

Lasso peptides from Actinobacteria - Chemical diversity and ecological role

Jimmy Mevaere

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Lasso peptides from Actinobacteria

Chemical diversity and ecological role

Par Jimmy Mevaere

Thèse de doctorat de biochimie et biologie moléculaire

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Abbreviations

ABC transporter	ATP-binding cassette transporter
ACN	Acetonitrile
ACP	Acyl carrier protein
ACT	Actinorhodin
AT	Acyltransferase
ATP	Adenosine triphosphate
BGC	Biosynthetic gene cluster
bp	Base pair
С	Cytosine
CDA	Calcium-dependent antibiotics
cDNA	Complementary cDNA
CoA	Coenzyme A
Dha	2,3-didehydroalanine
DHB	2, 5-dihydroxybenzoic acid
Dhb	(Z)-2,3-didehydrobutyrine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FA	Formic acid
G	Guanine
GBAP	Gelatinase biosynthesis-activating pheromone
GlcNAc	N-acetylglucosamine
HCCA	α-cyano-4-hydroxycinnamic acid
НК	Histidine kinase
HPLC	High-performance liquid chromatography
HTH	helix-turn-helix
KS	β-ketoacyl synthase
Lan	meso-lanthionine

LC-MS	Liquid chromatography-mass spectrometry
LP	Lasso peptide
MeLan	3-methyllanthionine
MIC	Minimal inhibitory concentration
$M_{\rm w}$	Molecular weight
NGS	Next-generation sequencing
NMR	Nuclear magnetic resonance
NRP	Non-ribosomal peptide
NRPS	Non-ribosomal peptide synthase
OCS	One-component system
OD	Optical density
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PTM	Post-translational modification
qPCR	Real-time quantitative polymerase chain reaction
QS	Quorum sensing
Q-tof	Quadrupole-time of flight
RED	Undecylprodigiosin
RiPP	Ribosomally synthesized and post-translationally modified peptide
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Revolutions per minute
RR	Response regulator
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SAM	S-adenosylmethionine-dependent methyltransferase
SARP	Streptomyces antibiotic regulatory protein
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SET	SDS, EDTA, Tris buffer
ssDNA	Single-stranded DNA
TCS	Two-component system
TE	trishydroxyméthylaminométhane-EDTA
Tm	Melting temperature

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Introduction

Microbes produce a vast array of biological active molecules making them a rich source of potent pharmacological and biotechnological agents. These molecules are essentially generated via the secondary metabolism and thus termed secondary or specialized metabolites. The discovery of specialized metabolites has been greatly stimulated by the recent development of next-generation sequencing, which has revealed a rich diversity of unexplored biosynthetic gene clusters in microbial genomes and metagenomes. The challenge remaining for the discovery of novel specialized metabolites from genes to molecules is to activate the expression of these clusters, since many are silent (cryptic) in standard laboratory condition of cultivation.

In prokaryotes, the expression of specialized metabolites is triggered in response to environmental stimuli and controlled either by specific-pathway regulators encoded directly within the biosynthetic gene cluster, pleiotropic regulators, or both. Therefore, biotechnological strategies have emerged to manipulate these regulators in order to trigger the expression of cryptic biosynthetic gene clusters (1,2).

Specialized metabolites are exploited as antibiotics among others in therapeutic and biocontrol applications, as well as for other clinically-relevant biological activities. However, their role in the physiology of the producing strains remains most often elusive. While a role in microbial competition and allelopathy can be proposed for antibiotics, certain antibiotics have revealed unsuspected activity as low concentration, indicating that the ecological role of these molecules may be more complex and pleiotropic. Specialized metabolites can for example constitute signalling molecules involved in bacterial communications and activation of coordinated response (3,4). It is in these contexts that lasso peptides from *Streptomyces* are of particular interest.

Since mid 20th century, the genus *Streptomyces* is renowned as a prolific producer of bioactive metabolites, and genome mining approaches have shown an even larger biosynthetic arsenal. *Streptomyces* sp. are filamentous sporulating bacteria, showing a complex life cycle involving spore germination, production of vegetative mycelium and aerial mycelium, which is then converted into chains of spores. In *Streptomyces*, the production of most of the specialized metabolites occurs during the transition phase between the vegetative mycelium and aerial hyphae, through a network of complex interconnected pathways.

The ribosomally synthesized and post-translationally modified peptides (RiPPs) constitute a family of peptides with a wide diversity of structures (5). It is interesting to note that the phyla *Actinobacteria* and in particular the *Firmicute* (mostly *Bacilli*) displays the largest number of RiPP gene clusters, 13 % and 80% respectively, including lanthipeptides, thiopeptides and lasso peptides as flagships and for which there is an increased interest (6). Lasso peptides are RiPPs that harbour a remarkable mechanically-interlocked topology. The lasso structure consists of an N-terminal macrolactam ring threaded by a C-terminal tail. This entangled topology is maintained by a steric locking of the tail inside the ring by bulky amino acid(s) and/or disulphide bond(s). The highly constrained structure and set of biological activities reported for lasso peptides make this an attractive scaffold for drug design.

Genome mining has revealed a considerable number of lasso peptide biosynthetic gene clusters in *Actinobacteria*, some of them potentially showing additional post-translational modifications (PTMs), thus increasing the chemical diversity of this family. Sviceucin is a lasso peptide with two disulphide bridges produced by *Streptomyces sviceus* and discovered by genome mining. Given that the production of sviceucin by the native strain was very low, the peptide was produced heterologously in *Streptomyces coelicolor*, yielding to a significant improvement of its production yield. The sviceucin biosynthesis gene cluster harbours a gene encoding a histidine kinase and two genes encoding putative response regulators, which appear to be involved in the regulation production of the peptide. While sviceucin displays antibacterial activities against different Gram-positive bacteria, its role in the physiology of *Streptomyces sviceus* remains unknown (7).

In the first place, the work presented here aimed to explore the chemical repertoire of lasso peptides, in particular those harbouring PTMs, and thus in **Chapter I**, I present the work performed to develop a new heterologous expression system in *Streptomyces* allowing the production of lasso peptides from different *Actinobacteria*.

Chapter II presents the work performed studying the regulation mechanisms of sviceucin biosynthesis and its ecological role. The regulation mechanism was investigated by gene-inactivation of the response regulators present within the sviceucine biosynthesis gene cluster and the glucuronidase gene reporter system. Possible ecological roles of sviceucin was investigated by characterizing the morphology and the sensitivity to osmotic and oxidative stress of the wild-type *S. sviceus* and mutants inactivated for specific genes of the gene cluster.

Literature review

I. Microbial natural product discovery in the genomics era

Bacteria and fungi have provided a wealth of natural products, coined secondary metabolites, or more recently, specialized metabolites. Specialized metabolites are relatively small organic molecules (usually $M_w < 3000$), exhibiting a high structural diversity and wide biological activities including antibiotic, antitumoural, immunosuppressant, enzymatic inhibition, herbicidal and insecticidal (8,9). Natural products, and in particular antibiotics, have greatly increased human life span in the 20th century, in particular since the discovery in the 1940s of antibiotics from the *Actinobacteria* by Selman Waksman and co-workers, including streptomycin isolated from *Streptomyces griseus*. However, the massive use of antibiotics has led to a rise of antibiotic resistance, leading to the emergence of multidrug- resistant pathogens. Thus there is an urgent need for the development of novel antibiotics.

The development of next generation sequencing (NGS) technologies has permitted the exploration of multiple microbial genomes as well as metagenomes, revealing a large number of novel gene clusters potentially involved in the biosynthesis of specialized metabolites, expanding the potential for natural product discovery, from genes to molecules (10,11). The *Actinobacteria* phylum, and in particular, the genus *Streptomyces*, which has been historically the more important producer of known antibiotics, still constitutes a promising source for the discovery of new compounds (12–15).

I.1. Diversity of microbial specialized metabolites

There are four families of microbial specialized metabolites, with a particularly high structural diversity and relatively well know biosynthetic production routes (**Figure 1**). Those families are terpenes, polyketides, non-ribosomal peptides (NRPs) and ribosomally synthesized and post-translationally modified peptides (RiPPs).



Figure 1 – Representative structures of terpenes (geosmin), polyketides (erythromycin), NRPs (daptomycin) and RiPPs (thiostrepton).

Terpenes are molecules composed of multiples of isoprene units (C_5H_8) that may be acyclic, cyclic, saturated or unsaturated and can bear various functional groups such as hydroxyl. They are one of the largest family of natural products, particularly represented in plants (16), but also in fungi (17) and bacteria (18,19). One widely distributed example of bacterial terpene is geosmin produced by *Streptomyces*. Geosmin is a volatile degraded sesquiterpene alcohol known for its characteristic earthy smell (**Figure 2**) (20,21). Geosmin is biosynthesized from farnesyl diphosphate by an Mg²⁺ dependent germacradienol-geosmin bifunctional synthase encoded by the SCO6073 gene of *Streptomyces coelicolor*. The N-terminal part of the synthase catalyses the conversion of farnesyl diphosphate to germacradienol and the C-terminal part catalyses the conversion of germacradienol to geosmin (20).



Figure 2 – The biosynthesis of geosmin by bifunctional germacradienol-geosmin synthase from *Streptomyces coelicolor* (22).

Polyketides are composed from the successive linkages of hydroxyl and keto hydroxyl acids such as acetate, propionate, malonate and methyl malonate units, yielding a very high chemical diversity. They are biosynthesized by polyketide synthases (PKS) from acyl CoA precursors (23). The archetypal type I PKS consists of an assembly line of modules. Each module contributes to the addition of one single monomer to the growing polyketide chain. Three catalytic domains are often present in each module, an acyltransferase (AT), an acyl carrier protein (ACP) and a β -ketoacyl synthase (KS). The AT domain selects an activated acyl monomer and connects the substrate to the thiol group of a 4'-phosphopantetheine cofactor bound to the ACP domain by post-translational modification. The KS domains catalyse the formation of the linkage between a monomer and the growing acyl chain. Erythromycin A is a metabolite which biosynthesis involves a type I PKS (**Figure 3**) (24,25). It is produced by *Saccharopolyspora erythraea* and is encoded by a 55 kb gene cluster composed of three large PKSs and other genes involved in deoxysugar biosynthesis, macrolide tailoring, and resistance (25). The erythromycin skeleton is generated from one propionyl-CoA starter extended with 6 methylmalonyl-CoA monomers by three PKS consisting of 7 total modules.

Type II PKSs are involved in the biosynthesis of aromatic polyketide products. The domains in type II PKSs are typically consisted of a single ACP and KS, in contrast to type I PKSs. Thus, the polyketide chain is produced by an iterative series of condensation reactions until the specific length, then cyclization is catalyzed by aromatase and cyclase to yield the aromatic polyketide (26,27). One example of aromatic polyketide is actinorhodin, a pigmented antibiotic produced by *Streptomyces coelicolor* (see section I.3.b). Its biosynthesis involves a type II PKS together with several tailoring enzymes such as oxygenases (28,29). Finally, type III PKSs catalyse iterative condensation as in type II PKSs, but without an ACP and with cyclization and aromatization are performed in the same enzyme active site (23).



Figure 3 – Illustration of modular PKS function: The erythromycin biosynthesis.

The precursors of polyketides are small carboxylic acids, such as acetate, propionate and malonate, and are activated as their coenzyme A (CoA) thioesters. One starter unit of propionyl-CoA and six extender units of (2*S*)-methylmalonyl-CoA (carboxylated propionyl-CoA) are required for the biosynthesis of erythromycin A. The erythromycin PKS (6-deoxyerythronolide B synthase, DEBS) consists of a loading module, six chain-extension modules and a chain-terminating thioesterase (TE), distributed across three gigantic proteins which are named DEBS 1, 2 and 3. Each chain-elongation module contains an acyl transferase (AT), a keto synthase (KS) and an acyl carrier protein (ACP) domain, and variable numbers of reductive elements, as required. Following TE-catalysed release from the PKS, the aglycone 6-deoxyerythronolide B is hydroxylated twice, glycosylated twice and methylated on a sugar residue to form the fully active antibiotic erythromycin A. DH, dehydratase; ER, enoyl reductase; KR, ketoreductase (30).

Non-ribosomal peptides are synthesized by the successive addition of amino acid building blocks by multimodular enzymes termed non-ribosomal peptide synthetases (NRPSs), which perform an assembly-line synthesis analogously to type 1 PKS synthases (31–34). Each module consists of multiple domains that are enzymatic units catalysing the polypeptide extension of one building block via substrate activation and peptide bond formation. Four principal domains have been identified: the adenylation domain (A domain), the peptidyl carrier protein (PCP domain), the condensation domain (C domain) and the thioesterase domain (TE domain). The A domain recruits specific amino acids and activate them as an amino acyl adenylate, using ATP. The activated substrate is transferred onto the phosphopantetheinylated PCP domain. Subsequently, the C domain forms the peptide bond between the next activated amino acid and the peptidyl substrate. Finally, the TE domain releases the product and can also lead to peptide cyclization. Additional tailoring enzymes are often found, such as epimerization or *S*-adenosylmethionine-dependent methyltransferase domains, increasing the diversity of the NRPS products. As an example, daptomycin is a branched cyclic lipopeptide produced by *Streptomyces roseosporus*, which biosynthesis involves three NRPS synthetases DptA, DptBC, and DptD and one *in trans* acting enzyme (**Figure 4**) (35,36).

Ribosomally synthesized and post-translationally modified peptides (RiPPs) form a major class of natural products, continuously growing on account of genome mining efforts (5). RiPPs are initially produced as gene encoded precursor peptides, which are matured by post-translational modification enzymes and then generally transported out of the producing cell. Unlike NRPS and its several hundred potential amino acid substrates, the post-ribosomal peptide synthesis pathway is restricted to the 20 proteinogenic amino acids. The precursors are composed of a leader peptide usually attached to the N-terminus of the core peptide and the core peptides. The leader peptide is thought to play important roles for the recognition by post-translational modification (PTM) enzymes and in export. Once the precursor is produced, the core peptide is cleaved and modified by PTM enzymes, generally encoded by genes located close to the precursor gene, to yield the mature peptide. Peptides are remodelled with a high diversity of PTM structures (**Table 2** – p33). These modifications confer peptides with a fixed conformation, resulting in better target specificity and higher stability under environmental conditions. As the main subject of this thesis, this family will be further described in the section II.





Figure 4 – Schematic overview of daptomycin biosynthesis by S. roseosporus.

The assembly of the peptide core is governed by the three NRPSs DptA, DptBC, and DptD, comprising 43 catalytic domains. Initiation of daptomycin biosynthesis is mediated by DptE and DptF, both responsible for the activation and incorporation of the FA moiety. In this model, the N-terminal CIII-domain of DptA catalyses the transfer of the DptF-bound FA onto the α -amino group of Trp¹. The synthesis of MeGlu¹² is carried out by the S-adenosylmethionine (SAM)-dependent methyltransferase DptI and a currently unknown aminotransferase. Cyclorelease is mediated by the C-terminal TE domain of DptD. The cyclization position is shown in grey. A, adenylation; AL, acyl-CoA ligase; AT, aminotransferase; C, condensation; E, epimerization; MT, methyltransferase; PCP, peptidyl carrier protein (36)

I.2. From genes to molecules

Classically the approach used for the discovery of specialized metabolites consisted in the screening of supernatant cultures, or cell extracts for specific bioactivities (such as antibiotic or enzyme inhibition), followed by bio-guided fractionation and purification of the compound of interest, which are then structurally characterized by spectroscopic methods (UV/IR spectroscopy, mass spectrometry, nuclear magnetic resonance (NMR) and X-ray crystallography). Improvements to this approach have been developed such as dereplication (37,38) ,which scans crude extracts for the presence of known compounds in order to focus on the discovery of novel ones.

More recently and stimulated by developments in NGS and the resulting rich dataset of microbial genomes, genome mining strategies have emerged as an alternative to classical bioguided approaches for the discovery of microbial specialized secondary metabolites (39–41). The genome analysis is carried out by searching homologs of genes and proteins involved in established biosynthetic pathways. The screening is achieved with bioinformatics tools such as Blast, antiSMASH 3.0 or Bagel3 (42,43). This approach has revealed that genes involved in the biosynthesis of specialized metabolites are typically clustered in the genomes of bacteria and fungi. Furthermore, it is also clear that larger microbial genomes, such as those of *Actinobacteria*, appear to carry more biosynthesis gene clusters when compared to smaller ones, revealing that actinobacteria may produce even more specialized metabolite than expected (12,15,44–48).

Genomic sequences also revealed, many biosynthesis gene clusters, termed cryptic or silent, do not appear to be significantly expressed under standard laboratory conditions of cultivation (1), in which the producing organisms are generally cultivated in conditions lacking environmental cues such as chemicals and other microorganism. Although the factors that control the specialized metabolite production remain elusive, environmental factors have been shown to up- or down-regulate the expression of the biosynthetic genes via complex regulation networks (49). Therefore, one way for discovery of novel microbial natural products via genome mining is the activation of the expression of these untapped reservoirs of bioactive molecules. In that respect, two main strategies have been proposed: the global approach or the pathway-specific approach (1). The former consists of changing the standard conditions of cultivation (media composition, temperature of incubation, cocultures with competitors or host

cells), with the idea to mimic environmental condition or to stress the organisms. The latter relies on a specific strategy for each gene cluster, such as inactivation of repressor proteins, overexpression of transcriptional factors and replacement of the promoter by a strong promoter (**Figure 5**).



Figure 5 – Pathways-specific strategies to induce expression of silent biosynthetic gene clusters (BGC) (1). Promoter replacement and inactivation of repressor strategies are shown in **a** and **b**.

In this targeted approach, the heterologous expression of putative gene clusters can also be achieved (12,50–52). Such strategy lefts a host that can be more readily genetically engineered and also extends genome mining approaches towards the production of metabolites from uncultivable microorganisms for which genomic information exists. The strategies to improve the expression of cryptic gene clusters are be presented in more detail in the introduction of Chapter I. Finally, the detection and identification of the produced metabolites can also be facilitated with a biosynthetic gene knockout that permits a comparison between extracts of the wild type and mutant by liquid chromatography – mass spectrometry (LC-MS) profiling (53,54).

I.3. Streptomyces, a prolific metabolite producer

Streptomyces is a genus within *Actinobacteria*, a phylum of Gram-positive bacteria with a high G/C DNA base ratio (**Figure 6**) (55,56). *Actinobacteria* produce more than 70% of all

natural product scaffolds used in clinically-relevant antibiotic molecules (48). *Streptomyces* sp. are non-motile and aerobic bacteria that grow at neutral pH and are mesophilic (optimal growth temperature between 25 °C to 30 °C).



Figure 6 – Intraclass relatedness of the class *Actinobacteria* showing the presence of five orders based on 16S rRNA gene sequence comparison (57).

The phylogenetic relatedness of the families of the class *Actinobacteria* is outlined. Taxa newly described are highlighted in bold. Bootstrap values of 50% or more are indicated at branch points. Bar, 2 substitutions per 100 nucleotide positions.

Streptomyces are distributed both in soil and aquatic habitats, where they typically use complex organic compounds from the environment as a source of energy. Their genomes displays a 60-70% G/C and their 8-9 Mb chromosomes are linear, with a "core" containing essential genes and "arms" carrying conditionally adaptive genes (58). Since the discovery of actinomycin, streptomycin and other antibiotics from actinobacteria by Selman Waksman and co-workers in the 1940s, intensive screening of soil habitat for antibiotic producers has been performed, showing that *Streptomyces* is a prolific producer of specialized metabolites. The development of genetic methods initiated in the 1960s by David Hopwood (59,60), followed by the genomic area in the 2000s (61,62) has revolutionized the species classification (63) as well as the methods to discover new specialized metabolites and to explore the biotechnological potential of these bacteria. Genome mining of *Streptomyces* sp. has revealed as many as 30-50 gene clusters per strain that are potentially involved in the biosynthesis of specialized metabolites from all four major chemical classes presented in section I.1 (39,64,65).

I.3.a. The life cycle of *Streptomyces*

Streptomyces show a complex morphology consisting of highly differentiated mycelia that form spores under coordination of physiological processes (**Figure 7**) (66,67). There are three major steps in the life cycle of *Streptomyces*: vegetative growth, aerial mycelium erection and sporulation. The life cycle of *Streptomyces* starts from a dormant spore that meets suitable liquid or solid environment to form a vegetative mycelium (56,68,69). The spore germinates by a swelling process followed by the installation of cell polarity and apical growth. One or more hyphae arise from the spores and grow by tip extension and branching, in contrast to unicellular bacteria such as *E. coli* that grow by elongation of the lateral wall and fission. The vegetative *Streptomyces* cells are separated into compartments that are connected by cross-walls, each compartment containing multiple copies of the chromosome. The vegetative mycelium can differentiate into aerial hyphae which show a hair-like surface, generally white, on the top of the colonies. The events that trigger the differentiation from the vegetative mycelium to the aerial hyphae remain unclear, but it typically occurs under unfavourable conditions. This

differentiation induces a form of programmed cell death in which the vegetative mycelium is autolysed to release nutrient in order to help the development of aerial hyphae (70).



Figure 7 – The life cycle of *Streptomyces* (56)

The erection of aerial hyphae is helped by a hydrophobic sheath that enables the hyphae to break the surface water tension and to grow into the air. In *S. coelicolor*, this sheath is composed of two proteins, the chaplins and the rodlins and one surfactant peptide SapB (67,69). Eventually, the multigenomic apical cell of aerial hyphae is converted into unigenomic spores by several synchronously coordinated biological processes such as chromosome segregation and sporulation septation (69).

I.3.b. Regulation of specialized metabolite biosynthesis in *Streptomyces*

In many bacteria, antibiotic production responds to stress situations in coordination with primary metabolic responses (71). One of the main pathways linking a specific type of environmental stimulus to a transcriptional response involves two-component systems (TCSs), which consist of a sensor histidine kinase (HK) and a cognate response regulator (RR) (72,73). Each HK responds to specific types of environmental stimuli via an extracytoplasmic sensor

domain. The signal is transferred via a phosphorelay that involves the autophosphorylation of a conserved His residue of the HK, which is then transferred to an Asp residue in the receiver domain of the cognate RR, which then activates transcription of target genes (**Figure 8 b**). Generally, two-component systems regulate gene expression at the level of transcription, through the DNA-binding domain of the RR. The analysis of prokaryote genomes also revealed the presence of unpaired HKs and RRs. In addition, certain TCSs components, although not encoded by adjacent genes, have been functionally associated (74). Finally, the occurrence of genes encoding single proteins containing sensor and output domains and lacking phosphotransfer domains typical of TCSs has conducted to propose an additional signal transduction pathway involving one-component systems (OCSs) (75) (**Figure 8 a**).



Figure 8 – One-component and two-component signal transduction systems.

a. A prototypical one-component system consists of one protein with a sensor domain and a regulator domain. **b**. A typical two-component system includes a sensor histidine kinase (HK) and cognate response regulator (RR), usually encoded by a pair of adjacent genes. ATP or GTP is used for autophosphorylation by the HK, and the phosphoryl group is transferred to the receiver domain of the RR (blue hexagon), which then controls the transcription of target genes through its output domain (in green) (76).

The biosynthesis of specialized metabolites in *Streptomyces* is subjected to subtle and precise regulatory events, involving a network of complex interconnected pathways of pathway-specific regulatory proteins or pleiotropic regulators or both (71,76–79) (77,79,80). Many studies on the biosynthesis and the regulation of specialized metabolites were performed in *S. coelicolor* A3(2) (64,73,81), which produces several specialized metabolites with diverse structures (**Figure 9**): three groups of chromosomally-encoded antibiotics: actinorhodin (ACT), undecylprodigiosin (RED) and calcium dependent antibiotics (CDA) and a group of plasmid-

encoded antibiotics: methylenomycins. More recently, a yellow pigment, coelimycin P1, has been isolated upon activation of a cryptic gene cluster (82).



Calcium-dependant antibiotics (CDA)

Figure 9 – Specialized metabolites produced by *Streptomyces coelicolor* A3(2) (78).

ACT is an aromatic polyketide synthesized by a type II PKS that displays a pH-sensitive colour (red at pH below 8.5, blue at pH above), from which S. coelicolor gets its name (81,82). RED are red hydrophobic tripyrroles made by a fatty acid synthase-like pathway involving a 22-genes (83). CDA are acidic lipopeptides synthesized by the NRPS pathway containing an N-terminal 2,3-epoxyhexanoyl fatty acid side-chain (84–86). Methylenomycins are cyclopentanone antibiotics made by an unusual pathway involving the condensation of a diketide with an pentulose, encoded by the gene cluster Mmy located on the plasmid SCP1 (87,88). Coelimycin P1 is a yellow alkaloid containing a unique functionalized 1,5-oxathiocane heterocycle, recently associated with a type I PKS cluster (cpk) (80).

Here, I use the regulatory mechanism of these molecules among other as example to illustrate the regulation of specialized metabolites by specific-pathway regulators and pleiotropic regulators.

I.3.b.i Pathway-specific regulators

The pathway-specific regulators are typically encoded within the gene cluster they controlled and in *Streptomyces* most of them belong to the *Streptomyces* antibiotic regulatory protein (SARP) family, which group paralogous proteins involved in antibiotic production only found in actinomycetes (mainly within streptomycetes) (77,83). SARPs bind to sequences in the target promoters via their N-terminal winged Helix-Turn-Helix (HTH) domain, and activate transcription through a C-terminal activation domain. SARPs can be subdivided into three classes in function of their size: small SARPs (< 300 aa), medium SARPs (around 600 aa) which share end-to-end similarity to each other and large SARPs (around 1000 aa) which share an N-terminal SARP domain, an AAA domain (<u>A</u>TPase <u>A</u>ssociated with diverse cellular <u>A</u>ctivities), and a C-terminal domain of unknown function (78).

Five pathway-specific regulators have been characterized in *S. coelicolor* A3(2) (**Table 1**). The proteins ActII-ORF4, RedD, CdaR and KasO, controlling ACT, RED, CDA and coelimycin P1 production, respectively, are representants of the SARP family. ActII-ORF4 and RedD are small SARPs binding to a repeated TCGA motif spaced by 11 bp at the -35 nt region of their target promoters while CdaR is a medium SARPs (78,83).

Pathway-specific regulator	Specialized metabolite controlled	Туре	Reference
ActII-ORF4	ACT	SARP	(84)
RedD	RED	SARP	(85)
RedZ	RED	OCS	(86)
KasO (also designated CpkO)	Coelimycin P1	SARP	(87)
CdaR	CDA	SARP	(88)

 Table 1 – Pathway-specific regulators in S. coelicolor A3(2)

An example of large SARP is PimR involved in the biosynthesis of pimaricin, a tetraene produced by *Streptomyces natalensis* (89). The SARP domain of PimR was shown to bind three heptameric direct repeats of the consensus CGGCAAG spaced by 4 bp and interacts with the - 55 nt region instead of the classical -35 nt region of target promoters. Unlike most of other large SARPs, PimR targets the promoter of another regulator *pimM*, which in turn activates the expression of the biosynthetic gene of primaricin (90). Interestingly, PimM orthologues are encoded and are functionally conserved in all known biosynthetic gene clusters of antifungal polyketides (91).

In addition to the SARP model described above, the biosynthesis of RED is controlled by two pathway-specific transcriptional activators, RedZ and RedD, with RedZ activating RedD, the direct activator (86). RedZ, is an atypical orphan RR lacking a set of conserved residues for phosphorylation, is an example of one-component system. Note that the mRNA of *redZ* present a very rare leucine codon, UUA, which can be only read by the specific tRNA BldA that is actively expressed when culture enters the stationary phase and initiates programmed cell death (86,92).

I.3.b.ii Pleiotropic regulators

A pleiotropic regulator function over a larger set of genes in the genome and in prokaryotes many are related to the metabolism of carbon, nitrogen and/or phosphate or correlated with morphological differentiation. Indeed, nutrient limitation is one of the principal signal to activate specialized metabolism inducing the developmental differentiation (56,77,80). An important representant of pleiotropic regulators in *S. coelicolor* is DasR, which is primarily involved in the metabolism of N-acetylglucosamine (GleNAc) (**Figure 10**) (93–95). GleNAc is the building block of the abundant natural polymer chitin and a major constituent of peptidoglycan constituting a primary source of carbon and nitrogen for streptomycetes. GleNAc released during programmed cell death (see section I.3.a), activates a cascade response leading to expression of specific-pathway-regulatory genes such as *actII-ORF4* or *redZ* (78,93,96)





(a) A complete signalling cascade from extracellular nutrients to the activation of ACT and RED production, from (79,93). N-acetylglucosamine (GlcNAc) enters the cell and is subsequently phosphorylated via the GlcNAc-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS). N-acetylglucosamine-6-phosphate (GlcNAc-6P) is then deacetylated by GlcN-6P deacetylase NagA to glucosamine-6-phosphate (GlcN-6P), the effector molecule that inhibits DasR DNA-binding. This results in the derepression of *actII-ORF4* and *redZ/redD*, the pathway-specific transcriptional activator genes for the ACT and RED biosynthetic gene clusters, respectively. (b) Summary of the DasR-controlled processes, from (95). Red lines indicate direct binding and transcriptional repression of the target genes by DasR. Derepression of control by DasR in response to the accumulation of GlcN-6P results in activation (green lines) of morphological development and antibiotic production, among other processes.

II. Ribosomally synthesized and post-translationally modified peptides

II.1. Diversity

As discussed previously in section **I.1**, RiPPs share a common biosynthetic strategy that starts from a linear precursor synthesized by classical transcription/translation processes, which then undergoes different levels of PTM, that results in a great diversity of molecules (5,97) (**Table 2**). Lanthipeptides and thiopeptides will be described the next sub-section given that they are a great source of information to understand the biosynthesis and the regulatory mechanism of RiPPs and followed by the description of lasso peptides as they are the subject of this thesis.

Subfamily	Characteristic features	res Representant (Reference)	
	Macrocyclized peptides		
Cyanobactins	N-to-C macrocyclic peptides produced by cyanobacteria sometimes further decorated with azole(in)es and/or prenylations	Patellamide A (98)	
Thiopeptides	A central six-membered nitrogen-containing ring additional PTMs including dehydrations and cyclodehydrations	Thiostrepton (99)	
Lasso peptides	An N-terminal macrolactam ring, with the C-terminal tail threaded and trapped within the ring	Microcin J25 (100)	
Bottromycins	An N-terminal macrocyclic amidine The precursor shows a C-terminal follower peptide instead of a N-terminal leader peptide	(101)	
Microviridins	Lactones made from Glu/Asp and Ser/Thr side chains and lactams made from Lys and Glu/Asp residues	(102)	
Streptide	A Trp-to-Lys carbon crosslink	(103,104)	
Head-to-tail cyclized peptides	N-to-C macrocyclic peptides produced by bacteria, fungi or cyanobacteria	Enterocin AS-48 (105)	
	Non-macrocyclized peptides		
Lanthipeptides	Lan and/or MeLan thioether bis-amino acids	Nisin (106)	
Linaridins	Dehydroamino acids containing peptides similar to lanthipeptide but lacking Lan and/or MeLan	Cypemycin (107)	
Linear azol(in)e-containing peptides (LAPs), also termed thiazole/oxazole-modified microcins (TOMMs)	(Methyl)oxazol(in)e and/or thiazol(in) heterocycles	Microcin B17 (108)	
Proteusins	D-amino acids and C-methylation	(109)	
Sactipeptides	Intramolecular thioether linkages between Cys sides chains and a carbons of other amino acids	(110)	
Glycocins	S-glycosylated peptides	(111)	
Methanobactins	Copper chelators used by methanotrophic bacteria	(112)	
Siderophore peptides	Peptides linked to a siderophore at the C-terminus	Microcin E492 (113)	
Peptide nucleotides	Short peptides linked to a nucleotide by a phosphoramide bond	Microcin C (114)	
PQQ-like	Small molecules generated from PTM of a precursor peptide or protein	Pyrroloquinoline quinone (PQQ) (115)	

Table 2 – Overview of microbial RiPPs subfamilies based on structural features, adapted from (97)
II.2. Lanthipeptides

II.2.a. Structure

Lanthipeptides (also called lantibiotics for those with antibacterial activities) are a major class of RiPPs containing *meso*-lanthionine (Lan) and 3-methyllanthionine (MeLan) (106). The first reported lanthipeptide was nisin in 1928 but their ribosomal origin was only confirmed in 1988 with the sequencing of the epidermin gene cluster (116,117). Lan and MeLan are residues formed by intramolecular connection of a cysteine and the post-translational modified amino acid 2,3-didehydroalanine (Dha) or (Z)-2,3-dehydrobutyrine (Dhb) formed by dehydration of a Ser and Thr residue, respectively (**Figure 11**) (118). Nisin is a lanthipeptide produced by *Lactococcus lactis* and is considered as a model to illustrate the biosynthesis of lanthipeptides, their regulation and their biological activities.



Figure 11 – Basic posttranslational modification for the lantibiotic family (118)

II.2.b. Biosynthesis

The gene cluster for the production of nisin consists of 11 genes: *nisABTCIPRKFEG* encoding the 57-amino acids long precursor NisA, the maturation enzymes NisB, -C and –P, two ABC transporter family NisT and NisFEG, an immunity lipoprotein involved in the self-protection NisI and a two-component system that comprises a sensor kinase (NisK) and a response regulator (NisR) (118–122). The nisin gene cluster is under the control of three promoters P_{nisA} , P_{nisR} and P_{nisF} that promote the transcription of three operons *nisABTCIP*, *nisRK* and *nisFEG*, respectively. A terminator is probably present after *nisA* that allows restricted read-through of the downstream genes *nisBTCIP* (Figure 12).



Figure 12 – Organization of nisin biosynthetic gene clusters.

The structural lantibiotic genes (filled black), the genes encoding proteins involved in precursor maturation and transport (vertical hatching), processing (backward tilted hatching, producer immunity (horizontal hatching), and regulation (forward tilted hatching) are indicated. The positions of the promoters that have been identified within the nisin gene clusters are indicated (123).

The precursor NisA is composed of a 23 amino acid long leader peptide and the 34 amino acid long structural peptide. The leader peptide has been shown to be requisite for secretion and complete maturation (124–126). NisA undergoes a series of dehydrations by the lanthipeptide dehydratase NisB, which utilizes glutamyl-tRNA^{Glu} as the glutamate donor to activate Ser/Thr. Then, glutamate elimination results in DhA/DhB residues (**Figure 13**) (127,128).





For Lan and MeLan structures, the segment derived from Ser/Thr are in red and those derived from Cys are in blue.

Cyclization is performed by NisC through thioether crosslinks, by addition of Cys thiols to the resulting DhA or DhB to form Lan or MeLan, respectively. The modified NisA is then transported across the cytoplasm membrane by NisT and the leader is cleaved by the extracellular peptidase NisP (129,130).

II.2.c. Classification

Lanthipeptides are subdivided into 4 classes depending on the post-translational modification enzymes which install (Me)Lan motif. For class I lanthipeptides, (Me)Lan motif is installed by the action of LanB and LanC enzyme, a lanthipeptide dehydratase and a lanthipeptide cyclase, respectively. For class II, III and IV lanthipeptides, dehydration and cyclization are carried out by bifunctional lanthionine synthetase LanM, LanKC and LanL, respectively. LanM is a large protein with an N-terminal dehydration domain that does not show homology to LanB proteins and a C-terminal cyclase domain which displays weak homology to LanC proteins. LanKC and LanL carry out the dehydration by successive action of a central kinase domain and an N-terminal phosphoSer/phosphoThr lyase domain, but differs in the cyclase domain (5,118). Although LanKC show LanC-like cyclase domain, it lacks the three zinc ligands binding domains conserved in the other cyclase domains.

