparse aerial hyphae and spore chains without significant delay as compared to the parental strain.

Antibiotic production by OK3 strain

Genomic potential of *S. albus* J1074 for secondary metabolism is significant (Zaburannyi et al. 2014), yet, to date, its expression required genetic interventions (Olano et al. 2014; Brana et al. 2014). Compounds of paulomycin family are the only known antibacterially active metabolites to be accumulated by J1074, although their production pattern was unstable (Olano et al. 2014; Gonzalez et al. 2016) We tested over a dozen of the most common agar media used for *Streptomyces* cultivation, and found those that reproducibly elicit the production of antibiotic compounds by J1074 (Figs. S6, S7). Particularly, *S. albus* accumulated antibacterial and (to a lesser extent) antifungal compounds when growing on SG2 agar. Using SG2, we compared antibiotic activity of SAM2, *bldA* mutant OK3 and OK3 + bldA. The results are summarized in Fig. 2. OK3 did not accumulate detectable amounts of antibacterial compounds and introduction of *bldA* restored antibiotic production to the mutant. The antifungal activity of OK3 was not impaired and even slightly increased as compared to SAM2. Using HPLC–MS (as detailed in ESM), we confirmed that production of candicidin-like compounds by OK3 is increased as compared to parent strain, while paulomycins were not found in OK3 (Fig. S8).

Next, we compared the ability of SAM2 and OK3 to heterologously produce antibiotics whose biosynthesis depend on TTA codon-containing genes. These were moenomycin and aranciamycin biosynthesis gene clusters, carried on

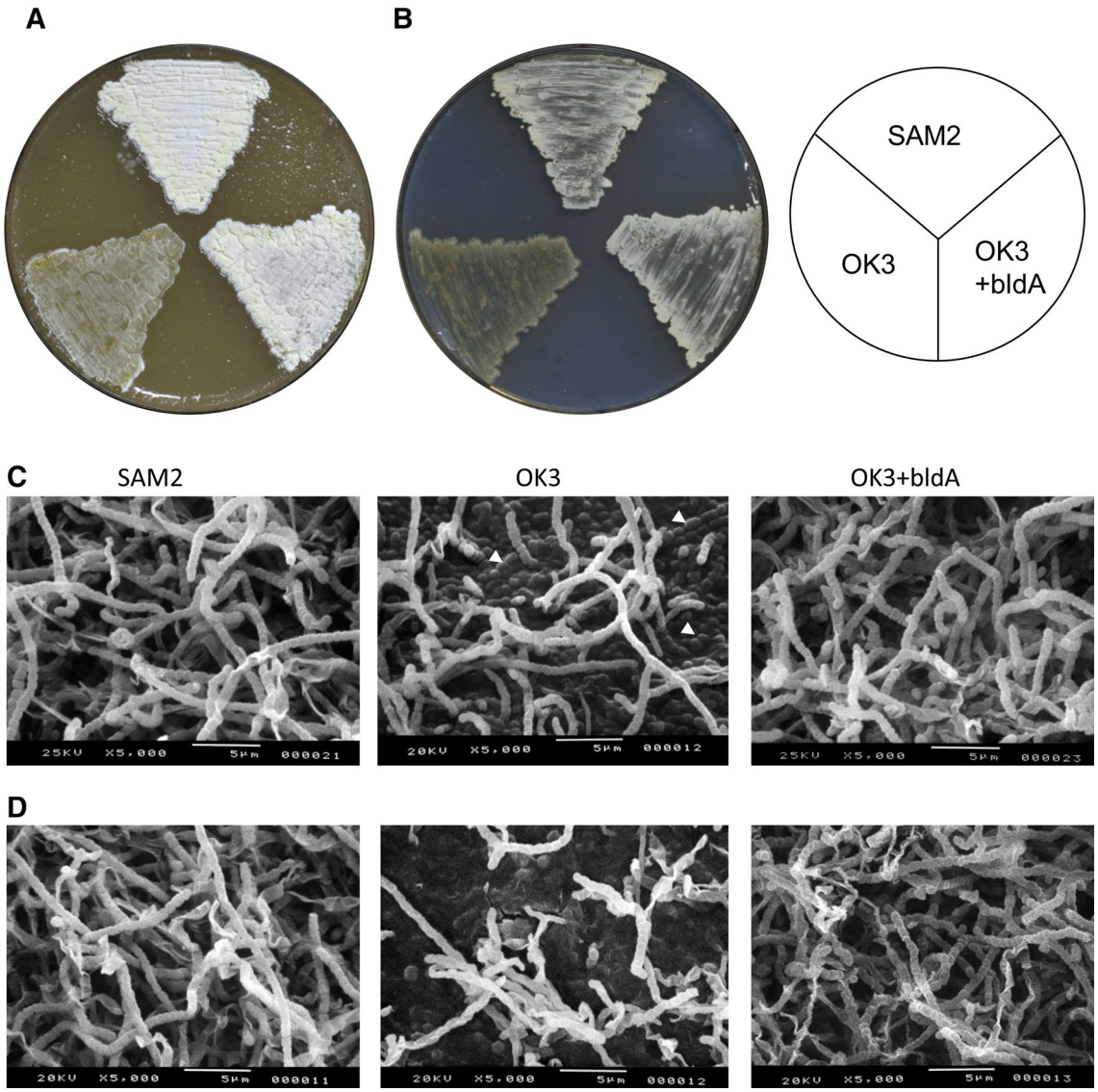


Fig. 1 *Streptomyces albus* J1074 *bldA* mutant phenotype. Parental (SAM2), mutant and complemented strains were grown for 3 days on SFM (**a**) and SMMS (**b**). The same lawns were used to obtain scanning electron microscopic views of colonial surfaces of SFM (**c**) and SMMS-grown cultures (**d**). The substrate mycelium of OK3 strain on SFM (**c**) appeared to be fragmented into spore-like chains (*white triangles*)

cosmids moeno38-6 (Lopatniuk et al. 2014) and pOJ436ara (Luzhetskyy et al. 2007), respectively. As can be judged from bioactivity and TLC-based assays, moeno38-6^{+} and pOJ436ara^{+} transconjugants of the parental strain abundantly produced respective antibiotics, while OK3 strain did not (Figs. 3, S9–S10).

The β-galactosidase reporter system to detect and elucidate *bldA*-based regulation

The above-described results imply that, in line with what is known for the other *Streptomyces* (Chater and Chandra 2008; Liu et al. 2013), failure to translate codon UUA in transcripts of secondary metabolism genes is the primary reason for the inability of OK3 to produce endogenous and heterologous antibiotics. Yet, with two exceptions (Wang et al. 2009; Makitrinskyy et al. 2013), direct evidence for involvement of *bldA* in regulation at the level of UUA translation is absent for all studied cases. In *S. albus* J1074, the *bldA* mutation and the observed phenotype (lack of antibacterial activity of the strain) are bridged by a complicated cascade of regulatory and structural genes. Codon-swap reporters based on β-galactosidase and luciferase genes are used in enterobacteria and yeast to peer into regulatory events at the level of translation of a certain codon (Urbonavicius et al. 2003; Lamichhane et al. 2013). We set out

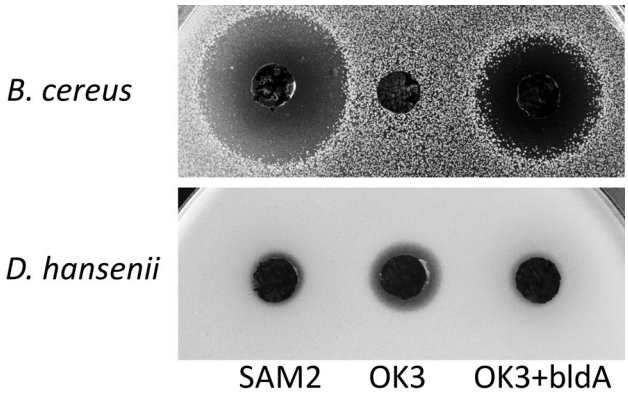


Fig. 2 The *bldA* knockout in *S. albus* abolished the production of antibacterial compound(s), but not antifungal one(s). For the bioassay, agar plugs were cut off 5-day lawns of *S. albus* strains grown on SG2 agar, and stacked on top of plates with test cultures. Note the small halos of *D. hansenii* growth inhibition around SAM2 and OK3 + *bldA* plugs, and increased halo around OK3 plug

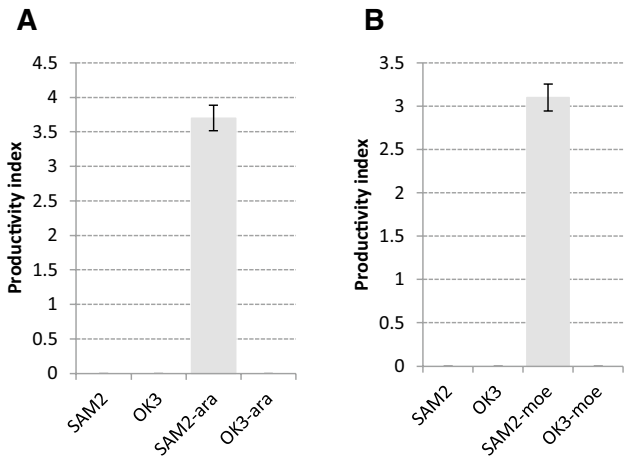


Fig. 3 Aranciamycin (**a**) and moenomycin (**b**) production is blocked in *bldA* mutant of *S. albus*. Strains were grown in SG1 medium, and productivity indices were calculated from disc diffusion assays as described in "Materials and methods". SAM2, OK3—strains carrying empty vector pSET152; SAM2/OK3-ara, SAM2/OK3-moe—*S. albus* strains carrying either cosmid pOJ436ara or cosmid moeno38-6, respectively

to develop an analogous reporter system for *Streptomyces albus* J1074, which would enable straightforward phenotypic detection of translation of UUA. Here, we took advantage of the fact that J1074 is one of the rare streptomycetes having no detectable intra- or extracellular enzymatic activity towards lactose analog, X-Gal (King and Chater 1986). Indeed, our analysis confirmed that none of the seven annotated *S. coelicolor* β-galactosidases had counterparts within J1074. Using reciprocal best BLAST hit strategy (Kuzniar et al. 2008), *S. coelicolor* M145 protein Sco3479 (referred hereafter to as $LacZ_{sc}$) was identified as an ortholog of *E. coli* LacZ. Several $lacZ_{sc}$-carrying plasmids were prepared (Fig. 4a) and expression of functional β-galactosidase from all of them (except for pOOB114 which carries in-frame stop codon in position 19 of $lacZ_{sc}$) was confirmed. Particularly, gene $lacZ_{sc}$ conferred *lacZ*-deficient *E. coli* to the ability to hydrolyze X-Gal; in liquid and solid media, different $lacZ^{+}_{sc}$ *S. albus* strains produced blue color of different intensity (Fig. S11). The latter most likely reflects different strength of transcription of $lacZ_{sc}$.

We introduced TTA, CTC, and stop codon-containing $lacZ_{sc}$ reporter plasmids (pRV3, pRV4, pOOB114, respectively) individually into OK3 strain. As expected, the plasmids did not cause the production of blue color (X-Gal hydrolysis) in the absence of inducer (cumate). In the presence of 50 mM cumate only the strain carrying CTC-specific reporter (pRV4) turned blue (Fig. 4). The ability of OK3-pRV3 to hydrolyze X-Gal was restored upon introduction of *bldA* (Fig. S12). All available data suggest that *bldA* deletion directly and specifically impaired the translation of TTA codon in $lacZ_{sc}$ reporter gene.

Discussion

In this work, we have carried out a set of experiments aimed to study the effects of *bldA* deletion on *S. albus* J1074 and to develop a simple reporter system for further investigation of *bldA*-mediated regulation in J1074.

Perhaps, the most striking property of Bld phenotype (displayed by *bldA* mutants of streptomycetes, vide supra) is its conditionality. That is, the degree of morphological and antibiotic synthesis defects strongly depend on growth conditions and level of transcription TTA codon-containing (TTA^{+}) genes (Guthrie and Chater 1990; Leskiw et al. 1993; Gramajo et al. 1993; Pope et al. 1996; Xu et al. 2008). The conditionality of Bld phenotype of *S. albus* OK3 has extreme manifestation. Indeed, we were able to identify media, where OK3 exhibits impaired morphological development, but under no conditions did we observe complete cessation of aerial mycelium and spore formation. To the best of our knowledge, there is no experimentally validated information on gene networks that govern morphogenesis in *S. albus* J1074. However, as far as we can judge from in silico analysis of J1074 genome (Zaburannyy et al. 2014), the *bld*- and *whi*-cascades are essentially the same in J1074 and other model streptomycetes. Gene *XNR4181* encoding ortholog of *S. coelicolor* AdpA (also known as BldH), a master regulator of colonial differentiation and (often) antibiotic biosynthesis (Chater and Chandra 2008), contains TTA codon, and thus should be under *bldA* control. Hence, either UUA within *adpA* mRNA is mistranslated efficiently enough to allow colonial development, or other (as-yet-unknown) genes compensate/bypass the loss of AdpA. In

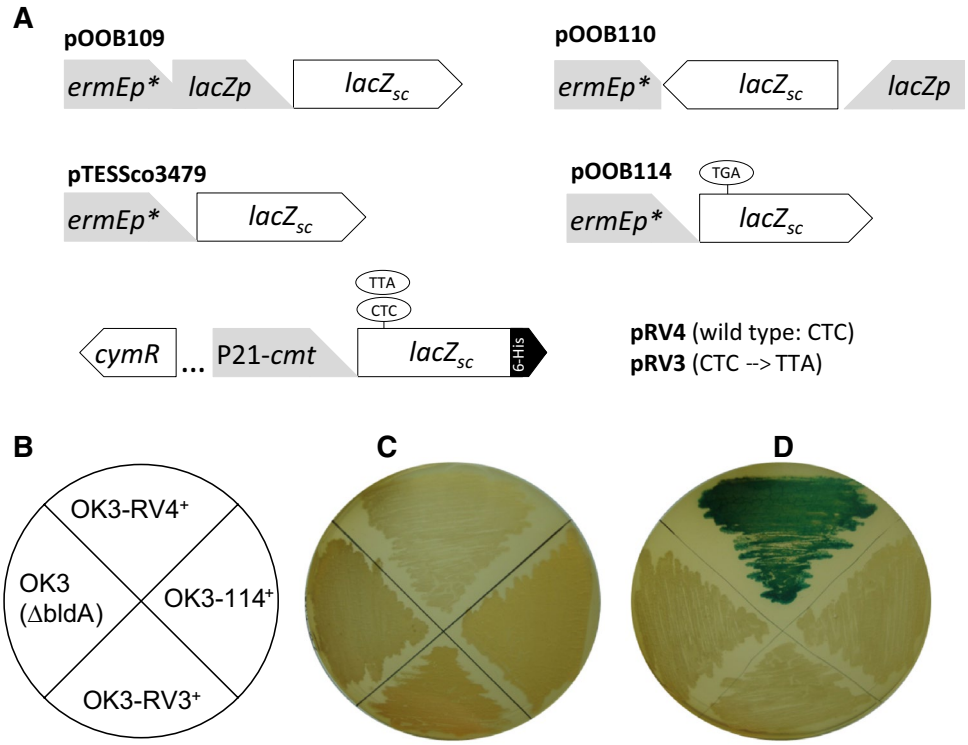


Fig. 4 Codon-specific β-galactosidase reporter for *S. albus* visualizes arrested TTA codon translation in *bldA* mutant OK3. All plasmids (**a**) carrying *S. coelicolor* M145 β-galactosidase gene *SCO3479* ($lacZ_{sc}$) contain actinophage ϕC31 *int-attP* integration module and apramycin resistance marker *aac(3)IV*. They differ in the type of promoter (*shaded gray*) that drives $lacZ_{sc}$ transcription, and presence of distinct codons (shown as *ovals*) in positions 8 and 19 of $lacZ_{sc}$ ORF (if not shown, then CTC codon is present). In pOOB109 and pOOB110, *ermEp* is rendered nonfunctional through truncation of its last 30 nts. In pRV3 and pRV4 $lacZ_{sc}$, transcription is cumate-inducible (Horbal et al. 2014). Four OK3 derivatives (**b**) were streaked onto TSA X-Gal-containing plates in the absence (**c**) and in presence (**d**) of cumate. CTC^+ reporter is expressed in OK3, while TTA^+ is not. The latter is expressed in wild type (SAM2) strain (see ESM, Fig. S11)

Streptomyces, there are several reports on mistranslation of various UUA^+ mRNAs, including *adpA*, in the absence of *bldA*-encoded $tRNA^{Leu}_{UAA}$ (Leskiw et al. 1991; Makitrinskyy et al. 2013). Although existence of a subset of TTA^+ genes capable of translation in *bldA* mutants appears to be paradoxical, an explanation for this was offered (Trepanier et al. 2002). Briefly, propensity for mistranslation is attributed to the nature of the first nucleotide downstream of the TTA codon. If either A or G follow TTA, then the ribosome is able to mistranslate the codon; otherwise (C/T downstream of TTA) translation is aborted. Thus, in the absence of $tRNA^{Leu}_{UAA}$, only mRNA containing the quadruplets TTAC/T will not be translated, and so regulatory effect will be attained. There is TTAC quadruplet in case of *S. albus* J1074 *adpA*; thus, codon context hypothesis does not seem to apply. The position of TTA within the coding sequence could be important. In most streptomycete genes, it is located close to the start codon (Zaburannyy et al. 2009), while in *adpA* gene family, it is positioned roughly in the middle of the ORF. Also, enhancement of UUA translation in *adpA* mRNA in the presence of *S*-adenosylmethionine was reported (Xu et al. 2008). These facts underscore the importance of environmental (rather than genetic) factors in translation of *adpA* mRNA, and they might play a crucial role in shaping the morphology of OK3 strain.

The *bldA* knockout abolished production of two heterologous antibiotics, moenomycin and aranciamycin, the biosynthesis of which is controlled by TTA-containing genes. Moreover, OK3 no longer produced endogenous antibacterial compounds. According to all available data, glycosylated antibiotics, paulomycins, are the principal source of antibacterial activity exhibited by *S. albus* J1074 (Majer and Chater 1987; Olano et al. 2014). We also believe that paulomycins accounted for most (if not all) of the antibacterial activity of SAM2, given the characteristic reddish coloration of *S. albus* lawns on SG2 agar, and the absence of that color in OK3, although detailed analysis was not pursued. Cessation of their production by OK3 strain is most likely due to presence of TTA codon within regulatory gene *SSGH_05342* (*plm30*) encoding LuxR family regulator essential for paulomycin production (Gonzalez et al. 2016). At the same time, level of antifungal activity of OK3 is not affected as compared to SAM2, and even appears to be elevated. *S. albus*

genome carries several gene clusters for biosynthesis of compounds that might target eukaryotic cells (Zaburannyy et al. 2014). One of these clusters (around gene *XNR_RS29020*), directing the production of candicidin-like compound, is free of TTA codons, and so could be expressed in *bldA*-minus background.

Our work has once again highlighted all the challenges associated with interpretation of complex, conditional, and composite phenotypes of *bldA* mutants. For example, since TTA codons are embedded into genes for pleiotropic and pathway-specific transcriptional factors, any observable defect of *bldA* mutant would be the net result of deregulated transcription and translation. It is thus difficult to account for and disentangle the relative contribution of each genetic and environmental factor to a final *bldA*-minus phenotype. This calls for development of a simple reporter system where effects of *bldA* knockout on translation can be reliably detected and studied in the most direct way. Several such systems (mostly based on antibiotic resistance genes) were employed in the past, and they gathered valuable information about *bldA* function (Leskiw et al. 1991; Pope et al. 1996). Nevertheless, available reporter genes are not without shortcomings, such as unnatural (for *Streptomyces*) codon usage biases; poor control of transcription; and absence, in most cases, of TTA-free version of the reporter gene. We developed, for *S. albus* J1074, a specialized codon reporter system based on *S. coelicolor* M145 β-galactosidase gene *SCO3479* ($lacZ_{sc}$). It allows tight transcriptional control over *SCO3479* expression, ease of phenotypic detection (in presence of chromogenic substrates), and His-tagged protein tracking. The simplicity of our reporter system lends itself to a rigorous elucidation of the regulatory role of TTA codons at the level of translation, as our initial experiments (vide supra) suggest.

A number of recent studies suggest that it is not the delayed expression of *bldA* gene but formation of mature (translationally competent) tRNA holds the key to its regulatory function (Leskiw et al. 1991; Xu et al. 2008; Pettersson and Kirsebom 2011). Yet, besides initial in silico predictions (Rokytskyy et al. 2016) nothing is known about the genetics of tRNA maturation in *Streptomyces*. Likewise, there is a growing body of data on the crucial role of environmental factors in tRNA biology (Laxman et al. 2013; Moukadiri et al. 2014; Sakai et al. 2016), although this area remains unexplored in case of *bldA* gene. We envision that the above-described reporter system will facilitate the screening of growth conditions and genes that modulate UUA translation efficiency, both in wild type and *bldA*-minus *S. albus* backgrounds.

Acknowledgements This work was supported by grant BG-41Nr from the Ministry of Education and Science of Ukraine (to B.O.).

References

Anagnostopoulos C, Spizizen J (1961) Requirements for transformation of *Bacillus subtilis*. J Bacteriol 81(5):741–746

Baltz RH (2010) *Streptomyces* and *Saccharopolyspora* hosts for heterologous expression of secondary metabolite gene clusters. J Ind Microbiol Biotechnol 37(8):759–772. doi:10.1007/s10295-010-0730-9

Bilyk B, Luzhetskyy A (2014) Unusual site-specific DNA integration into the highly active pseudo-*attB* of the *Streptomyces albus* J1074 genome. Appl Microbiol Biotechnol 98(11):5095–5104. doi:10.1007/s00253-014-5605-y

Braña AF, Rodríguez M, Pahari P, Rohr J, García LA, Blanco G (2014) Activation and silencing of secondary metabolites in *Streptomyces albus* and *Streptomyces lividans* after transformation with cosmids containing the thienamycin gene cluster from *Streptomyces cattleya*. Arch Microbiol 196:345–355. doi:10.1007/s00203-014-0977-z

Chater K, Chandra G (2008) The use of the rare UUA codon to define "expression space" for genes involved in secondary metabolism, development and environmental adaptation in *Streptomyces*. J Microbiol 46:1–11

Chater KF, Wilde LC (1980) *Streptomyces albus* G mutants defective in the SalGI restriction-modification system. J Gen Microbiol 116(2):323–334

Datsenko K, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645

Fedoryshyn M, Petzke L, Welle E, Bechthold A, Luzhetskyy A (2008) Marker removal from actinomycetes genome using Flp recombinase. Gene 419(1–2):43–47. doi:10.1016/j.gene.2008.04.011

González A, Rodríguez M, Braña AF, Méndez C, Salas JA, Olano C (2016) New insights into paulomycin biosynthesis pathway in *Streptomyces albus* J1074 and generation of novel derivatives by combinatorial biosynthesis. Microb Cell Fact 21:56. doi:10.1186/s12934-016-0452-4

Gramajo H, Takano E, Bibb M (1993) Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor*A3(2) is transcriptionally regulated. Mol Microbiol 7(6):837–845

Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 100:1541–1546

Guthrie EP, Chater KF (1990) The level of a transcript required for production of a *Streptomyces coelicolor* antibiotic is conditionally dependent on a tRNA gene. J Bacteriol 172:6189–6193. doi:10.1128/jb.172.11.6189-6193.1990

Herrmann S, Siegl T, Luzhetska M, Petzke L, Jilg C, Welle E, Erb A, Leadlay PF, Bechthold A, Luzhetskyy A (2012) Site-specific recombination strategies for engineering actinomycete genomes. Appl Environ Microbiol 78(6):1804–1812. doi:10.1128/AEM.06054-11

Horbal L, Fedorenko V, Luzhetskyy A (2014) Novel and tightly regulated resorcinol and cumate-inducible expression systems for *Streptomyces* and other actinobacteria. Appl Microbiol Biotechnol 98(20):8641–8655. doi:10.1007/s00253-014-5918-x

Iqbal HA, Low-Beinart L, Obiajulu JU, Brady SF (2016) Natural product discovery through improved functional metagenomics in *Streptomyces*. J Am Chem Soc 138(30):9341–9344. doi:10.1021/jacs.6b02921

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. John Innes Foundation, Norwich

King AA, Chater KF (1986) The expression of the *Escherichia coli lacZ* gene in *Streptomyces*. J Gen Microbiol 132(6):1739–1752. doi:10.1099/00221287-132-6-1739

Kuzniar A, van Ham RC, Pongor S, Leunissen JA (2008) The quest for orthologs: finding the corresponding gene across genomes. Trends Genet 24:539–551. doi:10.1016/j.tig.2008.08.009

Lamichhane TN, Blewett NH, Crawford AK, Cherkasova VA, Iben JR, Begley TJ, Farabaugh PJ, Maraia RJ (2013) Lack of tRNA modification isopentenyl-A37 alters mRNA decoding and causes metabolic deficiencies in fission yeast. Mol Cell Biol 33:2918–2929. doi:10.1128/MCB.00278-13

Laxman S, Sutter BM, Wu X, Kumar S, Guo X, Trudgian DC, Mirzaei H, Tu BP (2013) Sulfur amino acids regulate translational capacity and metabolic homeostasis through modulation of tRNA thiolation. Cell 154:416–429. doi:10.1016/j.cell.2013.06.043

Leskiw BK, Lawlor EJ, Fernandez-Abalos JM, Chater KF (1991) TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative, *Streptomyces* mutants. Proc Natl Acad Sci USA 88:2461–2465

Leskiw BK, Mah R, Lawlor EJ, Chater KF (1993) Accumulation of *bldA*-specified tRNA is temporally regulated in *Streptomyces coelicolor* A3(2). J Bacteriol 175:1995–2005. doi:10.1128/jb.175.7.1995-2005.1993

Liu G, Chater KF, Chandra G, Niu G, Tan H (2013) Molecular regulation of antibiotic biosynthesis in *Streptomyces*. Microbiol Mol Biol Rev 77(1):112–143. doi:10.1128/MMBR.00054-12

Lopatniuk M, Ostash B, Luzhetskyy A, Walker S, Fedorenko V (2014) Generation and study of the strains of streptomycetes—heterologous hosts for production of moenomycin. Russ J Genet 50(4):360–365

Luzhetskyy A, Mayer A, Hoffmann J, Pelzer S, Holzenkämper M, Schmitt B, Wohlert SE, Vente A, Bechthold A (2007) Cloning and heterologous expression of the aranciamycin biosynthetic gene cluster revealed a new flexible glycosyltransferase. ChemBioChem 8(6):599–602

Majer J, Chater KF (1987) *Streptomyces albus* G produces an antibiotic complex identical to paulomycins A and B. J Gen Microbiol 133:2503–2507. doi:10.1099/00221287-133-9-2503

Makitrynskyy R, Rebets Y, Ostash B, Zaburannyi N, Rabyk M, Walker S, Fedorenko V (2010) Genetic factors that influence moenomycin production in streptomycetes. J Ind Microbiol Biotechnol 37:559–566

Makitrynskyy R, Ostash B, Tsypik O, Rebets Y, Doud E, Meredith T, Luzhetskyy A, Bechthold A, Walker S, Fedorenko V (2013) Pleiotropic regulatory genes *bldA*, *adpA* and *absB* are implicated in production of phosphoglycolipid antibiotic moenomycin. Open Biol 3:130121. doi:10.1098/rsob.130121

Moukadiri I, Garzón MJ, Björk GR, Armengod ME (2014) The output of the tRNA modification pathways controlled by the *Escherichia coli* MnmEG and MnmC enzymes depends on the growth conditions and the tRNA species. Nucleic Acids Res 42:2602–2623. doi:10.1093/nar/gkt1228

Myronovskyi M, Rosenkränzer B, Luzhetskyy A (2014) Iterative marker excision system. Appl Microbiol Biotechnol 98(10):4557–4570. doi:10.1007/s00253-014-5523-z

Nguyen KT, Tenor J, Stettler H, Nguyen LT, Nguyen LD, Thompson CJ (2003) Colonial differentiation in *Streptomyces coelicolor* depends on translation of a specific codon within the *adpA* gene. J Bacteriol 185:7291–7296. doi:10.1128/JB.185.24.7291-7296.2003

Olano C, García I, González A, Rodriguez M, Rozas D, Rubio J, Sánchez-Hidalgo M, Braña AF, Méndez C, Salas JA (2014) Activation and identification of five clusters for secondary metabolites in *Streptomyces albus* J1074. Microb Biotechnol 7:242–256. doi:10.1111/1751-7915.12116

Ostash B, Campbell J, Luzhetskyy A, Walker S (2013) MoeH5: a natural glycorandomizer from the moenomycin biosynthetic pathway. Mol Microbiol 90:1324–1338. doi:10.1111/mmi.12437

Pettersson BM, Kirsebom LA (2011) tRNA accumulation and suppression of the *bldA* phenotype during development in *Streptomyces coelicolor*. Mol Microbiol 79:1602–1614. doi:10.1111/j.1365-2958.2011.07543.x

Pope MK, Green BD, Westpheling J (1996) The *bld* mutants of *Streptomyces coelicolor* are defective in the regulation of carbon utilization, morphogenesis and cell–cell signalling. Mol Microbiol 19:747–756. doi:10.1046/j.1365-2958.1996.414933.x

Rebets YV, Ostash BO, Fukuhara M, Nakamura T, Fedorenko VO (2006) Expression of the regulatory protein LndI for landomycin E production in *Streptomyces globisporus* 1912 is controlled by the availability of tRNA for the rare UUA codon. FEMS Microbiol Lett 256(1):30–37

Rodriguez L, Oelkers C, Aguirrezabalaga I, Braña AF, Rohr J, Méndez C, Salas JA (2000) Generation of hybrid elloramycin analogs by combinatorial biosynthesis using genes from anthracycline-type and macrolide biosynthetic pathways. J Mol Microbiol Biotechnol 2(3):271–276

Rodríguez A, Olano C, Vilches C, Méndez C, Salas JA (1993) *Streptomyces antibioticus* contains at least three oleandomycin-resistance determinants, one of which shows similarity with proteins of the ABC-transporter superfamily. Mol Microbiol 8(3):571–582

Rokytskyy I, Koshla O, Fedorenko V, Ostash B (2016) Decoding options and accuracy of translation of developmentally regulated UUA codon in *Streptomyces*: bioinformatics analysis. Springerplus 5:982. doi:10.1186/s40064-016-2683-6

Sakai Y, Miyauchi K, Kimura S, Suzuki T (2016) Biogenesis and growth phase-dependent alteration of 5-methoxycarbonylmethoxyuridine in tRNA anticodons. Nucleic Acids Res 44:509–523. doi:10.1093/nar/gkv1470

Sambrook J, Russell DW (2001) Molecular cloning, a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New-York

Takano E, Tao M, Long F, Bibb MJ, Wang L, Li W, Buttner MJ, Bibb MJ, Deng ZX, Chater KF (2003) A rare leucine codon in *adpA* is implicated in the morphological defect of *bldA* mutants of *Streptomyces coelicolor*. Mol Microbiol 50:475–486

Trepanier N, Jensen S, Alexander D, Leskiw B (2002) The positive activator of cephamycin C and clavulanic acid production in *Streptomyces clavuligerus* is mistranslated in a *bldA* mutant. Microbiology 148:643–656

Urbonavicius J, Stahl G, Durand JM, Ben Salem SN, Qian Q, Farabaugh PJ, Björk GR (2003) Transfer RNA modifications that alter +1 frameshifting in general fail to affect −1 frameshifting. RNA 9:760–768. doi:10.1261/rna.5210803

Wang J, Schully KL, Pettis GS (2009) Growth-regulated expression of a bacteriocin, produced by the sweet potato pathogen *Streptomyces ipomoeae*, that exhibits interstrain inhibition. Appl Environ Microbiol 75:1236–1242. doi:10.1128/AEM.01598-08