II.2.d. Biological activities

Nisin displays antibacterial activity against Gram-positive bacteria and has been used for more that 50 years as food preservative. Its mechanism of action is dual: nisin binds to the peptidoglycan intermediate building block lipid II, thus causing inhibition of cell wall biosynthesis and can insert into the membranes, leading to pore formation (131). Cytolysin is produced by *Enterococcus faecalis* and belongs to the group of two-component lanthipeptides. By name, cytolysin, consists of two modified peptides, which together display strong antibiotic activity but have weak or no antimicrobial activity when separated (118,132). Cytolysin displays antibacterial activity against Gram-positive bacteria and also functions as a virulence factor, lysing erythrocytes and polymorphonuclear leukocytes (133). Interestingly, the matured precursors of lantibiotics do not show antimicrobial activity. Thus, for those that are processed extracellularly, the leader peptide may prevent the activity of the mature peptide until it is outside the cell (120,134).

II.3. Thiopeptides

The first thiopeptide isolated was micrococcin in 1948, but thiostrepton isolated from *Streptomyces azureus* in 1954 is referred as the archetype compound (5,99). Although thiopeptide are mainly isolated from soil bacteria, novel representative has been isolated from marine environment (135).

II.3.a. Structure

Thiopeptides, also termed thiazolyl peptide, contain a nitrogenous macrocycle which present different oxidative states, fully saturated piperidine, didehydropiperidine, imidazopiperidine, pyridine and pyridine with hydroxyl at position 5 (**Figure 16**). Thiopeptides can harbour additional modifications such as dehydroamino acids similar to those of lanthipeptides (5,99).

II.3.b. Biosynthesis

The thiostrepton biosynthetic gene cluster identified from whole-genome scanning of *Streptomyces laurentii* is one example demonstrating that thiopeptides are ribosomally-synthesised then posttranslationally modified (135,136). Like the other RiPPs, thiopeptide biosynthetic gene clusters contain one gene encoding a precursor and other genes encoding the enzymatic machinery. For example, the gene cluster of thiomuracins contains one gene encoding the precursor TpdA, and 6 other genes encoding TpdB-G proteins (**Figure 14**).



Figure 14 – The biosynthetic gene cluster of thiomuracins (135).

In the precursor peptide sequence, the structural peptide is numbered with positive figures and the leading peptide with negative ones. Residues that appear in the mature thiopeptide are underlined.

In this biosynthesis, oxazoles, thiazoles, thiazoline rings and DhA and Dhb are first installed. Then, the cycloaddition between distant Dha residues is performed and finally further side-chain modifications are installed (**Figure 15**) (137). Dehydroamino acids residues are

probably installed by TpdB and TpdC which show weak similarities with LanB dehydratase from class I lanthipeptides (5,138,139).



Figure 15 – Biosynthetic pathway of thiomuracin I. LP, leader peptide (135).

Thiazole rings present in thiopeptides are also present in other RiPPs such as the cyanobactins and the linear azol(in)e-containing peptides (LAPs) (140,141). Additional genes are likely to be present in the different clusters to incorporate modification such as hydroxylation or methylation (137).

II.3.c. Classification

Thiopeptides have been regrouped into five series according to the oxidative state of the nitrogenous macrocycle, fully saturated piperidine, didehydropiperidine, imidazopiperidine, pyridine and pyridine with hydroxyl at position 5, respectively series a, b, c, d, and e (Figure 16).

II.3.d. Biological activities

The main biological activity of thiopeptide is the antibacterial. Thiopeptides efficiently inhibit protein synthesis in Gram-positive bacteria by either direct interaction with the 50S ribosomal subunit or by inhibiting elongation factor Tu (EF-Tu), whereas they display almost no activity against Gram-negative bacteria (5,99,135). Interestingly, some have been shown to possess other pharmacological activities such as anticancer (142) or immunosuppressive (143).



Figure 16 – Classification of thiopeptide antibiotics into different series (135). Their characteristic central six-member ring is highlighted in bold.

Thiopeptides were showed to be widely distributed in genomes and metagenomes of the human microbiota, with one thiopeptide was characterized recently from the human microbiome (144).

Interestingly, a thiopeptide containing a Dha in the tail close to six-membered central scaffold, such as thiostrepton, appears to induce the *tipA* gene promotion by binding to the regulatory protein TipAL (135,145,146). TipAL is a transcriptional regulator which contains a N-terminal helix-turn-helix DNA-binding domain and a C-terminal drug recognition domain. The binding of thiostrepton-like molecule to TipAL induce the recruitment of RNA polymerase (RNAP) to the promoter *tipAp*, thus resulting in autogenous transcriptional activation of the *tipA* gene. The feature was widely used in *Streptomyces* as an inducible promoter.

II.4. Lasso peptides

II.4.a. Diversity

Lasso peptides represent an emerging class of RiPPs. They possess a unique topology reminiscent to a lasso and were isolated from Proteobacteria, Actinobacteria and more recently from Firmicutes (100,147-149). The topology of a lasso peptide consists of an N-terminal macrolactam ring threaded by the C-terminal chain. The macrolactam ring is formed by the condensation of the N-terminal amino group of the core peptide with the side chain carboxylate of an Asp or Glu at position 7, 8 or 9. The N-terminal amino acid involved in the isopeptide bond is mostly a Gly and less frequently a Cys, Ser or Ala. The C-terminal tail is locked inside the ring by large amino acid(s) and/or disulphide bond(s) stabilizing the lasso topology (150-153). The size of isolated lasso peptides range from 15 to 24 residues with varied amino acid composition (147). The minimal gene cluster for lasso peptide biosynthesis consists of three genes encoding the precursor A and two processing enzymes B and C (154-156). Lasso peptides show diverse biological activities: antimicrobial, antiviral, receptor antagonist or enzyme inhibitor. Lasso peptides are subdivided into 3 classes according to the number of disulphide bonds (Figure 17 and Table 3) (5,100,147,148). Class I lasso peptides present two disulphide bonds while class II and III contain none or one, respectively. Class I lasso peptide are represented by five members, sviceucin, aborycin (RP71955), siamycins and humidimycin which share highly similar amino acid compositions and are only produced by Streptomyces species (7,157–162). Moreover, the structural elucidation of aborycin, siamycins and sviceucin showed that disulphide bonds Cys¹-Cys¹³ and Cys⁷-Cys¹⁹ connecting the ring to the C-terminal tail are strictly conserved. The structure for humidimycin is not available but the primary sequence showed exact same position of the cysteine residues suggesting the same secondary structure. Class II contains over 20 structurally diverse lasso peptides from different bacteria ranging from Proteobacteria to Actinobacteria and Firmicutes (163-165). A typical class II lasso peptide is represented by microcin J25 (MccJ25). Its structure consists of an 8-residue long N-terminal macrolactam ring, closed by an isopeptide bond formed between N-terminal amine of Gly¹ and the γ -carboxyl group of Glu⁸, which is threaded by the 13 amino acids C-terminal tail (150,151,166). The structure is stabilized by bulky amino acid(s) located on each side of the ring, such as in the case of Mccj25 where C-terminal tail is trapped by residues Phe¹⁹ and Tyr²⁰. Finally, BI-32169 is the sole member of the class III lasso peptide containing a single disulphide bond.





Class I, II and III assemble lasso peptides with two, none or one disulfide bond(s). Macrolactam ring is shown in green, C-terminal tail in blue, disulfide bonds in black. The bulky amino acids acting as plugs of the lasso topology are shown in pink.

The length of the N-terminal macrolactam ring of the known lasso peptides varies from 7 to 9 amino acids and the C-terminal tails length varies from 8 to 15 amino acids. The size of the tail above the ring, termed loop, can vary greatly between lasso peptides. For example, the loop of MccJ25 consists of 11 amino acids whereas the loop of capistruin consists of 4 amino acids.

The lasso topology is a very constrained structure and is reported to be remarkably stable against degradation by protease, heat shock and denaturating agents (166–169). While, thermal stability has been re-evaluated with the identification of new lasso peptides with different physicochemical properties. Indeed, several lasso peptides, such as caulosegnins, astexins, caulonodins, rhodanodin and rubrivinodin, can unthread to a branched-cyclic topology upon heating (163,169–171).

Table 3 – Overview of identified lasso peptides.

Residues involved in the isopeptide bond are shown in red. Cysteines involved in disulphide bond are underlined. "Bulky" residues that were shown to be involved in the stabilization of the tail into the ring are shown in orange. Residues not observed in the isolated lasso peptides but encoded by the structural gene are shown in blue. Extra post-translational modifications are shown in green.

Name	Producing organisms	Sequence	Class	Ref
Actinobacteria				
Aborycin, RP71955	Streptomyces griseoflavus	CLGIGSCNDFAGCGYAVVCFW	1	(157,172)
Siamycin I (MS-271/NP- 06)	Streptomyces sp. MS-271	CLGVGSCNDFAGCGYAIVCFW	1	(158,159)
Siamycin II	Streptomyces sp. MS-271	CLGIGSCNDFAGCGYAIVCFW	1	(158,159)
Sviceucin, SSV-2083	Streptomyces sviceus DSM 924	<u>C</u> VWGGD <u>C</u> TDFLG <u>C</u> GTAWI <u>C</u> V	1	(7,173)
Humidimycin	Streptomyces humidus F-100.629	C LGIGSCD D FAGCGYAIVCFW	1	(162)
Anantin	Streptomyces coerulescens	G FIGWGNDIFGHYSGDF	2	(174,175)
Lariatin	Rhodococcus jostii K01-B0171	G SQLVYR EW VG <mark>H</mark> SNVIKPGP	2	(153)
Sungsanpin	Streptomyces sp.	GFGSKPIDSFGLSWL	2	(176)
Lassomycin	Lentzea kentuckyensis sp.	GLRRLFADQLVGRRNI-CH3	2	(177)
Chaxapeptin	Streptomyces leeuwenhoekii C58	GFGSKPLDSFGLNFF	2	(178)
RES-701-1	Streptomyces sp. RE-701	GNWHGTAPDWFFNYYW	2	(179)
RES-701-3	Streptomyces sp. RE-701 and RE-896	GNWHGTSPDWFFNYYW	2	(180)
Propeptin	Microbispora sp. SNA-115	GYPWWDYRDLFGGHTFISP	2	(181)
SRO15-2005	Streptomyces roseoporus NRRL 15998	GYFVGSYKEYWSRRII	2	(173)
Streptomonomicin	Streptomonospora alba YIM 90003	S LGSSPYNDILGYPALIVIYP	2	(182)
BI-32169	Streptomyces sp. DSM14996	GLPWGCPSDIPGWNTPWAC	3	(164,165)

Proteobacteria

Astexin I	Asticcacaulis excentricus CB48	GLSQGVEPDIGQTYFEESRINQD	2	(170,183)
Astexin II	Asticcacaulis excentricus CB48	GLTQIQALDSVSGQFRDQLGLSAD	2	(184)
Astexin III	Asticcacaulis excentricus CB48	GPTPMVGLDSVSGQYWDQHAPLAD	2	(183)
Burhizin	Burkholderia rhizoxinica HKI 454	GGAGQYKEVEAGRWSDRIDSDDE	2	(163)
Capistruin	Burkholderia thailandensis E264	GTPGFQTPDARVISRFGN	2	(152,185)
Caulonodin I	Caulobacter sp. K31	GDVLNAPEPGIGREPTGLSRD	2	(171)
Caulonodin II	Caulobacter sp. K31	GDVLFAPEPGVGRPPMGLSED	2	(171)
Caulonodin III	Caulobacter sp. K31	GQIYDHPEVGIGAYGCEGLQR	2	(171)
Caulonodin IV	Caulobacter sp. K31	SFDVGTIKEGLVSQYYFA	2	(171)
Caulonodin V	Caulobacter sp. K31	SIGDSGLRESMSSQTYWP	2	(171)
Caulonodin VI	Caulobacter sp. K31	AGTGVLLPETNQIKRYDPA	2	(171)
Caulonodin VII	Caulobacter sp. K31	SGIGDVFPEPNMVRRWD	2	(171)
Caulosegnin I	Caulobacter segnis DSM 7131	GAFVGQPEAVNPLGREIQG	2	(169)
Caulosegnin II	Caulobacter segnis DSM 7131	GTLTPGLPEDFLPGHYMPG	2	(169)
Caulosegnin III	Caulobacter segnis DSM 7131	GALVGLLLEDITVARYDPM	2	(169)
Microcin J25	Escherichia coli AY25	G GAGHVP E YFVGIGTPIS F YG	2	(150,151,155,16 6,186)
Rhodanodin	Rhodanobacter thiooxydans LCS2	GVLPIGNEFMGHAATPGITE	2	(163)
Rubrivinodin	Rubrivivax gelatinosus IL44	GAPSLINSEDNPAFPQRV	2	(163)
Sphingonodin I	Sphingobium japonicum UT26	GPGGITGDVGLGENNFGLSDD	2	(163)
Sphingonodin II	Sphingobium japonicum UT26	GMGSGSTDQNGQPKNLIGGISDD	2	(163)
Sphingopyxin I	Sphingopyxis alaskaensis RB2256	GIEPLGPVDEDQGEHYLFAGGITADD	2	(163)

Firmicute				
Zucinodin	Phenylobacterium zucineum HLK1	GGIGGDFEDLNKPFDV	2	(163)
Xanthomonin III	<i>Xanthomonas citri</i> pv. <i>Magniferaeindicae LM941</i> ATCC 19865	GGAGAGEVNGMSPIAGISEE	2	(187)
Xanthomonin II	Xanthomonas gardneri ATCC 19865	GGPLAGEEMGGITTLGISQD	2	(187)
Xanthomonin I	Xanthomonas gardneri ATCC 19865	GGPLAGEEIGGFNVPGISEE	2	(187)
Syanodin I	Sphingobium yanoikuyae XLDN2-5	GISGGTVDAPAGQGLAGILDD	2	(163)
Sphingopyxin II	Sphingopyxis alaskaensis RB2256	GEALIDQDVGGGRQQFLTGIAQD	2	(163)

Paeninodin

Paenibacillus dendritiformis C454

AGPGTSTPDAFQPDPDEDVHYDS-2 **PO**3²⁻

(188)

It appears that thermal stability of lasso peptides is dependent of the size of the ring and the nature and the position of the amino acid acting as a plug (189,190). For example, a variant of capistruin with the amino acids plugs Arg^{15} and Phe^{16} substituted by alanine remains lasso folded but can be unthreaded when heated to 95°C (185). Other examples are xanthomonins identified in *Xanthomonas gardneri* that possess the smallest macrolactam ring identified so far, with 7 residues and which showed no alteration after 8 h at 95°C (187). *Caulobacter segnis* can produced three lasso peptides, from three different precursors, within one biosynthetic gene cluster yielding CsegI, -II and –III (169,189). Interestingly, only Cseg-II shows thermal stability although it shares high level of homology with Cseg-III. The Pro⁸ within the ring appears to be a key residue in the thermal stability of Cseg-II. Thermal stability of a lasso peptide at high temperature (*e. g.* 95°C) is an important feature from pharmacological and biotechnological perspective; nevertheless it may not reflect the stability in native environment.

II.4.b. Discovery

The lasso topology was described for the first time in 1995 with the characterization of the structure of RP-71955 (aborycin) which was isolated from *Streptomyces griseoflavus* during anti-HIV-1 screening in 1994 (**Figure 18**) (172). However, the first lasso peptide identified was anantin, isolated in 1991 from *Streptomyces coerulescens* during bio-guided screening of natural products for antagonist of the atrial natriuretic factor receptor, but its structure is not yet known (174,175).

In 1992, MccJ25, a plasmid-encoded lasso peptide isolated from the faecal strain *E. coli* AY25 was found during antimicrobial screening (186). The MccJ25 structure was first described as a head-to-tail cyclized peptide (167,168,191) but revised as lasso topology by multiple studies (150,166,186). MccJ25 became the model for biosynthesis and structure/activity relationships studies of lasso peptides because of its high level of production (1-10 mg/L) and its plasmid encoded genetic system. The genetic basis of MccJ25 biosynthesis consists of four genes named mcjA, -B, -C and -D encoding the precursor McjA, the maturation enzymes McjB and McjC and an ABC-transporter McjD for export and immunity (**Figure 19**) (154,155,167,186).



RP 71955

Figure 18 – RP-71955 lasso peptide structures (100). Left side, lasso peptides backbones. Right side, surface representation of ring plug residues.



Figure 19 – Organization of the MccJ25 gene cluster (192).

The genes mcjA (black arrow), mcjB and mcjC (white arrows), and mcjD (hatched arrow) encode the MccJ25 precursor McjA, putative maturation enzymes, and the immunity/export protein, respectively. The promoters are indicated by flags.

Initially, lasso peptides were identified through bioactivity-guided approaches such as antimicrobial activity, which led to the discovery of siamycins I and –II, propeptin or BI-32169 first described as a bicyclic compound (157,159,164,181). However, the lasso peptides were mainly identified using genome mining approaches since the discovery of the capistruin by Marahiel and co-workers using genome mining based on the maturation enzyme genes (7,152,183). In 2011, a mass spectrometry guided genome mining method termed peptidogenomic approach identified two new putative lasso peptides and theirs biosynthetic gene clusters, SRO15-2005 and SSV-2083 (173). In 2012, Link and co-workers performed a precursor centric genome mining study based on a small number of conserved amino acids in the precursor peptides. This approach resulted in the identification of 98 open reading frames (ORF) coding putative precursors and located in 79 gene clusters over 3000 prokaryotic genomes. Subsequently, this study allowed the heterologous expression in *E. coli* and the characterization of astexin-1 from the freshwater bacterium *Asticacaulis excentricus* (163,183).

In october 2016, Skinner and co-workers identified 1466 putative lasso peptide gene clusters in prokaryote genomes available in the national center for biotechnology information (NCBI) (6). These clusters were encoded in the genomes of Gram-positive (mainly *Actinobacteria* and *Clostridia*) and Gram-negative bacteria (α/γ *Proteobacteria*). Around 500 unique lasso peptides were predicted, some with new characteristics such as 10 amino acids length macrolactam ring, three disulphide bonds or putative post-translational modifications, demonstrating that lasso peptide family remains largely unexplored (6).

II.4.c. Biosynthesis

II.4.c.i Gene cluster organization

Link and co-workers identified in the astexins genes clusters a gene encoding a lasso peptide isopeptidase, which is not essential for the biosynthesis of astexins, and genome mining revealed that putative isopeptidases were present in many lasso peptide gene clusters (100,163,169,184). They generated a phylogenetic tree using the C-terminal domain of B or a short region of C homologues from 81 clusters and showed that it segregates into two different clades with cluster containing either almost exclusively isopeptidases or ABC transporter as well as biosynthesis only (**Figure 20**) (184). While the clusters containing ABC transporters are similar to that of MccJ25, those encoding an isopeptidase presented a conserved GntR-type regulator gene upstream and less frequently a set of conserved genes, one of which encoding TonB-dependent receptor protein downstream (**Figure 21 A**) (100,163,184).

The isopeptidase AtxE2 was shown to hydrolyse the isopeptide bond between Gly^1 and Asp^9 of astexin 2 but not astexin 1, demonstrating the specificity of the isopeptidase (**Figure 22**) (184). Moreover, AtxE2 does not hydrolyse the unthreaded astexin 2, suggesting that it recognizes and functions only on lasso folded peptide. In similar a fashion Atx1 functions only on astexin 1. Furthermore, these clusters do not encoded ABC transporter-like genes, indicating that the lasso peptides from these clusters might have another function than chemical weapon and could act intracellularly (100,184).



Figure 20 – Phylogenetic tree of known and putative lasso B homologues in 2013 (184). Clusters containing a peptidase are highlighted in blue, while clusters with an ABC transporter are shown in red. Clusters containing only an A, B, and C homologue are presented in black. Thicker line width indicates higher values of the Bayesian posterior probability. Branches with posterior probabilities <0.6 are indicated in the figure.



Figure 21 – Organization of ABC-transporter type gene clusters (**A**) and isopeptidase type gene clusters (**B**).

Gene encoding the precursors are in blue, the B proteins are in black and orange, the transporter in green, the proteins with unknown function in grey and the isopeptidase in brown.



Figure 22 – Lasso peptide isopeptidase AtxE2 hydrolyses the isopeptide bond (in blue) of astexin 2 (184). The macrolactam ring is shown in grey and the C-terminal tail is shown in yellow.

II.4.c.ii Maturation process

The biosynthesis of lasso peptides was mostly studied using MccJ25 as model (154,155,186). The biosynthesis of MccJ25 starts with the production of a 58 amino acid precursor called McjA which is consisted of a 37 amino acids N-terminal part and a 21 amino acid C-terminal part, forming the leader peptide and the core peptide, respectively (**Figure 23**). Previous studies have shown that both McjB and McjC act conjointly to convert McjA into MccJ25 and could be carried *in vitro* in the presence of Mg²⁺ and ATP (154,156,192). Mutational studies of precursors of several lasso peptides, including MccJ25, capistruin and caulosegnins, showed that a conserved Thr at the penultimate position in the leader sequence is essential for lasso peptide production (169,185,193,194). Moreover, it has been shown that this Thr can be replaced by Ser, Cys, Val or Ile demonstrating that the size and shape of the amino acid is more likely a recognition element rather as a catalytic residue for the biosynthetic machinery. Nevertheless, Thr is remarkably conserved (194).

The residues involved in the isopeptide linkage to form the macrolactam ring are essential for the production of lasso peptides. Substitution of either the N-terminal amino acids Asp/Glu were poorly not tolerated by the biosynthetic machinery or or (169,185,171,195,170,187). Similarly, mutations Glu⁷Ala, Glu⁷Asp and Glu⁷Ala/Glu⁸Asp in the precursor peptide abolished the production of xanthomonin demonstrating the tight specificity of the biosynthetic machinery regarding the macrolactam synthesis (187).



Figure 23 – Proposed mechanism of lasso peptide biosynthesis (100).

The maturation enzymes B and C act in concert to convert the precursor peptide A into the lasso peptide. B is a transglutaminase homologue that cleaves the leader peptide and function as a chaperone to prefold the precursor. Then, C catalyse the formation of the isopeptide bond to close the ring.

The lasso topology is installed by McjB and McjC in successive steps (**Figure 23**). Other studies suggest that McjB and McjC would form a structural complex named MccJ25 synthetase (156,194,196). Since McjA can be converted into MccJ25 using inner membrane protein extracts from *E. coli* harbouring the MccJ25 gene cluster, this complex might be membrane associated (196).

McjB and McjB-like proteins possess a C-terminal domain homologous to the transglutaminase core domain that displays a Cys-His-Asp catalytic triad conserved in cysteine proteases that catalyse either peptide bond formation or hydrolysis (192). The role of the putative triad (Cys¹⁵⁰–His¹⁸²–Asp¹⁹⁴) of McjB in cleavage of the leader peptide from the precursor was confirmed *in vitro* and *in vivo* by mutational studies (156,194,197,198). Studies also revealed an ATP-dependency of McjB suggesting a putative chaperone function of the N-terminal domain of McjB in the prefolding of the precursor in addition to the cleavage of the leader peptide although experimental proofs are still lacking, although the similarity of this domain to PqqD (analogue with chaperone function) seems to reinforce this hypothesis (156).

McjC-like proteins activate the γ -carboxyl group of Glu or Asp residue and thus forming the isopeptide bond with the newly freed N-terminal amine (151,156,192,194). McjC contains a C-terminal motif involved for ATP-binding reminiscent of the ATP/Mg²⁺-dependent enzyme asparagine synthetase B but lacking its N-terminal catalytic domain which catalyses the hydrolysis of Gln into Glu and NH₃ (192). The residues Ser¹⁹⁹ and Asp²⁰³ were believed to be important for the Mg²⁺ coordination and ATP interaction respectively. Mutational analysis confirmed that the residues are important for enzymatic activity of McjC (192).

II.4.c.iii Additional modifications

Genes encoding putative regulators and post-translational modification enzymes are present at the vicinity of the biosynthesis gene cluster of some lasso peptides (**Figure 24**). For example, the paeninodin and lassomycin gene clusters encode a kinase and an O-methyltransferase respectively while sviceucin and siamycin possess TCS encoded next to their gene clusters (**Figure 24**).

Lassomycin and paeninodin are currently the only examples of lasso peptide possessing extra PTM. Lassomycin is a short lasso peptide isolated from *Lentzea kentuckyensis* (177). Initially, the 3D structure of lassomycin was showed to be an unthreaded lasso topology but a study later demonstrated that lassomycin has probably a standard lasso peptide conformation (199). The structure elucidation showed that lassomycin possesses a methyl at the C-terminal, probably installed by the putative O-methyltransferase encoded downstream of the biosynthetic gene cluster (**Figure 25**) (177).



Figure 24 – LP gene clusters containing putative gene encoding PTM enzyme (A) or two-component System (B).



Figure 25 – Lassomycin structure and gene cluster (177)



Figure 26 – Proposed schematic of paeninodin biosynthesis.

Precursor peptide PadeA is phosphorylated at its C-terminal Ser by PadeK and then processed into the mature, phosphorylated lasso peptide by PadeB1, PadeB2 and PadeC. Cellular export of the lasso peptide is accomplished by ABC transporter PadeD (188).

Paeninodin, a lasso peptide isolated from the firmicutes *Paenibacillus dentritiformis* C454, was found to be phosphorylated at the C-terminal serine residue. A novel kinase responsible for this modification is encoded by *padeK* downstream of the precursor gene *padeA* (**Figure 26**) (188). Genome mining centred on PadeK identified many homologous proteins encoded by lasso peptide clusters in *Firmicutes* and *Proteobacteria*. Some of these clusters contain genes coding for new putative PTM enzymes that have been identified such as

nucleotidyltransferase and sulfotransferase (188). Numerous clusters feature additional biosynthetic genes such as PTM, especially in *Actinobacteria* and will probably be described in the future (183).

II.4.d. Biological activities

Lasso peptides, and in particular those isolated from actinobacterial sources, have been shown to be capable of diverse biological activities: some possess antimicrobial and antiviral properties as those observed for aborycin, siamycin I and II while others can antagonize receptors or inhibit enzyme functions such as propeptin inhibiting the prolyloligopeptidase, anantin antagonizing the atrial natriuretic factor or BI-32619 antagonizing the human glucagon receptor (5,147,148,100,157,159,164,174,181,200).

Antimicrobial activity of lasso peptides is generally limited to closely related-producing strains such as displayed by microcin J25, capistruin or lariatin. Microcin J25 exhibit minimum inhibitory concentration (MIC) from nanomolar to low micromolar range selectively against the Enterobacteria including the Escherichia coli, Salmonella newport and Shigella flexneri (186). MccJ25 from E. coli E. coli AY25 uses the outer membrane iron uptake receptor FhuA, the TonB/exbB/ExbD energy transduction complex and the inner membrane protein SbmA to penetrate the cells and to reach its target, the RNA polymerase (201–206). Similarly, capistruin act as inhibitor of RNAP (152,207). Astexin-1 from Asticacaulis excentricus displays antibacterial activity against the close related strain Caulobacter crescentus (170). Lariatin A and B were isolated from Rhodococcus jostii K01-B0171 and present growth inhibition against Mycobacterium smegmatis and M. tuberculosis (153). Lassomycin, isolated from Lentzea kentuckyensis, displays bactericidal activity against Mycobacterium tuberculosis (177). Lassomycin enhances by 7- to 10-fold the ATP hydrolysis the ClpC1 ATPase involved in protein degradation thus resulting in inhibition of proteolysis (177). Streptomonomicin, isolated from S. alba, displays low micromolar antimicrobial activity against Gram-positive strains, including Bacillus anthracis, but no activity on Gram-negative bacteria and fungi organisms (182). B. anthracis mutants resistant to streptomonomicin display phenotypic differences such as clumping cells and unusual long chaining as a result of mutation on the response regulator walR (182). WalR forms a TCS system with the histidine kinase WalK and that control genes involved in cell wall metabolism and cell division, explaining the growth defects of B. anthracis (208). Humidimycin, isolated from S. humidus F-100.629, enhance by 4.5-fold the antifungal activity against Aspergillus fumigatus of caspofungin, a lipopeptide that inhibits the 1,3-β glucan synthase involved in the generation of beta-glucan that is used for cell wall biosynthesis in fungi. A deletion mutant of genes skn7 and sakA in A. fumigatus displayed decreased resistance to humidimycin. Skn7 is a response regulator in the TCS controlling cell wall biosynthesis and osmoregulation, and SakA is a mitogen-activated protein kinase involved in osmotic and oxidative stress responses suggesting that humidimycin might affect a TCS. Siamycins from Streptomyces MS-271 exhibit antimicrobial activities against Enterococcus faecalis at micromolar concentration (209). Interestingly, submicromolar concentrations of siamycin reduced the production of gelatinase and gelatinase biosynthesis-activating pheromone (GBAP) and attenuated biofilms formation. Ma and co-worker demonstrate that siamycin inhibits auto phosphorylation of the FsrC protein but also effect on Vick sensor kinase and 8 others membrane sensors from E. faecalis V583. All of these proteins have different sensory domains but share a common ability to bind and hydrolyse ATP. Moreover, auto phosphorylation of sensor histidine kinase PrrB (RegB) from the Gram-negative strain Rhodobacter sphaeroides was also inhibited by siamycin I. Sviceucin, isolated from Streptomyces sviceus, shares similar structure and antimicrobial activities against E. faecalis with siamycin I and inhibits fsr quorum sensing of E. faecalis (7). Similarly, sviceucin shows moderate activity against E. faecalis (MIC above 10 µM) and can reduce the gelatinase production by *E. faecalis* at low micromolar concentrations (7).

II.4.e. Bioengineering using lasso peptides

The bioengineering potential of lasso peptides for drug design has been demonstrated by employing the lasso peptide as a highly compact and stable scaffold for a biologically active peptide epitope (210,211). In 2011, the integrin binding motif Arg-Gly-Asp (RGD) was grafted onto the Mccj25 loop using site-direct mutagenesis of the precursor generating a nanomolar integrin inhibitor (**Figure 27**). Moreover, the stability of this bioactive moiety was increased from total degradation in 4 h, to less than 50% degradation in 30 h for the grafted lasso peptide. Finally, in 2014, Thr¹⁵ residue of the RGD variant of MccJ25 was substituted by a Phe to increase target selectivity (211).



MccJ25(RGDF) Figure 27 – Epitope grafting in MccJ25 (100,210,211)

III. Ecological role of RiPPs

Research on specialized metabolites from microorganisms has long been oriented towards the exploitation of their antibiotic properties, while nearly neglecting the roles and functions of these molecules in nature (3,212). The most obvious roles of specialized metabolites reside in defence by the producing strains against competitions. However, many antibiotics have revealed unsuspected activity as low concentration, indicating that the ecological role of these molecules may be more complex and pleiotropic (4). This trend is also true for RiPPs, which also might play a key role as allelochemicals or antimicrobials but also contribute to the important processes such as bacterial communication or signalling for morphological changes.

Quorum sensing is a mechanism of cell-to-cell communication between bacteria, mediated by small diffusible signal molecules that trigger changes in gene expression in response to fluctuations in population density (213,214). In Gram-positive bacteria, and in particular across the *Firmicutes* phylum (low GC % Gram-positive bacteria), this process is mainly mediated by autoinducing peptides (AIPs). Two main signalling pathways have been characterized in Gram-positive bacteria, involving extracellular sensing through TCS or import followed by intracellular sensing (**Table 4**).

Staphylococcus aureus produces a set of 7–9 amino acids long RiPPs encoded by the gene cluster *agr*, containing a characteristic thiolactone ring in the C-terminal region, called AIP (for autoinducing peptides). These peptides are secreted and activate the AgrC-AgrA TCS, inducing a regulatory cascade that triggers autoinduction of the peptide production as well as expression of virulence factors (**Figure 28**) (215–217).



Figure 28 – AIP-mediated quorum sensing in *Staphylococcus aureus* (218). The AgrD precursor peptide, processed N-terminally by SpsB and C-terminally by AgrB, is secreted via the transporter AgrB in the form of a tailed thiolactone ring (AIP), which acts on the HK AgrC, inducing phosphorylation of the cytoplasmic domain. The phosphate is transferred to the RR AgrA, which

activates transcription from the two *agr* promoters. The P2 promoter drives the autoactivation circuit, and the P3 promoter drives transcription of RNAIII, which is the regulatory effector of the system. Inhibitory noncognate AIP molecules (red) compete with the cognate AIP (blue) for receptor binding.

AIPs and QS pathway	Example of precursor and processed AIP (gene cluster)	Associated sensors / regulators	Bacteria	Main processes activated	References
Agr-like cyclic peptides / TCS	$AgrD \rightarrow AIP (agr)$	AgrC-AgrA (TCS)	Staphylococcus aureus	Virulence	(218)
Lanthipeptides / TCS	NisA \rightarrow nisin (nis)	NisK-NisR (TCS)	Lactococcus lactis	Autoinduction of bacteriocin production	(123,219)
RNPP-mediated	NprX → heptapeptide (<i>npr</i>) H ₂ N- SKPDIVG- COOH	NprR	Bacillus cereus group	Virulence ? Necrotrophism	(220,221)
	Short hydrophobic peptide, SHP (<i>str</i>) H ₂ N- EGIIVIVVG- COOH	Rgg	Streptococcus thermophilus	Streptide production Competence Virulence Bacteriocin production Oxidative stress response	(103,104)

Table 4-Typical autoinducing peptides and QS pathways in Gram-positive bacteria

Several lanthipeptides such as nisin, microbisporicin, planosporicin, cytolysin, mersacidin have also been shown to autoregulate their biosynthesis via signal transduction by TCSs (222–225). For example, subinhibitory concentrations (nM) of extracellular nisin can be detected by the TCS NisK-NisR encoded in the peptide biosynthetic gene cluster, resulting in an autoactivation of nisin production.

Another pathway for QS regulation, reported in bacilli, enterococci and streptococci, involves cytoplasmic sensing of the AIPs. The peptides are secreted and proteolytically maturated to serve as intercellular signal. The cleaved peptides are then imported by an oligopeptide transporter and they interact with a family of cytoplasmic sensors called RRNPP (acronym given from the different sensors described: Rgg, Rap, NprR, PlcR and PrgX) (221). One example is the NprR/NprX system present in the *Bacillus cereus* group (B. anthracis, B. cereus, and B. thuringiensis species) (220). The pro-signalling peptide NprX is secreted and hydrolysed to its active form, identified as a heptapeptide. The heptapeptide, imported through the oligopeptide transporter Opp, interacts with the RRNPP sensor NprR, thus activating the expression of genes encoding the metalloprotease NprA, proposed to constitute a virulence factor (226) as well as degradative enzymes such as lipases or peptidases, which participate in the necrotrophism process in an insect larval model (227). Another example is provided by the short hydrophobic peptide (SHP) described in streptococci (103). At threshold concentrations, SHP is imported into the cytoplasm by the Ami oligopeptide transporter, where it modulates gene expression upon binding to the transcriptional regulator Rgg. The genes shp and rgg genes constitute the regulatory genes of a biosynthetic cluster (str) involved in the biosynthesis of streptide, a RiPP of unknown function recently characterized as a macrocyclic peptide containing an unprecedented Lys to Trp crosslink (104,228). In addition to streptide biosynthesis, Rgg regulates different processes such as competence, virulence, bacteriocin production and oxidative stress response (229).

AIP-mediated QS illustrates the role of RiPPs in the regulation of diverse and interconnected biological processes, such as activation of bacteriocin production and virulence factors. Antimicrobial RiPP production and host colonization or virulence can be intimately linked, when antimicrobial RiPP produced by bacteria influences the infected host microbiota. A direct link between antibacterial RiPP and virulence has been provided for lysteriolysin S (LLS), a bacteriocin containing thiazole, oxazole and methyloxazole heterocycles produced by *Listeria monocytogenes* (230). This bacteriocin is specifically expressed in the intestine of mice

orally infected with the producing strain, where it the mice microbiota, thus increasing *L*. *monocytogenes* persistence and virulence (231). Finally, thiopeptides also appear to contribute to shape the host microbiota (144). Biosynthetic gene clusters encoding thiopeptides are widely distributed in genomes and metagenomes of the human microbiota, and certain are expressed *in vivo*.

Another role attributed to RiPPs is to contribute to morphogenesis or biofilm formation. Many bacteria are known to form complex multicellular communities composed of specialized cell types, such as the fruiting bodies of myxobacteria or the aerial mycelium of streptomycetes. Such behaviour involves a complex coordination through extracellular diffusible signals, and RiPPs may contribute to these processes as signalling molecules. In addition, the surfactant properties of certain RiPPs have been shown to more directly contribute to the morphogenesis. As an example, the lanthipeptides SapB and SapT, produced by *Streptomyces coelicolor* and *Streptomyces tendae*, respectively, have been shown to contribute to the formation of aerial mycelium (232,233). SapB and SapT are hydrophobic peptides forming an amphipathic layer at the air-water interfaces. They are thought to function as biosurfactants that decrease the surface tension at the air/colony surface interface and thus facilitate the emergence of aerial hyphae.

Finally, an important role of certain RiPPs is related to metal chelation. In particular, methanobactins are copper-binding peptides, or chalkophores, produced by methane-oxidizing bacteria are expressed and secreted in response to copper limitation (112). These peptides contain a pair of heterocyclic rings with associated thioamide groups that form the copper coordination site. Methane is the sole carbon source for most methane oxidizing bacteria and the abundant membrane-associated methane monooxygenase involved in methane oxidation (pMMO) needs copper as cofactor. Methanobactins directly contribute to copper acquisition and have been also proposed to play a role in other processes such as signalling, membrane development and detoxification of reactive oxygen species.

IV. Objectives of the thesis

Genome mining of *Actinobacteria* has revealed a considerable number gene cluster likely involved in lasso peptide biosynthesis and which also present genes encoding for putative additional PTM enzymes such as O-methyltransferases or kinases, which would raise the chemical complexity of these lasso peptides and add new physicochemical properties. While the production of lasso peptides appears to be efficient in rich media for many *Actinobacteria*, the development of universal heterologous expression tools allowing the heterologous expression is till needed for some cluster to allow efficient production of the large (mg) amount generally needed for NMR characterization. Therefore, in the chapter I, this work deals with production and characterization of novel lasso peptides from *Actinobacteria* to increase the chemical repertoire of lasso peptides with structures harbouring additional PTMs.

As discussed above, the mechanism regulation, the expression of actinobacterial lasso peptide as well as the ecological role of lasso peptides remains elusive. In our laboratory, the previous results with the lasso peptide sviceucin demonstrated the essential role of an atypical regulation system for its production in the heterologous host *S. coelicolor*. Furthermore, sviceucin appears to act as an antimicrobial at high concentration, whereas at subinhibitory concentration, it probably interacts directly with the *fsr* QS of *E. faecalis*, suggesting that sviceucin might be involved in signalling network. Therefore, in the chapter II, we investigated the regulatory mechanisms of sviceucin expression and present some insights into the ecological role of sviceucin.

Materials and methods

I. Chemicals and biological materials

All chemicals were either from Sigma-Aldrich or Carl Roth, unless specified in the text. All molecular biology reagents were from Thermo Fisher (Fermentas) unless otherwise stated. Antibiotics used, bacterial strains, plasmids and primers used are listed in Annex 1, Annex 2, Annex 3 and Annex 4, respectively. Primers were designed using Geneious 8.0.5 with the following parameters: 17-22 bp in length, annealing temperature at 56-64 °C, and 55-65 % GC content (234).

The compositions of solid and liquid bacterial culture media are listed in **Annex 5** and **Annex 6**, respectively. For growth, *Streptomyces sviceus* DSM-924 and *Streptomyces venezuelae* J1CD were routinely maintained on ISP4 agar plates, whereas *Streptomyces coelicolor* M1146, *Streptomyces albus* J1074, and *Streptomyces lividans* TK24 were maintained in SFM agar plates. The strains produced aerial mycelium at 3-4 days of incubation at 30 °C and spores appeared after 5 days. Spores were scratched off and passed through non-absorbent cotton-wool in a syringe. Spores were pelleted by centrifugation, resuspended with 20 % glycerol and stocked at -20 °C. For liquid culture, *S. sviceus, S. coelicolor* and *S. lividans* spores from stock at -20 °C or clones from freshly grown plates were inoculated in 50 mL GYM medium in a 250 mL baffled flask and incubated with shaking at 30 °C. *S. venezuelae* and *S. albus* were inoculated into MYM and TSB respectively as above. *Escherichia coli* strains were grown in LB medium at 37 °C with shaking. For long term storage, 700 μ L of culture were mixed with 700 μ L of 50 % glycerol and stocked at -80 °C.

II. General DNA and microbiology methods

II.1. Purification of plasmids and cosmids

Plasmids < 10 kb were purified using the GeneJETTM Plasmid Miniprep Kit (Fermentas) according to manufacturer's instruction, whereas plasmids > 10 kb and cosmids were purified using alkaline lysis method (235). Briefly, a 4 mL of LB culture was inoculated and incubated overnight at 37 °C. Culture was split into 2 mL tubes and was pelleted at 13000 rpm for 1 min. Supernatants were discarded and each pellet were resuspended in 200 µL of

solution I (50 mM Tris-HCl, pH 8, 10 mM EDTA) and 5 μ L of RNase A (10 mg/mL). Cells were complemented with 400 μ L of solution 2 (200 mM NaOH, 1% SDS) and mixed gently by inversion before adding 300 μ L of solution 3 (3 M KOAc pH 5.5, adjusted with acetic acid). After 10 min incubation at room temperature, lysates were centrifuged at 16000 x g for 10 min at 4 °C and supernatant was transferred to a new 1.5 mL tube. DNA was precipitated using 600 μ L of isopropanol and pelleted by centrifugation at 16000 x g for 10 min at 4 °C. Supernatant was discarded and pellet was washed twice with 200 μ L of 70 % ethanol. Finally, the DNA pellets were air dried 5 min under laminar flow and resuspended in 30 μ L ddH2O or TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA).

II.2. Isolation of genomic DNA

Genomic DNA was extracted following the salting out procedure (236). Cell pellets from 50 to 500 μ L of culture were mixed with 500 μ L of SET buffer (20 mM Tris-HCl, pH 7.5, 75 mM NaCl, 25 mM EDTA) followed by the addition of 10 μ L lysozyme (50 mg/mL) and incubated at 37 °C for 30 min. A volume of 14 μ L of proteinase K (20 mg/mL) and 60 μ L of 10% SDS were added and the reaction was incubated at 55 °C for 60 min. Afterwards, 200 μ L of 5 M NaCl and 500 μ L chloroform were added sequentially followed by centrifugation at 12000 g for 10 min. The aqueous layer was transferred to a clean tube and 0.6 volume of isopropanol was added to precipitate DNA. The precipitated DNA was washed twice with 70 % ethanol and air-dried before being dissolved in 100 μ L of TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) or ddH2O. Purified genomic DNA was stored at -20 °C.

II.3. General Polymerase Chain Reaction methods (PCR)

For standard PCR, one reaction of 20 μ L was prepared with 0.2 μ L of 5 U/ μ L Taq DNA polymerase (VWR), 10-100 ng of template, 0.4 μ L of 10 mM dNTP, 1.6 μ L of 25 mM MgCl₂, 2 μ L of 10 X key buffer and 0.5 μ M of each primer. For DNA template of high GC content such as that from actinobacteria (60-70 % GC), DMSO was made up to 10 %. A standard program was used consisting of denaturation of DNA template for 2 min at 95 °C prior to 32 PCR cycles. One cycle consists of denaturation at 95 °C for 30 sec, annealing at 56-64 °C for 30 sec and elongation at 72 °C for appropriate time (1 min/kb). A final extension step was run for 10 minutes at 72 °C.

For cloning purpose, Phusion High Fidelity DNA polymerase was used. Denaturation temperature was raised up to 98 °C and time for elongation was calculated according to the high-speed of Phusion (15-30 sec/kb).

For colony PCR, a single colony of *E. coli* was picked from agar plates using sterile toothpick or sterile tip smeared onto the wall of a PCR tube containing water. PCR tubes were placed into the thermal cycler and heated at 95 °C for 10 min, then standard PCR reaction mix was added and PCR was performed as described above.

II.4. Agarose gel electrophoresis

Typically, 1 % agarose gel were cast in 1 x TAE buffer (40 mM Tris-HCl, 0.1 % acetic acid, 50 mM EDTA, pH 8) supplemented with 1 x SYBR® Safe DNA gel stain (Invitrogen). ExactLadder® DNA PreMix 100 bp Plus and 2-log (Ozyme) were used as a size marker. Agarose gel electrophoresis was performed in 1 x TAE buffer at 90-120 V for 25-45 min.

II.5. General cloning procedure

DNA fragments to be inserted were generated by PCR. Double digestion with appropriate restriction enzymes was carried out at 37 °C overnight in a total volume of 10 μ L for standard analysis and 50-70 μ L for preparative digest. Digested vectors were treated with FastAP thermosensitive alkaline phosphatase at 37 °C for 1 hour. The vectors and inserts were treated with an heat inactivation at 75 °C for 10 min, gel purified and retrieved using NucleoSpin® Gel and PCR Clean-Up kit (Macherey-nagel) following manufacturer's instructions. Briefly, digests were subjected to a standard 1 % agarose gel electrophoresis. Fragments of interest and vectors were sliced from agarose gel using a SafeXtractor tool (5 Prime) and purified. DNA was eluted with 15 μ L of elution buffer. Typically, ligation was carried out in a total volume of 10 μ L overnight at 15 °C or at room temperature using 1 μ L of T4 DNA ligase 5 U/ μ L and with ten times more insert than vector. The ligation mixture was heat inactivated at 70 °C for 10 min and the whole volume was used to transform 100 μ L of chemical competent *E. coli* DH10B cells.

II.6. Preparation and transformation of competent E. coli cells

II.6.a. Electrocompetent cells

E. coli was grown in a 10 mL LB culture overnight at 37 °C with shaking. A volume of 200 mL of LB medium was inoculated with 2 mL of preculture and incubate at 37 °C with shaking until OD₆₀₀ reached 0.4-0.6. Cells were pelleted at 3000 g for 10 min at 4 °C. Pellets were washed twice with 200 mL of 10 % glycerol. Finally, cells were spun down as above and resuspended gently into 2 mL of 10 % glycerol. Aliquots (50 μ L/tube) were immediately used or flash frozen in liquid N₂ and stored at -80 °C for long term storage. For transformation, electro-competent cells were thawed on ice and mixed with DNA. Electroporation was performed at 1250 V (5-6 ms) using Eppendorf Eporator® and 500 μ L of LB medium were added to the cells immediately after the electro shock. After 60 min incubation at 37 °C with shaking, 100 μ L of cells were plated on LB agar plate containing appropriate antibiotics except for transformation with ligation mixture where all cells were recovered by centrifugation and plated on plate. Plates were incubated overnight at 37 °C or 30 °C for larger plasmids or cosmids.

II.6.b. Chemical competent cells

E. coli was grown in a 10 mL LB culture overnight at 37 °C with shaking. A volume of 200 mL of LB medium was inoculated with 2 mL of preculture, and incubated at 37 °C with shaking until OD₆₀₀ reached 0.4. The culture was cooled down on ice for 15 min and then centrifuged at 3000 g for 10 min at 4 °C. The pellet was gently washed with 200 mL cold solution containing 0.1 M CaCl₂ and 10 % glycerol and left on ice for 30 min. Cells were spun down as above and gently resuspended in 2 mL of the same solution and then aliquoted into 50 μ L per tube. Aliquots were immediately used or flash frozen in liquid N₂ and stored at -80 °C for future use. For transformation, chemical competent cells were thawed on ice for 10 min and mixed with DNA. Cells were incubated at least 20 min on ice followed by heat shock at 42 °C for 1 min and subsequent incubation on ice for 10 min. Then 500 μ L of LB medium were added and cells were incubated at 37 °C with shaking for 60 min. After short spin centrifugation, supernatant was discarded and cells were plated on LB agar containing appropriate antibiotics and incubated overnight at 37 °C or 30 °C for larger plasmids or cosmids.

II.7. Site-directed mutagenesis

Site-directed mutagenesis was performed according to the protocol of QuikChange kit (Qiagen) to modify dsDNA plasmid using mutagenic oligonucleotides. Oligonucleotides were designed to get the desired modification in the centre of the sequence of at least 27 bp lengths. The primers were used in PCR reaction with dsDNA plasmid to form the mutant vector. Then, the parental vector was digested using DpnI restriction enzyme that cleaves methylated DNA resulting in nicked ssDNA plasmid. Finally, *E. coli* DH10b cells were transformed with the nicked plasmid which subsequently repaired it into dsDNA plasmid.

II.8. Modification of cosmids using PCR targeting

The modification of cosmids was carried out using REDIRECT® Technology (237). Briefly, the strategy consists of replacing the target sequence within a dsDNA vector, typically cosmid, with an antibiotic resistance cassette generated by PCR using primer with homology extension in *E. coli*.

In order to replace a gene of interest in the cosmid by the apramycin resistance gene, the disruption cassette *aac(3)IV* was generated by PCR using pIJ773 as template. Homologous sequences of 38 or 39 nucleotides to the upstream and downstream regions of the gene to be deleted were introduced into the primers. High-fidelity PCR was carried out in a 100 µL volume. PCR products were treated with 10 U of DpnI for 1 h at 37 °C to remove the template DNA and gel purified. The cosmid to be modified was transferred to E. coli Bw25113 harbouring pIJ790. The plasmid pIJ790 encodes phage-mediated homologous recombination systems λ RED increasing greatly the efficiency of ssDNA recombination in addition to a temperature sensitive origin of replication that requires 30 °C for replication (238-240). The transformed strain was grown overnight at 30 °C with shaking in 10 mL LB medium containing 25 μ g/ μ L chloramphenicol and appropriate antibiotic. A volume of 500 μ L μ L of the preculture was used to inoculate 50 mL of SOB or LB medium containing appropriate antibiotics and supplemented with 750 µL of 10 % L-(+) arabinose. This culture was grown at 30 °C with shaking for 3-4 h until OD₆₀₀ reached 0.5. Cells were pelleted at 3000 g for 5 min at 4 °C and washed twice with 50 mL of 10 % glycerol. Cells were resuspended in the remaining supernatant (100-120 µL) and were split in two fractions. One was added with 4-10 µL of desalted linear PCR product and the other was added with 4-10 µL ddH₂O as negative control. Linear PCR products were desalted on 0.025 µM VSWP membrane filters (Millipore) against

ddH2O for 30 min. Electroporation was performed at 2500 V followed by addition of 500 μ L of LB medium. After 1 h of incubation at 37 °C with shaking, cells were all plated on LB agar containing the corresponding antibiotic to select integration of the cassette and at 37 °C overnight which promoted the loss of pIJ790. Clones were picked into 4 mL of LB medium containing appropriate antibiotics and grown overnight at 37 °C. Cosmids were extracted by alkaline lysis and modification was confirmed by PCR and digestion by restriction enzymes. The confirmed modified cosmid was further transferred to *E. coli* DH10B and re-extracted in order to obtain single population of modified cosmids.

II.9. Conjugation methods for *Streptomyces* strains

The E. coli ET12567 strain harbouring pUZ8002 and the plasmid or the cosmid to be transferred was grown in 10 mL of LB medium containing appropriate antibiotics overnight at 37 °C with shaking. A volume of 25 mL of LB medium containing appropriate antibiotics was inoculated with 250 µL of preculture and incubated at 37 °C with shaking until OD₆₀₀ reached 0.6. Cells were pelleted by centrifugation at 3000 g for 10 min at 4 °C and washed twice with 25 mL of LB medium to remove antibiotics and resuspended in 500 µL of fresh cold LB medium and left on ice. For each conjugation, around 10⁸ Streptomyces spores were resuspended in 2xYT medium and heat shocked at 50 °C for 10 min for germination. Spores were cooled down on ice for 5 min and mixed with E. coli cells prepared above. The mixture was centrifuged, most supernatant withdrawn and the pellet was resuspended in the remaining supernatant. Cells were diluted 100 and 1000 times in LB medium and 100 µL of each dilution were plated on SFM agar plate containing 10 mM MgCl₂ and incubated 16-20 h at 30 °C. After incubation, 5 mL of top SNA agar containing 30 µL of nalidixic acid 50 mg/mL to kill E. coli and appropriate antibiotics for selection were overlaid onto SFM agar plates. Incubation was continued for 3-5 days at 30 °C and exconjugants were streaked onto new SFM agar containing appropriate antibiotics. Correct exconjugants were confirmed by PCR.

As an alternative method, mycelium from *Streptomyces* was used for conjugation. A volume of 25 mL culture of GYM medium in 250 mL baffled flask were inoculated with 5 mL of 2 days old preculture and incubated at 28 °C with shaking. Cells were spun down by centrifugation at 3000 g for 10 min at 4 °C, the pellet was washed with LB medium and resuspended in 1 mL of LB. A volume of 50 μ L mycelium cells suspension was mixed with 500 μ L *E. coli* cells suspension prepared as above by inversion. Then, 50, 100 and 200 μ L of

this mix were plated on SFM agar plates containing 10 mM MgCl₂. Selection of exconjugants was as described above.

III. RNA methods

III.1. RNA extraction

Around 10⁸ spores of *Streptomyces sviceus* were suspended into 50 mL liquid GYM medium in 250 mL conical baffled flasks for 2 days at 30 °C with shaking to allow dispersed mycelial growth. A volume of 1 mL of preculture was used to inoculate 50 mL liquid GYM in 250 conical flask. Culture was grown for 108 hours at 30 °C with shaking. Then, 1 mL culture (70-80 mg) was mixed with one volume of RNAprotect (QIAGEN) or RNAlater (Ambion) reagent before centrifugation. The pellet was harvested by centrifugation at 12000 rpm for 10 min at 4 °C and stocked at -80 °C or immediately processed.

Three methods were used to homogenize *S. sviceus* mycelia: 1) The pellet was ground in liquid nitrogen using a pre-cooled RNase-free sterile pestle and mortar. The resulting powder was scraped into 2 mL RNase-free Eppendorf and 1.5 mL of TRIzol® Reagent (Ambion) was added and allowed to stand for 5 min at room temperature. 2) Mycelia were resuspended with 1 mL of TRIzol® Reagent and sonicated on ice for 6 cycles of 5 sec on and 10 s off at 10-30 % amplitude using Branson digital sonifier model 450. 3) Mycelia were resuspended with 1 mL of TRIzol® and lysed by FastPrep®-24TM 5G using lysing matrix B (MP biomedicalsTM). RNA was then first isolated using TRIzol reagent following manufacturer's recommendation (AmbionTM).

Before further purification, the precipitated RNA was treated with 5 U of DNase I for 30 min at 37 °C to eliminate genomic DNA in a volume of 50 μ L. The reaction was quenched with 5 μ L of 50 mM EDTA and incubated at 65 °C for 10 min. The solution was made up to 650 μ L of RLT buffer and then added 350 μ L of 100 % ethanol. The RNA was further purified on RNeasy mini spin column (Qiagen) following the manufacturer's instructions. RNA was eluted twice with 50 μ L of RNase-free water. The concentration of RNA was measured using a Nanovue plusTM spectrophotometer (GE Healthcare) and typically ~100 μ g was obtained. The sample was diluted 1 in 10 μ L H₂O. The 260/280 ratio was around 1.9 and the 260/230 ratio was greater than the 260/280 reading, as expected for pure RNA. To check RNA quality, 1 μ g was separated on a 1% TAE agarose gel by electrophoresis. Gel tanks, trays and combs were
treated with RNAzap (Ambion) and rinsed with DPEC-treated water. RNA was visualized using SYBR® Safe stain. To confirm the absence of genomic DNA, a control PCR reaction was performed using 100-200 ng RNA as template.

III.2. Reverse transcription PCR (RT-PCR)

Before reverse transcription, 2.5 μ g of RNA were digested with 2 U of TURBOTM DNase (Ambion) in a volume of 25 μ L following the manufacturer's recommendation. First strand cDNA synthesis was performed using 200 U of RevertAid Reverse Transcriptase following manufacturer's recommendation for GC-rich template with 2 μ g of RNA and 0.2 μ g of random hexamer in 20 μ L final volume. In addition a no enzyme blank (RT-) and no template blank (RT₀) were run for each sample. Then, the reaction was chilled on ice and RNA was digested using 1 μ L of RNase H (Roche) at 37 °C for 20 min. The obtained cDNA was diluted 10 fold and 1 μ L was used as a template in standard PCR with Taq DNA polymerase and gene specific primers. PCR products were analysed by 2 % agarose gel electrophoresis. In parallel, PCR reactions with RT- and RT0 samples treated the same manner were set up, serving as negative controls.

III.3. Real-time quantitative PCR (qPCR)

QPCR was performed on a LightCycler® 2.0 (Roche) using LightCycler® FastStart DNA Master PLUS SYBR Green I (Roche). The PCR mix was composed of 4 μ L of diluted 1:100 cDNA as a template, 2 μ L of 5 X Master Mix, 2 μ L of 25 % DMSO and 2 μ L of 2.5 μ M primer mix. PCR Program consists of a denaturation step of DNA template for 10 min at 95 °C prior to 45 cycles. One cycle consist of a denaturation step at 95 °C for 20 sec, annealing at 60 °C for 20 sec and elongation of 15 sec at 72 °C. Finally, a melting curve was performed from 65 °C to 95 °C at 0.1 °C/sec speed.

IV. Heterologous expression of lasso peptide gene clusters

IV.1. Generation of lasso peptide expression system

IV.1.a. Cosmid libraries

Sau3AI fragments of genomic DNA of *Streptomyces sviceus, Stackebrandtia* nassauensis and Nocardiopsis alba were dephosphorylated and ligated with BamHI-cleaved

pWED4. The cosmids were encapsulated in phage particles using MaxPlaxTM lambda packaging extracts (Epicentre Biotechnologies), which were used to infect *E. coli* DH10b cells. Clones were probed for lasso peptide genes using a DIG-labelled DNA probe.

Two cosmids P4H7 and P17G4 were identified as containing the sviceucin gene cluster. Although, P4H7 contains the whole gene cluster, P17G4 lacks the TCS *sviR1-R2-G* and the beginning of *sviD1* (**Table 5**). The cosmids P1N3 and P3W14 were found to contain the lasso peptide gene clusters from *Stackebrandtia nassauensis* and *Nocardiopsis alba* respectively.

P4H7 derivatives were generated using PCR-targeting to replace the genes *sviD3D4* and *sviD1D2* by an apramycin resistance cassette yielding P4H7D1D2 and P4H7D3D4. Disruption cassettes for *sviD3D4* and *sviD1D2* were generated by PCR with Phusion high fidelity DNA polymerase using primers JM109/JM110 and JM111/JM112 respectively. The 1.2 kbp EcoRI fragment of pW60 was used as template. P4H7 Cosmid and disruption cassette were transferred to *E. coli* Bw25113 harbouring pIJ790 by electroporation as described above. Cosmid from clones resistant to apramycin were extracted and verified by PCR and restriction enzyme digestion.

Table 5 – Lasso peptide cosmid expression vectors

Origin	Name	Selection marker
Streptomyces sviceus DSM-924	P4H7, P17G4	Carb Hyg
Stackebrandtia nassauensis DSM 44728	P1N3, P1AF19	Carb Hyg
Nocardiopsis alba DSM 43377	P3W14	Carb Hyg

IV.1.b. Plasmid vectors

The plasmids used for lasso peptide expression were provided by Prof. Sergey Zotchev and are listed in **Table 6**.

Table 6 – Lasso peptide plasmid expression vectors used in this study.

Origin	Name	Selection marker
Streptomyces sp ADI94-01	p9401-LP1	Apra Ts
Streptomyces sp ADI94-01	p9401-LP2	Apra Ts
Streptomyces sp ADI98-10	p9810	Apra Ts
Streptomyces noursei ATCC 11455	pSnou	Apra Ts
Streptomyces venezuelae ATCC10712	pSven	Apra Ts

IV.2. Lasso peptide production in heterologous host

Lasso peptide expression vectors were transferred by conjugation described in the corresponding section to the heterologous host listed in **Table 7**. The media tested for the production of lasso peptides are listed in the same tables.

Table 7 – Lists of the heterologous host and the media used for lasso peptide production screening. *Minimal Media.

Heterologous host	Liquide media	Agar media
Streptomyces coelicolor M1146	GYM	ISP2
Streptomyces lividans TK24	MYM	GYM
Streptomyces albus J1074	MYMg	MS
Streptomyces venezuelae J1CD	MP5	R5
	Pi-SMM*	ISP4 [*]

IV.3. Lasso peptide production and extraction

Lasso peptide producer strains, heterologous or native host were usually grown in 250 mL baffled flaks with 50 mL of media. Cultures were inoculated 1 in 100 with a late preculture, typically 3 days of growth at 30 °C. Cultures were incubated at 30 °C with shaking for 5 to 7 days. Then, the mycelia were separated from the supernatant by centrifugation at 4000 rpm for 5 min at room temperature. Lasso peptides were extracted both from the mycelium and the supernatant.

The mycelium harvested from 30 mL of culture was extracted with 50 mL of methanol at room temperature with stirring for 3 hours. Extracts were filter through Whatman paper n°2 or alternatively cleared from debris by centrifugation prior to evaporation using a Rotavapor rotative evaporator. Dried extracts were resuspended with 1 mL of ACN/0.1 % FA in H2O mQ 1:1 (v/v). The supernatants were treated by solid phase extraction using Sep-Pak® Plus C18 cartridges (Waters). First, the stationary phase was conditioned with 20 mL methanol and 20 mL 0.1 % FA in H₂O mQ. After loading 10 mL of supernatant, the cartridge was washed with 30 mL of 0.1 % FA in H₂O mQ and with 20 mL of ACN/0.1 % FA in H₂O mQ 1:5 (v/v) prior to elution with 3 mL of ACN/0.1 % FA 1:1 in H₂O mQ (v/v). The eluate was dried under and vacuum, resuspended with 500 μ L of ACN/0.1 % FA 1:1 in H₂O mQ (v/v).

V. Liquid chromatography mass spectrometry (LC-MS)

Analysis of lasso peptides were performed on a micro HPLC Ultimate 3000 (Dionex) coupled on line with an ESI Qq-TOF Q-Star Pulsar mass spectrometer (Applied Biosystems). Samples were separated on ACE 5 C18-300 column (1 x 150 mm) (Interchim). Solvent A was composed of 0.1 % FA and solvent B was composed of ACN. Separation was performed with a flow of 40 μ L/min. Liquid chromatography program used was 10 % B for at least 7 min, then solvent B was increased to 80 % B in 40 min and hold for 5 min. Finally, solvent B was decreased to 10 % in 3 min and hold for 7 min.

The data were analysed using Analyst software (SCIEX). The extracted-ion chromatogram (XIC) for sviceucin was generated using the range 1042,0 - 1042,7 m/z.

VI. Generation of deletion mutants in S. sviceus-

VI.1. Inactivation using pKGLP2-based suicide plasmids

The plasmid pKGLP2 is a suicide vector carrying the reporter system that is based on gusA which encodes the β -glucuronidase enzyme in its backbone (241). This vector was initially chosen to inactivate sviR1 and sviR2 gene in S. sviceus. Sequences adjacent to the genes to be deleted were amplified using Phusion High-fidelity PCR. The primers used were JBR151/JBR152 and JBR153/JBR154 yielding upR1 and dnR1, and JBR155/JBR156 and JBR157/JBR158 yielding dnR2 and upR2. The PCR products upR1 and dnR2 were digested using XbaI and MfeI whereas dnR1 and upR2 were digested with BamHI and MfeI. The restriction sites were introduced into the primers. Digested upR1/dnR1 and upR2/dnR2 fragments were cloned into BamHI/XbaI sites of pKGLP2 using tripartite ligation, respectively, yielding pKGLP2.R1 and pKGLP2.R2. E. coli ET12567/pUZ8002 was transformed with the suicide plasmid and subsequently used for biparental conjugation with S. sviceus. Positive clones were selected on SFM agar plates supplemented with hygromycin. The plasmid pKGLP2 cannot replicate in S. sviceus, thus only clones which integrated the constructed plasmids into their genomes were resistant to hygromycin. Exconjugants were grown in GYM medium for 15 passages without antibiotic to promote the loss of the plasmid backbone by homologous recombination. After cultivation, cultures were plated onto SFM medium for sporulation. Spores were harvested, diluted and plated onto GYM agar medium to produces single colonies. After 3-4 days incubation at 30 °C each plate was overlaid with 4 mL of 0.4 mM 5-bromo-4chloro-3-indolyl- β -D-glucuronide (X-Glu) solution. After 1 h incubation at 30 °C, clones remaining white were picked from GYM plates onto MS agar medium and deletion was confirmed by PCR using primer JM13/JM14.

VI.2. Inactivation using modified cosmids

VI.2.a. Generation of P4H8, a P4H7 based suicide cosmid

The region containing the $\Phi C31$ integrase and the oriT of the sviceucin expression cosmid P4H7 was replaced by a kanamycin resistance cassette by PCR targeting, yielding P4H8. Disruption cassette was generated by Phusion High-fidelity PCR using primers JM15/JM16 and pKD4 as template. Disruption cassette and P4H7 were transferred to *E. coli* BW25113 harbouring pIJ790 by electroporation. P4H8 was extracted from clones resistant to kanamycin and confirmed by PCR and restriction enzyme digestion.

VI.2.b. Modification of P4H8 by PCR Targeting

The genes *sviA*, *sviR1*, *sviR2*, *sviD1D2*, *sviD3D4*, *sviF* and *sviG* within P4H8 cosmids were replaced individually with an apramycin resistance cassette yielding P4H8A, P4H8R1, P4H8R2, P4H8D1D2, P4H8D3D4, P4H8F, P4H8G and P4H8Gv2. Disruption cassette were generated by Phusion High-fidelity PCR using pIJ773 as template. Each disruption cassettes were transferred to *E. coli* BW25113 harbouring pIJ790 and P4H8. Cosmids from *E. coli* clones resistant to apramycin were extracted and modifications were confirmed by PCR and restriction enzyme digestion.

VI.2.c. Inactivation using P4H8-based cosmids

Modified cosmids were transferred to *S. sviceus* by conjugation and exconjugants were selected on SFM agar with apramycin. Colonies were double picked on SFM plates containing apramycin and hygromycin, respectively. Those *S. sviceus* clones resistant to apramycin but sensitive to hygromycin were picked and verified. Gene replacement was confirmed by PCR.

VI.2.d. Gene complementation in the deletion mutants of S. sviceus

S. sviceus inactivated for *sviR1*, *sviR2* and *sviA* were complemented using pMS82-based vector. The genes *sviR1*, *sviR2* and *sviA* were cloned under the control of *ermE** between KpnI/HindIII sites of pMS82 yielding pMS82cR1, -cR2 and –cA, respectively.

VII. Gene reporter assays

The respective intergenic regions (*i.e.* putative promoters of *sviA*, *sviD1*, *sviR1*, *sviG* and *sviC*) were fused with *gusA* which encodes the β -glucuronidase enzyme in pGUS plasmid. The promoters were amplified by PCR and inserted between the XbaI and KpnI site of pGUS yielding pAGUS, pDGUS, pR1GUS, pGGUS and pCGUS. For positive control, the promoter of *ermE** was amplified by PCR using pSNAxre as template and cloned into the same sites as mentioned above yielding pEGUS. The pGUS constructs and pGUS were transferred to *S. sviceus* by conjugation. These recombinant *S. sviceus* strains were cultivated on MS plate containing appropriate antibiotics for 4 days at 30 °C. Then 4 cm² of mycelia were transferred to 50 mL GYM liquid preculture containing the appropriate antibiotics and incubated 3 days at 30 °C under agitation. One mL of preculture was used to inoculate 50 mL GYM culture without antibiotics and incubated at 30 °C under agitation. At different point, 2 aliquot of 1 or 2 mL of culture were harvested and mycelia were pelleted by centrifugation. One aliquot was lyophilised to measure the dry weight of mycelium and the other was lysed using 1 mL of lysis buffer (50 mM phosphate buffer, pH 7, 0.1 % triton X-100, 0.27 % β-mercaptoethanol and 4 mg/mL lysozyme) for 1 h at 37 °C under agitation (223).

Then, cell lysate was cleared by centrifugation and 100 μ L was assayed in triplicate in a 96-well assay plate. One hundred μ L of Z-Buffer (16.1 g/L Na₂HPO₄ 7H₂O, 5.5 g/L NaH₂PO₄ H₂O, 0.75 g/L KCl, 0.246 g/L MgSO₄-7H₂O, adjusted to pH 7, supplemented with 2.7 mL/L βmercaptoethanol immediately before use) containing 20 μ L of 4 mg/mL p-nitrophenyl-β-Dglucuronide was added to each well. GusA activity was monitored by measuring OD₄₂₀ of the product 4-nitrophenol every 2 minutes at 30 °C for at least 30 minutes on a POLARstar omega plate reader (BMG LABTECH).

Alternatively, GusA activity was assayed on R2YE agar media containing 100 μ L of 40 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-glucuronide. Plates were inoculated with high concentration of spores and incubated at 30 °C.

VIII. Oxidative sensibility assay

Sensibility of *S. sviceus* strains to oxidative reagent listed in **Table 8** was tested using a disk diffusion assay. Square petri plates ($120 \times 120 \times 17 \text{ mm}$) were filled with 60 mL of DNA medium. DNA plates were overlaid with 10 mL of SNA containing 5.10^6 spores of the tested

strains. Six mm paper disk were lay on the top of SNA and 10 μ L of the different component were added. Inhibition diameters were measured after incubation at 30 °C for 36 h.

Letter	Name	Storage	Solvent	Stock (M)
А	Tert-butyl-hydroperoxide	2-8 °C	dH ₂ O	1
В	Cumene hydroperoxide	2-8 °C	DMSO	0.1
С	Diethylenetriamine/nitric oxide adduct	-20 °C	dH ₂ O	0.1
D	Plumbagin	-20 °C	DMSO	0.1
Е	Menadione (Protect from light)	Room Temp	DMSO	0.1
F	Phenazine methosulphate	-20 °C	dH ₂ O	0.1
G	Diamide	-20 °C	dH ₂ O	0.1
Н	Hydrogen peroxide solution	2-8 °C	dH ₂ O	1

Table 8 – Oxidative components used in this study.

IX. Morphology characterization

IX.1. Optic microscopy

Pictures of growing cultures were taken using a digital camera and were generally processed using ImageJ software.

IX.2. Scanning Electronic microscopy (SEM)

S. sviceus wt, Δ sviA, Δ sviR1 and Δ sviR2 were grown on R5 and R5- agar media for 7 days at 30 °C. Eight mm diameter pieces of the sample were cut off from the plate using tips and fixed with glutaraldehyde. Working solution of glutaraldehyde (3 %) was prepared by mixing 12 mL of 50 % glutaraldehyde solution with 88 mL of 0.1 M phosphate buffer (pH 7.4) and then complemented with 100 mL of dH₂O.