Xu D, Kwon HJ, Suh JW (2008) S-Adenosylmethionine induces BldH and activates secondary metabolism by involving the TTA-codon control of *bldH* expression in *Streptomyces lividans*. Arch Microbiol 189:419–426. doi:10.1007/s00203-007-0336-4

Zaburannyi N, Rabyk M, Ostash B, Fedorenko V, Luzhetskyy A (2014) Insights into naturally minimised *Streptomyces albus* J1074 genome. BMC Genom 15:97. doi:10.1186/1471-2164-15-97

Zaburannyy N, Ostash B, Fedorenko V (2009) TTA Lynx: a web-based service for analysis of actinomycete genes containing rare TTA codon. Bioinformatics 25:2432–2443. doi:10.1093/bioinformatics/btp402

3 Discussion

NPs from microorganisms are the primary source of drug leads starting from the time of their discovery and until now. Emergence and spread of pathogens resistant to antibiotics raises the need to screen for new drug leads. The classical strain-centered drug discovery approaches faced their limits at the end of the 20th century, making screening programs less efficient and profitable. Changing the drug discovery pipeline, together with more judicious use of antibiotics, is necessary to deal with the antibiotic crisis and to delay the development of resistance. Shifting from old fashioned strain-centered to more advanced cluster-centered approach is currently accepted as the most promising way to make drug discovery more efficient. Identification of unique natural product biosynthetic pathways in the DNA sequence databases and their directed expression in optimized host strains with simple metabolic background facilitates the discovery of the new compounds, simplifies greatly their analysis and purification, and allows performing biological activity screening using crude extracts. Development of methods to efficiently construct large-insert genomic libraries and the construction of the host strains for efficient and high-level expression of NP pathways are important prerequisites for implementation of the cluster-centered approach in drug discovery. In this dissertation, we deal with the construction and optimization of the *Streptomyces* heterologous host strains for the discovery and derivatization of natural products. In particular, the effect of ribosome engineering on the expression of indigenous and heterologous secondary metabolite pathways as well as utilization of these mutations for the construction of chassis strains was studied. Additionally, an *S. albus*-based heterologous host strain for the diversification of ribosomally synthesized natural products through incorporation of the unnatural amino acids was developed in this work.

rpsL mutations as a tool to increase antibiotic production yields in *Streptomyces*

The positive effect of streptomycin resistance mutations on antibiotic production was first observed in 1996 by the group of Kozo Ochi [1]. Many of these mutations were located in the gene *rpsL,* which encodes the S12 ribosomal protein [2, 3]. Other part of streptomycin resistance mutations were located in *rsmG* gene that encodes for a 16S rRNA methyltransferase G. Deletion of this gene was often associated with antibiotic overproduction phenotype and low level of streptomycin resistance [4].

The approach for increasing antibiotic production through the introduction of mutations into *rpsL* or *rsmG* genes was named – ribosome engineering. Until now, numerous *rpsL* mutations were generated, and their effect on antibiotic production was described [1, 2, 5-9]. However, the generated data have several weak points, which prevent us from understanding the effect of *rpsL* mutations on the physiology of the strain. The vast majority of the *rpsL* mutations were induced spontaneously by growing the studied strains on the media supplemented with streptomycin. The *rpsL* mutants were selected among resistant colonies by amplifying and sequencing the *rpsL* gene. Since the genomes of the selected mutant strains were never sequenced, such approach does not exclude the possibility that additional mutations reside elsewhere in the genome of the selected mutants. Such apprehension is supported by generating of streptomycin-resistant mutants with antibiotic overproducing phenotype, however without mutations in the *rpsL* gene [4]. The existence of the numerous mutations in the genome does not allow linking the overproducing phenotype to any of them.

Many of the reported *rpsL* and *rsmG* mutations were generated in different *Streptomyces* strains, which differ in their physiology and regulatory networks.

The genomes of Streptomycetes encode for dozens of different natural products and readily produce only few of them under laboratory conditions. When studying the *rpsL* mutations, in a prevalent number of cases, only the effect on the production of one single compound was described. The description of how the introduction of the *rpsL* mutations influenced the production of other remaining compounds is missing.

Many of the described *rpsL* mutations were studied in merozygous state. The mutated versions of the *rpsL* gene were introduced in *Streptomyces* strain on the plasmid, and their effect on antibiotic production was studied in the presence of the wild type chromosomal copy of the gene. In the generated strains, the ribosomes population in the cell would be heterogeneous, with some part represented by native ribosomes and another part by ribosomes with the mutated S12 protein. This can hamper the explanation of the observed phenotypes.

In order to close the gaps in the existing data on the role ribosome engineering in antibiotic production and to further our understanding how to utilize it for rational overproduction of natural products, we set out to introduce different described *rpsL* mutations alone and in combination with deletion of *rsmG* gene into a single heterologous host *S. albus* J1074 and to

study their effect on the production on various indigenous as well as heterologous gene clusters. A technology was developed for the introduction of point mutations into the *rpsL* gene without a risk to obtain additional mutations elsewhere in the genome.

The platform for efficient introduction of point mutations into essential genes of *Streptomyces sp*.

Introduction of point mutations into the genes was traditionally performed in two steps. First, the gene of interest was substituted with a resistance gene, and then, in the second step, the marker was substituted back with a mutated variant of the gene with a desired point mutation. Both, deletion of the gene and substitution of the marker were achieved through double crossover using natural homology recombination of the strain. Utilization of the resistance marker simplified the screening procedure in both steps by allowing direct selection of the mutant strains according to their resistance phenotype. A modification of this two-step method exists, where the substitution of the gene with marker is skipped, and the natural gene is substituted directly with its mutated copy. In the case of this one-step approach, no possibility exists to select double-crossover mutants with point mutations directly, as their antibiotic resistance phenotype is identical to that of the wild type strain. To select the mutant strain with the desired point mutation time-consuming PCR screening followed by sequencing has to be blindly applied to hundreds to thousands of clones. Due to difficulties in screening for mutant clones, the latter approach was used only rarely.

The classical one- and two-step approaches can be applied for the introduction of mutations into the genes, which are not essential. In the case of essential genes introduction of the mutations in a two-step approach cannot be applied, since it is not possible to substitute an essential gene with an antibiotic marker. The difficult and time-consuming one-step approach can be applied for introduction into essential genes only in a limited way. The introduction of mutations into essential genes often reduces the fitness of the strain, what makes the complicated screening procedure even more tedious. To study the effect of *rpsL*-mutations on the antibiotic production in *S. albus*, numerous individual point mutations should be introduced into the essential gene *rpsL,* which encodes S12 ribosome protein. For this purpose, a new approach for efficient introduction of point mutations into essential genes was developed.

The introduction of point mutations into essential gene requires the construction of a strain where the essential gene is flanked by two resistance markers and of a library of BACs containing variants of the essential gene with point mutations. As *rpsL* gene forms an operon with another essential gene encoding a ribosome protein S7, this two gene operon should be manipulated as one functional unit. Apramycin resistance gene was introduced upstream of the operon and hygromycin resistance gene – downstream of the operon. Introduction of two markers allows direct screening for the mutants with point mutations in the *rpsL* gene. In these mutants, the whole operon together with both resistance genes is substituted with the operon containing mutated *rpsL*. These mutants show apramycin- and hygromycin–sensitive phenotype compared to the parental strain, which is resistant to both antibiotics.

For the introduction of point mutations into essential genes utilization of BACs instead of plasmids is beneficial. Compared to plasmids, BACs usually contain much longer DNA inserts which serve as homology regions during genome modification procedures. This allows crossover to occur at regions, which are quite distant to the essential gene to be modified and therefore minimizes the risk of unwanted polar effect on the essential gene. Because of short homology regions used in plasmid vectors, one has to consider such polar effects when using plasmids for manipulating of essential genes. In order to construct a library of the BACs with mutated variants of the *rpsL* gene, the corresponding BAC containing chromosomal fragment with the *rpsL* gene was selected. The constructed BACs with the mutated *rpsL* gene were introduced into the previously constructed strain with antibiotic markers flanking the operon with *rpsL* gene and gene encoding ribosome protein S7. The introduction of the BACs was performed by conjugation and the exconjugants were selected by the presence of resistance marker in the backbone of the BAC backbone. As the BAC backbone is not replicative, in order to give raise to exconjugants it should be integrated via single-crossover into the chromosome of the strain. The double crossover strains with point mutations were selected in two steps. First, double crossover and revertant strains were selected as white colonies in a blue-white screen due to a loss of the beta-glucuronidase gene encoded by the BAC backbone. Double crossover mutants were selected from revertant clones by analyzing their antibiotic resistance phenotype. The revertant strains were resistant to antibiotics while the double crossover strains with mutations in the *rpsL* gene were sensitive to both antibiotics.

The developed technology allows introduction of point mutations into essential genes in a straightforward way. Due to the utilization of BACs instead of conventional plasmids, a high rate of

crossover is reached. The presence of the beta-glucuronidase gene in the BAC backbone allows fast preselection of clones where either reversion of double crossover occurred. The substitution of the chromosomal antibiotic markers during the introduction of point mutations allows distinguishing between revertant and desired double crossover strains by checking resistance profile without the need to perform PCR and sequencing. This all allows introduction of the point mutations into essential genes, which might substantially lower the fitness of the mutant strain.

The effect of *rpsL* and *rsmG* mutations on antibiotic production

Numerous *rpsL* mutations were described in the literature; however, their effect on antibiotic production was studied to a different extent. In this study, we aimed to introduce mutations K88E, GI92, P91S, R86P, R94G, K88R, and L90K into *rpsL* gene of *S. albus* strain and to study their effect on the production of indigenous and heterologously produced natural products. Of all mentioned mutations we failed to introduce two mutations into the *rpsL* gene – K88R and L90K. The K88R mutation was reported to increase salinomycin production in industrial producer *Streptomyces albus* SAM-X. The salinomycin producer is not related to *S. albus* strains used in this study. It is not clear why all attempts to introduce K88R mutations into the *rpsL* gene of *S. albus* failed. A possible explanation could be that the K88R mutation is lethal for *S. albus*. In the case of salinomycin producer, other mutations in the chromosome induced previously by several rounds of UV and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis might have smoothened the lethal effect of the K88R mutation. In the case of the L90K mutation, its effect was described only in the background of the wild type *rpsL* gene. Therefore there was no evidence provided that the strains harboring L90K mutation in the *rpsL* gene remain viable.

The effect of the introduced mutations K88E, GI92, P91S, R86P, R94G on the production of both indigenously encoded candicidins, antimycins, antimycic acid, paulomycins and desferrioxamines, and heterologously produced aranciamycin, griseorhodin, cinnamycin, tunicamycin, pamamycin and moenomycin was studied (Table 3.1 and 3.2). In contrast to published data, which imply that *rpsL* mutations have exclusively positive effect on antibiotic production, we clearly demonstrate two phenomena which differ from generally accepted opinion:

1 – *rpsL* mutations, in general, have different effects on antibiotic production, ranging from negative to positive.

2 – each single *rpsL* mutation has a very different effect on the production of various natural products independently from their nature or used biosynthetic precursors.

Table 3.1 The production levels of heterologously expressed biosynthetic gene clusters by *rpsL*- and *rsmG*-mutant strains, compared to the original *S. albus*.

Mutation / BGC	Δ*rsmG*	K88E	GI92	K88E-GI92	K88E-GI92 Δ*rsmG*	P91S	K88E-P91S	R86P	R94G	R94G Δ*rsmG*
ara	↑4-fold	↑3,5-fold	↑3,5-fold	↑2,5-fold	↑4-fold	↑2,5-fold	↑2-fold	~	↑3-5-folds	↑5,5-fold
gris	↑2-fold	↑2-fold	↑2-fold	↑3-5-folds	↑6,5-fold	↑2-fold	↑2-fold	↑2-fold	↑2-fold	↑3-fold
cin	↑4-fold	↑2-fold	↑1,5-fold	↑1,5-fold	**↑7-fold**	↑3,5-fold	↑3-fold	↑3-fold	↑3-fold	**↑9-fold**
tun	↑2-fold	↓20 %	↓60 %	↓70 %	↓50 %	~	~	~	~	~
pam	~	~	~	~	~	~	~	~	~	~
moe	~	~	~	~	~	~	~	~	~	~

Ara stands for aranciamycin, gris – griseorhodin, cin – cinnamycin, tun – tunicamycin, pam – pamamycin, moe – moenomycin. ~ - the production level of the mutant strain does not differ from the original *S. albus.*

For instance, K88E mutation increased production of aranciamycin, griseorhodin, and cinnamycin, while showing a negative effect on tunicamycin and desferrioxamine production and no effect on pamamycin, moenomycin, candicidin and antimycin production. GI92 mutation instead, exerted a positive effect on the production of aranciamycin, griseorhodin, cinnamycin, candicidin, antimycin, antimycic acid, and paulomycin. Production of pamamycin and moenomycin were not affected by the mutation, while the production of desferrioxamine was reduced. The highest overproduction effects observed with single *rpsL* mutations were 8-fold increase in production of antimycic acid and 3,5-fold increase of aranciamycin in case of GI92 mutation, 3- to 5-fold increase of aranciamycin production in case of R94G mutation, and 3,5-fold increase in production of cinnamycin in case of P91S mutation. Interestingly, the production of pamamycins and moenomycins was not affected by the introduced *rpsL* mutations.

Table 3.2 The production levels of indigenous biosynthetic gene clusters by *rpsL*- and *rsmG*-mutant strains, compared to the original *S. albus*.

Mutation / BGC	Δ*rsmG*	K88E	GI92	K88E-GI92	K88E-GI92 Δ*rsmG*	P91S	K88E-P91S	K88E-P91S Δ*rsmG*
ant	↓2-fold	↓5-fold	↓3-fold	↓20-fold	not detected	↓3-fold	↓5-fold	↓4-fold
cnd	↓25 %	↓4-fold	↓2-fold	↓4-fold	↓5-fold	↓3-fold	↓3-fold	↓25 %
ant. acid	not detected	first detected	↑4-fold *	↑8-fold *	↑13-fold *	↓25 % *	↑40 % *	not detected
plm	not detected	↓40 %	↑2,5-fold	↑40 %	↑35 %	↑30 %	↑15 %	not detected
desf	↓20 %	not detected	not detected	↓25-fold	↓50-fold	↓50-fold	↓20-fold	↓46 %

Ant stands for antimycin, cnd – candicidin, ant. acid – antimycic acid, plm – paulomycin, desf – desferrioxamine.*- the production level of antimycic acid was compared to *S. albus* K88E strain, as in the wild type it was not detected.

Mostly only a moderate positive effect on antibiotic production was described after the introduction of *rpsL* mutations [10]. However, the introduction of several *rpsL* mutations or combination of them with the deletion of the *rsmG* gene were reported to have synergistic effect on the production of natural products [5, 11]. To study how combinations of the *rpsL* mutations alone and with the deletion of *rsmG* gene affect the production of various antibiotics under the same genetic background, the following mutant strains were constructed: K88E-GI92, K88E-P91S, K88E-GI92-Δ*rsmG*, K88E-P91S-Δ*rsmG,* R94G-Δ*rsmG,* and Δ*rsmG*. This choice of the mutation combinations have been made due to a number of reasons. The K88E mutation is the best studied *rpsL* mutation, which led to the moderate antibiotic overproduction in our experiments. The K88E mutation was therefore combined with the mutations GI92 and P91S, which also positively affected antibiotic production. To investigate the effect of the *rsmG* gene deletion on the antibiotic production in the background of *rpsL* mutations, the strains K88E-GI92-Δ*rsmG,* K88E-P91S-Δ*rsmG,* and R94G-Δ*rsmG* were constructed. As a control, the *rsmG* gene was deleted separately in *S. albus* strain.