Samples were placed into 2 mL perforated Eppendorf and incubated in 3 % glutaraldehyde solution overnight at 4 °C. Samples were washed 3 times with phosphate buffer for 10 min, 3 times with 70 % ethanol for 10 min, 3 times with 95 % ethanol and finally with 3 times with absolute ethanol for 10 min. Samples were stocked in absolute ethanol and processed via critical point drying before analysis. Images were generated with a Hitachi SU3500 SEM at 15 kV.

X. Autoinduction of sviceucin

X.1. Gene reporter assay

Gene reporter assay was performed to compare gene transcription levels of *S. sviceus* with and without sviceucin. Culture of *S. sviceus strains* were prepared as above and induction was realized using 50 nM sviceucin after 36 h of growth. Then, GusA was assayed at different time after induction.

X.2. qPCR

To quantify and compare gene transcription levels of *S. sviceus* cultivated with or without sviceucin, qPCR was performed using 16S rRNA as the reference gene. Spores of *S. sviceus* were germinated 5 min at 50 °C into 500 µL of 2xYT medium at a final concentration of 10⁶ spores/100 µL. One hundred µL of spore solution were used to inoculate 3 cultures of 50 mL GYM liquid medium. After 108 hours incubation at 28 °C, cultures were split in two parts of 25 mL. One part was treated with 0.04 % methanol and the second part was treated 500 nM sviceucin and incubation was continued. After 20 min, 1 h and 3 h of treatment, 1 ml of each culture was harvested and RNA was extracted and reverse transcribed into cDNA. QPCR was performed with diluted 1:100 cDNA using primers YL213/YL214, JM35/JM36, JM53/JM54, JM78/JM79, YL201/YL202, JM82/JM83 and JM84/JM85 to compare transcription of 16S rRNA, *sviA*, *sviC*, *sviD1*, *sviD3*, *sviR1* and *sviG*, respectively, in induced/non-induced cultures of *S. sviceus*. Specificity of primers was checked using melting curves and 2 % agarose gel electrophoresis. Calibration curves were performed on cDNA from *S. coelicolor* P4H7 that produced sviceucin or on cDNA pools of *S. sviceus* samples.

Chapter I: Heterologous expression of lasso peptides from *Actinobacteria*

I. Introduction

Genome mining has revealed that microbes, and in particular *Streptomyces*, have a great potential for the production of bioactive SMs (44). However, traditional methods for the discovery of bioactive metabolites, involving bioactivity-guided screening of culture extracts, have allowed the discovery of only a subset of the bacterial SMs. Most of these new BGCs appear to be expressed at low level or not at all under standard laboratory conditions of cultivation (40). To explore this untapped reservoir of new bioactive molecules, many innovative strategies to trigger or to improve their expression levels have been developed over the last decades (2). These approaches either target a global effect (see I.1.a optimization of the cultivation conditions) or are pathway-specific (I.1.b-c).

I.1. Common strategies to trigger and improve the expression of cryptic or silent BGCs

I.1.a. Optimization of cultivation conditions

The expression of many, if not most, BGCs is controlled by regulatory elements in response to environmental changes used by the producing microorganisms to adapt and to survive. Therefore, the cultivation condition such as nutrients or environmental factors (*e. g.* temperature, aeration), can have a strong effect on the biosynthesis of SMs. For example, jadomycin B production was shown to be stimulated in *S. venezuelae* ISP5230 upon heat shock at 42°C or treatment with ethanol 6% (vol/vol) (242,243). The composition of the carbon source is also a well-known factor that interferes with SMs expression. For example, glucose prevents the production of actinorhodin in *S. lividans* by repressing the expression of the regulatory activator *afsR2* (244). Zeeck and co-workers set up a non-selective approach termed OSMAC (One Strain Many Compounds) which consists in the manipulation of media composition and environmental factors. Using this approach they succeeded to identify more than 100 metabolites from 6 microorganisms including bacteria and fungi in one screening campaign (245). Other approaches consist in complementing the media with chemicals, such as rare earth elements or sub-inhibitory antibiotics which was shown to trigger the expression of cryptic gene

clusters (246–249). The presence of a competing microorganism can also help to trigger the expression of specialized metabolites. For examples, the physical contact of *Aspergillus nidulans* and *Streptomyces hygroscopicus* induced the production of new aromatic polyketides in the fungi (250,251). Although being successful, optimisation of culture conditions is limited to the microorganisms that are amenable to cultivation.

I.1.b. Heterologous expression

Heterologous expression is a powerful approach for natural product discovery, particularly useful for microorganisms that are impossible or difficult to be grown under laboratory conditions. The heterologous hosts are genetically amenable stains for which the knowledge of metabolic pathways are available and genetic tools are well developed. Genetic manipulation in these hosts are sometimes required to facilitate the expression of exogenous BGCs (51), including inactivation of their own BGCs in order to decrease the metabolic competition/complexity and/or insertion of site-specific integration sites for foreign DNA fragments. Among established hosts, E. coli is commonly-used for proteobacterial BGCs and Streptomyces strains such as S. coelicolor or S. albus are used for gene clusters of actinobacterial origin. Recently Bacillus subtilis is shown to be also a useful host for Firmicutes clusters (252). Of particular interest are several modified S. coelicolor strains in which four antibiotic clusters (Actinorhodin, undecylprodigiosin, calcium-dependent antibiotic, and cryptic polyketide) were deleted, have been developed, allowing successfully heterologous productions of new actinobacterial metabolites (51). The workflow of heterologous expression involves the cloning of the whole BGC from source genomic DNA into a suitable vector followed by its transfer into the chosen host. To facilitate the first BGC capture step, innovative techniques have been developed over the last decades, including circular polymerase extension cloning (253), Gibson assembly (254), lamda-RecET-mediated direct cloning (255) and transformation-associated recombination in yeast (256).

I.1.c. Metabolic engineering

Metabolic engineering is a method that aims to trigger production of metabolites and/or increase the yield of production by optimizing biosynthesis pathways using genetic manipulation (257). The expression of SMs is often controlled by pathway-specific regulators, such as repressors or activators. Manipulating these regulators by overexpressing the activator or deleting the repressor are promising methods. For example, AveR, a members of the large

ATP-binding regulators of the LuxR family which contain an N-terminal NTP binding domain and a C-terminal LuxR-like helix-turn-helix DNA-binding domain, activates the transcription of genes involved in avermectin biosynthesis in Streptomyces avermitilis. Overexpression of aveR enhanced avermectin production (258). A similar example is the improved production of stambomycins in S. ambofaciens by the constitutive expression of the regulator SamR0484 encoded within the gene cluster (259). It worth noting that pathway-specific regulators are often controlled by global regulation. For example, in many Streptomyces, a global regulation occurs at the transcription level with the use of the rare leucine codon UUA that can only be read by one tRNA encoded by *bldA*. The TTA codon is present in about 2-3 % of genes essentially involved in developmental process, such as aerial mycelium, and production of many SMs, suggesting a positive regulation role of *bldA* in these processes (260). It has been shown that exchanging TTA to an alternative leucine codon present in the activator actII-ORF4 restored the production of actinorhodin in a bldA mutant (261). In fungi, epigenetic regulation can control the transcription of genes via the use of histone deacetylation and DNA methylation. The inactivation of the histone deacetylase encoded by hdaA in Aspergillus fumigatus resulted in the production of two SMs, a toxin and an antibiotic (262). Alternatively, manipulation of immunity proteins is a useful method. These are proteins that protect the producing organisms from their own toxic compounds, (e.g. by active transport) that are often present in BGCs.

I.1.d. Synthetic biology

Synthetic biology methods apply the principle of modularity and orthogonality in engineering to biology. In contrast to metabolic engineering, synthetic biology aims to design and construct a biosynthetic pathway that will generate the desired product (257,263–265). The biosynthetic pathway is constructed by a rational combination of standard parts such as promoters, genes and/or terminators forming a core platforms, which can be further improved with new parts, (e.g. aiming to diversify the final product structure). It can be as simple as a promoter-biosynthetic genes-terminator construct. Synthetic biology is typically performed in three steps: design, construction and optimization. The successful design of a pathway relies heavily on the knowledge of the native biosynthetic mechanisms as well as the properties of parts such as promoters and terminators (146) (266). Recent advances in DNA cloning techniques as mentioned above in I.1.b facilitate the construction of synthetic pathways.

I.2. Heterologous expression of lasso peptides

Lasso peptides were initially isolated as bioactive metabolites using bioactivity-guided screening. The production of several lasso peptides was optimized following an OSMAC-like approach. For example, the production of Mccj25 in *E. coli* AY25 was optimal in M63 medium (minimal medium) but hardly detectable in the rich LB medium seemingly because of the yeast extract (186). Moreover, substituting glucose by lactose or glycerol resulted in 2-fold increase of Mccj25 production, demonstrating that a catabolite repression might affect the biosynthesis of MccJ25. Another example is that of capistruin which was produced in trace amounts by *B. thailendensis* E524 in M9 medium but increased by 300-fold in M20 medium at 42°C (152,185). It appeared that the production of LPs from *Proteobacteria* has a preference for minimal media whereas that of LPs from *Actinobacteria* are more efficient in rich media (163). However, it is commonly observed that many LPs are difficultly produced under laboratory conditions (personal communication, Y. Li, M. Marahiel).

With the discovery of capistruin in 2008 guided by genome mining and heterologous expression (152), heterologous expression of proteobacterial LP clusters in *E. coli* has become a standard process and allowed the production of many new LPs, such as astexins, sphingopixins, caulonodins and caulosegnins (163,183). Typically, the whole operon containing LP biosynthetic genes was cloned under the control of a well-characterized promoter used in *E. coli*. Genetic engineering was sometimes necessary to improve the yield of these LPs. Replacement of the intergenic region between genes encoding the processing enzymes and the precursor by an *E. coli* specific ribosome binding site was shown to enhance the production level of LPs (163,183,194).

Lasso peptides expression, as most of the specialized metabolites, appears to be cryptic. To overcome this problem, the group of Prof. Marahiel performed heterologous expression of lasso peptide gene clusters from *Proteobacteria* in the heterologous host *E. coli*. First, new gene clusters of lasso peptide were identified using genome mining based on maturation enzyme of microcin J25. Then, these gene clusters were cloned under the control of a T7 promoter and a T7 terminator (152) and the intergenic regions between the gene encoding the precursor peptide and the genes encoding the biosynthetic machinery were replaced with a strong *E. coli* ribosomal binding site (163), leading to the identification of 26 lasso peptides from *Proteobacteria* and from *Firmicutes* (**Table 3**).

In contrast to proteobacterial LPs, most of LPs from *Actinobacteria* were identified through bioactivity-guided screening. This process often involves laborious culture screening because of the complex life cycles of *Actinobacteria*. The only example of successful heterologous production of actinobacterial LP is that of the discovery of sviceucin (7). Previously identified by a peptidogenomic study in *S. sviceus* (173), the sviceucin gene cluster was transferred to the genome of *S. coelicolor* by a cosmid-based approach, which led to the production of sviceucin up to 15 mg/L culture in a rich medium (7). Given that LPs from *Actinobacteria* present very interesting properties for pharmaceutical and biotechnology purposes (*e. g.* siamycins, aborycin, humidimycin, lassomycin) (157,159,162,177,209) and that genome mining has revealed novel LP BGCs from this phylum encoding additional PTM enzymes, it is of particular interest to develop an approach to be able to explore the chemical diversity of such LPs. The aim of this study was thus to establish a universal heterologous expression strategy for actinobacterial LPs.

II. Results and discussion

II.1. Genome mining

To select LP clusters of interest to study, genome mining was performed using the sviceucin biosynthetic gene *sviB* from *S. sviceus* as a probe to interrogate the publicly available *Actinobacteria* genomes using the Basic local alignment search tool (BLAST). Moreover, in collaboration with Prof. Sergey Zotchev (University of Vienna, Austria), LP gene clusters from draft genomes of a collection of *Actinobacteria* isolated from marine sponges in Norway were identified using a combination of antiSMASH and manual searches (**Figure 29**) (see publication in annex) (42).

Selection of representative gene clusters for further study was based on the novel features of the putative LP products. The selected LP gene clusters followed the conserved organization *A-C-B(B1/B2)*. Genes encoding transporter and/or putative PTM enzymes were frequently found downstream of the biosynthetic genes, seemingly forming an operon. On the contrary, regulatory genes were commonly seen upstream of the precursor A gene and appeared to belong to a different operon (**Figure 29**). The amino acid composition of putative LPs indicated that they display new features including non-canonical residues involved in the macrolactam linkage (Ala or Tyr) and the presence of one disulfide bond (*i. e.* encoded in *N. alba, Actinoalloteichus* sp. ADI127-17, *Streptomyces* sp. ADI94-01). Interestingly, the

sequences of the putative LPs encoded by *N. alba* and *Actinoalloteichus* sp. ADI127-17, belong to type III LP family whose members have only one disulphide bond, are very similar, although the DNA sequence homology of the encoding the biosynthetic enzyme between these two strains is low (30-50 %). Moreover, accessory genes at the vicinity of their BGCs are different (**Figure 29**). The cysteine pattern of these two putative LPs is unique among type III molecules, in that both cysteines are present at the C-terminal tail whereas other members of class III possess one cysteine involved in the macrolactam ring (**Table 3**). One can imagine that the resulting disulphide bond can forms a large plug below the macrolactam ring that blocks the C-terminal tail to unthread. Finally, *S. venezuelae* potentially produces a lasso peptide which contains a single cystein at the C-terminal end (**Table 9**).



Figure 29 – Organization of the putative LP gene clusters identified using genome mining.

Table 9 – Selected lasso peptides used in this study.Residues potentially involved in the isopeptide bond are underlined and residues potentially involved in the macrolactam ring are shown in red.

Producing organism	Sequence	Length	Ring size	Class	РТМ
Stackebrandtia nassauensis DSM 44728	G GQYNDTH <mark>D</mark> SRQYYY	15	9	2	Putative methylation
Nocardiopsis alba DSM 43377	GRPNWGFENDWSCVRVC	17	8	3	Putative methylation
Actinoalloteichus sp ADI127-17	GRPSWGFEADWSCVMVC	17	8	3	
Streptomyces noursei ATCC 1455	YFGLTGYENLFHFYDKLH	18	8	2	Putative phosphorylation
Streptomyces venezuelae ATCC 10712	GQGGGQSEDKRRAYNC	16	8 or 9	2	Putative acetylation
Streptomyces sp ADI98-10	GYFVGSYKEYWTRRIV	16	9	2	
Streptomyces sp ADI94-01	AFGPCVENDWFAGTAWIC	18	7 or 9	3	
Streptomyces sp ADI94-01	G SSPNGTA <mark>D</mark> ANAQYYY	16	9	2	

Table 10 - List of the putative lasso peptide identified by genome mining with their expected mass.

The expected mass was calculated considering the cysteines as forming a disulphide bond. Residues potentially involved in the isopeptide bond are underlined and residues potentially involved in the macrolactam ring are shown in red.

Producing organism	Sequence	Additional PTM	[M +H] ⁺	[M+2H] ²⁺	[M+3H] ³⁺
Stackebrandita nassauensisDSM-44728	G GQYNDTH <mark>D</mark> SRQYYY		1848.757	924.883	616.924
		Methylation M+CH ₃	1863.792	932.400	621.936
		2 x Methylation M+2(CH ₃)	1878.826	939.917	626.947
Nocardiopsis alba DSM-43377	GRPNWGFENDWSCVRVC		2004.859	1002.933	668.958
		Methylation M+CH ₃	2019.894	1010.451	673.970
		2 x Methylation M+2(CH ₃)	1878.826	939.917	626.947
Actinoalloteichus sp ADI127-17	GRPSWGFEADWSCVMVC		1909.782	955.395	637.266
Streptomyces noursei ATCC 1455	YFGLTGYENLFHFYDKLH		2246.071	1123.539	749.362
		Phosphorylation M+PO ₃ ²⁻	2326.037	1163.522	776.018
Streptomyces venezuelae ATCC 10712	G QGGGQS ED KRRAYNC		1707.762	854.385	569.926
		Acetylation M+CH ₃ CO	1750.780	875.894	584.265
		2 x Acetylation M+2(CH ₃ CO)	1878.826	939.917	626.947
Streptomyces sp ADI98-10	G YFVGSYK E YWTRRIV		2006.028	1003.518	669.348
Streptomyces sp ADI94-01	AFGPCVENDWFAGTAWIC		1966.825	983.916	656.280
	G SSPNGTA <mark>D</mark> ANAQYYY		1660.687	830.848	554.234

Based on this information the mass of the putative LP were calculated in respect with the gene cluster environment and are shown in **Table 10**. **Table 10** – List of the putative lasso peptide identified by genome mining with their expected mass. Moreover, genes potentially involved in PTMs are present in certain clusters, suggesting these lasso scaffolds will be further functionalized. Detailed analyses of these PTM genes are as below.

II.1.a. Stackebrandtia nassauensis cluster

The *S. nassauensis* LP gene cluster (Sna-LP) encodes a putative methyltransferase annotated as a protein-L-isoaspartate(D-aspartate) *O*-methyltransferase (PIMT) downstream of *snaB2* with an intergenic region of 44 bp. Using the structural prediction tool HHPred (267), SnaO shows similarity to a PIMT from *Thermotoga maritima* (Annex 7) (268). PIMT play a role in the repair of damaged proteins that contain isomerized Asp residues in the peptide backbone. They catalyse the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to the free carboxyl of an isoaspartyl residue forming a methyl ester, which facilitates subsequent regeneration of aspartate after a non-enzymatic reaction.

The presence of a PIMT suggests that the final LP could present a methylation or that PIMT might regenerates damaged LP which contains two Asp residues. In addition, the Sna-LP cluster encodes three proteins potentially involved in regulation, SnaR2, SnaG and SnaR1. The genes *snaR1* and *snaR2* encode a small putative transcriptional regulator that possesses a specific Xenobiotic respond element (Xre) HTH DNA binding domain at the N-terminus, respectively (Annex 7). Structural prediction of SnaG using HHPred showed that the C-terminal domain presents a similar structure with the tetracycline transcriptional repressor (TetR) family (Annex 7). Based on the proximity with the biosynthetic genes, it is most likely that SnaR1 is involved in the regulation of the LP production.

II.1.b. Nocardiopsis alba cluster

The *N. alba* LP gene cluster, Nal-LP, harbours two genes coding putative SAMdependent methyltransferases (SAM-MT), *nalM1* and *nalM2* at the flanking regions of the biosynthetic genes. NalM2 is encoded in the same operon as the LP biosynthetic genes, therefore likely to be associated with this LP pathway. Sequence analysis by BLAST revealed that NalM2 harbours conserved domains of phospholipid N-methyltransferase and ribosomal RNA adenine methyltransferase. Moreover, HHpred analyses showed that it has structural similarity with the phosphoethanolamine methyltransferase from *Plasmodium falciparum* and a tRNA methyltransferase CmoA involved in the modification of the anticodon triplet in *E. coli* (269). Therefore NalM2 is likely to perform methylation on the LP product. Given that methylation changes greatly the property of the parental molecule and that protein methylation plays an essential role in biology, such methylation event would have relevance with the physiological function of LPs. In terms of regulatory genes, a TCS composed of a response regulator (NalR1) and a histidine kinase (NalG) is encoded downstream of *nalM1* (Annex 7). NalR1 contains an N-terminal signal receiver domain (REC) and a C-terminal HTH DNA binding domain related to the LuxR family. Structure prediction indicated that it is most related to the nitrate/nitrite response regulator protein NarL involved in TCS.

II.1.c. Actinoalloteichus sp. cluster

The A127-LP gene cluster from *Actinoalloteichus* sp. encodes a thiol oxidoreductase, *aspT*, possibly connected to the disulphide bond formation or isomerization. A similar gene is present in the sviceucin gene cluster and was shown to be important although not essential for sviceucin biosynthesis (7). A gene *aspR* encoding a DNA binding transcriptional activator from the SARP family is present in the cluster. BlastP analysis of AspR revealed bacterial transcriptional activation domain (BTAD) related to SARP, suggesting that it activates gene transcription in this LP cluster (**Annex 7**).

II.1.d. Streptomyces noursei cluster

The *S. noursei* LP gene cluster, Snou-LP, encodes a putative Ser/Thr protein phosphatase SnoG (**Annex 7**). It possesses a conserved catalytic domain of bacterial Ser/Thr kinases at the N-terminus and one transmembrane domain at the C-terminus; therefore it is likely membrane-bound. SnoG could be involved in the phosphorylation of the LP product. Upstream of the biosynthetic genes, a transcriptional regulator SnoR is present which has sequence homology with LysR family transcriptional regulator found in *Actinobacteria*.

II.1.e. Streptomyces venezuelae cluster

The *S. venezuelae* LP gene cluster, Sven-LP, encodes a N-acetyltransferase-like protein (SveN). HHpred analysis revealed that SvenN is structurally related to spermidine N-acetyltransferase or a protein N-acetyltransferase of the RimJ/RimL family that transfers an acetyl group to the N-terminal AA or the amino group of an internal lysine of a protein substrate

(270). Genome mining using this putative N-acetyltransferase revealed many other putative LP BGCs, in particular in *Streptomyces griseus* subs. *griseus* strain NRRL WC-3480 and *S. albus* DSM41398, encoding putative LPs with a very high sequence homology (Annex 8). The presence of an N-Acetyltransferase gene suggests that Sven-LP bears acetylation as PTM. The Sven-LP cluster encodes two transcriptional regulators, *sveR1* and *sveR2*. SveR1 shows similarity to GntR-type transcriptional regulators frequently found in *Streptomyces* (Annex 7). Both SveR1 and SveR2 possess UbiC transcription regulator-associated (UTRA) domain, which is believed to modulate activity of transcriptional factors in response to the binding of small molecules.

II.2. Heterologous production based on native gene clusters

The heterologous production of lasso peptide focused on the Sna_LP and Nal_LP, as previously the laboratory was attempted to produce related LPs from *S. nassauensis* and *N. alba.* Culture of both strain in various media did not lead to the detection of expected LPs, suggesting these clusters were either silent or cryptic under these laboratory conditions. Therefore heterologous expression of these putative lasso peptides was pursued.

II.2.a. Cosmid-based method

The first strategy used was to transfer the native gene cluster cloned in a cosmid into a heterologous *Streptomyces* host. This method had allowed the production of sviceucin in *S. coelicolor* M1146 with a high yield (10-15 mg/L culture) (7). Cosmid libraries based on the pWED4 cosmid of *S. nassauensis* and *N. alba* genomic DNA were generated and among 2000 clones two cosmids were identified to contain the Sna_LP cluster, named P1N3 and P1AF19, whereas one cosmid among 1500 clones screened was confirmed to contain the Nal_LP cluster named P3W14. This work had been previously performed by Haiyan Ma. These positives cosmids were transferred by conjugation *Streptomyces* strains such as *S. coelicolor* M1146 and *S. venezuelae* J1CD (**Table 11**) (51,271–273). These strains are commonly used as heterologous hosts for the expression of SM BGCs from *Actinobacteria* and in particular those of *Streptomyces*. *S. coelicolor* M1146 has inactivated BGCs for Act, Red, Cpk and CDA, and *S. venezuelae* was inactivated for the BGC of chloramphenicol (Cml) and for the master regulator JadR1 involved in the biosynthesis of jadomycin B (51,273). Upon conjugation, the pWED4 cosmid allowed integration of the whole gene cluster into the chromosome of the heterologous host via a phiC31 site.

 Table 11 – Production of lasso peptides in different heterologous host containing cosmid based expression system.

Broducing organism	Vootor	Heterologous host			
r roducing organism	vector	S. coelicolor M1146	S. venezuelae J1CD		
S. nassauensis	P1N3	+ / ND	+ / ND		
	P1AF19	+ / ND	+ / NA		
N. alba	P3W14	+ / ND	+ / ND		

S. coelicolor was cultivated in GYM liquid medium and *S. venezuelae* was cultivated in MYM liquid medium for 5 days at 30 °C. **ND**: not detected. NA: not analysed.

S. coelicolor M1146 and *S. venezuelae* containing the Sna_LP and Nal_LP clusters, respectively, were cultivated for 5 days at 30 °C in liquid cultures. GYM was used for *S. coelicolor* and MYM medium was used for *S. venezuelae*. Culture supernatants and mycelia were extracted using SPE and methanol extraction, and the presence of lasso peptides was analysed by LC-MS. The expected masses of the putative lasso peptides were not detected in any of the combination.

I decided to examine the possible reasons for this results and observed that the gene clusters of Sna-LP and Nal-LP encode respective regulatory proteins suggesting that the production of these LPs is controlled by specific conditions (**Figure 29**). The failure of heterologous production could be also due to the lack of other regulatory elements that are encoded in the native genome elsewhere and thus not available within the BGC. Moreover, the heterologous hosts are *Streptomyces* belonging to the family of *Streptomycetaceae* which is phylogenetically distant from the native producers belonging to the family of *Nocardiopsaceae* (for Nal_LP) and *Glycomycetaceae* (for Sna_LP). Previously, it has been shown that expressing the microbisporicin gene cluster from *Microbispora corallina* using a cosmid-based method failed in several *Streptomyces* hosts, but was successful in a *Nonomuraea* strain that was closer to the producing organism (274). Native elements such as promoters and regulators from the native LP cluster would function better in a host closer to the source strain.

II.2.b. Co-expression with the pathway-specific regulator

Given that some chosen LP clusters encode regulatory elements, it would be a useful strategy to overexpress potential active regulators with the LP BGCs in order to improved LP production. As a proof-of-principle, Sna-LP cosmids were coexpressed with its putative regulator SnaR1. Prof S. Zotchev and collaborators cloned *snaR1* in pUWLoriT plasmid under

the strong promoter *ermE*p*, yielding pSNAxre. This allowed constitutive expression of the regulator gene. The vector pSNAxre was transferred to *S. venezuelae* J1CD harbouring the Sna_LP cluster and cultivated in different media for 7 days at 30 °C (**Table 12**). Cultures were extracted and analysed by LC-MS, however, no expected mass of Sna_LP was observed. It thus appeared that a complex regulatory network controls the expression of the LP BGC for which knowledge is greatly lacking. To circumvent this and to achieve LP production, genetic engineering such as exchange the native promoter to a characterized one would be necessary and hold promise.

 Table 12 – Co-expression of pSNAxre and P1AF19 in S. venezuelae in liquid and agar medium.

 ND : Not detected

Hotorologous Host	Vootors	Liquid rich medium				Liquid minimal media
Heterologous Host	Vectors	GYM	MYM	MYM- g	MP5	Glycerol (Pi-SMM)
S. venezuelae J1CD	P1AF19 pSNAxre	ND	ND	ND	ND	ND
	1					
Heterologous Host	Vectors		Agar ricl	n medium		Agar minimal media
Heterologous Host	Vectors	ISP2	Agar ricl	n medium R5		Agar minimal media ISP4

II.3. Heterologous expression using genetically-engineered clusters

II.3.a. Promoter exchange strategy

Prof. Zotchev and co-workers have identified a promoter *cmlf*p whose activity can be enhanced up to 5 times upon ethanol shock in *S. venezuelae* J1CD (273). Therefore, the biosynthetic genes of Sna_LP (*A-C-B1B2-O*) which seemingly formed an operon was cloned in a plasmid under the control of *cmlf*p in pSOK806, yielding pSNA, and transferred to *S. venezuelae* J1CD (this work was conducted by Prof. Zotchev and collaborators). The resulting recombinant strains were cultivated in TSB overnight and were seeded in different media at 30 °C (**Table 13**). After 9 hours of incubation, the cultures were supplemented with 6% ethanol and cultivated for 2 days at 30 °C. The supernatant and the mycelium were analysed by LC-MS, however, the putative LP remained undetected. Finally, all these failures about Sna_LP production in heterologous hosts prompted us to search for a more universal and applicable strategy.

Hotovologous host	Vootors		Liquid ric	Liquid minimal media		
Heterologous nost	vectors	GYM	MYM	MYM- g	MP5	Glycerol (Pi-SMM)
S. venezuelae J1CD	pSNA	ND	ND	ND	ND	ND

Table 13 – Expression of pSNA vector in *S. venezuelae* J1CD in liquid media and upon ethanol shock. **ND**: Not detected

II.3.b. Orthogonal two-plasmid expression system

In collaboration with Prof. Sergey Zotchev, we aimed to develop a method to be able to explore the chemical diversity of LPs from exotic sources. Bearing this in mind, LP genome mining was performed on a collection of marine *Actinobacteria* isolated from sponges (see II.1 section and publication in annex) as well as some terrestrial *Streptomyces* reference strains. Six gene clusters were chosen for the study including that of *Streptomyces* sp ADI94-01, *Actinoalloteichus* sp ADI127-17, *Streptomyces* sp ADI98-10, *Streptomyces noursei* ATCC 1455 and *Streptomyces venezuelae* ATCC 10712.

The work in Prof. Zotchev's laboratory had showed that assembly of selected LP genes involved in the biosynthesis (A-C-B1/B2(B)) and in the transport (D) under the control of the strong and constitutive promoter *ermE*p* in *E. coli* resulted in vectors containing mutated clusters, suggesting a toxicity issue (data not shown). To circumvent this problem, they have proposed to replace the promoter *ermE*p* with a promoter that is unlikely to be recognized in *E. coli*. They observed that the promoter region upstream of the LP precursor gene of the A127-LP cluster from the rare actinobacterium *Actinoalloteichus* sp is regulated by the regulator SARP within the gene cluster. Therefore, the gene clusters of 9401-LP, 9401-LP2, A127-LP, 9810-LP, Snou-LP and Sven-LP were assembled individually in *E. coli* under control of A127-LPp using Gibson assembly and a pSOK809-based template (work performed by Sergey Zotchev and coworkers). The resulting constructs and the plasmid overexpressing the regulator SARP, called pSARP, were transferred by conjugation into various heterologous *Streptomyces* hosts. In addition to the abovementioned *S. coelicolor* and *S.venezuelae*, tested strains included S. lividans TK24 and *S. albus* J1074, which both have been used as useful hosts for the expression of secondary metabolites from *Actinobacteria* (271,272,275–277).

The conjugation efficiency for the LP expression plasmids was generally low, suggesting a potential toxicity problem in *Streptomyces* even without SARP. In particular, no exconjugants could be obtained for the Sven-LP cluster. Conjugation of pA127-LP and pSnou-LP vectors into *S. albus* also failed. The production of the putative LP were tested in four commonly-used rich media and one phosphate-limiting defined medium (278). The latter choice was based on the observation that production of some antimicrobial peptides was triggered by phosphate starvation, (e.g. in the case of microcin J25) (279).

LC-MS analyses of cell extracts indicated that MYM or MYM-g medium are the most suitable medium for LP production since the expected masses of three peptides (9401-LP1, 9810-LP and Snou-LP) were detected, albeit in trace amount (**Table 14**). In parallel, seven solid media were tested for the production of LP and only 9401-LP1, 9810-LP and Snou-LP, which were also detected in liquid media, were detected by LC-MS (**Table 14**). The MSMS experiments confirmed the primary structures predicted for these peptides. The fragmentation patterns were typical of other LPs, which resulted mainly from breakage within the tail regions (**Figure 30**). Moreover, reduction of 9401-LP1 with DTT yielded a mass increase of 2 Da, confirming the presence of one disulfide bond (**Figure 31**).

In terms of growth media, solid cultures were clearly found to be more adapted to the liquid ones for LP biosynthesis, consistently with previous studies (173). However, a universal production medium was not achieved making culture optimization of recombinant strains still necessary. *S. albus* appeared a slightly better heterologous expression host because LP production was observed in a wider range of conditions or higher yields (**Table 14**).

With this strategy three lasso peptides could not be detected, 9401-LP2, A127-LP and Sven-LP. Actinobacterial LPs are the most bioactive with several cellular targets identified, in particular the RNA polymerase that has been shown to be a common target of LPs despite their different antibacterial spectra (177,202,207,280), therefore the toxicity of produced LPs to the expression host is suspected to be a major issue

LP name	940	1-LP1	9401	-LP2	A127	7-LP	981	0-LP	Snou-LP
Expression strain	S. albus	S. lividans	S. albus	S. lividans	S. coelicolor	S. lividans	S. albus	S. lividans	S. lividans
				Liqu	id medium				
GYM	ND	ND	ND	ND	ND	ND	ND	ND	ND
MYM	trace	ND	ND	ND	ND	ND	ND	trace	trace
MYM-g	trace	trace	ND	ND	ND	ND	ND	trace	trace
MP5	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pi-limiting defined medium	ND	ND	ND	ND	ND	ND	ND	ND	ND
				Soli	d medium				
GYM	ND	trace	ND	ND	ND	ND	trace	ND	ND
SFM	ND	trace	ND	ND	ND	ND	+	+	+
ISP2	+	trace	ND	ND	ND	ND	trace	ND	trace
ISP4	ND	ND	ND	ND	ND	ND	+	ND	trace
Marine Broth	ND	ND	ND	NG	NG	NG	ND	ND	NG
R5	trace	trace	ND	ND	ND	ND	trace	trace	+
TSB	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 14 – Summary of heterologous production using the two-plasmid based system.ND: not detected; NG: no growth; +: promising for scaling-up.



Figure 30 – Characterization of produced lasso peptides by tandem MS (a) 9401-LP1, (b) 9810-LP, (c) Snou-LP.

The striking difference in production between 9401-LP1 and 9401-LP2 indicates that the lack of a pathway specific ABC transporter in the 9401-LP2 gene cluster could account for the failure of 9401-LP2 synthesis in the heterologous host. Without the cognate transporter that functions as an immunity mechanism, the production of 9401-LP2 could be too toxic for the producing cell. In its native host, 9401-LP2 might be exported by a dedicated ABC transporter encoded elsewhere in the genome.

Similar reason could explain the failure of A127-LP BGC expression. It is likely that *Streptomyces* hosts strains cannot install effectively the biosynthetic machinery, in particular the ABC transporter, originating from a phylogenetically distant actinobacterium. Worth of note, the Snou-LP could be produced although no transporter genes were found in the cluster suggesting that Snou-LP might exhibit another role than that of a typical antimicrobial peptide.



Figure 31 – LC-MS analysis of the native (A) and the reduced 9401-LP1 (B). Extracted ion chromatograms and the mass spectra are shown.

It is remarkable that the Sven-LP genes could not be conjugated into *Streptomyces*. One particularity of this cluster, in addition to the lack of transporter genes, is the presence of a gene whose product shows similarity to ribosomal protein serine acetyltransferases. A previous study , showed that genes encoding ribosomal proteins prevented the conjugation of the gene cluster of GE2270, a thiopeptide identified in *Planobispora rosea*, in *Streptomyces* (281). The reason is unknown. It is therefore possible that *SveN1* involved in protein acetylation plays a role in the conjugation process or in the resistance mechanism.

III. Conclusion and perspectives

In conclusion, in a collaboration with Prof. Serguey Zotchev this work developed a SARP-controlled orthogonal expression system for the production of actinobacterial LPs in *Streptomyces*. Three out six lasso peptides, 9810-LP, Snou-LP and 9401-LP1, were successfully produced and detected using LC-MS. To date Snou-LP is the first LP to have a Tyr as the first residue and 9401-LP1 represents the second example of a type III LP. This expression system is suitable for LP bioprospecting from untapped *Actinobacteria*, and opens a convenient way to produce and engineer LPs with new features that would hold promise for peptide-based drug discovery. However, our data indicate that the success of this heterologous expression is mainly determined by the intrinsic properties of the LP cluster of interest and LP pathway engineering is most likely required in order to have a universal application for the developed strategy. Understanding the regulatory mechanisms and factors that trigger LP biosynthesis in the native producers will help to improve SARP-controlled expression system.

Currently the yield of LP production using this SARP-based system is far from being satisfying. At this stage, one could not reliably estimate the amount of peptides produced. Further work is required in order to achieve high level production that is necessary for the characterization and application of novel LPs. The most promising strategy would be genetic engineering. For proteobacterial LP clusters, exchange of the intergenic region between the precursor A gene and the genes coding maturation enzymes to a well-characterized ribosome binding site greatly increased LP yields in *E. coli* (163,183). Indeed, a previous study has identified a terminator-like structure as a common feature in this region of several RiPPs clusters, including those of LPs (198). Recently it has been demonstrated that the related terminator in the microcin C gene cluster controls the production of the final metabolite (282). Therefore eliminating this region reduces the complexity of regulation. Moreover, given that toxicity is a frequent issue, it is envisaged to overexpress immunity genes, i.e. transporter genes in LP clusters, together with biosynthetic genes. These strategies coupled with screening of more culture conditions (media and temperature) will hold promise for heterologous production of actinobacterial LPs.

Chapter II: Regulation mechanism and ecological role of sviceucin in *Streptomyces sviceus*

I. Introduction

I.1. Sviceucin as a good model to study LP regulation and ecological roles

Currently very few data are available regarding the regulation of lasso peptides production (except for microcin J25) as well as their ecological roles (283). There are only assumptions that some lasso peptides could function as cargo molecules based on the presence of an isopeptidase encoded in the gene cluster such as in astexin-type lasso peptides from proteobacteria (183). The aim of this chapter is to use sviceucin as a model to study its regulatory mechanism and to investigate its function for the producing strain.

Sviceucin is a class I lasso peptide produced by S. sviceus. It was first detected during peptidogenomic study using IMS by Dorrestein and co-worker in 2011 and later characterized by our group (7,173). The sviceucin gene cluster organization is similar to that of lariatins (A-C-B1/B2-D3/D4) and manual annotation revealed genes involved in regulation (sviG/R1/R2), transport (sviD1/D2) and putatively in disulphide bond formation (sviE/F) (Figure 32) (7). Sviceucin displayed moderate antimicrobial activity against Gram-positive bacteria including Bacillus megaterium and Staphylococcus aureus subsp. aureus ATCC6538 but no antimicrobial activity against Gram-negative bacteria. Interestingly, 1 µM of sviceucin was shown to inhibit gelatinase production in Enterococcus faecalis after 6 h of growth whereas MIC was above 10 μ M. This suggest that sviceucin might share similar mode of action with that of siamycins which were demonstrated to reduce gelatinase production via inhibiting the fsr quorum sensing at sublethal concentrations (209,280). Sviceucin was produced at low level by the S. sviceus under laboratory conditions thus a heterologous expression system was developed in S. coelicolor. It has been shown in the heterologous expression experiments that cosmids lacking the three regulatory genes (sviGR1R2) could not lead to sviceucin production. SviG shows homology to histidine kinases of the two-component system (TCS) and both SviR1 and R2 are related to the response regulators.

Α		В	
Stre (A	eptomyces sviceus Actinobacteria)		3D structure of sviceucin (NMR) (2,4 mM, d3-MeOH, 298 K, 600 MHz)
С			
Sviceucin	sviR1 sviR2 sviG sviD1 sviD.	2 sviC	sviB2 sviD3 sviD4 sviE sviF ABC ABC ABC SviE sviF sviB1

Figure 32 – Streptomyces sviceus (A), 3D structure of sviceucin (B) and its gene cluster (C).

(A) Streptomyces sviceus sporulating on SFM agar with aerial mycelium in white and spores in grey. (B) Schematic representation of sviceucin structure and the 3D structure modelled with after NMR experiments. The macrolactam ring, the tail and the disulphide bonds are shown in green, blue and black streak, respectively. Disulphide bonds are shown as gold yellow bond and the side chain of tryptophane residue is shown in blue. (C) Response regulators, *sviR1* and *sviR2*; Sensor histidine kinase, *sviG*; ABC transporter, *sviD1/D2* and *sviD3/D4*; Sviceucin precursor, *sviA*; macrolactam synthetase, *sviC*, PqqD-like protein, *sviB1*; Cystein protease, DoxX-like protein, *sviE*; Thiol-disulphide-oxidoreductase-like protein, *sviF*. The putative promoters were annotated with dark arrow.

The importance of this TCS system for sviceucin production makes sviceucin a good model to study the regulatory mechanism. Moreover, the possibility to grow and to genetically manipulate the producing strain together with the complex life of *Streptomyces* renders sviceucin system suitable to study the ecological roles of LPs.

I.2. Overview of growth and development in *Streptomyces*

Streptomyces are soil-dwelling bacteria ubiquitous in the environment. They show a complex developmental morphology with three distinct stages in their life cycle when they are grown on solid substrate: vegetative growth, aerial growth and sporulation (68). In liquid media, the aerial mycelia or aerial hyphae do not differentiate and sporulation does not occur for most of species. The development of *Streptomyces* starts with a dormant spore which meets a suitable environment. This triggers influx of water by an unknown mechanism resulting in swelling process followed by the growth of one or two germination tubes. The germination tubes elongate through the incorporation of cell wall materials at the apical tips and forme the vegetative mycelia. Branching filaments appear regularly resulting in a rapid growth of the vegetative mycelia.



Figure 33 – Germination of spores, vegetative growth, aerial growth and sporulation of *Streptomyces* sp.

The germination of spores of *Streptomyces* results in the vegetative growth and biomass accumulation. Exposure to stress such as nutrient limitation induces PCD and aerial mycelia formation, which coincides with the production of many antibiotics. Eventually, the apical cells of aerial hyphae are converted into chains of spores (68).

In contrast to the vegetative mycelia which grow deep into the surrounding substrate, the aerial hyphae erect to the air conferring a white and fuzzy appearance to the colony. An aerial hypha grows also by tips extension but does not branch. The emergence of aerial hyphae towards the air may involve influx of water generated by turgor pressure. Turgor pressure might be induced by the increase concentration of osmotically active solutes formed by the enzymatic cleavage of polymers such as glycogen or polyphosphate. A form of a programmed cell death (PCD) occurs in vegetative mycelia prior to the erection of aerial hyphae and is the starting point of the development of aerial hyphae. PCD involves autolysis of a part of the vegetative mycelia to release nutrient in the form of building blocks which are used by the remaining cells to differentiate into aerial hyphae. The biosynthesis of many antibiotics occurs during PCD probably in order to protect the nutrient released from uptake by other microorganisms. It could be also at the origin of the increased concentration of the osmotically active solutes. The erection of aerial hyphae is assisted by the production of hydrophobic metabolites such as the lanthipeptide SapB or proteins such as the chaplins and the rodlins, which ensure the filament to break tension surface at the air-colony interface.

Eventually, the apical cells of the multigenomic aerial hyphae are turned synchronously into unigenomic spores by several biological processes. The chromosomes are partitioned at regular interval within the apical cell and unigenomic compartments are formed through a synchronous septation. The cell wall of the compartments gets thicker and the grey polyketide pigments associated with the spores are produced. As *Streptomyces* are non-motile, spores are a great solution to colonize new environment. The spores of *Streptomyces* are resistant to desiccation and physical forces allowing bacteria to survive harsh conditions. They are, however, less resistant to chemical, UV and heat than endospores from *Bacillus subtilis* for example.

II. Results and discussion

II.1. Determining the operon structure in the sviceucin gene cluster

Inspection of the sviceucin gene cluster suggested that it could be transcribed in four operons: *sviR1*, *sviGR2*, *sviD1D2* and *sviA-F* (Figure 34). To prove the operon structure of *sviD1D2* and *sviA-F*, RT-PCR was performed on RNA extracted from the heterologous expression strain *S. coelicolor* P4H7 which produced a high amount of sviceucin. Primers were designed to amplify the intergenic regions of *sviD2-A*, *sviA-C* and *sviD4-E*. The PCR amplification of the intergenic region between *sviD2* and *sviA* failed to produce PCR products suggesting that *sviD2* and *sviA* are not transcribed in a single mRNA (Figure 34). The PCR amplification of the intergenic regions of *sviA-C*, and *sviD4-E* resulted in products

corresponding to the positive control. These results suggest that sviC and sviE are present in the same transcript and highly likely under the control of a sviA promoter, whereas sviD2 is transcribed separately.



Figure 34 – RT-PCR on S. coelicolor P4H7 cDNA.

PCR was performed with primers to amplify the intergenic regions of *sviD2-sviA*, *sviA-sviC* and *sviD4-sviE*. RNA was used as control for genomic contamination (-) and P4H7 cosmid DNA was used as positive control (g). Freshly 10x diluted cDNA was used as a template (c).

II.2. Growth dependence of sviceucin production

Based on the hypothesis that sviceucin is an autoinducing peptide that may be involved in the quorum sensing process, it is essential to establish the relationship of sviceucin production with regard to cell density (growth). Moreover, the knowledge of the stage of development when sviceucin is produced in *S. sviceus* would provide insights into its potential function.

Sviceucin was first identified in sporulating *S. sviceus* cultivated on agar media by a peptidogenomics study (173). In our laboratory, the production of sviceucin was typically observed after stationary phase and at low level in liquid culture. The production of sviceucin was monitored over time in *S. sviceus* cultivated in GYM medium using LC-MS analysis. Spores of *S. sviceus* was plated on MS agar media and incubated for 3 days at 30 °C and 4 cm² of white aerial mycelium was scratch off to inoculate 50 mL of GYM as a preculture. After 3 days, 3 mL of the preculture was used to inoculate 300 mL of GYM medium in 1 L Erlen with baffles and incubated 7 days at 30 °C (**Figure 35**). *S. sviceus* showed a typical *Streptomyces* growth with the generation of small clumps of cells that started to produce a grey pigment at high cell density at about 36-48 h. Subsequently, the cell density progressively decreased over time. Because of the non-homogenous growth of *S. sviceus*, it was difficult to measure the mycelia weight and the production of sviceucin before 36 h. Nevertheless, the production of sviceucin was observed from 36 h growth which might correspond to the beginning of the stationary phase and remained constant over time.



Figure 35 – Time course production of sviceucin in *S. sviceus*. The growth curve (black dot) was obtained by measuring the weight of dry mycelia and the production of sviceucin (triangle) was monitored by LC-MS analysis. (n=2)

In parallel, the expression of *svi* genes was investigated using a gene reporter system where the promoter of sviA (sviAp) encoding the precursor peptide was transcriptionally fused to gusA encoding the β -glucuronidase (GusA), yielding pA. This construct was inserted into the chromosome of S. sviceus via the ϕ C31 integration site. GusA activity was measured over time. The promoter activity of *sviA* was concomitant with the production of sviceucin which reached almost the maximum intensities at the stationary phase, but decreased upon prolonged growth (Figure 36 A). In the same manner, the activity of other putative promoters was probed, including those of *sviR1*, *sviD1*, *sviG* as well as *sviC* (Figure 36 C, E, D and B, respectively). Worth of note, although RT-PCR showed that sviA-F are transcribed in one operon, the intergenic region of *sviA*-*C* is 123 bp long and could harbour a regulatory element. Intergenic region between genes encoding the precursor peptide and the biosynthetic machineries are likely used to regulate the ratio between substrate and enzyme. For example, during the biogenesis of a RiPP antibiotic microcin C, the transcription of genes encoding maturation enzymes are decoupled from that of the precursor gene A under microcin production conditions, in order to have a high abundance of gene A transcript (282). Indeed, the gene reporter assay showed that the intergenic region between *sviA* and *sviC* could drive the expression of *gusA* to a level comparable of *sviA*p (Figure 36 B), suggesting that this intergenic region harbours a promoter (*sviC*p). Finally, the activity of *sviA*p and *sviC*p was similar and followed the same trend, which maximized at the beginning of the stationary phase and then decreased progressively. Gene reporter assays also indicated that the promoters of *sviR1* and *sviG* have higher activity compared to *sviA*p or *sviC*p, and remain rather stable during the stationary phase.



Figure 36 – Activity of the promoter of *sviA*, *sviC*, *sviR1*, *sviD1*, and *sviG*. Promoters were cloned upstream of the promoterless *gusA* in the integrative plasmid pGUS. These plasmids were introduced in *S. sviceus*, yielding *S. sviceus* pA, pC, pR1, pD, pG and pGUS. *S. sviceus* strains harbouring the gene reporter systems were grown in GYM medium for 9 or 11 days and GusA activity was measured and normalized with the DWM (dry mycelium weight) (n=2).

Due to technical difficulties, the relationship of growth vs. sviceucin production or gene transcription was not available before 36 h's growth, making it impossible to conclude if sviceucin is produced in response to the cell density. However, these experiments indicated that the highest activity of *svi* biosynthetic genes transcription occurs when cells enter the stationary phase leading to high amount of sviceucin. This is similar to the production of other secondary
metabolites in *Streptomyces*. In order to establish at which developmental stage the cells start to synthesize sviceucin, in the future it will be necessary to perform experiments on *S. sviceus* grown on solid media.

II.3. Probing the regulation mechanism of sviceucin biosynthesis

II.3.a. Is sviceucin biosynthesis controlled by SviR1 and SviR2?

Previous studies in our laboratory has demonstrated that the atypical TCS (*sviGR1R2*) is required for sviceucin production (7). The gene *sviG* encodes a histidine kinase which contains four transmembrane segments at the N-terminus, the dimerization and phosphoacceptor domain of the sub-family histidine kinase at the centre as well as an ATPase domain at the C-terminus. SviR1 and SviR2 are similar to response regulators, each containing a receiver domain at the N-terminus and a HTH DNA binding domain of the LuxR-like protein at the C-terminus. SviG would autophosphorylate, upon sensing of a signal in the extracellular medium, and modulate the activity of one or both response regulator (SviR1 and/or SviR2) via a phosphorelay. In order to address whether SviR1 and/or SviR2 regulate the expression of sviceucin, the genes *sviR1* and *sviR2* were inactivated in *S. sviceus* individually by allelic exchange. The inactivated strains were also complemented in *trans* using ϕ BT1 integrative vectors pMS82 containing respective regulator under the control of the strong and constitutive promoter *ermE*p*.



Figure 37 – Sviceucin production in different *S. sviceus* wt and mutant strains. Sviceucin was extracted from pellet and supernatant of *S. sviceus* wild type, *S. sviceus* sviR1::FRT-oriTaac(3)IV-FRT (Δ R1), *S. sviceus* Δ R1 pMS82cR1 (cR1), *S. sviceus* sviR2::FRT-oriT-aac(3)IV-FRT (Δ R2), *S. sviceus* Δ R2 pMS82cR2 (cR2). (n=3).

The strains were cultivated for 7 days in GYM medium and sviceucin was extracted. The production of sviceucin was analysed using LC-MS and compared to a titration curve for quantification (**Figure 37**).

In *S. sviceus* wt, sviceucin was mainly detected in the supernatant and a minor fraction remained in the mycelium. In *S. sviceus* ΔRI sviceucin production appeared to be abolished or was too low to be detected, while complementation of *sviR1* in *trans* in *S. sviceus* ΔRI restored the production of sviceucin. *S. sviceus* $\Delta R2$ produced reduced amounts of sviceucin compared to the wild type. Complementation of *S. sviceus* $\Delta R2$ with *sviR2* indeed restored sviceucin production to certain degree. It is interesting to note that sviceucin was present mainly in the mycelia in $\Delta R2$ mutant, whereas the corresponding complemented and the wild type strain secreted sviceucin to the extracellular medium. These experiments demonstrated that both SviR1 and SviR2 are involved in the regulation of sviceucin biosynthesis in a differentiated manner and SviR1 that seems to play a more important role than SviR2 since sviceucin is completely absent in $\Delta R1$.



Figure 38 – Putative phosphorylation site of SviR1 (Asp77) and sequencing of pMS82vR1. The wt sequence is at the top and the mutated sequence is at the bottom. The codon encoding the putative phosphorylation site is indicated with an orange bar.



Figure 39 – Putative phosphorylation site of SviR2 (Asp59) and sequencing of pMS82vR2. The wt sequence is at the top and the mutated sequence is at the bottom. The codon encoding the putative phosphorylation site is indicated with an orange bar.

In order to confirm the role of SviR1 and SviR2 as response regulators involved in TCS, the respective phosphorylation site Asp77 in SviR1 and Asp59 in SviR2 were mutated into an Ala using site-directed mutagenesis in the complementation vector pMS82cR1 and pMS82cR2, yielding pvR1 and pvR2, respectively (**Figure 38** and **Figure 39**). The constructs were transferred into the corresponding *S. sviceus* mutants and sviceucin production was tested after 7 days of growth in GYM medium as previously. Contrary to the previous experiment (**Figure 37**), a basal production of sviceucin was observed in *S. sviceus* $\Delta R1$ (**Figure 40**).



Figure 40 – Total production of sviceucin in *S. sviceus* Δ R1 pvR1 and *S. sviceus* Δ R2 pvR2. The total production of sviceucin corresponds to the addition of sviceucin extract from the supernatant and the mycelia. (n= 3)

Nevertheless, complementation with the wildtype sviR1 resulted in an increase in the amount of sviceucin, whereas complementation with $sviR1_D77A$ did not change the basal production level. These experiments confirm that SviR1 is indeed a response regulator, that functions via a phosphorelay and that it is the major regulator of sviceucin biosynthesis. The results with sviR2 complementation were rather challenging (**Figure 40**). The production of sviceucin in *S. sviceus* $\Delta R2$ reached a higher level when compared to the previous experiment and sviceucin was efficiently produced in the supernatant.

This unfortunately reflects the variability of *Streptomyces* cell cultures that are generally hard to control. Nevertheless, complementation with *sviR2* wild type and D59A variant did not lead to significant increase in sviceucin level. Taken together these data point out that SviR2 contributes to the regulation of sviceucin biosynthesis but with a minor effet.

II.3.b. Attempts to determine the SviR1 and SviR2 regulon in the svi cluster

In order to have insights into how SviR1 and SviR2 regulate gene transcription of the *svi* cluster, the activity of the promoters of *sviA* and *sviC* were measured at different genetic backgrounds: *S. sviceus* $\Delta sviR1$ and $\Delta sviR2$ using the gene reporter system described above. The cultures were prepared as previously and the activity of GusA was measured after 36 h of growth. The activity of the promoters of *sviA* and *sviC* were not affected by the inactivation of *sviR1* or *sviR2* individually (**Figure 41**). Further experiments are required to conclude, and particularly it remains to be seen if the gene transcription of the *sviA*-F operon is affected in a double deletion background $\Delta sviR1R2$. It also cannot be excluded at this stage that SviR1 or SviR2 control the transcription of other operon in the *svi* cluster.



Figure 41 – Promoter activity of *sviA* and *sviC* in *S*. *sviceus* Δ *sviR1* and Δ *sviR2*.

II.3.c. Effects of deleting other genes on sviceucin production

To investigate how genes other than the biosynthetic ones (*sviAB1B2C*) contribute to sviceucin production, genes encoding the histidine kinase putatively involved in TCS (*sviG*), the transporter (*sviD1D2* and *sviD3D4*) and the thiol oxidoreductase (*sviF*) were deleted individually by allelic exchange in *S. sviceus*. Care had to be taken so that gene inactivation did not cause polar effects for the expression of downstream genes. These mutant strains were subjected to analysis for sviceucin production by LC-MS. Sviceucin level in all mutant strains was several order of magnitude lower than in the wild type (**Figure 42**). Interestingly, deletion of *sviG* did not *completely* abolish sviceucin synthesis, indicating that other histidine kinases encoded in the genome may also activate SviR1 or SviR2.



Figure 42 – Sviceucin production in *S. sviceus* $\Delta sviD1D2$, $\Delta sviD3D4$, $\Delta sviF$ and $\Delta sviG$. Wild-type n=1; other n=3.

It also seems more likely that SviG forms a cognate TCS with SviR1, because sviceucin production level of $\Delta sviG$ and $\Delta sviR1$ are most similar. However, experimental proof is necessary to support this hypothesis. The effects of transporter gene deletion were not

surprising, as these transporters function generally as immunity proteins to pump the toxins out of the producing cells. At this stage it is not yet possible to discern the different roles of the two transporter systems. As for SviF, the thiol oxidoreductase, it had previously shown to be not essential in the disulphide bond formation in sviceucin (7), so its effect on sviceucin level is likely linked to the physiological function of the peptide (see later section).

To summarize, the response regulator SviR1 is a major player regulating sviceucin biosynthesis, while SviR2 is involved in the regulation but to lesser extent, although its exact function remains elusive. Interestingly, sviceucin is the only class I lasso peptide that harbour a second putative response regulator in its BGC (Figure 43). Substantial further work is required to finally determine the molecular mechanism of regulation.

II.3.d. Is sviceucin an autoinducing peptide?

The involvement of a TCS (SviG/R1 and/or R2) in the regulation of sviceucin biosynthesis leads to the hypothesis that sviceucin is an as autoinducing peptide. The biogenesis process of sviceucin is reminiscent to those of autoinducing peptides, such as nisin, which are post-translationally modified peptides and are exported outside the cell by dedicated ABC transporters as well as whose expression is generally modulated by a regulation mechanism involving TCS (176,244). The working hypothesis was that sviceucin would function as an extracellular signalling molecule which would induce its own expression by interacting with SviG, and subsequently leading to the activation of SviR1 and/SviR2 (**Figure 44**).

To test this hypothesis, the autoinduction effect of sviceucin was first investigated using real-time qRT-PCR experiments. *S. sviceus* was grown in GYM medium from 10^6 spores for 4 and half days at 30 °C. The spores were used to inoculate media in order to obtain a more reproductive growth. The apparently low concentration of spores used resulted in a considerable delay in the beginning of exponential phase. Therefore, the induction of sviceucin was performed at 108 h when the growth was in exponential phase and cellular-density was sufficient to extract RNA. The quantity of transcript 1 h after the induction with sviceucin was measured using qRT-PCR (**Figure 45**).

Under this condition, the *sviA* transcript was around 100-1000 times more abundant than the transcript encoding the biosynthetic enzymes and the transporter based on the ΔC_t (Figure 45 A).



Figure 43 – BGC of the class I lasso peptides.



Figure 44 – Hypothesis of work: sviceucin is an autoinducing peptide. The colour code used is the same as **Figure 32** (c).

High number of precursor gene transcripts appears to be general scenario for RiPPs biosynthesis (282). Nonetheless, the addition of sviceucin in the culture did not lead to significant increase of the expression of the biosynthetic genes compared to the non-induced controls (Figure 46).



Figure 45 – Sviceucin gene expression in *S. sviceus* in presence or absence of 0.5 μ M sviceucin. *S. sviceus* was grown in 50 mL GYM broth in triplicate for 108 h at 28°C. Cultures were split into two. One was treated with methanol (control) and the other was treated with sviceucin (induced). After 1 h incubation, cells were harvested and RNA extracted. QPCR was performed on 1 in 100 diluted cDNA. Quantity of PCR product for each transcript are shown in **A** and relative expression of each transcript are presented in **B**. (n=3)

The autoinduction effect of sviceucin was also tested in parallel using the *gusA* reporter system and the activity of GusA was measured 30, 120 and 1440 min after sviceucin induction. The results also showed no significant differences between the induced and non-induced conditions with regard to *sviA* and *sviC* expression (**Figure 46** and **Figure 47**).



Figure 46 – Autoinducing assay in *S. sviceus* wild type using gene reporter system pA. *S. sviceus* strain was grown for 36 h in GYM at 30 °C and treated with or without 50 nM sviceucin. (n=3)



Figure 47 – Autoinducing assay in *S. sviceus* wild type using gene reporter system pC. *S. sviceus* strain was grown for 36 h in GYM at 30 °C and treated with or without 50 nM sviceucin. (n=3)

We reasoned that sviceucin produced by the wild type could potentially interfere with the autoinduction tests, as observed for nisin (219). Thus, the *sviA* gene encoding the precursor peptide of sviceucin was deleted in *S. sviceus* by allelic exchange. The promoter activity of *sviA* and *sviC* was probed in this Δ *sviA* mutant strain. Two approaches were used to study the sviceucin effet were used: 1) sviceucin was added to the culture at early growth phase where the *svi* genes transcription is expected to be low; 2) sviceucin was added at the beginning of the cell culture. As shown in the **Figure 49-50**, in neither case, *sviA* nor *sviC* gene transcription was significantly affected by sviceucin.



Figure 48 – Autoinduction assays in *S. sviceus* $\Delta sviA$ using gene reporter system pA and pC. *S. sviceus* strains were grown for 36 h in GYM at 30 °C and subsequently treated with or without 50 nM sviceucin. (n=3)



Figure 49 – Autoinduction assay in *S. sviceus* $\Delta sviA$ using gene reporter system pA cultivated in media with or without 50 nM sviceucin. (n=2)

Next, we reasoned that the culture conditions we used may not be optimal for this autoinduction. The used GYM medium that is rich and contains glucose known to repress the expression of many SMs, therefore the feed-forward mechanism could have been inhibited in this condition of cultivation resulting in basal expression of sviceucin. Finally, it is commonly found in the literature that in many systems, quorum sensing mechanism is better demonstrated in particular appropriate media. Therefore, we set out to look for a medium by notably varying the carbon source so that the expression of *sviA* gene (thus the biosynthetic genes of sviceucin) would be activated.

The first mineral medium base we used was Pi-SMM (Phosphate-limited streptomycete minimal medium) (236). *S. sviceus* pA was cultivated in GYM medium as previously described and used to inoculate Pi-SMM with different carbon sources (236). A volume of 1 mL of preculture was washed twice with the minimal medium without carbon sources and resuspended in 30 mL with the minimal medium containing one of the carbon source. The cultures were incubated at 30 °C for 7 days (**Figure 50**). Under these conditions, *S. sviceus* grew poorly except for the cultures with sucrose and glycerol. However, the promoter activity of *sviA* in both media was similar as that in the GYM medium, with GUS units around 40-80 (**Figure 36 A** and **Figure 46**). These results showed that sucrose could be an interesting carbon source for the analysis of sviceucin expression.



Figure 50 – Growth (**A**) and GusA activity (**B**) of *S. sviceus* pA in Pi-SMM with different carbon source. (Pi-limited phosphate minimal medium) 7 days of growth at 30 °C. The red line indicates the threshold where no growth was observed. One culture of *S. sviceus* grown in Pi-SMM sucrose and one culture of *S. sviceus* grown in Pi-SMM glycerol gave aberrant measure for mycelium weight and subsequently for *sviA* p activity. Thus, they were omitted in this graph. (n=2)

In parallel, *S. sviceus* pA was grown in a second mineral media base PGMM (Pridham and Gottlieb modified minimal medium) and cultured analogously with different carbon sources (**Figure 51**) (284). In contrast to the previous experiments, *S. sviceus* pA grew well in most cultures except in the culture containing sorbitol of which no growth was observed.



Figure 51 – Growth (**A**) and GusA activity (**B**) of *S. Sviceus* pA in PGMM with different carbon source. (Pridham and Gotlieb modified minimal medium) after 7 days of growth at 30 °C. The red line indicates the threshold where no growth was observed. (n=2)

S. sviceus grew the fastest in the PGMM medium containing glucose, sucrose, arabinose and mannose, respectively. Therefore, GusA activity was tested at 4 days and at 8 days of growth when the remaining cultures reached stationary phase. The activity of GusA measured in most of the cultures reached almost 10 times the level obtained in Pi-SMM and in GYM. The culture containing maltose, xylose and sucrose exhibited the highest activity of GusA, although

large variability between each replicates was observed. The culture containing glycerol, arabinose, mannitol and galactose exhibited a similar activity than the culture containing glucose. *S. sviceus* grew well in mannose but did not exhibit intense GusA activity. This result is reminiscent to the previous experiment performed in Pi-SMM with mannose in which growth was observed but no GusA activity was observed. Thus, it suggests that mannose inhibits, somehow, the expression of *sviA*. Thus, the induction assay by sviceucin was finally performed in *S. sviceus* wt grown in PGMM containing sucrose and mannose, respectively, using the *sviAp* gene reporter system (**Figure 52**). The induction assay was conducted at 3 and 4 days of growth and the GusA activity was measured one hour after the addition of sviceucin. There were no significant differences on the 3 day-culture between induced and non-induced culture at both media. On the contrary, a clear trend of increased *sviA* gene transcription appeared upon induction under both conditions. This is a promising direction to pursue.



Figure 52 – Autoinduction assay in *S. sviceus* pA grown in PGMM sucrose or mannose. The autoinduction was tested after 1 h of induction using 50 nM of sviceucin at 3 and 4 days of growth. (n=3)

It is worth to note that, during the previous experiments (**Figure 48** and **Figure 49**), the activity of *sviA*p was shown to be about 10 times greater in *S. sviceus* Δ *sviA* as generally observed in wild type. The same trend was observed in PGMM media supplemented with sucrose and mannose (**Annex 10**). These results suggest that the inactivation of *sviA* increased the activity of the promoter of *sviA*. To confirm this observation, *S. sviceus* Δ *sviA* harbouring pA was complemented with *sviA* gene cloned under the control of *ermE*p* in the Φ BT1 integrative plasmid pMS82, yielding pMS82A, and cultivated in GYM medium at 30 °C. The empty vector pMS82 was used as control. The GusA activity was measured at early exponential phase, 19 h, and at the stationary phase, 40 h (**Figure 53**). At 40 h growth, the presence of *sviA*

gene indeed seemed to decrease the *sviA* gene transcription as compared to the control. This indicates that at least one form of the sviA gene product, such as mRNA, precursor peptide or the final product sviceucin, could have a negative effect on its own gene transcription.



Figure 53 – GusA activity of *sviA*p in *S. sviceus* Δ *sviA* complemented with pMS82A. The activity of GusA was measured in *S. sviceus* Δ *sviA* pA harbouring pMS82 or pMS82A. (n=3)

Due to the time constraint, this work could not be pursued further within the frame of this thesis. Current data, sometimes non-conclusive, demonstrate that the regulation of sviceucin production involves a complex mechanism, for which further knowledge is required in order to propose a revised hypothesis. One main technical difficulty associated with this work was the inability to reliably determine the growth phase of Streptomyces because of its non-homogenous growth. Since sviceucin production is apparently growth-dependent, it is essential that results obtained from wild type and mutant strains can be compared with confidence. The key questions to address in the immediate future are to determine which genes in the *svi* cluster are regulated by SviR1 and SviR2 as well as to experimentally prove that SviG forms a TCS with SviR1 or SviR2.

II.4. Morphological characterization of *S. sviceus* mutants

In order to get insights into the functional role of sviceucin in *S. sviceus*, the morphology and the growth of mutant strains of *S. sviceus* including $\Delta sviA$, $\Delta sviR1$ and $\Delta sviR2$, were characterized in solid media. The agar medium was preferred rather than liquid medium since *S. sviceus* undergoes full developmental cycles (*i. e.* vegetative growth, aerial mycelium and sporulation) on solid media. The strains of $\Delta sviR1$ and $\Delta sviR2$ were the first inactivated strains obtained, therefore, S. sviceus wt, $\Delta sviR1$ and $\Delta sviR2$ were cultivated on the SFM, GYM, TSB, ISP2 rich agar media and the ISP4 minimal agar medium for 17 days at 30 °C. Pictures of the colonies were taken at 4, 10 and 17 days representing early, mid- and late growth (**Figure 54**, **Figure 55**, **Figure 56**, **Figure 57** and **Figure 58**). Typical growth for S. sviceus strains was observed on every media except for TSB on which S. sviceus surprisingly arrested at a vegetative mycelium stage that looked like a bald phenotype (**Figure 57**). This phenotype was not observed for other streptomycetes such as S. coelicolor or S. venezuelae on TSB suggesting that this feature is typical of S. sviceus.

Otherwise, on the other media, the aerial mycelium has already emerged at 4 days on every media and the sporulation was clearly observed at 10 days of growth. On SFM, all strains produced yellowish droplets (**Figure 54**). On ISP4 agar media, the *S. sviceus* strains showed a precocious sporulation event that could be observed at 4 days of growth (**Figure 58**). In contrast to *S. sviceus* wt, $\Delta sviR1$ exhibited scarce sporulation on SFM agar medium at 10 days and the central patch of grey spores turned white at 17 days suggesting that a second PCD round was in progress (**Figure 54**). *S. sviceus* $\Delta sviR2$ exhibited an early sporulation, starting at 4 days, and a more abundant production of yellowish droplets. Moreover, the colony displayed a more wrinkled morphology than the wild type, although it could be hidden by its fuzzy appearance. The three strains showed similar growth on GYM, ISP2 and ISP4 agar media (**Figure 58**). Altogether, there were no major morphological and developmental differences between the wild type and mutant strains on these tested conditions.

The characterization of the *S. sviceus* strains including *S. sviceus* $\Delta sviA$ (which was not available previously) was further performed on R5 agar medium (**Figure 59**). *S. Sviceus* $\Delta sviA$ exhibited a slight delay in growth and the sporulation of *S. sviceus* $\Delta sviR1$ was also scarce compared to the wild type, as previously observed in SFM. The most striking difference was the growth of *S. sviceus* $\Delta sviR2$ that was blocked in the vegetative mycelia growth (**Figure 59**). Complementation of *sviR2* back to the $\Delta sviR2$ mutant restored the normal phenotype as the wild-type on R5 media (**Figure 60**). Different components such as CaCl₂ or proline were omitted in R5 medium, and neither of them had effects on the morphology of $\Delta sviR2$.



Figure 54 – Characterization of morphology of *S. Sviceus* wt, *sviR1* and *sviR2* cultivated on SFM medium. Scale bar = 1 cm



Figure 55 – Characterization of morphology of *S. Sviceus* wt, sviR1 and sviR2 cultivated on GYM medium. Scale bar = 1 cm



Figure 56 – Characterization of morphology of *S. Sviceus* wt, *sviR1* and *sviR2* cultivated on ISP2 medium. Scale bar = 1 cm



Figure 57 – Characterization of morphology of *S. Sviceus* wt, *sviR1* and *sviR2* cultivated on TSB medium. Scale bar = 1 cm



Figure 58 – Characterization of morphology of *S. Sviceus* wt, *sviR1* and *sviR2* cultivated on ISP4 medium. Scale bar = 1 cm



Figure 59 – Morphologies of the different *S. sviceus* strains grown on R5 media. *S. sviceus* wt, $\Delta sviA$ $\Delta sviR1$ and $\Delta sviR2$ were grown on R5 media 5 days at 30 °C.



Figure 60 – Morphology of *S. sviceus* cultivated on R5 agar medium and R5 derivatives agar media for 8 days at 30 °C.

The morphology of the *S. sviceus* strains was investigated using scanning electronic microscopy (Figure 61 and Figure 63). Figure 61 shows the vegetative mycelium of the different strains of *S. sviceus* after 3 days at 30 °C. The strains $\Delta sviA$ and $\Delta sviR1$ displayed thinner filaments than *S. sviceus* wild-type, which could be the result of the absence of sviceucin. $\Delta sviR2$ displayed only few segmented cells, although the lawn was initially far more

abundant as observed in the photographs and it could suggests that a part of the colony was ripped of the agar during the preparation of the samples. It was first thought that it was a contamination due to the bacille-like morphology but some long filament reminiscent to *Streptomyces* could be observed (**Figure 62**).

S. sviceus AsviA

S. sviceus wt





Images of the strains $\Delta sviR1$ and $\Delta sviR2$ complemented with pcR1 and pcR2 were generated after 2 days of growth in R5 medium (**Figure 62**). We clearly observed a difference in the length and the branching of the filament of $\Delta sviR1$ complemented with pcR1 when compared to $\Delta sviR1$, suggesting a role in sviceucin in the development of *S. sviceus* at early stage. In *S. sviceus* $\Delta sviR2$, the complementation with pcR2 restored the fragmented phenotype to the wild-type phenotype.