As evident from our results combination of separate *rpsL* mutations proved to have an unpredictable effect on antibiotic production, as the single *rpsL* mutations do. When compared to single mutations, the combination of K88E with GI92 reduced production of aranciamycin, did not affect the production of cinnamycin, further decreased production of tunicamycin and increased production of griseorhodin. A similar effect also showed a combination of mutations, K88E and P91S. An interesting effect showed the deletion of the *rsmG* gene and the combination of this deletion with other *rpsL* mutations. Deletion of the *rsmG* gene alone caused a 4-fold increase in the production of aranciamycin and cinnamycin, a 2-fold increase of griseorhodin and tunicamycin production, and completely abolished the paulomycin production. High overproduction rates caused by *rsmG* deletion were further improved by combination with *rpsL* mutations. For instance, a 9-fold and 7-fold increase of cinnamycin production was observed in R94G-Δ*rsmG* mutant and K88E-GI92-Δ*rsmG* mutant, respectively. Therefore our results indicate that a combination of *rpsL* mutations with the deletion of *rsmG* gene has a much better synergistic effect on antibiotic production than a combination of *rpsL* mutations alone.

The effect of *rpsL* and *rsmG* mutations on the transcriptome in *Streptomyces*

Understanding of how *rpsL* and *rsmG* mutations stimulate antibiotic production in *Streptomyces* is important for the rational design of overproducer strains. Several hypotheses were developed to explain the effect of ribosome engineering on the physiology of the strain. According to one of them, the *rpsL* mutations confer ribosomes higher stability, especially at the late growth phases [6, 12]. Since the onset of antibiotic production often takes place in the late stationary phase, increased translational activity at late growth phases could explain the positive effect of *rpsL* mutations on antibiotic production. The increased expression of a ribosomal recycling factor was also reported for *rpsL* mutants [13]. This “ribosome-centered” hypothesis would mean that the productivity of the strains is stimulated at the translational level. This hypothesis, however, cannot explain the increased transcription levels of the pathway-specific regulatory gene *actII-ORF4* observed in *rpsL* mutants of *S. coelicolor,* which overproduce actinorhodin [14]. The product of *actII-ORF4* activates transcription of the actinorhodin biosynthetic genes. Activation of silent gene clusters triggered by different *rpsL* mutations also implies the involvement of transcriptional regulation in the formation of *rpsL*-phenotype. The effect of *rpsL* mutations on the transcriptional state of *Streptomyces* cells is poorly studied. It was hypothesized that the mutations in the *rpsL* gene mimic the stringent response, which usually is

triggered as cell answers to nutrient starvation. During stringent response, the intracellular concentration of the alarmone guanosine-3',5'-tetraphosphate (ppGpp) rises through the action of ppGpp synthase encoded by the gene *relA* [15, 16]. In *E. coli* cells, this alarmone was reported to bind to RNA-polymerase and to change its affinity to transcriptional promoters [17, 18]. However, in contrast to *E. coli*, Gram-positive bacteria and members of the genus *Streptomyces,* in particular, lack the ppGpp-binding site in an RNA-polymerase. Therefore, it is difficult to explain the upregulation of antibiotic biosynthesis in *rpsL*-mutants of *Streptomyces* through the action of the alarmone ppGpp. It was proposed that increased activity of ppGpp synthase RelA, reduces the intracellular concentration of GTP, what in turn downregulates transcription of the genes with guanine as transcriptional start nucleotide [19]. Since the transcriptional start points for *Streptomyces* genes are not determined, and the impact of different *rpsL* mutations on transcription in *Streptomyces* was not studied, it is impossible to prove this hypothesis.

To further our understanding of how ribosome engineering (introduction of *rpsL* mutations and deletion of *rsmG* gene) influences the gene transcription in *Streptomyces* transcriptome sequencing of the following strains was performed: *S. albus*, *S. albus* K88E, *S. albus* GI92, *S. albus* K88E/GI92, *S. albus* P91S, *S. albus* P91S/K88E, *S. albus ΔrsmG*, *S. albus* K88E/GI92/*ΔrsmG*, *S. albus* P91S/K88E/*ΔrsmG, S. albus* ara*, S. albus* K88E/GI92 ara, *S. albus* R94G ara, *S. albus* R86P ara. Our results demonstrate for the first time profound impact of ribosome engineering on the whole transcriptome of streptomycetes. More than 50% of the genes of *S. albus* changed their transcriptional level in consequence of *rpsL* mutations or *rsmG* deletion. These substantial transcriptome changes of *S. albus* mutants imply that the whole regulatory network of the strain is affected by *rpsL* and *rsmG* mutations. Indeed, numerous genes encoding sigma factors and regulatory proteins altered their transcription levels as a result of introduced mutations. More than 70% of all genes encoding for transcriptional sigma factors, which determine promotor specificity of RNA polymerase, altered their transcription more than twofold in mutant strains. Our results provide clear evidence that despite the lack of ppGpp binding site in the RNA polymerase of streptomycetes, *rpsL* and *rsmG* mutations elicit physiological changes through altered gene transcription. Our data do not refute the hypothesis that the enhanced stability of ribosomes harboring *rpsL* mutations plays a role in the formation of mutant phenotype. In the context of antibiotic production, the results of transcriptome sequencing data demonstrate that transcription of 23 from 26 *S. albus* indigenous secondary metabolite clusters were affected by *rpsL* and *rsmG*

mutations. In contrast to the mostly positive effects of ribosome engineering published in the literature, our results demonstrate both positive and negative effects of *rpsL* and *rsmG* mutations on cluster expression. Interestingly, the antibiotic production levels in *rpsL* mutants linearly correlated with the transcriptional levels of corresponding clusters. This phenomenon was detected in the case of paulomycin, desferrioxamine, and aranciamycin clusters. The production of candicidin and antimycin did not correlate well with the transcriptional activity of their biosynthetic clusters. This is likely an exception, since the phosphopantetheinyl transferase gene that is necessary for the biosynthesis of candicidin and antimycin is located within the paulomycin cluster which is regulated in the opposite way to the candicidin and antimycin clusters. The upregulated transcription of candicidin and antimycin was accompanied by downregulated transcription of paulomycin gene cluster and of phosphopantetheinyl transferase gene what prevented enhanced production levels of candicidin and antimycin. Linear correlation between cluster expression and antibiotic production in *rpsL* and *rsmG* mutants raises the question to which extent the enhanced stability of mutated ribosomes contributes to antibiotic overproduction. Altogether our results indicate that the extent of physiological changes *rpsL* and *rsmG* mutation elicit in *Streptomyces* is far broader than was expected before and the existing ribosome- and RNA-polymerase-centered hypotheses have to be improved in order to explain these changes.

rpsL and _rsmG_ mutations in the construction of _Streptomyces_ hosts for heterologous expression of biosynthetic gene clusters

Heterologous host strains play an important role in the discovery of new natural products. Their development and utilization allows changing the drug discovery strategy from old-fashioned screening of complex culture extracts containing numerous bioactive compounds minute amounts to up-to-date targeted expression of unique biosynthetic gene clusters in strains with optimized production and simplified metabolic background. Ideally, heterologous host strains should be easy to modify genetically, should not produce indigenous secondary metabolites and should provide high expression rates of heterologous clusters and high production yields. Since *rpsL* and *rsmG* mutations often have a positive effect on antibiotic production, they are regarded as a valuable tool during the construction of optimized heterologous host strains. In this work, we studied the effect of different *rpsL* mutations and *rsmG* gene deletion on antibiotic production regarding their potential use in the construction of *Streptomyces* heterologous host strains. The effect of

ribosome engineering on the production of various natural products was studied in the same strain – *S. albus*, which is widely used for heterologous expression of biosynthetic gene clusters. Our results demonstrate that ribosome engineering influences the production of both indigenously encoded and heterologously expressed natural products. In this regard, the introduction of the *rpsL* mutations and the deletion of the *rsmG* gene can be applied for the development of chassis strains in order to increase their production yields. Of particular interest is the fact that ribosome engineering showed both, positive and negative effect on antibiotic production in *S. albus*. This contradicts the widely accepted opinion that ribosome engineering mostly enhances production yields in streptomycetes. Among numerous studied mutations, there was none which influenced antibiotic production in the same manner. Every single mutation elicited a positive effect on the production of one to several compounds while inhibiting the production of the other compounds. No correlation between the type of mutation elicited effect and nature of the compound could be detected. Such an unpredicted manner of how *rpsL* and *rsmG* mutations affect the production of natural products complicates their utilization during the heterologous host construction. Improvement of the production yields of various compounds cannot be achieved by the introduction of single *rpsL* or *rsmG* mutation or even by their combinations. The effect of various mutations on the production of a particular compound has to be checked in order to improve its yields. In order to unfold the potential of ribosome engineering for the improvement of the antibiotic production yields, construction of the panel of heterologous strains with different single *rpsL* and *rsmG* mutation seems to be necessary.

S. albus J1074 as a host for derivatization of ribosomally synthesized and post-translationally modified peptides (RiPPs)

The class of RiPPs is a relatively new group of NPs with high structural diversity and promising bioactivities. The RiPPs are produced through a short ribosomally synthesized precursor peptide. The precursor peptide usually consists of an N-terminal leader peptide and C-terminal core peptide. The core peptide is converted into the final molecule in the course of numerous post-translational modifications, while the leader peptide is removed. The leader peptide plays an essential role during the RiPP maturation. It serves as a substrate-recognition sequence for modification enzymes.

The RiPPs possess a tremendous chemical diversity which is reflected in the broad range of their biological activities. Nevertheless, the biosynthesis of RiPPs is restricted to proteinogenic amino acids. The non-proteinogenic amino acid residues present in the RiPP structures are generated by enzymatic post-translational modifications of proteinogenic residues [20, 21]. Direct incorporation (during translation) of non-canonical amino acids into the pre-peptide of RiPPs is of great interest, as it will not only generate compounds with altered activities but can also contribute to the identification of their biological targets. Two methods for the direct incorporation of non-canonical amino acids into the pre-peptide of RiPPs have been described [22]. One of the methods relies on the construction of a strain auxotrophic for a certain amino acid. The constructed auxotrophic strain is then cultivated in a minimal media supplemented with structural analogs of the amino acid, for which the strain is auxotrophic. Under these conditions, the codons, encoding for amino acid the strain is auxotrophic for, are mistranslated with incorporation of unnatural analogs [22]. The most important limitations of this method are low production yield usually observed in minimal media and inability to incorporate structurally unrelated amino acids. This approach for successfully used for incorporation of unnatural amino acids into lantibiotic lichenicidin [23] and lasso peptide capistruin [22] in *E. coli*.

The second approach for the introduction of non-canonical amino acids into RiPPs relies on the suppression of the stop-codon by the orthogonal suppressor tRNA (s-tRNA) and cognate aminoacyl-tRNA synthetase (aaRS) that charges the tRNA [22]. The genes from *Methanosarcina* sp. encoding aaRS and s-tRNA for the translation of amber stop-codon as pyrrolysine and the genes from *Methanococcus* sp. for incorporation of tyrosine are most widely used for the incorporation of non-canonical amino acids into RiPPs. The suppression of stop-codon was successfully used for the incorporation of non-canonical amino acids into the RiPPs capistruin, lacticin and nisin in *E. coli*, and into thiocillin in *Bacillus cereus* [22, 24, 25]. No system for the incorporation of non-canonical amino acids into RiPPs was adapted for streptomycetes. As evident from the genome sequencing studies the genomes of streptomycetes encode numerous biosynthetic clusters for RiPPs. Due to the high GC-content of *Streptomyces* DNA and complex regulatory mechanisms controlling the production of RiPPs, expression of RiPP clusters in other organisms like *E. coli* or *Bacillus* sp. is still challenging. The introduction of non-canonical amino acids into streptomycetal RiPPs can, therefore, be performed almost exclusively in their natural producers – streptomycetes.

In this work, we report the utilization of the system for the incorporation of unnatural amino acids into RiPPs in particular for *Streptomyces* species. The system is based on the codon-optimized gene of *Methanosarcina barkeri* encoding pyrrolysine aminoacyl-tRNA synthetase and the cognate s-tRNA gene [26]. These genes suppress the amber stop-codon with the incorporation of pyrrolysine residue into the peptide chain. The codon suppression experiments were performed in *S. albus* strain which is preferably used for heterologous expression of the biosynthetic gene clusters. The lantibiotic cinnamycin, which belongs to the family of RiPPs, was used for incorporation of non-natural amino acids. The cinnamycin gene cluster was successfully cloned from the natural producer using TAR-assembly in yeasts. Two positions in the structure of cinnamycin have been chosen for the incorporation studies: 2 and 10, which encode Arginine (Arg) and Phenylalanine (Phe) respectively. For this purpose, the amber stop codon was introduced into the respective positions of the pre-peptide gene within the cinnamycin gene cluster. The constructed cinnamycin clusters with stop codon in the 2^{nd} and the 10^{th} position were co-expressed separately with pyrrolysine aminoacyl-tRNA synthetase and s-tRNA in *S. albus*. When supplemented with pyrrolysine or its analogs, the amber stop-codon within the precursor peptide gene should be suppressed, and the derivatives of cinnamycin with non-canonical amino acids should be produced. Due to the absence of pyrrolysine on the market, its commercially available analogs were used for feeding purposes: H-Lys-Alloc–OH (Alloc), H-Lys-Cyc–OH (Cyc) and H-Lys-Boc–OH (Boc). The feeding with pyrrolysine analogs resulted in the production of cinnamycin derivatives with amino acid substitutions in positions 2 and 10. The structures of the generated cinnamycin derivatives were confirmed by partial acid hydrolysis and tandem mass spectrometry. All of the used pyrrolysine analogs were successfully incorporated in the cinnamycin structure, however with different efficiency. The overall efficiency of incorporation was high enough for purification of one digit milligram quantities of cinnamycin derivatives. The purified cinnamycin derivatives demonstrated different activity in biological studies. For instance, substitution of the Phe by Alloc in the 10^{th} position decreased the activity against *B. subtilis* by 2-fold while the substitution of Arg by Alloc in the 2^{nd} position improved the activity twice.

The successful development of the system for the incorporation of non-canonical amino acids into the RiPP antibiotics, presented in this study, allows generation of antibiotic derivatives with altered properties. Through directed introduction of an amber stop-codon into the precursor peptide gene, the developed system allows site-specific introduction of several pyrrolysine analogs

into the structure of antibiotics. In this way, the biological activity, stability, bioavailability, etc., of the parental compound can be substantially altered. Introduction of Alloc amino acid residues can be utilized for the further modification of the compounds through their bio-orthogonal conjugation with allyl-alcohol [22]. Changing the substrate specificity of the original aminoacyl-tRNA synthetase from pyrrolysine towards the amino acids with functional groups for click chemistry reactions will significantly facilitate the identification of RiPPs biological targets.