Images of the strains were also generated after 3 days of growth with or without 50 nM sviceucin added in the medium (Figure 63). Under SEM, *S. sviceus* wild-type long filaments

without branching corresponding to the aerial hyphae were observed. The strains $\Delta sviA$ and $\Delta sviR1$ displayed shorter filaments and their apical tip was swollen up. There were few branching points but they were not extending at this point. Interestingly, the addition of sviceucin in the medium restored the wild-type phenotype in *S. sviceus* $\Delta sviR1$ but not in $\Delta sviA$ neither in $\Delta sviR2$.

S. sviceus ∆sviR1





S. sviceus ∆sviR2

S. sviceus AsviR2 pcR2



Figure 62 – SEM photographs of *S. sviceus sviR1* and *sviR2* complemented with pcR1 and pcR2, respectively. Strains were cultivated on R5 medium for 2 days.

This would suggest that sviceucin and *sviA* must be present to restores the phenotype or that the sviceucin concentration used was not enough to restore the wild-type phenotype in *S. sviceus* $\Delta sviA$. In conclusion, the strains $\Delta sviA$ and $\Delta sviRI$ displayed thinner filaments at early growth and an atypical swollen up tips and few branching during aerial hyphae formation. The complementation with 50 nM of sviceucin in R5 medium restored the wild-type phenotype for

 $\Delta sviR1$ but not $\Delta sviA$, suggesting that sviceucin do play a role in the development of *S. sviceus* via an unknown mechanism that involves *sviA* gene products (mRNA, precursor peptide?).



Sviceucine 50 nM

Figure 63 – SEM photograph of *S. sviceus* and its derivatives cultivated on R5 medium with 50 nM sviceucin for 3 days. *S. sviceus* strains were grown on R5 agar medium for 3 days at 30 °C.



Figure 64 – Morphology of S. sviceus strains on R5 agar medium with 10 or 1% sucrose.

The precedent results also suggested that the phenotype of $\Delta sviR2$ is probably independent of sviceucin. Since the R5 medium, commonly used for manipulating *Streptomyces* protoplasts, contains high concentration of sucrose (10%) that is used to increase the osmolarity in order for the protoplasts to survive during regeneration in genetic manipulation experiment, we asked if sucrose had an effect on the phenotype of $\Delta sviR2$. The concentration of sucrose was thus decreased from 10% to 1% in order to observe whether the wild-type morphology was restored. The sucrose was also exchanged with another sugar osmotic stabilizer, maltose, in order to investigate whether the phenotype observed was specific to sucrose or to the osmotic stabilizer property. At 3 days and 10 days of growth, $\Delta sviR2$ showed its unique morphology while the other strains appeared to grow normally (**Figure 64**). It is worth to note that the complementation of the strain sviR2 with pMS82R2 restored the wildtype phenotype. On solid medium, the erection of the aerial hyphae is supported by the generation of the turgor pressure, thus osmoadaptation is essential. In *S. coelicolor* and *S. avermitilis*, the exposure of continual high external osmolarity induces the osmotic adaptation response which requires the response regulator OsaB (285–287). Indeed, *S. coelicolor* and *S. avermitilis* inactivated for *osaB* failed to develop aerial hyphae when grown on various media with 10% sucrose or 250 mM KCl. The phenotype displayed by $\Delta sviR2$ was similar to the phenotype exhibited by *S. coelicolor* inactivated for the sigma factor σ^{B} , which govern osmotic and oxidative stress response (288), when grown on MM supplemented with 500 mM KCl or R2YE (289). The *S. sviceus* strains were cultivated on ISP4 agar media supplemented with 500 mM KCl (37.28 g/L) to test their sensitivity to salt stress condition (**Figure 65**).

The strain $\Delta sviR2$ showed only vegetative growth, higher proliferation, as the diameter of the colony is bigger than wt, and a deep orange colour. Moreover, the colony dug into the agar suggesting agar decomposition as indicated by the more translucent part surrounding the colony. *S. sviceus* wild-type as well as the complemented strains cR1 and cR2 showed only vegetative growth suggesting that the aerial formation was impaired. The strains $\Delta sviA$ and $\Delta sviR1$ were not able to grow on ISP4 containing 500 mM KCl suggesting that they are sensitive to such hyperosmotic condition. The addition of 100 nM of sviceucin in the medium did not restore the aerial hyphae formation for *S. sviceus* wild-type or the growth of $\Delta sviA$ or $\Delta sviR1$.

The orange coloration and the morphology of the $\Delta sviR2$ colony developed on ISP4 plate supplemented with 0.5 M KCl was reminiscent to a similar phenotype observed in *Streptomyces natalensis* inactivated for *katA1*, encoding the mono-functional catalase KatA1, in the presence of sub-lethal oxidative stress (290). This study showed that the onset of aerial hyphae is probably associated with the emergence of intracellular oxidative stress caused by nutrient restriction. In this case, the inactivation of *katA1* increased the expression of antioxidant systems, one of which is an iron-chelator. They hypothesized that the decrease of iron-availability down regulates the expression of *bldN* and its regulatory cascade, including the hydrophobic sheath proteins rodlins and chaplins, resulting in the bald phenotype. Osmotic and oxidative stresses impaired the growth of *Streptomyces* and appear to be required for the transition from vegetative mycelium to aerial hyphae.

Sviceucin 100 nM



Figure 65 – Morphologies of *S. sviceus* strains cultivated 10 days on ISP4 with or without 0.5 M KCl and with or without sviceucin 100 nM.

II.5. Sensitivity to oxidative stress of *S. sviceus* mutants

Inspired by the similarity of $\Delta sviR2$ with the reported *Streptomyces* mutant deficient in the anti-oxidative response, we asked the question if there is a link between sviceucin and the sensitivity toward oxidative stress in *S. sviceus* (288). The strains were tested with different oxidative stressor reagents on using a diffusion assay disk of paper (**Figure 66**). A 10 mL top Soft-Nutrient-Agar (SNA) medium containing 5×10^7 spores was plated over the 60 mL of DNA in 12 cm² plastic plates. Then, the paper disks were laid on the plate and 10 µL the different oxidative reagents were added. The plates were incubated 48 h at 30 °C.

The sensitivity of *S. sviceus* wild-type was first assayed using different concentration of the oxidative reagents. *S. sviceus* wild-type was resistant toward high concentration of tertbutyl-hydroperoxide, cumene hydroperoxide, diethylenetriamine and diamide. However, inhibition zone was observed when exposed to 5 mM plumbagin, 25 mM menadione and phenazine methosulphate, and 25 mM hydrogen peroxide. The sensitivity of the *S. sviceus* mutant strains to the different oxidative reagent was then tested (**Figure 66**).

S. sviceus mutant strains exposed to 10 mM menadione and 50 mM H_2O_2 show a slightly higher sensitivity as indicated by the inhibition zone. The most striking difference was the higher sensitivity of all the mutant strains toward 25 mM and 50 mM phenazine methosulphate. Actually, the growth of all the strains were impaired by phenazine methosulphate at these concentration, however, *S. sviceus* wild-type could still grow close to the paper disk whereas all the mutant strain showed a true inhibition zone which was measured and reported into the **Figure 67**. Menadione, plumbagin and phenazine methosulphate are known to produce superoxide anion O_2 - which is involved in the generation of further reactive oxygen species (ROSs) that will cause oxidative damage to proteins, DNA, and lipids leading to cell death (291,292). The superoxide anions are maintained to a low level by superoxide dismutases (SODs) (293). The SODs are grouped accordingly to the metal species in the redox active centre, Mn^{2+} , Fe^{2+} , Cu^{2+} , Zn^{2+} , and Ni^{2+} . The later was characterized in *Streptomyces* and the genome of *S. sviceus* was found to encode one homologue of Ni²⁺-SOD.

Letter	Name	Storage	Solvent	Stock (M)
А	Tert-butyl-hydroperoxide	2-8 °C	dH_2O	1
В	Cumene hydroperoxide	2-8 °C	DMSO	0.1
С	Diethylenetriamine/nitric oxide adduct	-20 °C	dH_2O	0.1
D	Plumbagin	-20 °C	DMSO	0.1
Е	Menadione (Protect from light)	Room Temp	DMSO	0.1
F	Phenazine methosulphate	-20 °C	dH_2O	0.1
G	Diamide	-20 °C	dH_2O	0.1
Η	Hydrogen peroxide solution	2-8 °C	dH_2O	1



Figure 66 – Oxidative stress sensitivity of *S. sviceus* wt.

The high sensitivity of the *S. sviceus* mutant strains toward superoxide anion generated by phenazine methosulphate suggest that sviceucin could play a role in the protection of *S. sviceus* against oxidative stress. Moreover, the *svi* cluster encodes SviE, a DoxX protein with unknown function and SviF, a thiol-oxidoreductase. The homologs of this pair of proteins were recently identified in *Mycobacterium tuberculosis* as playing an essential role in coordinating a network of proteins involved in the thiol homeostasis (294). This strengths the hypothesis generated from our preliminary data, that sviceucin has a link with response to oxidative stress in *S. sviceus*.

III. Conclusions and perspectives

This part of work was exploratory as there were very few data previous results to formulate hypothesis. Our preliminary results demonstrate that sviceucin production involves a complex regulation mechanism with multiple levels. Further experiments are necessary to advance a more mature hypothesis. Key questions that can be answered in the near future are how SviR1 and SviR2 regulate the gene expression of the *svi* cluster and whether they really form a TCS with SviG. Subsequently experiments can be designed in order to identify the signals that activate SviG. Our data also pointed out a potential link of sviceucin to the oxidative stress response which would be also connected to the morphological development in *Streptomyces*.







Diethylenetriamine/nitric oxide adduct 50 mM







Hydrogen peroxide 50 mM







Phenazine methosulfate 50 mM



Figure 67 – Oxidative stress sensitivity of different S. sviceus deletion strains.

Conclusion and perspectives

Lasso peptides are RiPPs with a remarkable mechanically-interlocked topology (100,147,283). The structure, reminiscent to rotaxane, consists in an N-terminal macrolactam ring threaded by the C-terminal tail which is trapped by steric interaction through bulky amino acids and/or disulphide bonds. This endows them with highly compact and stable structure. Lasso peptides exhibit potent biological activities such as antimicrobial and these activities combined with their stability, make them good candidates for pharmacological and biotechnological purposes.

In the first part of this thesis, three new lasso peptides with unusual starting amino acids or new representative of the class III were heterologously expressed and characterized by mass spectrometry. In the second part, the physiological roles of sviceucin in *S. sviceus* was investigated and have shown that sviceucin production was growth dependent, that the regulator SviR1 and SviR2 were involved in the regulation mechanism of sviceucin and have raised the hypothesis that sviceucin might play a role in the osmotic and/or oxidative stress response.

I. Heterologous expression of lasso peptides from *Actinobacteria*

Genome mining of lasso peptide was performed using the sviceucin biosynthetic gene *sviB* from *S. sviceus* as a probe in actinobacterial genomes in NCBI and in a collection of *Actinobacteria* isolated from sponges in Norway of our collaborator Prof Zotchev. Among the gene clusters identified, 8 were selected for their particularity such as additional genes encoding putative PTM enzymes. The presence of genes encoding putative regulatory proteins suggests that the expression of the lasso peptide gene clusters are controlled by a complex regulatory network in the producing organism and that specific conditions must be required to trigger the expression of lasso peptides. Different approaches such as the cosmid-based vector used for the heterologous expression of sviceucin, the co-expression of pathway-specific regulator and promoter exchange were employed for the heterologous expression of lasso peptides. Most of the attempts failed to produce the predicted lasso peptides, except the orthogonal two-plasmid expression system developed by our collaborator, Prof Zotchev. This system is based on the characteristic of the lasso peptide gene cluster from *Actinoalloteichus* in which a gene encodes a SARP protein that positively regulates the expression of the lasso peptide genes.

Three out of six lasso peptides were identified and characterized by LC-MS using this method. The orthogonal two-plasmid expression system is suitable for lasso peptide prospecting, however, the production yield is insufficient for structural or biological activity characterization. The expression system needs to be improved by further genetic engineering. For example, the intergenic region between the precursor gene and the biosynthetic machinery could be exchanged with an *Streptomyces* ribosome binding site such as AAAGGAGG sequence (163,183,295). This region probably contributes to the regulation of the expression of lasso peptides genes and would be a good target for the first genetic modification (198). The ABC-transporter present in a large majority of lasso peptide gene clusters would be another promising target since they are involved in the export and the immunity of the producer toward the produced lasso peptides. One way to improve the production of lasso peptide would be to overexpress these ABC-transporters in order to increase the export of the putative lasso peptide and to reduce the toxicity.

The identification of the putative lasso peptides relied typically on the targeted search by LC-MS of the predicted masses of the lasso peptides according to the biosynthetic rules and truncated forms. Nevertheless, it might be possible that the putative lasso peptides were overlooked because of unexpected modifications. Therefore, untargeted LC-MS/MS peptidomic profiling of the extracts from the heterologous host with or without the expression system would be interesting to assess the peptides produced specifically for the strain carrying the expression system. Then, the mass spectra of peptides are compared to peptide databases which is generated by putative peptides derived from genomic information. The peptide natural products are known to present extensive modification and many are non-linear resulting in complex spectra and poor identification. However, this method was performed for the identification of more than 10 RiPPs from *Streptomyces*, one of which being sviceucin demonstrating that it does work with complex molecules such as lasso peptides containing disulphide bonds (173). Finally, molecular networking using mass spectra is an interesting additional method to identify peptides from the same family, their variants or truncated forms by comparing pairs of spectra since related peptides would generated related spectra (40,296).

II. Regulation mechanism and physiological role of sviceucin

New bioactive compounds are needed for medicine and in particular to fight against multidrug resistant bacteria. Bioactive molecules are generally identified during bioactivity guided-screening. Lasso peptides are not an exception, in fact most of them were initially identified during such screening. Although some lasso peptides were identified as receptor antagonist or enzyme inhibitor, most are reported as antibacterial molecules, which is not surprising since most of the bioactivity-guided screening test antimicrobial activity. There are few hypotheses of lasso peptide physiological functions, such as morphogen or cargo molecule, but no evidence to support them so far, except for microcin J25 which is considered as a true antimicrobial due to its low nM MIC and its target, the RNA polymerase. In other words, the physiological roles of lasso peptide screening elusive. In this context we carried out the study of the function of lasso peptide using sviceucin as a model presented as the second part of this work.

We reasoned that in order to understand the physiological role of sviceucin, we needed to decipher the expression and the regulation mechanism of sviceucin. Therefore, in the first place, the operon structure and the expression of sviceucin gene cluster were studied using RT-PCR and gene reporter systems. Secondly, the regulatory mechanism, and in particular the role of the response regulators SviR1 and SviR2, was investigated using gene inactivation and gene reporter systems. Finally, the morphology and the sensitivity to oxidative stress of the different mutant strains of *S. sviceus* were characterized.

In the second chapter of this thesis, we have shown that the expression and production of sviceucin were probably correlated to the growth phase of *S. sviceus*, reaching their maximum at the beginning of the stationary phase. We have also demonstrated that the response regulator SviR1 and SviR2 contributed to the regulation of sviceucin expression in *S. sviceus*, although SviR1 appears to play an essential role. Moreover, we observed that high concentration of KCl inhibited the growth of the *S. sviceus* mutants of *sviA* and *sviR1*, which do not produce sviceucin, and that every mutant presented a higher sensitivity to the oxidative compound phenazine methosulphate.

At first sight, the organization of the gene cluster of sviceucin expression appeared to be classic among RiPPs such as that of microcin C with a stem-loop between the genes encoding the precursor peptide and the biosynthetic machinery (282). Indeed, we have shown that the genes *sviA* and *sviC* to *sviF* can be transcriptionally linked, although the amount of PCR product in this experiment was low thus reducing the quality of the RT-PCR (**Figure 34**). However, we observed that the intergenic region between *sviA* and *sviC* present, surprisingly, a promoting activity using *gusA* gene reporter system, which suggests that the expression of *sviC-sviF* could

be controlled by a promoter in front of sviC. The presence of other internal promoters, in front of sviE and sviF for examples, cannot be excluded. Therefore, it would be interesting to test the DNA region upstream of the svi genes for promoter activity using the gusA reporter system. The fact that the sviceucin genes got activated at the end of the growth phase would suggest that the transition of the primary metabolism and the secondary metabolism would trigger the expression of sviceucin genes.

The role of the response regulators SviR1 and SviR2 was investigated by inactivating individually the genes *sviR1* and *sviR2* using suicide cosmids. We showed that the response SviR1 appears to play an essential role in the production of sviceucin. These results are supported by the fact that mutation of the putative phosphorylation site of SviR1 results in the decrease of the production of sviceucin similar to the *sviR1* inactivated strain while the variant of SviR2 has no significant effect. In the future, it would be interesting to study the interaction of SviR1 and SviR2 with the promoters present in the sviceucin gene cluster using electrophoretic mobility shift assay or two-hybrid experiments in order to clearly demonstrate that SviR1 and SviR2 directly control the expression of sviceucin genes (297). A more global approach such as RNA sequencing could be also performed with *S. sviceus* wild-type and inactivated strains for *sviR1* and *sviR2* in order to shed light on the regulation network controlled by these response regulators.

One main difficulty in chapter II was the sviceucin production level in *S. sviceus* and the mutant strains where a certain variability was observed. For example, basal production of sviceucin was sometimes detected or not in *S. sviceus* $\Delta sviR1$ or in *S. sviceus* $\Delta sviR2$ making the interpretation about the role of the response regulators challenging. Therefore, it appeared crucial to determine a condition in which sviceucin expression or production is fully activated, in order to unambiguously demonstrate the target of SviR1 and SviR2 or future experiment, such as RNA sequencing. Different conditions of culture were tested in particular in minimal media (Pi-SMM and PGMM) with different carbon sources, as it has been shown that glucose could affect the production of specialized metabolites (244). *S. sviceus* grew very well in the minimal media containing sucrose and strong expression of *sviA* using the *sviAp-gusA* gene reporter system was detected. At first, PGMM containing sucrose appears to be a good choice but the expression of sviceucin still remains highly variable. The expression and the production of sviceucin was exclusively tested in liquid media and it would be interesting to switch on solid media where growth is in my opinion more stable. The *gusA* gene reporter can be used in solid media as the substrate, 5-bromo-4-chloro-3-indolyl- β -D-glucuronide, can be added directly in the media or on the top on the media as a solution or top agar and is converted into blue compound in presence of GusA (241).

The signal that triggers the expression of sviceucin is still unknown. Since autoinduction was not demonstrated, we reasoned that the signal might be another molecule. In fact, the presence of genes encoding a second ABC-transporter, which is important for the production of sviceucin, suggest that a molecule, peptidic or not, could be exported from *S. sviceus*. This molecule would act as a ligand to the histidine kinase SviG in order to trigger sviceucin expression and other genes (**Figure 68**). To investigate this hypothesis, global approaches such as peptidomic and metabolomic, can be performed in the supernatant of *S. sviceus* and *S. sviceus* $\Delta sviD1D2$ during the growth phase in liquid media in order to identified the molecules exported by the ABC-transporter. Despite the fact that SviD3D4 is probably involved in the export of sviceucin, the supernatant of *S. sviceus* $\Delta sviD3D4$ could also be analysed.

The role of sviceucin in *S. sviceus* was investigated by characterizing the morphology of the different mutants of *S. sviceus*, in particular $\Delta sviA$, in different media at the colony level with digital camera but also with SEM at the cell level. In one hand, we observed that the mutants that do not produce sviceucin, *sviA* and *svR1* did not grow in in the medium ISP4 containing high concentration of KCl, whereas *S. sviceus sviR2* was not impaired but exhibited orange pigmentation. In the other hand, the growth of *S. sviceus* $\Delta sviR2$ was impaired in R5, whereas $\Delta sviA$ and $\Delta sviR1$ were not. These results suggest that sviceucin might have a role in the protection of *S. sviceus* against osmotic stress and raise questions about the role of SviR2 in the regulation of sviceucin genes expression.

In a similar manner, we investigate the effect of oxidative stress on the different mutants. First, it is worthy of note that the resistance towards oxidative stress of *S. sviceus* is very high compare to *S. coelicolor* as high concentration of oxidative reagent was needed to observe inhibiting zone. Then, we observed that the sensitivity of the mutant strains of *S. sviceus* toward phenazine methosulphate, which generates anion superoxide, was increased. Interestingly, other anion superoxide generator did not affect the mutants, suggesting that the mutants are specifically sensitive to the phenazine or to the methosulphate. The role of methosulphate could be rule out by using pure phenazine in this experiments.


Figure 68 – Induction of sviceucin production.

In addition, genomics and proteomics experiments could be highly informative on the effect of phenazine and sviceucin in the stress response, in particular the induction of the expression of the nickel superoxide dismutase present in *S. sviceus* and involved in the detoxification of superoxide anion. Preliminary experiments showed that the presence of subinhibitory concentration of phenazine methosulphate did not affect the production of sviceucin in *S. sviceus*.

In conclusion, this work has shown a new expression system to get access to lasso peptides encoded by *Actinobacteria* and that can be readily used in practice as three new lasso peptides were identified. Finally, from the research that has been carried out on the physiological role of lasso peptides, it is possible to conclude that sviceucin might play a role in osmotic and oxidative stress in *S. sviceus*. The findings, in this study and in the literature, suggest that most of lasso peptides probably another role than the typical antimicrobial peptides. Clearly, further research will be needed to understand the functions of lasso peptides, however their biological activities, relative to their native functions or not, present a great interest for pharmacology and biotechnology.

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Annexes

Annex 1 – Antibiotic used in this study.

Antibiotics were prepared in water and filtered sterilized unless specified.

Antibiotic	Abbreviation	Concentration in media (µg/mL)	Stock (mg/mL)
Hygromycin B	Hyg	50	50
Carbenicillin	Carb	50-100	50
Kanamycin	Kan	50	50
Chloramphenicol	Cm	25	25 (Ethanol)
Apramycin	Apra	50	50
Nalidixic Acid	Nal	25-50	50 (0.1 M NaOH)
Spectinomycin	Spc	50-100	100
Thiostrepton	Ts	50	17 (DMSO)
Neomycin	Neo	50	50

Annex 2 – Bacterial strains used in this study.

Name	Description	Selectable Marker	Source
<i>E. coli</i> DH10b	For plasmid propagation		Invitrogen
<i>E. coli</i> DH5α	For plasmid propagation		Invitrogen
<i>E. coli</i> ET12567/pUZ800 2	Donor strain for conjugation	Cm Kan	(237)
<i>E. coli</i> S17-1 λpir	DNA propagator with R6K origin of replication		
<i>E. coli</i> Bw25113	For plasmid propagation for λ RED; $\Delta(araD-araB)567$, $\Delta(rhaD-rhaB)568$, $\Delta lacZ4787$, (::rrnB-3), $hsdR514$ rph-1		(239)

Streptomyces coelicolor M1146 and derivatives

M1146	Streptomyces coelicolor $\Delta act \Delta red \Delta cpk \Delta cda$		(51)
JM1	Streptomyces coelicolor M1146 Ω P4H7 in ϕ C31 attB	Hyg	(7)
JM2	Streptomyces coelicolor M1146 Ω P17G4 in ϕ C31 attB	Hyg	(7)
JM3	Streptomyces coelicolor M1146 Ω P1N3 in ϕ C31 attB	Hyg	This work
JM4	Streptomyces coelicolor M1146 Ω P1AF19 in ϕ C31 attB	Hyg	This work
JM5	Streptomyces coelicolor M1146 Ω P3W14 in ϕ C31 attB	Hyg	This work
Scoe127	Streptomyces coelicolor Ω p127 in ϕ C31 attB	Apra	This work

Streptomyces venezuelae J1CD and derivatives

Sven	Streptomyces venezuelae J1CD $\triangle cml \Delta jadR1$		(273)
Sven1	Streptomyces venezuelae J1CD Ω P1AF19 in ϕ C31 attB	Hyg	This work
Sven1-x	Streptomyces venezuelae J1CD Ω P1AF19 in ϕ C31 attB + pSNAxre	Hyg Ts	This work

Sven2	Streptomyces venezuelae J1CD Ω P3W14 in ϕ C31 attB	Hyg	This work
Sven3	Streptomyces venezuelae J1CD Ω pSNA ₂ in ϕ C31 attB	Apra	This work

Streptomyces lividans TK24 and derivatives

Sliv	Streptomyces lividans TK24		(271)
Sliv pSNOU-1	Streptomyces lividans TK24 Ω pSNOU-1	Apra	This work
Sliv1	Streptomyces lividans TK24 Ω P4H7 in ϕ C31 attB	Hyg	This work
Sliv2	Streptomyces lividans TK24 Ω P17G4 in ϕ C31 attB	Hyg	This work
Sliv3	Streptomyces lividans TK24 Ω P1N3 in ϕ C31 attB	Hyg	This work
Sliv4	Streptomyces lividans TK24 Ω P1AF19 in ϕ C31 attB	Hyg	This work
Sliv5	Streptomyces lividans TK24 Ω P3W14 in ϕ C31 attB	Hyg	This work
Sliv6-1	Streptomyces lividans TK24 Ω pSNOU-1 in ϕ C31 attB	Apra	This work
Sliv6-3	Streptomyces lividans TK24 Ω pSNOU-1 in ϕ C31 attB	Apra	This work
Sliv7	Streptomyces lividans TK24 Ω pSNA1 in ϕ C31 attB	Apra	This work
Sliv8	Streptomyces lividans TK24 Ω pSNA6 in ϕ C31 attB	Apra	This work
Sliv94-1	Streptomyces lividans TK24 Ω p9401 (1) in ϕ C31 attB	Apra Ts	Provided by Zotchev
Sliv94-2	Streptomyces lividans TK24 Ω p9401 (1) in ϕ C31 attB	Apra Ts	Provided by Zotchev

Streptomyces albus J1074 and derivatives

Sal

Streptomyces albus J1074

Sal1	Streptomyces albus J1074 Ω P1AF19 in ϕ C31 attB	Hyg	This work
Sal2	Streptomyces albus J1074 Ω P3W14 in ϕ C31 attB	Hyg	This work
Sal3	Streptomyces albus J1074 Ω pSNA1 in ϕ C31 attB	Apra	This work
Sal94-1	Streptomyces albus J1074 Ω p9401 (2) in ϕ C31 attB	Apra Ts	Provided by Zotchev
Sal94-2	Streptomyces albus J1074 Ω p9401 (2) in ϕ C31 attB	Apra Ts	Provided by Zotchev
Sal98	Streptomyces albus J1074 Ω p9801 in ϕ C31 attB and replicative pSARP	Apra Ts	Provided by zotchev

Streptomyces sviceus DSM 924 and derivatives

SSV	Streptomyces sviceus DSM-924, Sviceucin native producing strain		(7,298)
SSV pA	Streptomyces sviceus Ω pA-GUS in ϕ C31 attB	Apra Spc	This work
SSV pAs	Streptomyces sviceus Ω pAs-GUS in ϕ C31 attB	Apra Spc	This work
SSV pC	Streptomyces sviceus Ω pC-GUS in ϕ C31 attB	Apra Spc	This work
SSV pD	Streptomyces sviceus Ω pD-GUS in ϕ C31 attB	Apra Spc	This work
SSV pE	Streptomyces sviceus Ω pE-GUS in ϕ C31 attB	Apra Spc	This work
SSV pG	Streptomyces sviceus Ω pG-GUS in ϕ C31 attB	Apra Spc	This work
SSV pGus	Streptomyces sviceus Ω pGUS in ϕ C31 attB	Apra Spc	This work
SSV pR	Streptomyces sviceus Ω pR-GUS in ϕ C31 attB	Apra Spc	This work
SSVoR1	Streptomyces sviceus Ω pMS82cR1 in ϕ BT1 attB	Hyg	This work
SSVoR2	Streptomyces sviceus Ω pMS82cR2 in ϕ BT1 attB	Hyg	This work
SSV1	Streptomyces sviceus Ω pKGLPR1	Hyg	This work

SSV2	Streptomyces sviceus Ω pKGLPR2	Hyg	This work
SSV3	Streptomyces sviceus $\Delta sviRI$		This work
SSVA	Streptomyces sviceus sviA::FRT-oriT-aac(3)IV-FRT	Apra	This work
SSVA pA	Streptomyces sviceus sviA::FRT-oriT-aac(3)IV-FRT Ω pA-GUS in ϕ C31 attB	Apra Spc	This work
SSVA pAs	Streptomyces sviceus sviA::FRT-oriT-aac(3)IV-FRT Ω pAs-GUS in ϕ C31 attB	Apra Spc	This work
SSVA pC	Streptomyces sviceus sviA::FRT-oriT-aac(3)IV-FRT Ω pC-GUS in ϕ C31 attB	Apra Spc	This work
SSVA pR1	Streptomyces sviceus sviA::FRT-oriT-aac(3)IV-FRT Ω pR1-GUS in ϕ C31 attB	Apra Spc	This work
SSVA pG	Streptomyces sviceus sviA::FRT-oriT-aac(3)IV-FRT Ω pG-GUS in ϕ C31 attB	Apra Spc	This work
SSVR1	Streptomyces sviceus sviR1::FRT-oriT-aac(3)IV-FRT	Apra	This work
SSVR1 pA	Streptomyces sviceus sviR1::FRT-oriT-aac(3)IV-FRT Ω pA-GUS in ϕ C31 attB	Apra Spc	This work
SSVR1 pC	Streptomyces sviceus sviR1::FRT-oriT-aac(3)IV-FRT Ω pAs-GUS in ϕ C31 attB	Apra Spc	This work
SSVR1 pR1	Streptomyces sviceus sviR1::FRT-oriT-aac(3)IV-FRT Ω pR1-GUS in ϕ C31 attB	Apra Spc	This work
SSVR1 pG	Streptomyces sviceus sviR1::FRT-oriT-aac(3)IV-FRT Ω pG-GUS in ϕ C31 attB	Apra Spc	This work
SSVR1 fR1	Streptomyces sviceus sviR1::FRT-oriT-aac(3)IV-FRT Ω pMS82FR1 in φBT1 attB	Hyg Apra	This work
SSVR1 cR1	Streptomyces sviceus sviR1::FRT-oriT-aac(3)IV-FRT Ω pMS82cR1 in ϕ BT1 attB	Hyg Apra	This work
SSVR1 vR1	Streptomyces sviceus sviR1::FRT-oriT-aac(3)IV-FRT Ω pMS82vR1 in φBT1 attB	Hyg Apra	This work
SSVR2	Streptomyces sviceus sviR2::FRT-oriT-aac(3)IV-FRT	Apra	This work
SSVR2 pA	Streptomyces sviceus sviR2::FRT-oriT-aac(3)IV-FRT Ω pA-GUS in ϕ C31 attB	Apra Spc	This work
SSVR2 pC	Streptomyces sviceus sviR2::FRT-oriT-aac(3)IV-FRT Ω pAs-GUS in ϕ C31 attB	Apra Spc	This work
SSVR2 pR1	Streptomyces sviceus sviR2::FRT-oriT-aac(3)IV-FRT Ω pR1-GUS in ϕ C31 attB	Apra Spc	This work
SSVR2 pG	Streptomyces sviceus sviR2::FRT-oriT-aac(3)IV-FRT Ω pG-GUS in ϕ C31 attB	Apra Spc	This work
SSVR2 cR2	Streptomyces sviceus sviR2::FRT-oriT-aac(3)IV-FRT Ω pMS82cR2 in φBT1 attB	Hyg Apra	This work

SSVR2 vR2	Streptomyces sviceus sviR2::F	T- <i>oriT-aac(3)IV</i> -FRT Ω pMS82	2vR2 in \phiBT1 attB	Hyg Apra	This work
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Annex 3 – Vectors used in this study.

Name	Description	Selectable marker	Source
P4H7	pWED4-based cosmid with whole sviceucin gene cluster	Carb Hyg	(7)
P17G4	pWED4-based cosmid with the partial sviceucin gene cluster (lacking sviR1, sviR2, and sviG)	Carb Hyg	(7)
P1N3	pWED4-based cosmid with the putative LP gene cluster of Stackebrandtia nassauensis	Carb Hyg	Hayian Ma
P1AF19	pWED4-based cosmid with the putative LP gene cluster of Stackebrandtia nassauensis	Carb Hyg	Hayian Ma
P3W14	pWED4-based cosmid with the putative LP gene cluster of Nocardiopsis alba	Carb Hyg	Hayian Ma
pKGLP2	Suicide based plasmid for gene inactivation	Hyg	(241)
pKGLPR1	Up and Dn sviR1 cloned into XbaI/BamHI sites of pKGLP2	Hyg	This work
pKGLPR2	Up and Dn sviR2 cloned into XbaI/BamHI sites of pKGLP2	Hyg	This work
P4H8	P4H7 $\phi C31$ -oriT::kan ^R	Carb Hyg Kan	This work
P4H8R1	P4H8 sviR1::aac(3)IV	Carb Hyg Kan Apra	This work
P4H8R2	P4H8 sviR2::aac(3)IV	Carb Hyg Kan Apra	This work
P4H8A	P4H8 sviA::aac(3)IV	Carb Hyg Kan Apra	This work
P4H8G	P4H8 sviG::aac(3)IV	Carb Hyg Kan Apra	This work
P4H8Gv2	P4H8 sviG::aac(3)IV	Carb Hyg Kan Apra	This work
P4H8F	P4H8 sviF::aac(3)IV	Carb Hyg Kan Apra	This work
P4H8D1D2	P4H8 sviD1D2::aac(3)IV	Carb Hyg Kan Apra	This work
P4H8D3D4	P4H8 sviD3D4::aac(3)IV	Carb Hyg Kan Apra	This work
pGUS	Promoter probe vector; pSETGUS with deleted KpnI fragment containing <i>tipA</i> promoter	Apra Spc	(237)
pEGUS	Promoter of <i>ermE</i> p* cloned into XbaI/KpnI of pGUS	Apra Spc	This work
pAGUS	Putative promoter of sviA cloned into XbaI/KpnI of pGUS	Apra Spc	This work

pAsGUS	Putative promoter of long version of sviA cloned into XbaI/KpnI of pGUS	Apra Spc	This work
pCGUS	Putative promoter of <i>sviC</i> cloned into XbaI/KpnI of pGUS	Apra Spc	This work
pDGUS	Putative promoter of sviD1 cloned into XbaI/KpnI of pGUS	Apra Spc	This work
pGGUS	Putative promoter of <i>sviG</i> cloned into XbaI/KpnI of pGUS	Apra Spc	This work
pRGUS	Putative promoter of sviR1 cloned into XbaI/KpnI of pGUS	Apra Spc	This work
pSNA	Complete <i>Stackebrandtia nassauensis</i> LP gene cluster cloned under control of <i>cmlF</i> promoter in an integrative vector pSOK806	Apra	Provided by Zotchev
pSNAxre	The gene <i>snaR1</i> (Xre-type transcriptional regulator) from LP gene cluster of <i>Stackebrandtia nassauensis</i> cloned into the pUWLoriT vector under the control of the promoter of <i>ermE</i> p*	Carb Ts	Provided by Zotchev
p9401-LP1	pSOK809-based cosmid with the putative LP-1 gene cluster of Streptomyces sp ADI94-01	Apra Ts	Provided by Zotchev
p9401-LP2	pSOK809-based cosmid with the putative LP-2 gene cluster of Streptomyces sp ADI94-01	Apra Ts	Provided by Zotchev
p9810-LP	pSOK809-based cosmid with the putative LP gene cluster of Streptomyces sp ADI98-10	Apra Ts	Provided by Zotchev
pSven-LP	pSOK809-based cosmid with the putative LP gene cluster of <i>Streptomyces venezuelae</i> ATCC 10712	Apra Ts	Provided by Zotchev
pSnou-LP	pSOK809-based cosmid with the putative LP gene cluster of Streptomyces noursei ATCC 11455	Apra Ts	Provided by Zotchev
pA127-LP	pSOK809-based cosmid with the putative LP gene cluster of Actinoalloteichus sp ADI127-17	Apra Ts	Provided by Zotchev
pKD4	Template for FRT-flanked kanamycin cassette	Kan Carb	(240)
pIJ773	Template for FRT-flanked <i>aac(3)IV-oriT</i> (Apramycin ^R)	Apra	(237)
pIJ777	Template for FRT-flanked <i>aphI-oriT</i> (Neomycin ^R)	Neo	(237)
pIJ790	[oriR101], replication temperature sensitive [repA101(ts)], araBp-gam-be-exo,	Cm	(4)
pCP20	flp, bla, cat, rep101 ^{ts}	Carb Cm	(299)
pMS82	φBT1 based integrative vector	Hyg	(300)

pW60	Template for FRT-flanked <i>aac(3)IV</i> (Apramycin ^R)	Carb Apra	Greg Challis, Univ of Warwich
pSET152	φC31 based integrative vector	Carb	(236)
pMS82FR1	sviR1 and sviR1 promoter cloned into HindIII site of pMS82	Hyg	This work
pMS82cR1	ermE* promoter and sviR1 cloned into KpnI/HindIII site of pMS82	Hyg	This work
pMS82cR2	ermE* promoter and sviR2 cloned into KpnI/HindIII site of pMS82	Hyg	This work
pMS82cA	ermE* promoter and sviA cloned into KpnI/HindIII site of pMS82	Hyg	This work
pCP20	flp, bla, cat, rep101 ^{ts}	Carb Cm	(237)
P4H7D1	P4H7 <i>sviD1::aac(3)IV</i>	Apra Carb Hyg	This work
P4H7D3	P4H7 <i>sviD3::aac(3)IV</i>	Apra Carb Hyg	This work
pvR1	sviR1 D77A mutant of pMS82cR1	Hyg	This work
pvR2	sviR2 D59A mutant of pMS82cR2	Hyg	This work

Annex 4 – Primers used in this study. Flippase recognition target (FRT) sites are shown in green. Restriction sites are underlined.