References:

1. Shima, J., et al., *Induction of actinorhodin production by rpsL (encoding ribosomal protein S12) mutations that confer streptomycin resistance in Streptomyces lividans and Streptomyces coelicolor A3(2).* Journal of Bacteriology, 1996. **178**(24): p. 7276-7284.
2. Hosoya, Y., et al., *Acquisition of certain streptomycin-resistant (str) mutations enhances antibiotic production in bacteria.* Antimicrobial Agents and Chemotherapy, 1998. **42**(8): p. 2041-2047.
3. Hesketh, A. and K. Ochi, *A novel method for improving Streptomyces coelicolor A3(2) for production of actinorhodin by introduction of rpsL (Encoding ribosomal protein S12) mutations conferring resistance to streptomycin.* Journal of Antibiotics, 1997. **50**(6): p. 532-535.
4. Nishimura, K., et al., *Mutations in rsmG, encoding a 16S rRNA methyltransferase, result in low-level streptomycin resistance and antibiotic overproduction in Streptomyces coelicolor A3(2).* Journal of Bacteriology, 2007. **189**(10): p. 3876-3883.
5. Wang, G., et al., *A Novel Insertion Mutation in Streptomyces coelicolor Ribosomal S12 Protein Results in Paromomycin Resistance and Antibiotic Overproduction.* Antimicrobial Agents and Chemotherapy, 2009. **53**(3): p. 1019-1026.
6. Okamoto-Hosoya, Y., T. Hosaka, and K. Ochi, *An aberrant protein synthesis activity is linked with antibiotic overproduction in rpsL mutants of Streptomyces coelicolor A3(2).* Microbiology-Sgm, 2003. **149**: p. 3299-3309.
7. Okamoto-Hosoya, Y., S. Okamoto, and K. Ochi, *Development of antibiotic-overproducing strains by site-directed mutagenesis of the rpsL gene in Streptomyces lividans.* Applied and Environmental Microbiology, 2003. **69**(7): p. 4256-4259.
8. Wang, G.J., et al., *Identification of a Novel Lincomycin Resistance Mutation Associated with Activation of Antibiotic Production in Streptomyces coelicolor A3(2).* Antimicrobial Agents and Chemotherapy, 2017. **61**(2).
9. Tamehiro, N., et al., *Innovative approach for improvement of an antibiotic- overproducing industrial strain of Streptomyces albus.* Applied and Environmental Microbiology, 2003. **69**(11): p. 6412-6417.
10. Baltz, R.H., *Strain improvement in actinomycetes in the postgenomic era.* J Ind Microbiol Biotechnol, 2011. **38**(6): p. 657-666.
11. Tanaka, Y., et al., *Antibiotic Overproduction by rpsL and rsmG Mutants of Various Actinomycetes.* Applied and Environmental Microbiology, 2009. **75**(14): p. 4919-4922.
12. Hosaka, T., et al., *The novel mutation K87E in ribosomal protein S12 enhances protein synthesis activity during the late growth phase in Escherichia coli.* Molecular Genetics and Genomics, 2004. **271**(3): p. 317-324.
13. Hosaka, T., J. Xu, and K. Ochi, *Increased expression of ribosome recycling factor is responsible for the enhanced protein synthesis during the late growth phase in an antibiotic-overproducing Streptomyces coelicolor ribosomal rpsL mutant.* Molecular Microbiology, 2006. **61**(4): p. 883-897.
14. Wang, G.J., T. Hosaka, and K. Ochi, *Dramatic activation of antibiotic production in Streptomyces coelicolor by cumulative drug resistance mutations.* Applied and Environmental Microbiology, 2008. **74**(9): p. 2834-2840.

15. Ochi, K., *From microbial differentiation to ribosome engineering.* Bioscience Biotechnology and Biochemistry, 2007. **71**(6): p. 1373-1386.
16. Hesketh, A., et al., *The global role of ppGpp synthesis in morphological differentiation and antibiotic production in Streptomyces coelicolor A3(2).* Genome Biol, 2007. **8**(8).
17. Doniselli, N., et al., *New insights into the regulatory mechanisms of ppGpp and DksA on Escherichia coli RNA polymerase-promoter complex.* Nucleic Acids Research, 2015. **43**(10): p. 5249-5262.
18. Ross, W., et al., *ppGpp Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on Transcription Initiation during the Stringent Response.* Molecular Cell, 2016. **62**(6): p. 811-823.
19. Krasny, L. and R.L. Gourse, *An alternative strategy for bacterial ribosome synthesis: Bacillus subtilis rRNA transcription regulation.* Embo Journal, 2004. **23**(22): p. 4473-4483.
20. Budisa, N., *Expanded genetic code for the engineering of ribosomally synthetized and post-translationally modified peptide natural products (RiPPs).* Current Opinion in Biotechnology, 2013. **24**(4): p. 591-598.
21. Goto, Y. and H. Suga, *Engineering of RiPP pathways for the production of artificial peptides bearing various non-proteinogenic structures.* Current Opinion in Chemical Biology, 2018. **46**: p. 82-90.
22. Al Toma, R.S., et al., *Site-Directed and Global Incorporation of Orthogonal and Isostructural Noncanonical Amino Acids into the Ribosomal Lasso Peptide Capistruin.* Chembiochem, 2015. **16**(3): p. 503-509.
23. Oldach, F., et al., *Congeneric Lantibiotics from Ribosomal In Vivo Peptide Synthesis with Noncanonical Amino Acids.* Angewandte Chemie-International Edition, 2012. **51**(2): p. 415-418.
24. Kakkar, N., et al., *Incorporation of Nonproteinogenic Amino Acids in Class I and II Lantibiotics.* Acs Chemical Biology, 2018. **13**(4): p. 951-957.
25. Luo, X.Z., et al., *Recombinant thiopeptides containing noncanonical amino acids.* Proc Natl Acad Sci U S A, 2016. **113**(13): p. 3615-3620.
26. Myronovskyi, M., et al., *beta-Glucuronidase as a Sensitive and Versatile Reporter in Actinomycetes.* Applied and Environmental Microbiology, 2011. **77**(15): p. 5370-5383.

4. Appendix. Unpublished results

I

Rational ribosome engineering to improve secondary metabolite production

1. Results

1.1. Heterologous expression of antibiotic biosynthetic gene clusters in S. albus mutant strains

To analyze the influence of *rpsL* mutations on the heterologous production levels of different classes of antibiotics, we chose to express several different gene clusters in *S. albus* strains carrying these mutations (Table 1). We compared the production levels of aranciamycin and griseorhodin (PKS 2nd type BGCs (Luzhetskyy et al., 2007) and (Li and Piel, 2002)), pamamycin (Rebets et al., 2015), cinnamycin (a ribosomally synthesized and post-translationally modified peptide (RiPP) (Lopatniuk et al., 2017)), moenomycin (a phosphoglycolipid (Ostash and Walker, 2010)) and nucleoside antibiotic tunicamycin (Wyszynski et al., 2012)) in *S. albus rpsL* mutants. To ensure reproducibility, we performed three biological replicates and two technical replicates in the expression experiments.

The production of aranciamycin was higher in almost all mutated strains, except for R86P (Figure 1). The highest production level was more than 5-fold higher than that in the reference strain and was achieved in the R94G mutation strain. An interesting effect was observed in strains with combinations of two different rpsL mutations, including K88E, which had GI92 (KE-GI), and K88E, which had P91S (KE-PS). Separately, each of those mutations increased aranciamycin production, while strains with combinations (KE-GI and KE-PS) exhibited lower production.

Despite the similar biosynthetic origin of aranciamycin and griseorhodin, the effect of the same mutations on the production level of these two compounds was substantially different. The production of griseorhodin was approximately 2-fold higher for all mutations but was more than 3-fold higher in double KE-GI mutant.

Table 1. Descriptions of the strains used in this study.

Name of the strain	Description
Streptomyces albus ΔpseB4	Initial strain (Bilyk and Luzhetskyy, 2014)
S. albus ΔrsmG	*S. albus ΔpseB4* strain with the *rsmG* gene knocked out
S. albus K88E	*S. albus ΔpseB4* strain with a K88E mutation in the *rpsL* gene
S. albus K88E ΔrsmG	*S. albus ΔpseB4* strain with a K88E mutation in the *rpsL* gene and the *rsmG* gene knocked out
S. albus GI92	*S. albus ΔpseB4* strain with a GI92 mutation in the *rpsL* gene
S. albus GI92 ΔrsmG	*S. albus ΔpseB4* strain with a GI92 mutation in the *rpsL* gene and the *rsmG* gene knocked out
S. albus K88E-GI92	*S. albus ΔpseB4* strain with K88E and GI92 mutations in the *rpsL* gene
S. albus K88E-GI92 ΔrsmG	*S. albus ΔpseB4* strain with K88E and GI92 mutations in the *rpsL* gene and the *rsmG* gene knocked out
S. albus P91S	*S. albus ΔpseB4* strain with a P91S mutation in the *rpsL* gene
S. albus P91S ΔrsmG	*S. albus ΔpseB4* strain with a P91S mutation in the *rpsL* gene and the *rsmG* gene knocked out
S. albus K88E-P91S	*S. albus ΔpseB4* strain with K88E and P91S mutations in the *rpsL* gene
S. albus K88E-P91S ΔrsmG	*S. albus ΔpseB4* strain with K88E and P91S mutations in the *rpsL* gene and the *rsmG* gene knocked out
S. albus R86P	*S. albus ΔpseB4* strain with an R86P mutation in the *rpsL* gene
S. albus R86P ΔrsmG	*S. albus ΔpseB4* strain with an R86P mutation in the *rpsL* gene and the *rsmG* gene knocked out
S. albus R94G	*S. albus ΔpseB4* strain with an R94G mutation in the *rpsL* gene
S. albus R94G ΔrsmG	*S. albus ΔpseB4* strain with an R94G mutation in the *rpsL* gene and the *rsmG* gene knocked out

Tunicamycin production was the highest in the *S. albus* strain with the P91S mutation, while the K88E and GI92 mutations decreased the production of this antibiotic, and a combination consisting of these mutations produced an even more detrimental effect (Figure 1). The other three mutations did not influence its production.

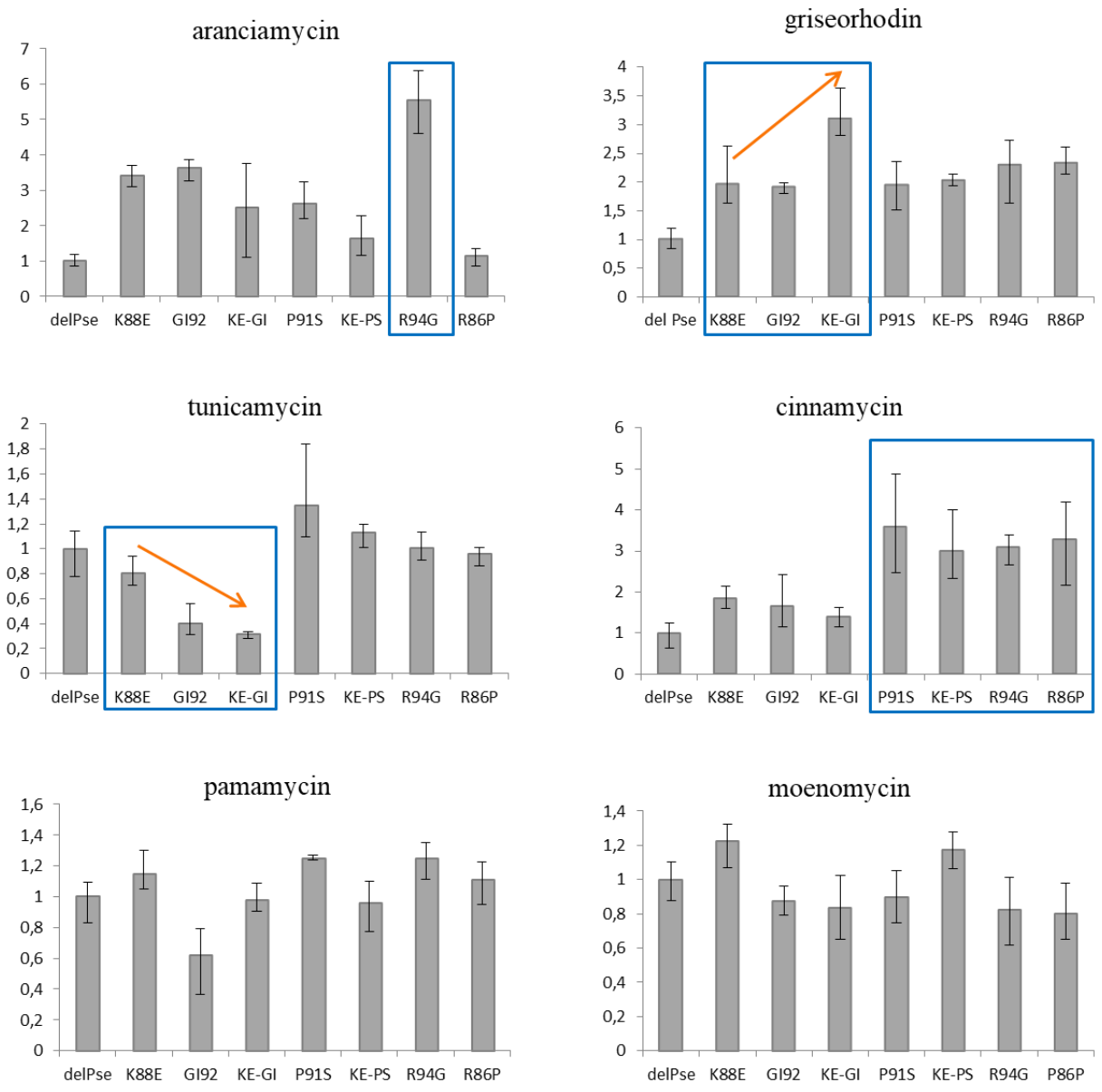


Figure 1. The level of production of antibiotics in the *S. albus delPse* and *rpsL* mutant strains. The production level in the original strain was defined as 1.

For cinnamycin, the P91S, K88E-P91S, R94G, and R86P mutations increased production by 3-4-fold. The K88E and GI92 mutations individually slightly increased cinnamycin production, but when combined, they decreased the cinnamycin yield to its original production level.

The mutations had almost no effect on the production of pamamycin or moenomycin.

1.2. Transcriptome analysis of S. albus mutant strains with the aranciamycin BGC.

Considering the significant influence of *rpsL* and *rsmG* mutations on the transcription of indigenous BGCs in *S. albus*, we wanted to check if this is valid for heterologous BGCs as well. To explore this issue, a transcriptome analysis was performed for three mutant strains and an original strain carrying aranciamycin BGC. Samples were collected on the 2nd and 3rd days of growth. The level of transcription of each gene in the aranciamycin BGC in the reference strain was defined as 1. On the second day of growth, the transcription levels of all genes but ORF 20 (a transporter gene) in the clusters in the mutant strains were lower than that observed in the original strain (Figure 2). On the third day of growth, the transcription of most of the genes in the aranciamycin BGC was increased by up to 3- to 4-fold in the mutant *S. albus R94G/ara* strain, which also produced the highest level of aranciamycin. In the *S. albus* strain *K88E-GI92/ara*, the transcription levels of only four genes (minimal PKS and aromatase) were upregulated, and the transcription of the remaining genes was lower or the same as that of the original strain. The lowest transcription level observed for the aranciamycin biosynthetic genes was in the *S. albus R86P*/*ara* strain, in which it was up to 3-4-folds lower than was observed in the *S. albus delPse/ara* strain (Figure 2). These transcriptome results partially explain the aranciamycin production levels. The increase in the production level that we observed in *S. albus R94G/ara* was, to a certain degree, due to an increase in the transcription of aranciamycin BGC, but this does not fully explain the observed changes. For example, this mechanism does not explain why the *S. albus R86P/ara* mutant produced a slightly higher amount of antibiotic than was produced by the *S. albus delPse/ara* strain or why the transcription of the aranciamycin BGC was lower in this strain. Our data are in agreement with the expectation that the mutations explored in this study also affect translation.

1.3. Heterologous expression of antibiotic BGCs in S. albus rpsL-ΔrsmG double-mutant strains

The R94G mutation and the KE-GI combination had the most pronounced effect on the level of antibiotic production. Therefore, strains that carried these mutations in combination with *rsmG* deletion were chosen to investigate antibiotic production. All of the vectors with BGCs that are mentioned in section 3.6 were transferred to three *S. albus* strains: *S. albus*

ΔrsmG, S. albus K88E-GI92 ΔrsmG, and *S. albus R94G ΔrsmG.* The level of production of the corresponding secondary metabolites was then measured.

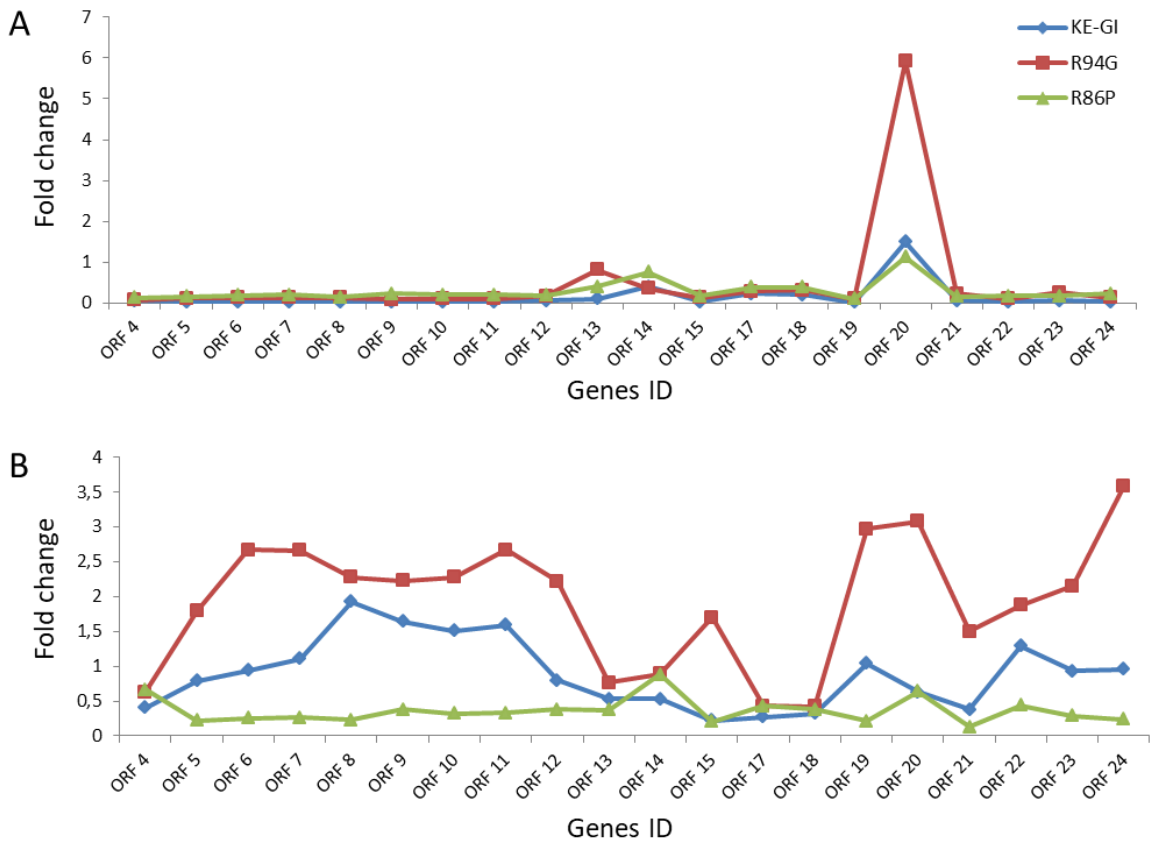


Figure 2. Comparison of the transcription levels of aranciamycin BGC genes in *S. albus delPse/ara, S. albus K88E-GI92/ara, S. albus R94G/ara* and *S. albus R86P/ara.* The transcription level in the original strain was defined as 1.

In the case of aranciamycin, the production level observed in the strain with the *rsmG* deletion was 4-fold higher, but when *rsmG* deletion was combined with both *rpsL* mutations, production was only 2-fold higher (Figure 3).

Increases in griseorhodin production were not as dramatic as those observed for aranciamycin. There was a 2-fold increase in the *S. albus ΔrsmG* strain and an approximately 30 % increase for the *rpsL*- ΔrsmG* combination. Nevertheless, we found that combining mutations in the *rpsL* gene with *rsmG* deletion increased the production of PKS II Type natural products. In the case of tunicamycin, we observed a 1.7-fold increase in production in the *S. albus ΔrsmG* strain, and the *rsmG* gene deletion also partially restored the negative effect of the KE-GI combination on the level of tunicamycin production. The most notable

impact of *rsmG* deletion was on the production of cinnamycin. We found that cinnamycin production was 2.36-fold higher in the *S. albus ΔrsmG* strain than in the original strain. When combined with *rpsL* mutations, antibiotic production was further increased by 4-fold for the KE/GI mutation and 3.3-fold for the R94G mutation.

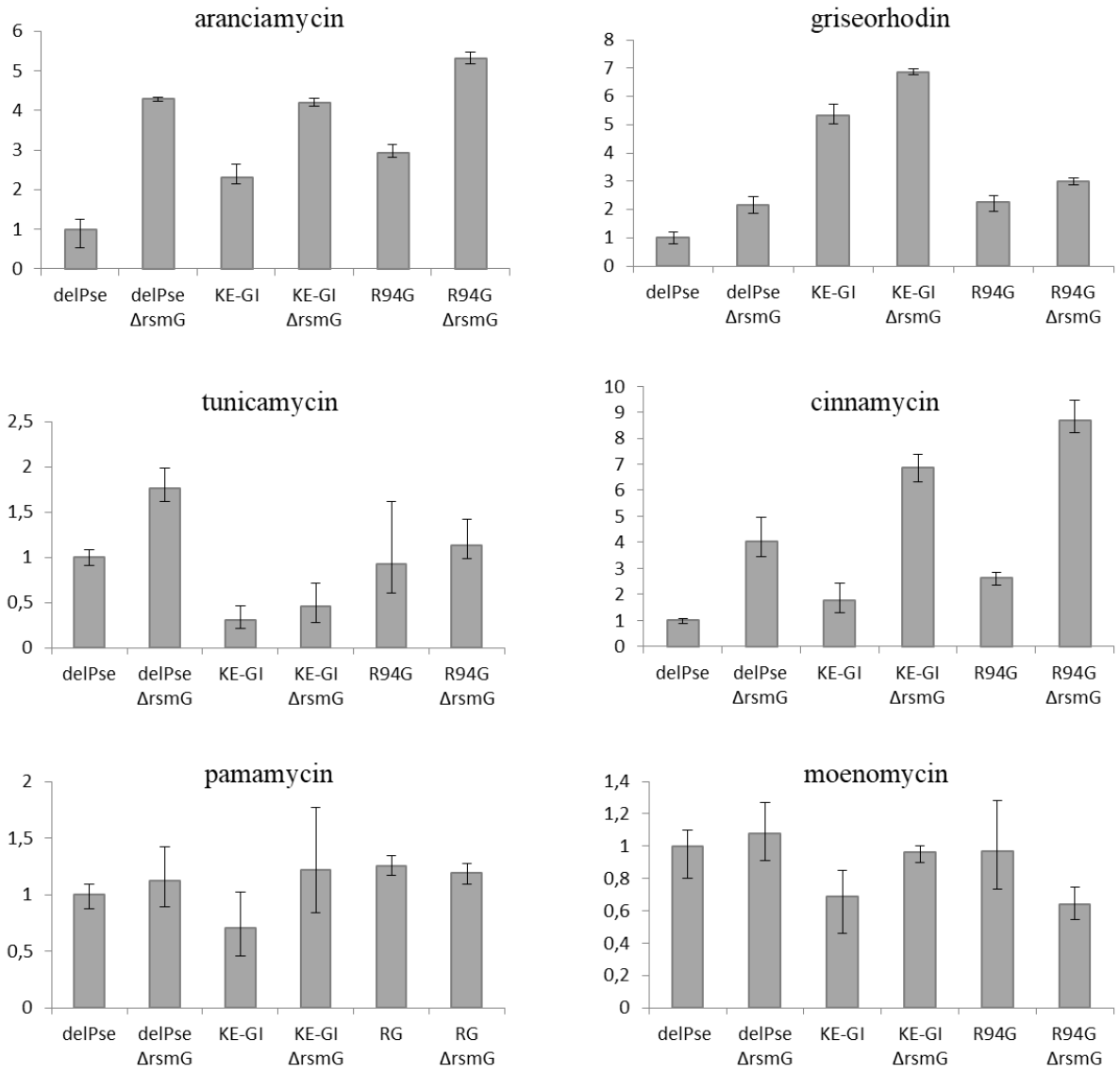


Figure 3. Comparison of antibiotic production levels in *S. albus* strains with *rpsL* mutations and strains with combinations of *rpsL* mutations and *rsmG* deletion. The production level of the original strain was defined as 1.

Similar to the effect of *rpsL* mutations, *rsmG* deletion did not significantly influence the level of production of pamamycin and moenomycin. While we observed slight changes, when we took into account the deviation between biological and technical replicates, these changes were not substantial (Figure 3).

2. Discussion

Several observations have been made after expressing heterologous BGCs in *S. albus rpsL* and/or *rsmG* mutants. First, all mutations have different effects on production levels that depend on the nature of the BGC. Second, the position of the mutation exerts an individual effect on BGC expression (Figure 1). In our study, we chose two different antibiotics in the PKS II Type family, aranciamycin and griseorhodin. In general, all mutations had a positive effect on the production of PKS II-type encoded products, except for the effect of the R86P mutation on aranciamycin. However, the results for both clusters were slightly different. While the R94G mutation had the most significant impact on the production of aranciamycin, inducing a 5- to 6-fold increase, it was a combination of two mutations, K88E and GI92 that produced the highest increase (3-fold) of griseorhodin titers. The same combination K88E-GI92 resulted in a 2.5-fold higher level of production of aranciamycin than was observed in the original strain even though each of these mutations separately produced a better yield (3.5-fold for both). The K88E mutation was previously found to improve the production of actinorhodin in *S. lividans* and *S. coelicolor* (Shima et al., 1996; Wang et al., 2009; Wang et al., 2008), as did the K88E-GI92 combination (Wang et al., 2009). In *S. coelicolor*, the K88E mutation was shown to influence antibiotic production levels by increasing translational activity during the late growth phase (Okamoto-Hosoya et al., 2003; Wang et al., 2009). This increased translational activity included features such as hyper-accuracy in ribosomes, a more stable 70S complex, aberrant protein synthesis and higher expression levels of ribosome recycling factor (RRF) (Hosaka et al., 2006). Yoshiko Okamoto-Hosoya compared actinorhodin production among *S. coelicolor rpsL* mutants carrying mutations such as K88E, P91S, K43N, K43T, K43R and K88R. While all of these mutations led to a hyper-accurate phenotype, only K88E and P91S caused antibiotic overproduction (Okamoto-Hosoya et al., 2003). These results demonstrated that inducing hyper-accuracy in ribosomes does not affect antibiotic overproduction in strains with *rpsL* mutations. The authors, therefore, proposed that aberrant protein synthesis caused by an increase in the stability of the ribosome complex and the enhanced expression of the RRF was responsible for antibiotic overproduction.

In our study, we show that enhanced BGC transcription is a major reason for corresponding natural product overproduction. Transcriptome analysis of strains with the

KE-GI, R94G and R86P mutations showed that compared to the original strain, strains with these mutations exhibited alterations in the transcription levels of the aranciamycin BGC (Figure 2). We observed that in all of the mutants, the level of transcription of the cluster was lower on the second day of growth. However, on the third day, the mutant strain with the KE-GI *rpsL* variant had slightly higher transcription levels of the four genes and minimal PKS and aromatase levels (Figure 2). At the same time, the strain with the R94G mutations exhibited enhanced transcription by 2- to 3-fold of almost all genes in the cluster. The transcription level of the aranciamycin BGC correlated with aranciamycin production, suggesting that the increased transcription of the aranciamycin BGC observed in strains with *rpsL* mutations was a major reason for the enhanced production levels observed in those strains (Figure 1 and 2).

Another antibiotic that was overproduced by the *rpsL* mutant strains was the lanthipeptide cinnamycin (Figure 1). We also observed differences in the effect of various *rpsL* mutations on cinnamycin production levels. A group of four mutants (*rpsL* P91S, KE-PS, R94G and R86P) produced 3- to 4-fold higher levels of cinnamycin, while the K88E and GI92 mutations were less efficient at improving the production of this antibiotic. We also noted that combinations including one of two *rpsL* mutations, KE-GI and KE-PS, did not increase production over that observed for each mutation separately (Figure 1). In addition to the enhanced transcriptional level of BGCs, these results could be due to an increase in the stability of ribosomes, which would increase protein synthesis. Because the pre-peptide molecules and all enzymes required for modifications to RiPPs are ribosomally synthesized, the enhanced ribosomal activity observed in *rpsL* mutant strains increases the pool of pre-peptide molecules and modification enzymes available in the cell and accordingly elevates RiPP production levels. Okamoto-Hosoya et al. (Okamoto-Hosoya et al., 2003) demonstrated that in *S. coelicolor*, the stability of ribosomes with the P91S mutation was slightly higher than in those with the K88E mutation (determined according to the ratio of 70S ribosome particles in the presence of various concentrations of Mg^{2+}). This finding might explain why cinnamycin was produced at higher levels by the *S. albus* strain with the P91S mutation than by the strain with the K88E mutation, but we should not exclude the effect of transcriptional regulation.

The production of three other antibiotics was not significantly increased in the *rpsL* mutant strains. Most of the mutations did not influence the production of the antibiotic tunicamycin, and the K88E and GI92 mutations reduced its production to 80 % and 40 %, respectively, of the level observed in the original strain. A ribosomal engineering approach has never been reported to improve the production of nucleoside antibiotics. No substantial changes were observed in the production of pamamycin and moenomycin, and their levels were approximately the same in the mutants as that observed in the original strain. Perhaps, the regulation of the production of these two antibiotics involves processes different from those involved in the regulation of the production of aranciamycin, griseorhodin and cinnamycin. Therefore, the effects of *rpsL* mutations on antibiotic overproduction will not be the same as their effects on all other classes of natural products. The rpsL-mediated mechanisms should be studied in more detail because they affect not only the stability of ribosomes and translation in general but also changes in the transcription patterns observed in the strains, and the consequences of these changes cannot yet be predicted.

The effect of the *rsmG* gene deletion was analyzed both separately and in combination with some of the *rpsL* mutations evaluated here. We found that *rsmG* deletion improved the positive effects of the *rpsL* mutations KE-GI and R94G on the level of production of aranciamycin, griseorhodin and cinnamycin (Figure 3). The deletion itself boosted the production of those antibiotics and surprisingly also had a positive effect on the production of tunicamycin, in which it caused an almost 2-fold increase. We did not notice any substantial changes in the production of pamamycin or moenomycin.

To conclude, *rpsL* mutations and *rsmG* deletion appear to affect both transcription and translation. At the transcriptional level, they change the expression of genes for pleiotropic regulators and sigma factors, leading to significant repercussions within the entire transcription regulatory network. However, they were also shown previously to increase ribosome stability and overall protein synthesis; and our data indicate that altered transcriptional regulation alone cannot explain the effects of *rpsL* mutations on secondary metabolome. Together, these changes in transcriptional regulation and protein synthesis significantly alter BGC expression and very often increase yields of heterologous natural products. Therefore, the generated panel of *S. albus rpsL* and *rsmG* mutants can be widely used as superior hosts for the improved expression of heterologous BGCs. The optimized

production of natural products in heterologous hosts will facilitate their supply for pharmacodynamics and pharmacokinetic studies and thus positively influence the entire drug discovery and development chain.

Acknowledgments

We thank Prof. Mervyn Bibb for providing the tunicamycin biosynthetic gene cluster, Prof. Jörn Piel, for providing the griseorhodin biosynthetic gene cluster, Prof. Andreas Bechthold for providing the aranciamycin gene clusters.

References

Bilyk, B., Luzhetskyy, A., 2014. Unusual site-specific DNA integration into the highly active pseudo-attB of the Streptomyces albus J1074 genome. Applied Microbiology and Biotechnology. 98**,** 5095-5104.

Hosaka, T., et al., 2006. Increased expression of ribosome recycling factor is responsible for the enhanced protein synthesis during the late growth phase in an antibiotic-overproducing Streptomyces coelicolor ribosomal rpsL mutant. Molecular Microbiology. 61**,** 883-897.

Li, A. Y., Piel, J., 2002. A gene cluster from a marine Streptomyces encoding the biosynthesis of the aromatic spiroketal polyketide griseorhodin A. Chemistry & Biology. 9**,** 1017-1026.

Lopatniuk, M., et al., 2017. Streptomyces albus: A New Cell Factory for Non-Canonical Amino Acids Incorporation into Ribosomally Synthesized Natural Products. Acs Chemical Biology. 12**,** 2362-2370.

Luzhetskyy, A., et al., 2007. Cloning and heterologous expression of the aranciamycin biosynthetic gene cluster revealed a new flexible glycosyltransferase. Chembiochem. 8**,** 599-602.

Okamoto-Hosoya, Y., et al., 2003. An aberrant protein synthesis activity is linked with antibiotic overproduction in rpsL mutants of Streptomyces coelicolor A3(2). Microbiology-Sgm. 149**,** 3299-3309.