Name	Sequence	Description
JM1	CATGGGTGTCCCCTGTCCGTTCGTTGTCGAGATGAGCAC TGTAGGCTGGAGCTGCTTC	sviA inactivation
JM2	GGACATCGGGCGACCTACGTTGCCGGAGGGCACCGGCCG ATTCCGGGGATCCGTCGACC	sviA inactivation
JM3	AAAGGGGCAGGCATGGCAGACACGACACCCGGCAAGGTGATTCCGGGGATCCGTCGACC	snaXREb inactivation
JM4	GGTGTGGCACGAACTCGGGAGTGCCTACCAATGGGGTCA TGTAGGCTGGAGCTGCTTC	snaXREb inactivation
JM5	CTCGCAAAAATCGGAACTGTATAGGAGTCCCGAAAGATG ATTCCGGGGATCCGTCGACC	snaXRE-TF inactivation
JM6	ACACCTTTTTACGAGGTGGCGGGCCGGTTTGAGTATCTA TGTAGGCTGGAGCTGCTTC	snaXRE-TF inactivation
JM7	GAGGGGCCACGCCCGCGCATGGTTTATTCGCGCGAGTTA TGTAGGCTGGAGCTGCTTC	snaHMP inactivation
JM8	TCGGTCACATCCTGTCACACAGAATCAAGAAACGCAATG ATTCCGGGGATCCGTCGACC	snaHMP inactivation
JM9	CGACAGGATCCGGGCTCCCCCGGCAGAGTCGTGGACATG ATTCCGGGGATCCGTCGACC	nalHMP inactivation
JM10	TGAACTTGGCGGCCAGCACCTCCCGCTCCGATCCGGTCA TGTAGGCTGGAGCTGCTTC	nalHMP inactivation
JM11	GGAAGCCTGGTTGTACGACA	PCR verification sviR1
JM12	GGATCGACCTGGTCATGGAC	PCR verification sviR1
JM13	TTCGTCATCGATCGTGACCC	PCR verification sviR2
JM14	AGGTGTTCGTCCTCGTGTTG	PCR verification sviR2
JM15	ACCCAACAAAGATCTCCCGTACTGACGGACACACCGAAGTG TGTAGGCTGGAGCTGCTTCG	<i>\phiC31-oriT</i> deletion of P4H7
JM16	TTTTTCACAAAACGGTTTACAAGCATAAAGCTGGTCGAC CATATGAATATCCTCCTTA	<i>\phiC31-oriT</i> deletion of P4H7
JM17	CATCGGGCGACCTACGTTGCCGGAGGGCACCGGCCGGTGG TGTAGGCTGGAGCTGCTTC	sviA inactivation
JM18	ACTCCGCCCTCAGGGGGGGGGTCAGCCGATCAGTGGGTCAATTCCGGGGATCCGTCGAC	sviA inactivation
JM19	CTTAAGGCTCTCCTGTATGTGGTCCGTGGGTGCGTCATGG TGTAGGCTGGAGCTGCTTC	sviR1 inactivation
JM20	TGCCTCGGGTAAGCGACGGTGCGGCGCGCGCAGGACGTCA ATTCCGGGGATCCGTCGAC	sviR1 inactivation
JM21	TCCTGCGGCGCCGCACCGTCGCTTACCCGAGGCACGTCAG TGTAGGCTGGAGCTGCTTC	sviR2 inactivation
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JM22	CCTGCCTGCCCGTCCGACGTCCCGGGAGTCCCTGTGACC ATTCCGGGGATCCGTCGAC	sviR2 inactivation in P4H8
JM23	CCTGTCGCACAGCAAAG	PCR verification $\Delta \phi C31$ -oriT
JM24	GCTGATCCGGTGGATGA	PCR verification $\Delta \phi C31$ -oriT
JM25	CCCTTGACGCTATGGCT	PCR verification Δ <i>snaXREb</i>
JM26	ACAAGGGCCGTCCATTT	PCR verification Δ <i>snaXREb</i>
JM27	GAACTTTTGCCGCACCA	PCR verification Δ <i>snaHMP</i>
JM28	TCTACGTGCGTGACTCC	PCR verification Δ snaHMP
JM29	TCGTGGGATGACTTCCG	PCR verification Δ <i>snaXRE-TF</i>
JM30	GGGGATTCGGAGACTGG	PCR verification Δ <i>snaXRE-TF</i>
JM31	CTTGCCGAGTTGATGGC	K1-aac(3)IV_Rev
JM32	ATGCAGCGTCGTGTTGG	K2-aac(3)IV_Fwd
JM33	CCGACCCAGGATCGTAC	PCR verification Δ <i>nalHMP</i>
JM34	CACGGTCCGGATACCTC	PCR verification Δ <i>nalHMP</i>
JM35	TCGACAACGAACGGACA	RT primer sviA
JM36	AAGTCGGTGCAGTCTCC	RT primer sviA
JM37	TGGGGTCTGTTCGCCAT	RT primer <i>sviC</i>
JM38	TCCTCGGGTCGATGCTC	RT primer <i>sviC</i>
JM39	AAAGGGGCAGGCATGGCAGACACCGACACCCGGCAAGGTG <mark>CTGACGCCGTTGGATACACC</mark>	snaXREb inactivation
JM40	CTCGCAAAAATCGGAACTGTATAGGAGTCCCGAAAGATG <mark>CTGACGCCGTTGGATACACC</mark>	snaHMP inactivation
JM41	TCGGTCACATCCTGTCACACAGAATCAAGAAACGCAATG <mark>CTGACGCCGTTGGATACACC</mark>	snaXRE-TF inactivation
JM42	CGACAGGATCCGGGCTCCCCCGGCAGAGTCGTGGACATG CTGACGCCGTTGGATACACC	nalHMP inactivation

JM43 JM44	GCGAAGACGGTGACCAC GGCGACGGTCTTCTTCG	Sseg_05887 S. Sviceus (HrdB homolog) Sseg_05887 S. sviceus (HrdB homolog)
JM45	GTGTCCCACCATCACGC	snaA sequencing
JM46	CACAGCACCGGATCAGC	snaA sequencing
JM47	ACGTCTCTTTCATCGCCG	nalA sequencing
JM48	TGCCCGTTCGGTCGTT	nalA sequencing
JM49	GGTCGAGGACAGGGCCGTA	PCR primer sviC
JM50	ATGTCTGGCGTCCGGCAC	PCR primer sviC
JM51	CGAGAAGGTCATCGAGGTC	PCR primer hsrB
JM52	GCAGGAGTGTGAAGCTGAC	PCR primer hsrB
JM53	AACTCTTCGGGATCATGCC	PCR primer sviC
JM54	TGGAACAGGTGAGGGTGTC	PCR primer sviC
JM55	GTAGTC <u>GGTACC</u> CGGCCGGTGCCCTCCGGCAA	KpnI sviA promoter cloning
JM56	ACTCCGCCCTCAGGGGGGGGGTCAGCCGATCAGTGGGTCAGGTAACTGATGCCGTATTTG	sviA inactivation in P4H8
JM57	GTAGTC <u>TCTAGA</u> CCCGGGACCGCTCCGGGACC	XbaI sviA promoter cloning
JM58	GTAGTC <u>GGTACC</u> GCGTGTGTTCACCTCGTCAC	KpnI sviC promoter cloning
JM59	GTAGTC <u>TCTAGA</u> CCCACTGATCGGCTGACCGC	XbaI sviC promoter cloning
JM60	GTAGTC <u>GGTACC</u> GCCGATCAGACTGTCGCGGC	KpnI sviD1 promoter cloning
JM61	GTAGTC <u>TCTAGA</u> CCGCGCTGGACCGTCCGCAC	XbaI sviD1 promoter cloning
JM62	GTAGTC <u>TCTAGA</u> GCCGATCAGACTGTCGCGGC	XbaI sviG promoter cloning
JM63	GTAGTC <u>GGTACC</u> CCGCGCTGGACCGTCCGCAC	KpnI sviG promoter cloning

JM64	ACCTCGCTCTGCTAATCCTG	PCR verification pUWL- snaXRE
JM65	GACACCATCGCAAATCCGTC	PCR verification pUWL- snaXRE
JM66	GTAGTC <u>GGTACC</u> GACGCACCACGGACCACAT	KpnI sviR1 promoter cloning
JM67	GTAGTC <u>TCTAGA</u> GCATGGTGGGCGTTGGTGGC	XbaI sviR1 promoter cloning
JM68	GTAGTC <u>GGTACC</u> GCCTGCCCCTTTTGGTGTGC	KpnI ermE* promoter cloning
JM69	GTAGTC <u>TCTAGA</u> GTACCAGCCCGACCCGAGCA	KpnI ermE* promoter cloning
JM70	GTAGTC <u>GGTACC</u> GGGTGTCCCCTGTCCGTTCG	KpnI Short <i>sviA</i> promoter <i>cloning</i>
JM71	GTCGTTGAAGCTGCCGGGGA	Sequencing of pGUS cloning
JM72	CGGTTGTCCTCTCACGGGTCCCGGAGCGGTCCCGGGTCA TGTAGGCTGGAGCTGCTTC	sviD1D2 inactivation in P4H8
JM73	ATGTGCCGTACACGGGGCCGCGACAGTCTGATCGGCATG <mark>ATTCCGGGGATCCGTCGACC</mark>	sviD1D2 inactivation in P4H8
JM74	CCGTGAAAACCCATGTCG	PCR primer sviR2
JM75	TCACCGTCCCTTACTCG	PCR primer <i>sviR2</i>
JM76	TCTGATGAGCGAGATGGC	PCR primer sviD1
JM77	ACCCTGACCTTGGAGACG	PCR primer sviD1
JM78	GACGTACCGCCTATCACC	PCR primer sviD1
JM79	GAGTCGCTGTCCCATGC	PCR primer sviD1
JM80	TTCCGTTTCTCACCGAGC	PCR primer sviR1
JM81	GATCGTTCACCTGGAGC	PCR primer sviR1
JM82	AGGAAGCCGTGATTGTCC	PCR primer sviR1
JM83	GTACCCACTCCGAAAGCC	PCR primer sviR1
JM84	TCCGTATGCCCGAACTG	PCR primer sviR2

JM85	CATGGTGAGGATGAGGAC
JM86	CTCGTGTCCCCTTACTCGGCTTACTCGCTACTCGGCTTA TGTAGGCTGGAGCTGCTTC
JM87	GGCTCGGGCGGGCCGTCACGAGGGGGGGGGGCAGCCGTGAGCATTCCGGGGGATCCGTCGACC
JM91	ATGACCTTGTTCCACACG
JM92	GTAGTC <u>AAGCTT</u> TATTCGCCGCTGGAATCC
JM93	ATGCGT <u>AAGCTT</u> ACGTCAACAGCCGAGTCC
JM94	ATGCGT <u>AAGCTT</u> TCGGGTAAGCGACGGTGC
JM95	ATGCGT <u>TCTAGA</u> TCTCCTGTATGTGGTCCG
JM96	ATGCGT <u>TCTAGA</u> GCCCGATCCTACCAACC
JM97	GCAGGC <u>GGTACC</u> AGCCCGAGCACG
JM98	ATGCGT <u>AAGCTT</u> CGCACCGTCGCTTACCC
JM99	ATGCGT <u>TCTAGA</u> GCGGATTCCAGGTGACC
JM100	TGCGTACTTATATGCGTC
JM101	GCCACAATTTGATATATT
JM102	CGGCGATCAGGATCCGGGTCGCGGCGGAGGCGGTCACAGATTCCGGGGATCCGTCGACC
JM103	ACGCGGTCCGCCTCGTTCGCGTATCTCACGATCCTCCTG TGTAGGCTGGAGCTGCTTC
JM104	GTGACATCATTCTGTGG
JM105	ATGCGT <u>TCTAGA</u> ATGACCTCGACCGACGAGC
JM106	ATGCGT <u>AAGCTT</u> GGGTCAGACACAGATCCAGG
JM107	CGGCGATCAGGATCCGGGTCGCGGCGGAGGCGGTCACAG TGTAGGCTGGAGCTGCTTC
JM108	CCGTTGGTGATCGCCGGTGCGGACGGTCCAGCGCGGGTGATTCCGGGGATCCGTCGACC
JM109	CTCGTGTCCCCTTACTCGGCTTACTCGCTACTCGGCTTA ATTCCGGGGATCCGTCGACC

PCR primer *sviR2* sviD3D4 inactivation in P4H8 sviD3D4 inactivation in P4H8 PCR verification $\Delta sviD3D4$ HindIII sviR1+promoter cloning HindIII sviR1 cloning HindIII sviR1 cloning XbaI sviR1 XbaI *ermE** promoter cloning KpnI *ermE** promoter cloning HindIII sviR2 cloning XbaI *sviR2* PCR verification *flp* PCR verification *flp* sviG inactivation in P4H8 sviG inactivation in P4H8 Sequencing up *aac(3)*IV XbaI sviA complementation HindIII sviA complementation sviGv2 inactivation in P4H8 sviGv2 inactivation in P4H8 sviD3D4 inactivation in P4H7

JM110	GGCTCGGGCGGGCCGTCACGAGGGGGGGGGGCAGCCGTGAGC TGTAGGCTGGAGCTGCTTC
JM111	CGTACACGGGGCCGCGACAGTCTGATCGGCATGATCGAGATTCCGGGGATCCGTCGACC
JM112	TCCTCTCACGGGTCCCGGAGCGGTCCCGGGTCACACGTC TGTAGGCTGGAGCTGCTTC
JM113	ATGCGT <u>AAGCTT</u> GCGGTCACAGGGACTCC
JM114	CGTGAAGCACGGGGCCATCACGCAGAC
JM115	GTCTGCGTGATGGCCCCGTGCTTCACG
JM116	CGTCGTCCTCATGGCCATCCGTATGCCCG
JM117	CGGGCATACGGATGGCCATGAGGACGACG
JM118	ATGCCCGACCAGCTCACG
JM119	CGATGCCCGCGATGAAGG
JM120	GTACTTCACCGCCATCG
JM121	CTTCCTCGGGTCCTTCG
JM122	ATGCGT <u>AAGCTT</u> GCCGATCAGACTGTCGC
JM123	GCTTGAAGTAGTCGCTCC
JM124	GTGCAGGAGAAGATGGC
JBR136	GGGTGACGGATCATCTCGTC
JBR139	CCCGGGACCGCTCCGG
JBR144	GGGGTTACCCTGGTAGATGGC
JBR151	TAGC <u>TCTAGA</u> CTCGTGACCGGCTGGC
JBR152	ATGG <u>CAATTG</u> GACGCACCACGGACCA
JBR153	ATGC <u>CAATTG</u> CCGTCGCTTACCCGAGGCA
JBR154	ATGTGGATCCTTCGACATCGACGAGTACGTCTTC

sviD3D4 inactivation in P4H7 sviD1D2 inactivation in P4H7 *sviD1D2* inactivation in P4H7 HindIII sviG Point mutation SviR1Asp-Ala77 Point mutation SviR1Asp-Ala77 Point mutation SviR2Asp-Ala59 Point mutation SviR2Asp-Ala59 PCR verification *sviE* PCR verification *sviE* PCR verification *sviF* PCR verification *sviF* HindIII sviG promoter PCR primer for Ni-SOD PCR primer for Ni-SOD PCR primer *sviC* PCR primer *sviA* promoter PCR primer *sviR1* promoter XbaI up *sviR1* MfeI up sviR1 MfeI dn sviR1 BamHI dn sviR1

JBR155	TAAT <u>TCTAGA</u> CCGGTCAGGTGCTCGATCGA	XbaI dn <i>sviR2</i>
JBR156	ATAT <u>CAATTG</u> CGTGCCTCGGGTAAGCGACG	MfeI dn <i>sviR2</i>
JBR157	ATCG <u>CAATTG</u> GACGGGCAGGCAGGAACGG	MfeI up <i>sviR2</i>
JBR158	ATATGGATCCGAGGCCATGACGGAGATGCG	Bam
delsviA	CCGGTGCTCATCTCGACAACGAACGGACAGGGGACACCCT TGTAGGCTGGAGCTGCTTCG	sviA inactivation
YL131	TCGTTCCGCGTCGCCCGTCACAGAAGGGAAGTAGACCATG ATTCCGGGGATCCGTCGACC	sviF inactivation
YL133	ACTGGGGGCCGCTCGCCGGTT	PCR verification sviE
YL134	TTGCGGGGCGGTCGCGGGG	PCR verification sviE
YL135	GCATTCGGCTCGTTCCGCGT	PCR verification sviF
YL136	GACATGAGCCGAGCAGGGC	PCR verification sviF
YL175	GAAGGTGTGGGGGGGGCGAAGACCGGGGGGGCGTCGCCGTCA TGTAGGCTGGAGCTGCTTC	sviF inactivation
YL198	TCGGTCGAGGTCATGGGT	RT primer inter <i>sviD-A</i>
YL199	ATCACCACCTGTTGTGTGTCG	RT primer sviD1
YL200	CTCGTCGAACAGGAGGATGTC	RT primer sviD1
YL201	GACCTTGTCGACCTCTCAGAC	RT primer sviD3
YL202	GATCAGGTACTCCCAGATCGC	RT primer sviD3
YL203	GGGGTGCGTGGTTATCTGAG	RT primer sviR1
YL204	AAAGTCGAGCACTTGCCTCTC	RT primer sviR1
YL205	CTCAGATGCAGTCGGTTGGAG	RT primer sviR2
YL206	CGTCTGATCGCCGAGTTCTG	RT primer sviR2
YL207	GGATCTGTGTCTGACCCACTG	RT primer inter <i>sviA</i> -C
YL208	CGGCAGAATCAGGAAGTCCAT	RT primer inter <i>sviA</i> -C

YL210GGCACACGGTACTGATGATGART primer inter sviD4-EYL211CCAAGGGCTACAAGTTCTCCRT primer hsrBYL212CGCGAGCTTGTTGATGACCRT primer hsrBYL213AATACCGGATACCACTCGCACRT primer 16S rRNAYL214AATATTCCCCACTGCTGCCTCRT primer 16S rRNAYL215AGGCTGTGGAAAGGGTTGACRT primer SSEG_07502YL216CAGGTACTTGTAGGCCTCGTCRT primer SSEG_07502	YL209	CTTCGCGGTCAACGTCAAC	RT primer inter <i>sviD4-E</i>
YL211CCAAGGGCTACAAGTTCTCCRT primer hsrBYL212CGCGAGCTTGTTGATGACCRT primer hsrBYL213AATACCGGATACCACTCGCACRT primer 16S rRNAYL214AATATTCCCCACTGCTGCCTCRT primer 16S rRNAYL215AGGCTGTGGAAAGGGTTGACRT primer SSEG_07502YL216CAGGTACTTGTAGGCCTCGTCRT primer SSEG_07502	YL210	GGCACACGGTACTGATGATGA	RT primer inter <i>sviD4-E</i>
YL212CGCGAGCTTGTTGATGACCRT primer hsrBYL213AATACCGGATACCACTCGCACRT primer 16S rRNAYL214AATATTCCCCACTGCTGCCTCRT primer 16S rRNAYL215AGGCTGTGGAAAGGGTTGACRT primer SSEG_07502YL216CAGGTACTTGTAGGCCTCGTCRT primer SSEG_07502	YL211	CCAAGGGCTACAAGTTCTCC	RT primer hsrB
YL213AATACCGGATACCACTCGCACRT primer 16S rRNAYL214AATATTCCCCACTGCTGCCTCRT primer 16S rRNAYL215AGGCTGTGGAAAGGGTTGACRT primer SSEG_07502YL216CAGGTACTTGTAGGCCTCGTCRT primer SSEG_07502	YL212	CGCGAGCTTGTTGATGACC	RT primer hsrB
YL214AATATTCCCCACTGCTGCCTCRT primer 16S rRNAYL215AGGCTGTGGAAAGGGTTGACRT primer SSEG_07502YL216CAGGTACTTGTAGGCCTCGTCRT primer SSEG_07502	YL213	AATACCGGATACCACTCGCAC	RT primer 16S rRNA
YL215AGGCTGTGGAAAGGGTTGACRT primer SSEG_07502YL216CAGGTACTTGTAGGCCTCGTCRT primer SSEG_07502	YL214	AATATTCCCCACTGCTGCCTC	RT primer 16S rRNA
YL216 CAGGTACTTGTAGGCCTCGTC RT primer SSEG_07502	YL215	AGGCTGTGGAAAGGGTTGAC	RT primer SSEG_07502
	YL216	CAGGTACTTGTAGGCCTCGTC	RT primer SSEG_07502

Medium	Composition (/L)	
International Streptomyces Project 4 (ISP4)	DIFCO [™] ISP medium 4	37 g
Soy Flour Mannitol (SFM)	Primeal Soy Flour	20 g
•	D-Mannitol	20 g
	Agar	20 g
Glucose Yeast Malt	D-(+)-Glucose	4 g
	Yeast extract	4 g
	Malt extract	10 g
	CaCO ₃	2 g
	pH 7	
	Agar	12 g
Lysogeny broth Miller (LB)	Tryptone	10 g
	Yeast extract	5 g
	NaCl	10 g
	pH 7	
	Agar	15 g
Soft Nutrient Agar (SNA)	DIFCO [™] Nutrient Broth	8 g
	pH 7	
	Agar	7.5 g
R5	Sucrose	103 g
	K_2SO_4	0.25 g
	MgCl ₂ ,6H ₂ O	10.12 g
	Glucose	10 g
	Difco Casaminoacids	0.1 g
	Trace element solution	2 mL
	Yeast extract	5 g
	TES buffer	5.73 g
	Agar	20 g
	Complement before use	
	KH_2PO_4 (5 g/L)	10 mL
	$CaCl_2 2H_2O(1M)$	20 mL
	L-proline (20%)	15 mL
	NaOH (1N)	5 mL
Basal medium (T. G. PRIDHAM)	(NH4) ₂ SO ₄	2.64 g
	KH ₂ PO ₄	2.38 g
		e

Annex 5 – Solid media	
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	K_2HPO_4	5.65 g
	MgSO ₄	1 g
	CuSO ₄	6.4 mg
	FeSO ₄	1.1 mg
	MnCl ₂	7.9 mg
	ZnSO ₄	1.5 mg
	pH 7	
	Agar	15 g
R2	Sucrose	129 g
	K_2SO_4	0.31 g
	MgCl ₂ ,6H ₂ O	12.65 g
	Glucose	12.5 g
	Difco Casaminoacids	0.125 g
	Complement before use	
	KH ₂ PO ₄ (0.5%)	12.5 mL
	CaCl ₂ 2H ₂ O (3.68% or 0.25M)	100 mL
	L-proline (20%)	18.75 mL
	TES buffer (5.73%, adjusted pH7.2)	125 mL
	Trace element solution	2.5 mL
	NaOH (1N)	6.25 mL
R2YE	R2	1000 mL
	Yeast extract (10%)	50 mL

Annex 6 – Liquid media

Medium	Composition (/L)	
Glucose Yeast Malt (GYM)	D-(+)-Glucose	4 g
	Yeast extract	4 g
	Malt extract	10 g
	pH 7	
Lysogeny broth Miller (LB)	Tryptone	10 g
	Yeast extract	5 g
	NaCl	10 g
	pH 7	
Malt Yeast Maltose (MYM)	Malt extract	10 g
	Yeast extract	4 g
	Maltose	4 g
	MOPS	1.9 g

Tryptic Soy Broth (TSB)	BACTO [™] Tryptic Soy Broth	30 g
Discussion in the LOC		0.41
minimal medium (Pi-SMM)	NaH ₂ PO ₄ , H ₂ U	0.41 g
	NH ₄ Cl	5.35 g
	KCL	0.74 g
	Na ₂ SO ₄	0.28 g
	Citric acid	0.38 g
	MES	9.76 g
	рН 7	
	Complement before use	
	Element solution	1 mL
	Vitamines solution	1 mL
	D-(+)-Glucose 30%	100 mL
	MgCl ₂ 1 M	1.25 mL
	CaCl ₂ 1 M	0.25 mL
Element solution (Filtered)	FeCl ₃ , 6H ₂ O	100 mM
	$CuCl_2$, $2H_2O$	50 mM
	ZnCl ₂	250 mM
	MnCl ₂	50 mM
	Na ₂ MoO ₄ , 2H ₂ 0	100 μM
	CoCl ₂ , 6H ₂ O	100 mM
	H ₃ BO ₄	50 mM
Vitamines solution (Filtered)	Biotin	50 mg
	Calcium pantothenate	1 g
	Nicotinic acid	1 g
	Myo-inositol	25 g
	Thiamine/HCl	1 g
	Pyridoxal/HCL	1.5 g
	Para-aminobenzoic acid	200 mg
2YT	Tryptone	16 g
	Yeast extract	10 g
	NaCl	5 g
	pH 7	
SOB	Tryptone	20 g
	Yeast extract	5 g
		-

	NaCl	0.6 g
	KCl	0.186 g
	MgCl ₂ 1M	10 mL
	MgSO ₄ 1M	10 mL
	рН 7	
Modified coshon utilization modium		264 ~
from Pridham and Gottlieb (PGMM)	(NH4)2504	2.04 g
	KH ₂ PO ₄ anhydrous	2.38 g
	K ₂ HPO ₄ 3H ₂ O	5.65 g
	MgSO ₄ 7H ₂ O	1 g
	FeSO ₄ 7H ₂ O	1 mL of
		0.11 %
	MnCl ₂ 4H ₂ O	1 mL of
		0.79 %
	$ZnSO_4 7H_2O$	1 mL of
	~ ~ ~ ~	0.15 %
	$CuSO_4 5H_2O$	l mL of
	11.7	0.64 %
	рн /	



Annex 7 – SMART annotation of different genes from lasso peptide cluster identified during genome mining.

Pink segments : low complexity region ; **Blue bars** : Transmembrane regions ; **Red segments** :Signal peptides ; **SnaO** : Pfam GCD14=tRNA methylation ; **SnaR1** : HTH_Xre=Helix-turn-helix Xre family like proteins ; **SnaR2** :HTH_Xre, Pfam DUF397=Domain of unknown function ; **SnaG** :Transmembrane regions and low complexity ; **SnoG** : S_TKc=Serine/Threonine kinase domain ; **NaIG** : Pfam HisKA_3= Histidine kinase involved in TCS, HATPase_c= Histidine Kinase-like ATPases ; **AspR** :Trans reg_C= Transcriptional regulatory protein, BTAD= Bacterial transcriptional activatior domain ; **SveR1** :HTH_GNTR= Helic-Turn-Helix gluconate operon transcriptional repressor, UTRA= UbiC transcription regulator-associated ; **SveR2** : UTRA.

Annex 8 – Example of Sven lasso peptides homologue and logo.

The organization of all the precursor are identical : *A-C-B1/B2-sveN-like*. The genes surrounding this core cluster are very different. Althought the amino acid sequences are similar or identical, the DNA sequence was not. The logo was performed with the 9 last amino acids of the leader peptide and of the core peptides. The Thr at position 8 here is conserved in all the lasso peptide precursor.

Producing organism	Sequence of the precursor	Length
Streptomyces venezuelae ATCC 10712	MTDLPRTEEAPAGAEVLDIGDAAELTQ-GQGGGQSEDKRRAYNC	16
Streptomyces albus DSM41398	MNLRRPQHPQVGALDGVTLHSHSERKIMDSLLSTETVISDDELLPIEVGGTAELTE- GQGGGQS <mark>ED</mark> KRRAYNC	16
Streptomyces griseus NRRL WC- 3480	MNPLQTTEHTDENDLLPVEIGEATVLTE-GQGGGQSEDKRRAYNS	16
Streptomyces Sp. CcalMP-8W	MNPLQTTEHTDENDLLPVEIGEATVLTE-GQGGGQS <mark>ED</mark> KRRAYNS	16
Streptomyces sp. AtexAB-D23	MIDTCNENSDTAPEKKSVAVDLGDVAELTE-GQGGGQSEDKRRAYNC	16
Actinosynnema mirum DSM43827	MALLPEQWNEPPEHTTDGTPSGHDAPVLLTSIGDMSSVTL-GQGKGSAEDKRRAYN-	15
Amycolatopsis alba DSM44262	METVNDQPESGQTTDAPVLLVSLGEASAVTL-GQGKGSSEDKRRAYN-	15



Name	Producer	Sequence	Ref
Astexin I	Asticcacaulis excentricus CB48	GLSQGVEPDIGQTYFEESRINQD	(170)
Astexin II	Asticcacaulis excentricus CB48	G LTQIQALDSVSGQFRDQLGLSAD	(184)
Astexin III	Asticcacaulis excentricus CB48	G PTPMVGL D SVSGQYWDQHAPLAD	(184)
Burhizin	Burkholderia rhizoxinica HKI 454	GGAGQYKEVEAGRWSDRIDSDDE	(163)
Capistruin	Burkholderia thailandensis E264	G TPGFQTP D ARVISRFGN	(152)
Caulonodin I	Caulobacter sp. K31	GDVLNAPEPGIGREPTGLSRD	(171)
Caulonodin II	Caulobacter sp. K31	GDVLFAPEPGVGRPPMGLSED	(171)
Caulonodin III	Caulobacter sp. K31	GQIYDHPEVGIGAYGCEGLQR	(171)
Caulonodin IV	Caulobacter sp. K31	SFDVGTIKEGLVSQYYFA	(171)
Caulonodin V	Caulobacter sp. K31	SIGDSGLRESMSSQTYWP	(171)
Caulonodin VI	Caulobacter sp. K31	A GTGVLLP E TNQIK <mark>RY</mark> DPA	(171)
Caulonodin VII	Caulobacter sp. K31	SGIGDVFPEPNMVRRWD	(171)
Caulosegnins I	Caulobacter segnis DSM 7131	GAFVGQPEAVNPLG<u>RE</u>IQG	(169)
Caulosegnins II	Caulobacter segnis DSM 7131	GTLTPGLPEDFLPGHYMPG	(169)
Caulosegnins III	Caulobacter segnis DSM 7131	GALVGLLLEDITVARYDPM	(169)
Rhodanodin	Rhodanobacter thiooxydans LCS2	GVLPIGNEFMGHAATPGITE	(163)
Rubrivinodin	Rubrivivax gelatinosus IL44	GAPSLINSEDNPAFPQRV	(163)
Sphingonodin I	Sphingobium japonicum UT26	G PGGITG D VGLGENNFGLSDD	(163)
Sphingonodin II	Sphingobium japonicum UT26	G MGSGST D QNGQPKNLIGGISDD	(163)

Annex 9 – Table of lasso peptides heterologously expressed in *E. coli*.

Sphingopyxin I	Sphingopyxis alaskaensis RB2256	GIEPLGPVDEDQGEHYLFAGGITADD	(163)
Sphingopyxin II	Sphingopyxis alaskaensis RB2256	GEALIDQD VGGGRQQFLTGIAQD	(163)
Syanodin I	Sphingobium yanoikuyae XLDN2-5	G ISGGTV D APAGQGLAGILDD	(163)
Xanthomonin I	Xanthomonas gardneri ATCC 19865	GGPLAGEEIGGFNVPGISEE	(187)
Xanthomonin II	Xanthomonas gardneri ATCC 19865	G GPLAG EEMGGITTLGISQD	(187)
Xanthomonin III	Xanthomonas citri pv. Magniferaeindicae LM941 ATCC 19865	GGAGAGEVNGMSPIAGISEE	(187)
Zucinodin	Phenylobacterium zucineum HLK1	G GIGGDF E DLNKPFDV	(163)
Paeninodin	Paenibacillus dendritiformis C454	AGPGTSTPDAFQPDPDEDVHYDS	(188)



Annex 10 – Activity of *sviA*p in *S. sviceus* strains grown in PGMM with sucrose (A) and mannose (B). The GusA activities were measured after 3 and 4 days of growth at 30° C. (n=2)



Annex 11 – Induction of sviceucin expression using culture supernatant of different *S. coelicolor* strains. *S. sviceus* pA was grown in GYM medium for 1 days and then supernatant from *S. coelicolor* M1146, P4H7 and P17G4 grown for 4 days was added. (n=3)

Signal that trigger sviceucin biosynthesis Annex 11

Since the hypothesis of which sviceucin could induce its own expression was ruled out, the signal that trigger the expression of sviceucin remains unknown. Given that sviceucin is highly expressed in the heterologous host *S. coelicolor* P4H7, the supernatant of a 4 days culture of this strain was used to induce the expression of sviceucin in *S. sviceus* pA. The induced cultures showed a slightly longer lag phase and a precocious GusA activity. The activity of GusA of the culture of *S. sviceus* pA induced with supernatant from *S. coelicolor* P4H7 and in particular P17G4 was shown to be significatively higher than the culture induced with the supernatant of the heterologous host *S. coelicolor* M1146 or without induction. The fact that the supernatant of *S. coelicolor* M1146 does not induce the expression of GusA showed that

molecules does not come from *S. coelicolor* but rather encoded in P4H7 or P17G4. Nevertheless, due to the impact of the developmental process on the expression of sviceucin, the involvment a putative QS molecule was not ruled out yet.



Annex 12 – S. sviceus strains grown on R5 media with different concentration of sucrose for 2 and 5 days at 30 °C.