Ostash, B., Walker, S., 2010. Moenomycin family antibiotics: chemical synthesis, biosynthesis, and biological activity. Natural Product Reports. 27**,** 1594-1617.

Rebets, Y., et al., 2015. Insights into the Pamamycin Biosynthesis. Angewandte Chemie-International Edition. 54**,** 2280-2284.

Shima, J., et al., 1996. Induction of actinorhodin production by rpsL (encoding ribosomal protein S12) mutations that confer streptomycin resistance in Streptomyces lividans and Streptomyces coelicolor A3(2). Journal of Bacteriology. 178**,** 7276-7284.

Wang, G., et al., 2009. A Novel Insertion Mutation in Streptomyces coelicolor Ribosomal S12 Protein Results in Paromomycin Resistance and Antibiotic Overproduction. Antimicrobial Agents and Chemotherapy. 53**,** 1019-1026.

Wang, G. J., et al., 2008. Dramatic activation of antibiotic production in Streptomyces coelicolor by cumulative drug resistance mutations. Applied and Environmental Microbiology. 74**,** 2834-2840.

Wyszynski, F. J., et al., 2012. Biosynthesis of the tunicamycin antibiotics proceeds via unique exo-glycal intermediates. Nature Chemistry. 4**,** 539-546.

II
The platform for biosynthetic engineering of RiPPs in *Streptomyces*

1. Introduction

Ribosomally synthesized and post-translationally modified peptides (RiPPs) represent one of the major classes of microbial natural products (Yang and van der Donk 2013; Santos-Aberturas, Chandra et al. 2019). RiPPs possess high structural diversity with a number of non-proteinogenic residues present in their architecture. These features endow them with the variety of biological activities and the great potential for pharmaceutical applications (Goto and Suga 2018). RiPPs are synthesized on ribosomes from 20 available proteinogenic amino acids, and all non-proteinogenic structures are introduced by a range of tailoring enzymes on the post-translational modification step. The ribosomally synthesized precursor peptide consists of two parts: N-terminal leader peptide and C-terminal core peptide. Leader peptide possesses recognition sequences for tailoring enzymes and serves for enzyme recruitment and activation (Goto and Suga 2018). Core peptide, in turn, undergoes a range of structural modifications and, after proteolytic cleavage of the leader peptide, is converted into a mature product. The fact that the amino acid sequence of the core peptide defines the structure of the final molecule provides the opportunity to change the final amino acids composition through simple codon substitutions in the precursor peptide. In drug development, the derivatization of natural products is the bottle-neck; therefore, methods for fast and efficient derivatization are in great need. The biosynthetic plasticity of RiPPs can help to overcome the problem of derivatization.

In the year 2006, a new RiPP, thioviridamide, characterized by five thioamide bonds and potent anticancer activity was described (Hayakawa, Sasaki et al. 2006; Hayakawa, Sasaki et al. 2006). In 2013 the thioviridamide biosynthetic gene cluster (BGC) was published (Izawa, Kawasaki et al. 2013) and since then a number of similar clusters have been identified, and their products have been reported (Izumikawa, Kozone et al. 2015; Frattaruolo, Lacret et al. 2017; Kawahara, Izumikawa et al. 2018). Thioviridamide-like compounds are extensively modified RiPPs, characterized by a presence of thioamide bonds, C-terminal macrocycle, histidine modifications, and permanent positive charge. Thioholgamide A is a thioamidated RiPP produced by *S. malaysiense* that differs from

thioviridamide only by three amino acids substitution and one less thioamide bond. Thioholgamide A possesses higher cytotoxicity against a range of cancer cell lines, compared to thioviridamide (Kjaerulff, Sikandar et al. 2017). Recently the target and proposed mechanism of action for thioviridamide-like compounds were reported (Takase, Kurokawa et al. 2019). The compound binds to the mitochondrial respiratory chain complex V (F_1Fo-ATP synthase), which leads to activation of the integral stress response in the cell via GCN2-ATF4 pathway (Takase, Kurokawa et al. 2019). However, the active site of the thioviridamide, as well as structure-activity relationships is still not revealed.

All reported thioviridamide-like compounds possess different cytotoxicity level, whereas the only difference between them is minor changes in amino acids composition of their core peptides (Frattaruolo, Lacret et al. 2017). Therefore, optimization of the amino acid composition of the compounds might yield even more active compounds. Moreover, the active site of the molecule and the structure-activity relationships may be revealed by changing the amino acid sequence of the core peptide. The directed derivatization of thioviridamide has been recently reported (Kudo, Koiwai et al. 2019). Here authors have compared the core regions of precursor peptides of thioviridamide-like compounds and selected 42 amino acids substitutions for derivatization of thioviridamide. The heterologous expression of the modified biosynthetic clusters in *S. avermitilis* host strain resulted in the successful production of 35 thioviridamide derivatives. The bioactivity assay revealed different levels of cytotoxicity for new compounds.

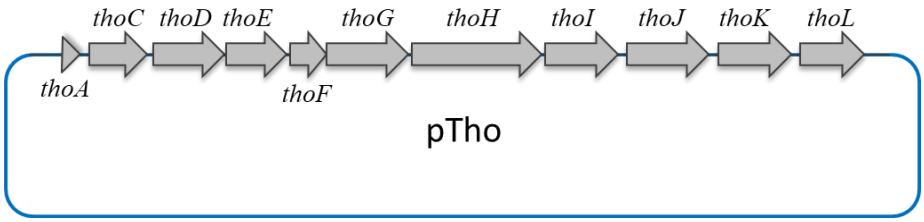
In this work, we aimed to develop a fast and efficient method for derivatization of RiPPs in *Streptomyces* host. The generated library of new thioholgamide derivatives with random amino acids at the preselected positions enables us to access the full chemical space of the compound and to evaluate the sensitivity of tailoring enzymes to amino acid substitutions.

2. Results and discussion

2.1 Cloning of the thioholgamide biosynthetic gene cluster and expression in Streptomyces hosts.

In this work, we intended to generate thioholgamide derivatives via random mutagenesis of different codons of the core region of the thioholgamide precursor peptide

gene. Such genetic manipulations with the precursor peptide in the chromosome of the *S. malaysiense* producer strain are difficult and time-consuming. Thus, we decided to clone the thioholgamide BGC and to perform all genetic manipulations in *E. coli*. The new constructs would be expressed in *Streptomyces* host strains. The thioholgamide BGC was captured directly from the genome of the strain *S. malaysiense* on the yeast/*E. coli*/*Streptomyces* shuttle vector pCLY10 by transformation associated recombination (TAR) in *S. cerevisiae* BY4742. The 1kb regions from the left and right side of the cluster were amplified and cloned into the pCLY10 vector. The mixture of linearized vector and chromosomal DNA of *S. malaysiense* was used to transform yeast spheroplasts. After 5-6 days of growth, the yeast colonies were picked on the selective media. The colony-PCR was used to find the positive yeast clones with the construct. The total DNA from positive colonies with pCLY10 vector containing thioholgamide BGC was isolated and transformed into *E. coli*. The presence of the thioholgamide BGC on the vector was confirmed by restriction mapping, and it was named pTho (Fig. 1).



ThoA – precursor peptide
ThoC – phosphotransferase; self-resistance?
ThoD – unknown;
ThoE – phosphotransferase; self-resistance?
ThoF – oxidative decarboxylase (AviMeCys formation)
ThoG – SAM-dependent histidine methyltransferase
ThoH – thiotransferase; thioamide bonds formation
ThoI – unknown;
ThoJ – histidine hydroxylase
ThoK – cysteine protease
ThoL – transmembrane protein

Figure 1. Tioholgamide BGC on the cloning vector. The putative function of genes from the cluster is described below the vector.

The pTho vector was transferred into four host strains *S. albus* J1074, *S. albus* del14, *S. lividans* TK24, and *S. lividans* del8 via conjugation, to analyze the expression of the

thioholgamide biosynthetic cluster. We observed the highest production level in *S. lividans* del8 chassis strain, with the yield of 30 mg/L (Fig. 2). The mass peak of thioholgamide A was the major peak on the mass chromatogram and the mass of the compound, 1305^{+}, corresponded to the one in the native producer strain *S. malaysiense* (Fig.3a and 3b). This fact, together with fragmentation pattern (Fig.3c), proves that the thioholgamide cluster in *S. lividans* del8:pTho is successfully expressed and the fully modified thioholgamide A is produced.

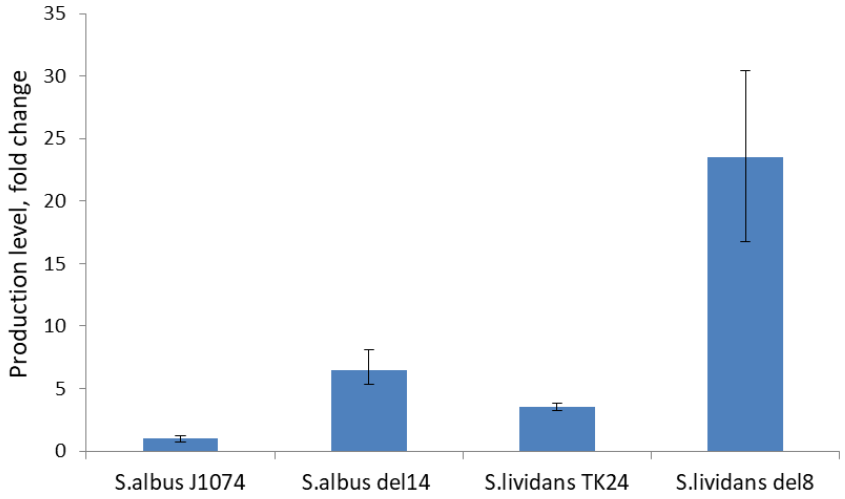


Figure 2. The production level of thioholgamide A in *S. albus* J1074, *S. albus* del14, *S. lividans* TK24, and *S. lividans* del8. The production level in *S. albus* J1074 is considered as 1, and the production level of the rest are compared to J1074.

2.2 Thioholgamide derivatization.

The comparison of the amino acid sequence of core peptide regions of precursor peptides from the BGCs similar to thioholgamide was performed. We could observe that amino acids Thr/Ser at position 1 and 8, and histidine and cysteine in positions 12-13 are very conservative (Fig. 4). Therefore, these four amino acid positions were excluded from randomization experiments. In our work, we aimed to substitute a single chosen amino acid of the core peptide with all possible amino acids. For this purpose, the site-saturation mutagenesis was used (Zheng, Baumann et al. 2004; Nov 2012). This technique provides the possibility to incorporate random amino acids at a certain position of the peptide by using the NNN codon for PCR mutagenesis (where N stands for any nucleotide). As *Streptomyces* have high GC-content in their genomes, the NNS codon (S stands for G or C) was used for

random mutagenesis. The utilizing of NNS codon instead of NNN reduces the library of strains that needs to be checked to identify new thioholgamide derivatives, which greatly simplify the search and makes it faster.

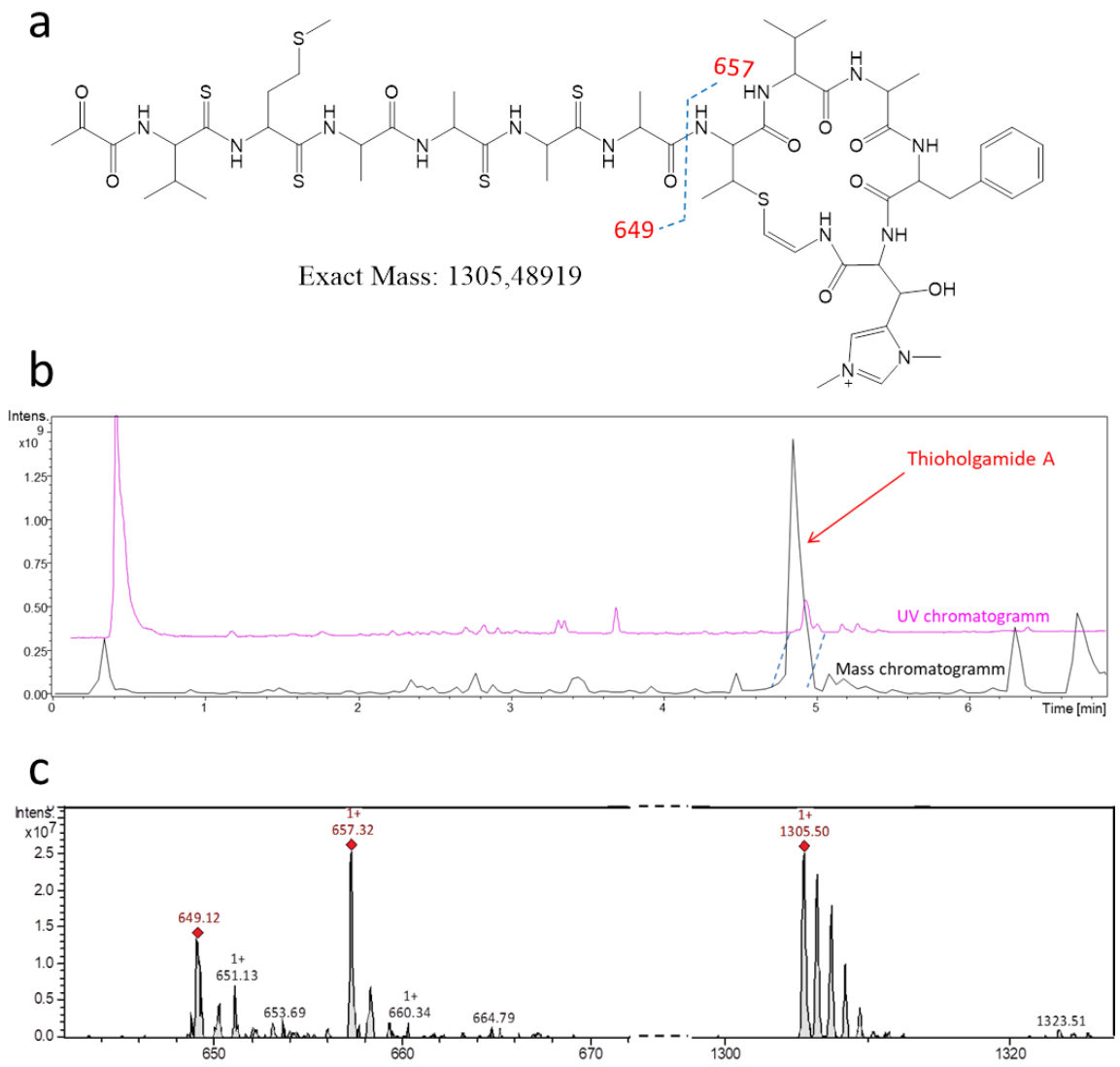


Figure 3. (a) Thioholgamide chemical structure. The fragmentation pattern is shown with a dotted line. (b) HPLC/MS analysis of crude extract from *S. lividans* pTho. (c) MS/MS fragmentation pattern of the thioholgamide A.

In the pTho vector, bearing the thioholgamide BGC, the precursor peptide gene was deleted, leaving the *Mss*I restriction site on the place of *thoA* gene. The new plasmid was named pTho:Δ*thoA*. Then the linearized pTho:Δ*thoA* at *Mss*I site was used for homologous recombination with the mixture of mutated variants of *thoA*-X^{NNS} by using RedET in *E. coli*. The mix of clones obtained after recombination was used for tri-parental conjugation with *S. lividans* del8. 200 exconjugants of *S. lividans* del8:pTho:*thoA*-X^{NNS} were picked on the MS

plates supplemented with Am50 and Phos200. Then 200 strains were plated on plates with solid production media. After 7 days of growth, a small scale extraction with butanol from the plates was performed. The extracts were analyzed on HPLC/MS for the presence of thioholgamide derivatives. After detecting the new derivatives, the producing strains were grown in liquid production media to verify the production level of new compounds. The chromosomal DNA from strains producing the new thioholgamides was isolated, and the pre-peptide gene was amplified to confirm the codon substitution by DNA sequencing (data not shown).

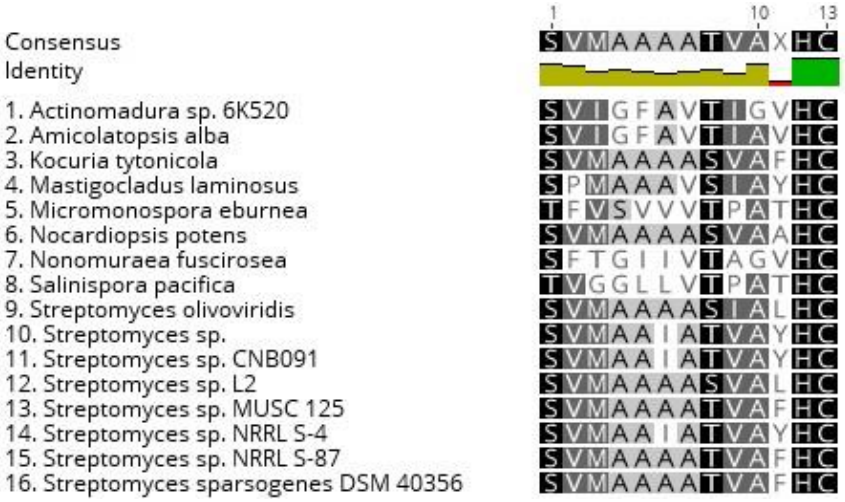


Figure 4. Comparison of the core peptide regions of precursor peptides from the BGCs similar to thioholgamide BGC.

The abovementioned approach was implemented for derivatization of 5 amino acids positions in the thioholgamide core peptide: Val-2, Met-3, Ala-7, Val-9, Ala-10. Only by randomizing these 5 amino acid positions, we succeed to detect 58 new thioholgamide derivatives (Fig. 5). 58 successfully incorporated amino acids versus 37 not incorporated shows very high success rate of incorporation. Those amino acids substitutions that we couldn’t detect possibly were not tolerated by tailoring enzymes, and such non-maturated precursor peptides underwent degradation.

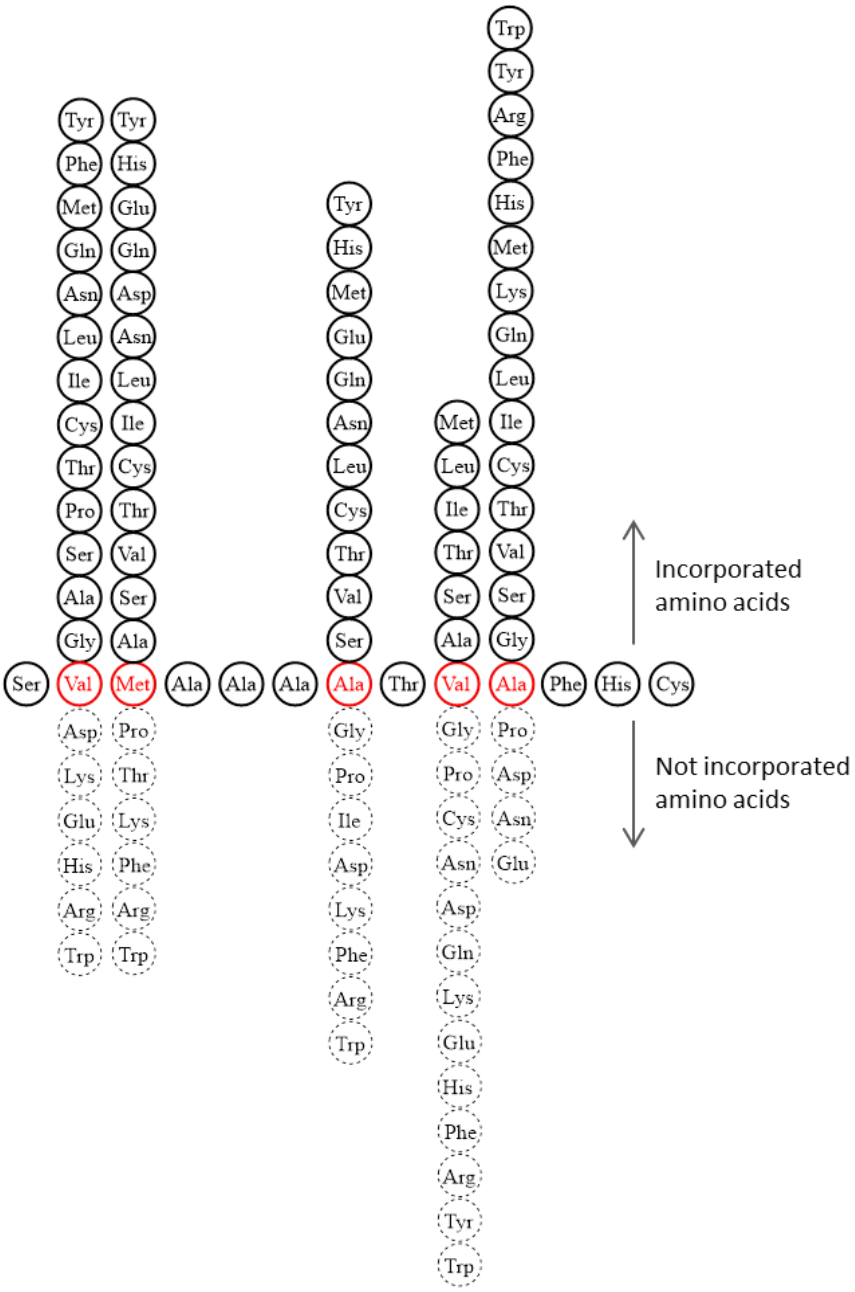


Figure 5. Summary of thioholgamide derivatives production. Amino acid positions that were derivatized are shown in red circles. Amino acids depicted with dotted circles were not incorporated in the corresponding positions.

Half of the derivatives at position Val-2 showed very low production level, compared to the original thioholgamide (Fig. 6). Only small non-polar amino acids, such as alanine, proline, leucine, isoleucine, and methionine had the production about twice less or similar to the native thioholgamide. The remaining detected derivatives of Val-2 had very low production yields. This fact implies the importance of the valine at position 2 for tailoring

enzymes, most likely for the protease that performs proteolytic cleavage of the leader peptide.

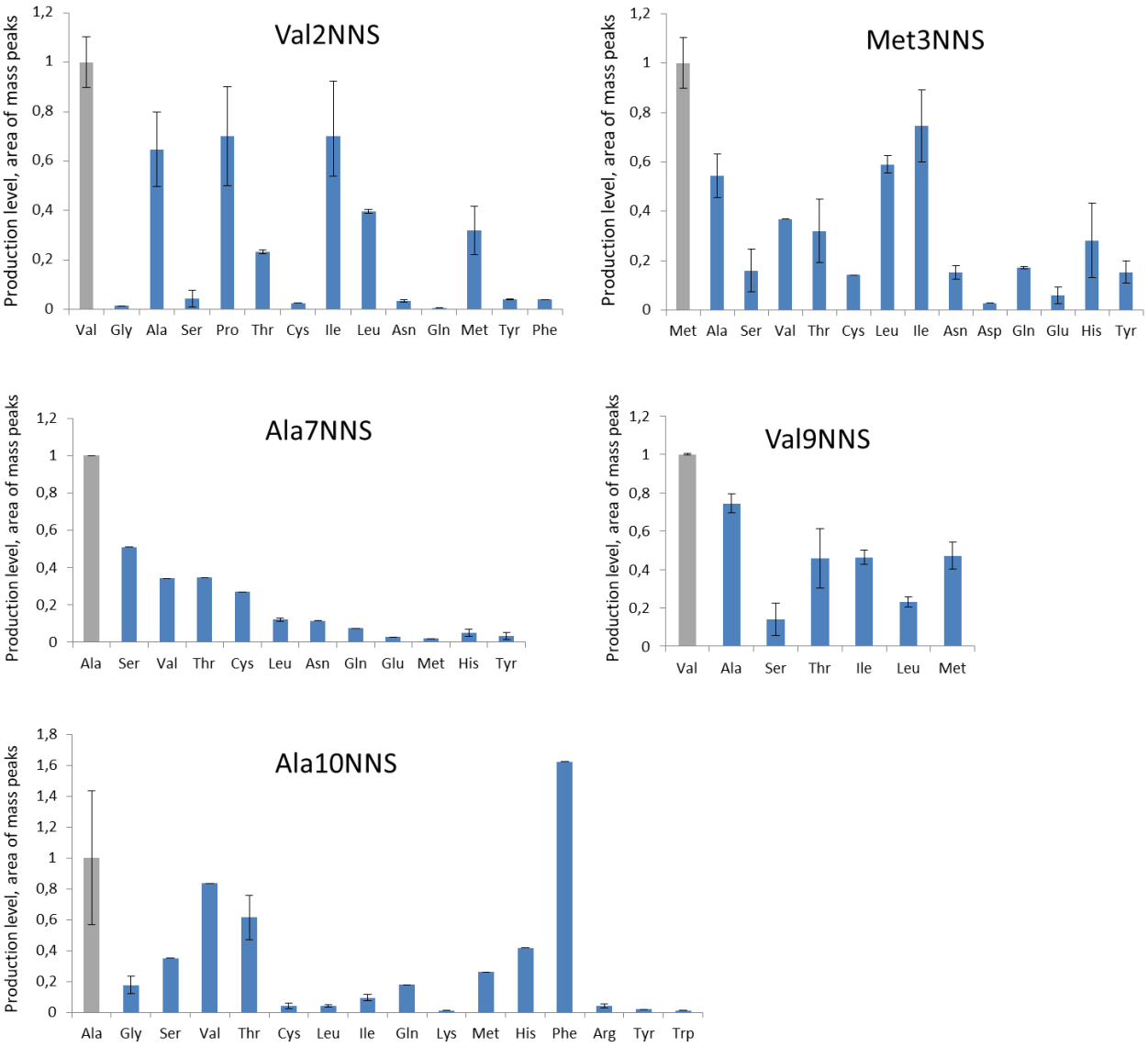


Figure 6. Comparison of the production levels of thioholgamide derivatives. The production level of original thioholgamide A is considered as 1.

Thioholgamide derivatives obtained by randomization of the Met-3 already have better production level than it was observed for position Val-2. We observed comparably good production level even for those thioholgamides with large amino acids incorporated, such as histidine, tyrosine, glutamine, and asparagine (Fig. 6). This shows the flexibility of amino acid at position 3, what can be used for derivatization or labeling of thioholgamide.

Only 6 thioholgamide derivatives were obtained through randomization of position Val-9 (Fig. 5). Solely small amino acids were incorporated at this position. This might be since Val-9 is present in the macrocycle and is located very close to the cyclization place. Another amino acid that is close to the cyclization spot is Ala-7. 11 thioholgamide derivatives were detected by randomization of this position (Fig. 5). However, we observe the clear correlation between the decrease in the production level of the new derivatives and the increase of the mass of incorporated amino acids (Fig. 6).

Interestingly, from the comparison of core regions of precursor peptides, it seemed that alanine in position 10 is very conservative (Fig. 4). But, we have obtained the most derivatives by randomization of the corresponding codon. We succeed to incorporate 15 different amino acids in position 10, including large amino acids, such as tryptophan, arginine, tyrosine, and phenylalanine (Fig. 5). Moreover, the production level of the derivative with phenylalanine was almost twice higher than of the original thioholgamide with alanine instead (Fig. 6). This shows that modification enzymes can tolerate the amino acid substitutions of Ala-10.

As a conclusion, we report here a method for fast and efficient amino acid substitution in core peptide through codons randomization of the precursor peptide gene. The efficiency of random amino acids incorporation overall was more than 60 %, but it varies depending on the position. For instance, the Val-2 and Met-3 had the incorporation success of 13 amino acids out of 19, when for Ala-10, we observed not predictable outcome of 15 amino acids incorporated. Randomization of Ala-7 codon yielded 11 new derivatives and Val-9 – only 6 derivatives. This fact we explain with the neighboring of Ala-7 and Val-9 with the Thr-8, which is involved in the macrocyclization. Amino acids with the bulky side chain might inhibit or even prevent this tailoring step, what, in turn, might block further modification steps. As a result of this, the unmodified non-mature compound is degraded and cannot be detected.

In our method, we don't select the positive *E. coli* clones carrying the plasmid with a mutated pre-peptide gene after recombination. Instead, we perform the conjugation with the whole mixture of clones obtained after RedET. This saves a lot of time and resources, and besides, we let the *Streptomyces* to pick a plasmid with the favorable codon for such

amino acids that are encoded by different codons. This strategy might be a reason for a good incorporation rate that we have observed in our experiments. Moreover, covering all possible amino acids incorporation, we access the full chemical space for thioholgamides. Such library of thioholgamide derivatives might serve as a great tool to understand the bioactivity and structure-activity relationship of this kind of compounds. Furthermore, it can be used for fast derivatization of any other RiPP to improve the activity of the compound or to generate designed compounds with improved pharmacokinetic properties needed for realization of the compound as a drug lead.

References:

Frattaruolo, L., R. Lacret, et al. (2017). "A Genomics-Based Approach Identifies a Thioviridamide-Like Compound with Selective Anticancer Activity." Acs Chemical Biology **12**(11): 2815-2822.

Goto, Y. and H. Suga (2018). "Engineering of RiPP pathways for the production of artificial peptides bearing various non-proteinogenic structures." Current Opinion in Chemical Biology **46**: 82-90.

Hayakawa, Y., K. Sasaki, et al. (2006). "Thioviridamide, a novel apoptosis inducer in transformed cells from Streptomyces olivoviridis." Journal of Antibiotics **59**(1): 1-5.

Hayakawa, Y., K. Sasaki, et al. (2006). "Structure of thioviridamide, a novel apoptosis inducer from Streptomyces olivoviridis." Journal of Antibiotics **59**(1): 6-10.

Izawa, M., T. Kawasaki, et al. (2013). "Cloning and Heterologous Expression of the Thioviridamide Biosynthesis Gene Cluster from Streptomyces olivoviridis." Applied and Environmental Microbiology **79**(22): 7110-7113.

Izumikawa, M., I. Kozone, et al. (2015). "Novel thioviridamide derivative-JBIR-140: heterologous expression of the gene cluster for thioviridamide biosynthesis." Journal of Antibiotics **68**(8): 533-536.

Kawahara, T., M. Izumikawa, et al. (2018). "Neothioviridamide, a Polythioamide Compound Produced by Heterologous Expression of a Streptomyces sp. Cryptic RiPP Biosynthetic Gene Cluster." J Nat Prod **81**(2): 264-269.

Kjaerulff, L., A. Sikandar, et al. (2017). "Thioholgamides: Thioamide-Containing Cytotoxic RiPP Natural Products." Acs Chemical Biology **12**(11): 2837-2841.

Kudo, K., H. Koiwai, et al. (2019). "Comprehensive Derivatization of Thioviridamides by Heterologous Expression." Acs Chemical Biology **14**(6): 1135-1140.

Nov, Y. (2012). "When Second Best Is Good Enough: Another Probabilistic Look at Saturation Mutagenesis." Applied and Environmental Microbiology **78**(1): 258-262.

Santos-Aberturas, J., G. Chandra, et al. (2019). "Uncovering the unexplored diversity of thioamidated ribosomal peptides in Actinobacteria using the RiPPER genome mining tool." Nucleic Acids Research **47**(9): 4624-4637.

Takase, S., R. Kurokawa, et al. (2019). "Mechanism of Action of Prethioviridamide, an Anticancer Ribosomally Synthesized and Post-Translationally Modified Peptide with a Polythioamide Structure." Acs Chemical Biology **14**(8): 1819-1828.

Yang, X. and W. A. van der Donk (2013). "Ribosomally Synthesized and Post-Translationally Modified Peptide Natural Products: New Insights into the Role of Leader and Core Peptides during Biosynthesis." Chemistry-a European Journal **19**(24): 7662-7677.

Zheng, L., U. Baumann, et al. (2004). "An efficient one-step site-directed and site-saturation mutagenesis protocol." Nucleic Acids Research **32**(14).