Annex 13 – S. sviceus strains grown on ISP4 with 0, 200 or 500 mM KCl for 2 and 5 days at 30 °C.



Annex 14 – Constructon of P4H8 : Replacement of *phiC31-oriT* in P4H7 by FRT-kan^R-FRT. **pKD4** : Kan^R template ; **P4H7** : Negative control ; **Clone 1-2-3-4** : Positive.



Annex 15 – Digestion of the cosmid P4H7 and P4H8.					
1: P4H7 – XhoI;	14000	12000 2300	1300	1000	OK
2 : P4H7 – HindIII	25000	3400 2400	973		OK
3 :P4H8 – XhoI	14600	11200 2300	1300	1000	OK
4 : P4H8- HindIII	25000	5500			OK
5 : Ladder					



Annex 16 – Inactivation of *sviA*, *sviR1* and *sviR2*. P4H8 :inactivation of *phiC31-oriT* in P4H7 ; Clone 1 and 2 ok P4H8A :inactivation of *sviA* in P4H8 ; Not correct here. P4H8R1 : inactivation of *sviR1* in P4H8 ; Clone 2 Ok. P4H8R2 : inactivation of *sviR2* in P4H8 ; Clone 2 Ok



Annex 17 – PCR verification of P4H8A. Wild-type :3076 bp ; $\Delta \phi C31$ -oriT :1759 bp OK Wild-type *sviA* : 604 bp ; $\Delta sviA$: 1481 bp ;double population of plasmids present in the miniprep.



Annex 18 – PCR verification of purified P4H8A. Non specific fragment very close to the *sviA* wild-type. PCR will be optimized.



Annex 19 – PCR optimization of purfied P4H8A. 58-60-62-64 °C Tm ; P4H8 $\Delta sviA$: Ok





Annex 21 – Selection of *S. sviceus sviA::aac(3)IV*. 32 clones were picked on MS with Apra and MS with Hygro. Clones sensitive resistant to apra but sensitive to hygro are probably inactivated for *sviA*. Clones 3 - 8 - 14 and 16 are correct.



Annex 22 – Inactivation of *sviA* in *S. sviceus*.
PM : Ladder
1 : P4H7 – control negatif (600 bp)
2 :*S. sviceus* Δ*sviA* gDNA (0 bp) OK
3 : P4H7 – control positif (900 bp)
4 : *S. sviceus* Δ*sviA* gDNA – control positif (900 bp) OK



Annex 23 – Selection of *S. sviceus* sviD1D2::aac(3)IV.

64 clones were picked on MS with Apra and MS with Hygro. Clones sensitive resistant to apra but sensitive to hygro are probably inactivated for *sviD1D2*. One clones is correct : 36.



MS Apra

MS Hygro

Annex 24 – Selection of *S.* sviceus *sviG*::*aac(3)IV*.

64 clones were picked on MS with Apra and MS with Hygro. And all were Apra^R and Hygro^S.



Annex 25 – PCR verification of *S. sviceus* Δ*sviD1D2* and Δ*sviG*. Δ*sviD1D2* : Primer JBR168/136 - : 2470 bp ; + : 2170 bp ; Clone 36 : 2170. Δ*sviG* : Primer JM85/JBR142 ; - : 1487 bp ; + : 1760 bp, Clone 5/19/38 : 1760 bp.



MS Apra

MS Hygro

Annex 26 – Selection of S. sviceus sviD3D4 :: aac(3)IV.

64 clones were picked on MS with Apra and MS with Hygro. Clones sensitive resistant to apra but sensitive to hygro are probably inactivated for *sviD3D4*. Four clones were correct : 8 - 17 - 13 - 32.



Annex 27 – PCR verification *S. sviceus sviD3D4 ::aac(3)IV*). Primers JM91/210YL ; **M** : Ladder - :2050 bp + : 1562 bp **8, 13, 17** and **32** : 1562 bp OK



MS Apra

MS Hygro

Annex 28 – Selection of *S. sviceus* svi*F*::*aac(3)IV*. 48 clones were picked on MS with Apra and MS with Hygro. Clones sensitive resistant to apra but sensitive to hygro are probably inactivated for *sviF*. Three clones were correct : 1 - 18 - 28.



Annex 29 – PCR verification *S. sviceus sviF::aac(3)IV*). Primers YL135/YL136 ; M : Ladder - : 921 bp + : 1479 bp 1, 18, and 28 : 1479 bp OK



Annex 30 – Cloning of *sviR1* and *sviR2* in pMS82.

M : Ladder

pfR1 : the gene *sviR1* and its native promoter was cloned in pMS82 (900 bp), clone 1 and 2 ok **pcR1** : *sviR1* was cloned in pMS82 under the control of *ermE*p* (900 bp), clone 1 and 2 ok **pcR2** : *sviR2* was cloned in pMS82 under the control of *ermE*p* (1,2 kbp), clone 1 and 2 ok



Annex 31 – Complementation of *S. sviceus* $\Delta sviR1$ pcR1 and $\Delta sviR2$ pcR2.

1 : Ladder

- **2** : S. sviceus wild-type (200 bp) **3** : S. sviceus \triangle sviR1(0 bp) **4** : S. sviceus \triangle sviR1 pcR1 (200 bp)
- **5** : *S. sviceus* wild-type (180 bp) **6** : *S. sviceus* $\Delta sviR2(0 \text{ bp})$ **7** : *S. sviceus* $\Delta sviR2$ pcR2 (180 bp)



Annex 32 – Complementation of *S. sviceus* $\Delta sviR1$ and $\Delta sviR2$.

 $1: Ladder, \mathbf{2}: H_2O$

3 : S. sviceus wild-type (0 bp) **4** : pcR1 (900 bp) **5** : S. sviceus Δ sviR1 pcR1 (900 bp) OK

6 : *S. sviceus* wild-type (0 bp) **7** :pcR2 (900 bp) **8** : *S. sviceus* Δ*sviR2* pcR2 (900 bp) OK



Annex 33 – PCR verification of pMS82vR1 and pMS82vR2. M : Ladder ; T : pMS82cR1 or -cR2 ; Clone 1/2/3; pMS82vR1 : clone 1 and 3 OK pMS82vR2 : clone 2 and 3 OK



Annex 34 – Cloning of *ermEp*-sviA* into pMS82. PCR verification of pMS82cA. *sviA* : Primer JM105/JM106 ; T+ : P4H8 – 162 bp ; cA : OK *ermEp** : Primer JM96/JM97 ; T+ : pUWL_SNAxre – 300 bp ; cA : OK



Annex 35 – PCR colony of *S. sviceus* containing gene reporter system. T : pGUS template ; g : *S. sviceus* gDNA ; 1 : clone 1 ; 2 : clone 2.

pA : clone 1 and 2	ok
pD1 : clone 1	ok
pG : clone1 and 2	ok
pR1 :clone 1 and 2	ok



Annex 36 – PCR verification of pC. Primers JM58/JM59 – 147 bp expected ; clone 1/2/3 : OK

Publication

An orthogonal system for heterologous expression of actinobacterial lasso peptides in *Streptomyces* hosts

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Abstract

Lasso peptides (LP) are ribosomally synthesized and post-translationally modified peptides produced by bacteria. They are characterized by an unusual lariat-knot structure. LPs from *Actinobacteria* are particularly promising for peptide-based drug discovery. Targeted genome scanning revealed a wide diversity of LPs encoded in *Actinobacteria*. To circumvent problems with cloning of such LP gene clusters, we developed an orthogonal expression system based on a newly-identified regulatory circuit from *Actinoalloteichus* sp. for heterologous production of actinobacterial LPs in *Streptomyces* hosts. Six LP gene clusters, mainly originating from marine actinobacteria, were chosen as proof-of-concept studies. By varying the *Streptomyces*

expression hosts and a small set of culture conditions, three new LPs were successfully produced and characterized by tandem MS. Among them, there are one rare type III LP with a disulfide linkage and two containing non-canonical first residues. The newly developed expression system thus sets the stage to uncover and bioengineer the chemo-diversity of actinobacterial LPs.

Keywords

Actinobacteria, lasso peptides, orthogonal heterologous expression system, *Streptomyces* hosts

Microbial natural products have been the major source of human medicines and agrochemicals for hundreds of years. Recent advances in DNA sequencing and genomicsrevealed that microorganisms harbor a wealth of natural product biosynthetic gene clusters, far exceeding the number of known molecules identified via bioactivity-guided screening. This discrepancy is attributed to the fact that a large number of such gene clusters are not expressed or yield low amounts of products that elude detection under standard laboratory conditions. To enable genomics-based discovery of new natural products, numerous strategies have been developed to activate otherwise cryptic gene clusters.¹ One important approach is to express the pathways in heterologous hosts in combination with cluster engineering or co-expression of a specific regulator.²

Lasso peptides (LPs) are an emerging family of ribosomally synthesized and posttranslationally modified peptides produced by bacteria.³ Composed of 15 to 24 amino acids, they are characterized by an interlocked structure formed by an N-terminal macrolactam ring and a C-terminal tail.⁴⁻⁶ The 7- to 9-residue macrolactam ring is closed between the amino group of the first residue (Gly, Cys, rarely Ser or Ala) and the carboxyl side chain of a Glu or Asp. The tail threads through the ring; unthreading is prevented by steric hindrance from bulky residues and, in some cases, by additional disulfide linkages (Figure 1). Lasso peptides are classified into three categories: those without disulfide bonds form the dominant type II group, whereas the type I and III group, defined by having two or one disulfide bonds, respectively, comprise very few representatives (two for type I and one for type III). Lasso peptides display interesting biological activities, such as enzyme inhibitors and antagonists of receptors, reflecting an advantageous binding of the constrained lasso scaffold to protein targets. Most of the bioactive lasso peptides are isolated from *Actinobacteria.*⁵ A few highlights include siamycins that attenuate the expression of virulence factors in *Enterococcus faecalis* by inhibiting a histidine kinase,⁷ lassomycin that displays anti-mycobacterial activity via inhibition of the Clp protease⁸ and streptomonomicin, which potently inhibits the growth of *Bacillus anthracis* by targeting a vital two-component system.⁹ Their broad range of bioactivities and novel modes of action thus motivate the search for new lasso peptides from *Actinobacteria*. In this regard, bioprospecting of untapped actinobacterial sources such as those from exotic environments seemed particularly promising in affording bioactive LPs.¹⁰⁻¹¹

Genomics-guided approaches greatly accelerated LPs discovery in the recent years. Core genes involved in LP formation encode a precursor peptide (A) that encompasses an Nterminal leader and a structural sequence, a cysteine protease (B) and a macrolactam synthetase (C). Many clusters alternatively contain split B genes: the encoded B1 protein resembles PqqD involved in pyrroloquinoline quinone biosynthesis, whereas the B2 protein is similar to cysteine proteases. The role of B1 in binding the leader peptide has been recently demonstrated.¹² Precursor A-¹³ or enzyme B-focused mining¹⁴ of bacterial genomes as well as mass spectrometry-based peptidogenomics¹⁵ revealed a wide distribution of LP clusters in diverse phyla. Subsequently, a substantial amount of efforts has been made to explore the production potential of Proteobacteria, yielding more than 25 new lasso molecules since 2008.⁶ With the exception of microcin J25 (an archetype of LPs produced by Escherichia coli), all proteobacterial LPs were produced by heterologous expression of the corresponding gene clusters in E. coli. Heterologous production of LPs is a preferred strategy, owing to the small size of lasso peptide gene clusters that is amenable to manipulation. Several expression plasmids were developed to allow facile cloning of these clusters, in which transcription of core biosynthetic genes were under the control of an inducible promoter. In combination with genetic engineering and culture optimization, this strategy permitted selected proteobacterial LPs, e.g. capistruin, to be produced in *E. coli* at a yield of up to 1.6 mg/L.¹⁶

Despite being a rich source of bioactive LPs, *Actinobacteria* have not yet been subject to such intensive exploitation guided by genome mining, with only three examples reported recently. These are the discovery of sviceucin from *Streptomyces* sviceus,¹⁷ chaxapeptin from *S. leeuwenhoekii*¹⁰ and SRO15-2005 from *S. roseosporus*.¹⁵ Sviceucin is the only case for which high level production was achieved by expressing the cosmid-borne, native cluster in *S. coelicolor*. Our previous work on other actinobacterial LPs showed thatsimply transferring the native cluster to *S. coelicolor* did not systematically lead to peptide production. There is a lack of a general strategy for heterologous expression of actinobacterial LPs, in contrast to those originating from *Proteobacteria*. We report here an effective orthogonal expression system designed to circumvent toxicity problems encountered during cloning of actinobacetrial LP

gene clusters in *E. coli*, and to produce these LPs in *Streptomyces* hosts. To demonstrate its utility, the system was successfully applied to bioprospecting of LPs from various actinomycete bacteria.

We first mined recently-acquired draft genome sequences from several both known and newly isolated actinomycete bacteria for putative LP biosynthesis gene clusters. In particular, draft genomes of the following actinobacteria were investigated: Streptomyces spp. ADI94-01 and ADI98-10 (isolated from the marine sponge Antho dichothoma, unpublished data), Actinoalloteichus sp. ADI127-17 (same source), S. noursei ATCC 11455, and S. venezuelae ATCC 10712. Using a combination of antiSMASH 3.04¹⁸ and manual searches, LP gene clusters were identified in all genomes (ADI94-01 genome contained two LP clusters). All LP gene clusters in these actinomycetes showed the conserved gene organization following the order A-C-B/(-B1-B2), and additional genes encoding transporters or putative modifications enzymes were identified in the same operons downstream of the B or B2 gene (Figure 2). In particular, the Sven-LP gene cluster from S. venezuelae contained a gene encoding a putative acetyltransferase. The A127-LP gene cluster from Actinoalloteichus sp. harbored a gene encoding a putative oxidoreductase, and also contained a putative regulatory gene encoding a "Streptomyces antibiotic regulation protein" (SARP) upstream of the precursor gene A (Figure 2). Comparative analysis of the amino acid sequences for the LP precursors revealed that they are quite different, and thus represent a considerable chemo-diversity (Table 1). Moreover, the putative LPs would display new features including non-canonical residues involved in the macrolactam linkage (Ala1 or Trp1), the presence of one disulfide bond (rare type III LPs) and post-translational modifications decorating the lasso scaffold (Table 1)

In an attempt to generate constructs for heterologous expression of these gene clusters, they were PCR-amplified from the respective genomic DNAs and subjected to Gibson assembly with the pSOK806 vector¹⁹, placing them under control of a strong constitutive promoter *ermE*p*. However, the analysis of recombinant constructs for all the clusters revealed multiple deletions, suggesting potentially toxic effect of the expressed peptides on the cloning host, *E. coli*. These cloning attempts were repeated several times, with the same outcome. In order to determine whether the *ermE*p* promoter is active in *E. coli*, the plasmid pSOK808¹⁹ carrying a *ermE*p* promoter::glucuronidase-encoding gene (*gusA*) fusion was introduced into this bacterium, and the GusA activity was measured. A considerable GusA activity could be confirmed in the crude extracts of *E. coli* DH5a carrying pSOK808 (data not shown). To circumvent this problem, an alternative orthogonal two-plasmid expression system was constructed. We reasoned that promoters from a rare actinobacterium, such as *Actinoalloteichus* sp., will unlikely be recognized by the *E. coli* sigma factors. Therefore, a putative promoter region upstream of the LP precursor gene in the A127-LP cluster was chosen for this experiment. Based on the gene organization, this promoter may be activated by the SARP regulator encoded by the gene located upstream, thus potentially providing an additional control over the expression level. In order to test this hypothesis, we first replaced the *ermE*p* promoter in pSOK808 with the abovementioned *A127-LP* promoter and tested the resulting construct, designated pSOK809, in *E. coli* for expression of GusA. As expected, almost no GusA activity was detected.

Next, we introduced the latter plasmid into *S. venezuelae*, where rather moderate (*ca.* 15 times lower compared to *ermE*p*-based pSOK808) level of GusA activity was observed (data not shown). In order to test our hypothesis about SARP-dependent upregulation of the *A127-LP*p promoter, a multi-copy plasmid pSARP carrying the *Actinoalloteichus* sp. SARP-encoding gene under control of *ermE*p* was constructed and introduced into the *S. venezuelae* strain carrying pSOK809. Comparative analysis of the GusA activity in the *S. venezuelae* (pSOK809) strains with or without pSARP revealed *ca.* 5 fold higher expression of the *gusA* gene in the latter. Taking together, these results strongly suggested that the developed two-plasmid system (Figure 3) can be useful for assembly and heterologous expression of LP biosynthetic gene clusters.

Indeed, selected LP gene clusters were successfully assembled in *E. coli* under control of the *A127-LP*p promoter using Gibson ligation and pSOK809-based template. The resulting constructs were integrated into the genomes of various heterologous *Streptomyces* hosts via the phage VWB attachment site of pSOK809. Subsequently, the pSARP plasmid was introduced into these recombinant strains. We mainly used *S. albus* J1074 and *S. lividans* TK24 as hosts, unless otherwise stated. It was observed that conjugation efficiency for the LP expression plasmids was generally low, implying a potential toxicity problem in *Streptomyces* even at the background expression level without SARP. In particular, no exoconjugants could be obtained for the Sven-LP cluster in both abovementioned strains, as well as in *S. coelicolor* M1146. Conjugation of pA127-LP and pSnou-LP into *S. albus* also failed. On the contrary, pA127-LP could be introduced into *S. coelicolor*.

With the *Streptomyces* strains carrying LP expression plasmids, four commonly-used rich media and one phosphate-limiting defined medium²⁰ were screened for LP production in
liquid cultures. The latter choice was based on the observation that production of some antimicrobial peptides was triggered by phosphate starvation, e.g. in the case of microcin J25.²¹ Liquid chromatography coupled to mass spectrometry (LC-MS) analyses of cell extracts indicated that the best culture condition used MYM or MYM-g medium, under which the expected masses of three peptides (9401-LP1, 9810-LP and Snou-LP) were detected in respective extracts, albeit in trace amount (Table 2).

In parallel, seven solid media were tested for LP production (Table 2). Three out of five expected peptides, 9401-LP1, 9810-LP and Snou-LP, were detected in extracts by LC-MS. They were produced in two or more culture conditions used for the respective strains. At least one combination of strain and culture condition gave a product yield suitable for tandem MS characterization. MSMS analyses confirmed unambiguously the primary structures predicted for these peptides. The fragmentation patterns were typical of other LPs, which were resulted mainly from breakage within the tail regions (Figure 3). Moreover, reduction of 9401-LP1 with DTT yielded a mass increase of 2 Da, confirming the presence of one disulfide bond (Figure S1). The best production medium was specific for each LP, and media preference did not show a general pattern. No significant difference among expression hosts for either peptide was noticed. On the contrary, the peptides A127-LP and 9401-LP2 could not be detected under any tested conditions.

The production of three new lasso peptides with a relatively small effort in culture screening showcased the utility of this heterologous expression strategy. To our knowledge, Snou-LP is the first LP to have a Tyr as the first residue and 9401-LP1 represents the second example of the type III LP. Nevertheless, their lasso topology awaits confirmation by NMR studies. To date, regulatory mechanisms and factors that trigger LP biosynthesis in the native producers remain largely unknown. The use of the SARP-responsive promoter *A127-LP*p to drive LP biosynthetic gene expression in *Streptomyces* bears the advantage of bypassing the native regulatory elements. This strategy is thus applicable to exploit LP production potential from unconventional actinobacteria and from metagenomics sources. Moreover, our heterologous expression approach opens a convenient way to generate LP variants by genetic engineering, as reported for LPs from proteobacteria.²²

S. albus J1074 and *S. lividans* TK24 used in this study are well established hosts for heterologous production of secondary metabolites, each being more suitable than the other for certain metabolites.²³ Our data suggest that genotypes of these two hosts do not have a significant influence on LP production level, at least for the LPs and conditions examined. *S.*

albus appeared slightly better because LP production in a wider range of conditions or higher yields were frequently observed for this strain (Table 2). In terms of growth media, solid cultures were clearly found to be superior to the liquid ones for LP biosynthesis, in consistence with previous studies.¹⁵ However, the most suitable production medium is LP-dependent and culture optimization of recombinant strains is still necessary. Since *S. albus* and *S. lividans* are common laboratory strains, the screening process can benefit from the abundant knowledge of their metabolism, and be simplified because of the availability of a range of tested media to choose.

Nevertheless, the strategy developed here failed to express three other LP clusters (9401-LP2, A127-LP and Sven-LP). We suspect that the toxicity of produced LPs to the expression host is a major issue. Several cellular targets of antibacterial LPs have been identified.^{8,24-25}. In particular, the RNA polymerase has been shown to be a common target of LPs including microcin J25,²⁴ siamycins and capistruin,²⁶ despite their different antibacterial spectra. The immunity mechanism of the native producer to its toxic LP is conferred by a dedicated ABC transporter, often encoded in the LP gene cluster. The striking difference in production between 9401-LP1 and 9401-LP2 clearly indicated that the lack of a resistance mechanism in the heterologous host could account for the failure of 9401-LP2 synthesis, as its gene cluster does not encode a pathway-specific transporter in contrast to that of 9401-LP1. For A127-LP, although transporter genes are present, it is likely that *Streptomyces* strains cannot install effectively the immunity mechanism originating from a phylogenetically distant actinobacterium. Similar phenomenon was observed when expressing the gene cluster of microbisporicin originating from *Microbispora coralline in Streptomyces*.²⁷ Worth of note, the 9810-LP could be produced although no transporter genes were found in the cluster. It is remarkable that the Sven-LP genes could not be conjugated into Streptomyces. One peculiarity of this cluster, in addition to the lack of transporter genes, is the presence of a gene whose product shows similarity to ribosomal protein serine acetyltansferases. A precedent example showed that genes encoding ribosomal proteins prevented the conjugation of the gene cluster of GE2270, a thiopeptide identified in *Planobispora rosea*, in *Streptomyces*.²⁸ It is therefore likely that this gene involved in protein acetylation plays a role in the conjugation process. Overall, our data indicate that the success of heterologous expression is mainly determined by the intrinsic properties of the LP cluster of interest and LP pathway engineering is required in order to have a universal application for the developed strategy.

In conclusion, this study developed a SARP-controlled orthogonal expression system for the production of actinobacterial LPs in *Streptomyces*. It is suitable for LP bioprospecting from untapped *Actinobacteria*, and opens a convenient way to produce and engineer LPs with new features that would hold promise for peptide-based drug discovery.

Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table S1. *Streptomyces* strains are routinely maintained on Soya-Flour-Mannitol (SFM) agar plates. For LP production, recombinant *Streptomyces* strains were screened in liquid media including GYM (4 g/L glucose, 4 g/L yeast extract and 10 g/L malt extract), MYM (4 g/L maltose, 4 g/L yeast extract, 10 g/L malt extract, 1.9 g/L MOPS), MYM-g in which maltose was replaced by 24 mL/L glycerol, MP5 (7 g/L yeast extract, *5* g/L NaCl, 1 g/L NaNO₃, 36 ml/L glycerol, 20.9 g/L MOPS, pH 7.5) and phosphate-limited defined medium in which glucose was replaced by 24 mL/L glycerol.²⁰ Solid media used for LP production were GYM, SFM, ISP2 (BD DifcoTM), ISP4 (BD DifcoTM), marine broth (Pronadisa), R5²⁹ and Tryptic Soy Broth (TSB, BD BactoTM) agar. Conjugation of recombinant plasmids into *Streptomyces* was performed as previously described by Flett et al.³⁰ Antibiotics used were apramycin (50 µg/mL) and thiostrepton (17 µg/mL) for selection of recombinant *Streptomyces* strains.

Construction and testing of SARP-based expression system

The pSOK809 reporter plasmid was constructed from PCR-amplified fragments using Gibson assembly.³¹ DNA fragment encompassing the vector part with *gusA* gene was amplified from the pSOK808 template¹⁹ using primers 808GUS-F and 808GUS-R (Table S2). Putative SARP-dependent promoter region from the *Actinoalloteichus* sp. ADI127-17 LP gene cluster upstream of the A gene was amplified from the genomic DNA of the organism using primers SARp-F and SARp-R (Table S2). The pSARP plasmid for expression of the SARP regulator was constructed by a similar procedure. The vector part was amplified from the pUWLoriT template³² using primers UWLS-F and UWLS-R (Table S2). The SARP-encoding gene was amplified from the genomic DNA of the *Actinoalloteichus* sp. ADI127-17 using primers SARPg-F and SARPg-R (Table S2). Both plasmids were introduced in *S. venezuelae* ATCC 10712 via conjugation from *E. coli* ET12567/pUZ8002 either alone or in combination. The resulting strains were tested for GusA activity after 48 h of fermentation in the MYM liquid medium as described in Sekurova et al.¹⁹

Gibson assembly was also used to construct all the plasmids for lasso peptide expression using the pSOK809 part without the *gusA* gene as a backbone and PCR-amplified genomic fragments encompassing respective gene clusters. Oligonucleotide primers used in construction of recombinant plasmids are listed in Table S2. Sequences of the LP gene clusters in this study were deposited in the GenBank. Accession numbers are KX379996 for 9401-LP1, KX379997 for 9401-LP2, KX379998 for 9810_LP, KX379999 for A127_LP, KX380000 for Snou_LP and KX380001 for Sven LP.

Oligonucleotide primers were designed using software j5³³ and Clone Manager 9 (Sci-Ed Software, USA). All constructs were sequenced to verify that no mutations were introduced during PCR amplification.

Production and extraction of LPs

The SARP-based LP expression plasmids were introduced into *Streptomyces* strains by conjugation with *E. coli* ET12567/pUZ8002 as donor strain. Exconjugants were selected on SFM agar supplemented with 10 mM MgCl₂ and apramycin. Integration of the LP gene cluster into the chromosome was confirmed by PCR. The pSARP plasmid was subsequently transferred to the resulting recombinant strain in a similar manner by conjugation. Exconjugants were selected with thiostrepton supplemented in the medium.

Growth on liquid medium for production: A seed culture of each recombinant *Streptomyces* strains harboring the LP cluster was grown in the liquid medium of choice supplemented with apramycin and thiostrepton at 30°C for 2 days. This was used to inoculate 50 mL of the same medium and the culture was grown in the presence of 5% XAD-16 resin at 30°C for 7 days. The mycelia and the resin were extracted with MeOH at room temperature for 3 hours. The extracts were dried in a rotary evaporator and re-suspended in 60% acetonitrile before LC-MS analysis.

Growth on solid medium for production: A seed culture of each recombinant *Streptomyces* strains harboring the LP cluster was grown in liquid GYM medium supplemented with apramycin and thiostrepton at 30°C for 2 days. This seed culture was spread on the solid medium of choice. The plates were sealed and incubated at 30 °C for 7 days. Next, the agar with mycelia were cut into pieces and extracted in 100% MeOH at room temperature for 3 hours. The debris were removed by filtration; the resulting extracts were dried and resuspended in 60% acetonitrile. To scale up, selected strains were grown in square Petri dishes (10 cm x 10 cm) and extraction was performed as described above. Peptides were enriched from the crude extracts by solid phase extraction using a Waters C18 SPE cartridge. 9401_LP1 and 9810-LP

were produced in *S. albus* on ISP2 and ISP4 plates, respectively, and Snou-LP was produced in *S. lividans* on SFM agar.

LC-MS analysis

LP characterization by LC-MS was performed as described previously.¹⁷ Briefly, extracts were separated on an ACE 5 C18-300 column (1×150 mm) (Interchim) operated by an Ultimate 3000 micro-HPLC system (Thermo Scientific) coupled to an ESI-Qq-TOF Q-STAR mass spectrometer (AB Sciex). The gradient used was as follow: 10% to 90% B in 40 min followed by an increase to 100% B in 5 min at a flow-rate of 40 µL/min at 40 °C (A: water with 0.1% formic acid; B: AcCN with 0.1% formic acid). For LC-MS/MS experiments, the doubly charged ion of the LP of interest was selected for collision-induced dissociation with an excitation voltage of 25-30 eV. In the case of 9810-LP peptide, a higher excitation energy of 40 eV was used.

Associated Content

Supporting Information: Strain/plasmid list, primer list and additional MSMS spectra

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SBZ and YL designed the study, JM, CG, OS, ONS and HM performed the experiments, SZ, SR, SBZ and YL supervised the project, SR, SBZ and YL wrote the manuscript.

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Table 1. Chosen lasso peptide gene clusters. Leader peptides are in italic; residues involved in the macrolactam linkage are in bold; Cys residues involved in the disulfide bond formation are underlined.

Name	Precursor peptide sequence	LP size	LP novel	Original strain
		(aa)	features	
9401-	METNDVYEPPALEEIGGFAELTM-	18	type III	Streptomyces sp.
LP1	A FGP <u>C</u> VEN D WFAGTAWI <u>C</u>		Ala1	ADI94-01
9401-	MKAGAVPSLEEPAAYEPPMVVDLGHVR	16	highly	Streptomyces sp.
LP2	<i>EVTL-G</i> SSPNGTA D ANAQYYY		hydrophobic	ADI94-01
A127-	MTESLGSYEPPALEEVGIFDEVTL-	17	type III	Actinoalloteichus sp.
LP	G RPSWGF E ADWS <u>C</u> VMV <u>C</u>			ADI127-17
9810-	MKLQKKAYVKPSLFKQGDFSKKTA-	16	Glu9	Streptomyces sp.
LP	G YFVGSYK E YWTRRIV			ADI98-10
Snou-	MQGNELQETDKLQTYEAPKMIECGSFQ	18	Tyr1	S. noursei ATCC
LP	<i>EDTG-YFGLTGYENLFHFYDKLH</i>			11455
Sven-	MTDLPRTEEAPAGAEVLDIGDAAELTQ	16	potential	S. venezuelae ATCC
LP	- G QGGGQS E DKRRAYNC		acetylation	10712

Table 2. Summary of heterologous production of selected lasso peptides.

LP name	940	1-LP1	940	1-LP2	A127	'-LP	9810-LP		Snou-LI
Expressio	S.	S.	S.	S.	S.	<i>S</i> .	S.	S.	S.
n strain	albus	lividans	albus	lividans	coelicolor	lividans	albus	lividans	lividans
Liquid medium									
GYM	ND	ND	ND	ND	ND	ND	ND	ND	ND
MYM	trace	ND	ND	ND	ND	ND	ND	trace	trace
MYM-g	trace	trace	ND	ND	ND	ND	ND	trace	trace
MP5	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pi-	ND	ND	ND	ND	ND	ND	ND	ND	ND
limiting defined									
medium									
	Solid medium								
GYM	ND	trace	ND	ND	ND	ND	trace	ND	ND
SFM	ND	trace	ND	ND	ND	ND	+	+	+
ISP2	+	trace	ND	ND	ND	ND	trace	ND	trace
ISP4	ND	ND	ND	ND	ND	ND	+	ND	trace
Marine	ND	ND	ND	NG	NG	NG	ND	ND	NG
Broth									
R5	trace	trace	ND	ND	ND	ND	trace	trace	+
TSB	ND	ND	ND	ND	ND	ND	ND	ND	ND
Calc.	19	65.9	16	59.7	191	0.8	20	05.2	2245.1
mass (Da)									
Obs. mass	98	34.0]	ND	NI)	66	59.4	749.4
(m/z)	[M+	-2H] ²⁺					[M+	-3H] ³⁺	[M+3H] ³

ND: not detected; NG: no growth; +: promising for scaling-up









Figure 4. Characterization of produced lasso peptides by tandem MS: (a) 9401-LP1; (b) 9810-LP; (c) Snou-LP.

Supplementary Information

An orthogonal system for heterologous expression of actinobacterial lasso peptides in *Streptomyces* hosts

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Name	Description	Origin
Strains	·	. –
E. coli	dam dcm hsdM hsdS hsdR cat tet; with plasmid	Gust et al.
12567/pUZ8002	pUZ8002; for conjugation	2003
<i>E. coli</i> DH10b	For plasmid propagation	Invitrogen
S. coelicolor M1146	$\Delta act, \Delta red, \Delta cpk, \Delta cda$	Gomez- Escribano and Bibb, 2011
S. lividans TK24	Heterologous expression host	Rückert C et al. 2015
S. albus J1074	Heterologous expression host	Chater and Wilde, 1976
Plasmids		
pSOK809	Reporter plasmid to probe the activity <i>A127-LP</i> p	This study
pSARP	Expression plasmid for SARP regulator under the control of P_{ermE^*}	This study
p9401-LP1	Expression plasmid of 9401_LP1 cluster	This study
p9401-LP2	Expression plasmid of 9401_LP2 cluster	This study
pA127-LP	Expression plasmid of A127-LP cluster with SARP encoded	This study
p9810-LP	Expression plasmid of 9810-LP cluster	This study
pSnou-LP	Expression plasmid of Snou-LP cluster	This study
pSven-LP	Expression plasmid of Sven-LP cluster	This study

Table S1. Plasmids and strains used in this study.

Name	Nucleotide sequence	DNA template	Final construct
808GUS-F	GGTGTCTGCTGTCCATCGATCGAAGGAG AGTTCACCATGCTGA	pSOK808	pSOK809
808GUS-R	CGAAGCGCCGAACCACGAAGTGCGAAGT TCACCGAAGAGCG	pSOK808	pSOK809
SARp-F	TTCGCACTTCGTGGTTCGGCGCTTCGGTG AACTCCG	Actinoalloteichus sp. ADI127-17 gDNA	pSOK809
SARp-R	ACTCTCCTTCGATCGATGGACAGCAGAC ACCAGGTCGCCGC	Actinoalloteichus sp. ADI127-17 gDNA	pSOK809
UWLS-F	GGTGCAGCCGAATGCGTATGGTGCACTC TCAGTACAATCTGCTCT	pUWLoriT	pSARP
UWLS-R	GCAGACACCAGGTCGATCCTACCAACCG GCACGATTGTCCA	pUWLoriT	pSARP
SARPg-F	CCGGTTGGTAGGATCGACCTGGTGTCTG CTGTCGGCCG	Actinoalloteichus sp. ADI127-17 gDNA	pSARP
SARPg-R	GAGTGCACCATACGCATTCGGCTGCACC ACGTCGC	Actinoalloteichus sp. ADI127-17 gDNA	pSARP
809-127-F	GCATCGAGCGGTCCTGCAGGTCGACTCT AGAGGATCCGCG	pSOK809	pA127-LP
809-127-R	GCAGACACCAGGTCGGTGAACTCTCCTT CGATCGATGGACAGC	pSOK809	pA127-LP
A127-LP-F	TCGAAGGAGAGTTCACCGACCTGGTGTC TGCTGTCGGCCG	Actinoalloteichus sp. ADI127-17 gDNA	pA127-LP
A127-LP-R	AGTCGACCTGCAGGACCGCTCGATGCAG CGAAGGC	Actinoalloteichus sp. ADI127-17 gDNA	pA127-LP
809-94011- F	CCAGGCGGGACGTGCAGGTCGACTCTAG AGGATCCG	pSOK809	p9401-LP1
809-94011- R	GTCTCACCTGCTCGTGAACTCTCCTTCGA TCGATGGACAG	pSOK809	p9401-LP1
94011-LP-F	TCGATCGAAGGAGAGAGTTCACGAGCAGGT GAGACGAACATGGAGACGAACG	<i>Streptomyces</i> sp. MP94-01 gDNA	p9401-LP1
94011-LP-R	CCTCTAGAGTCGACCTGCACGTCCCGCCT GGCCGGGTCCG	<i>Streptomyces</i> sp. MP94-01 gDNA	p9401-LP1
809-94012- F	TCGATCGAAGGAGAGTTCACCAGCTGAT GCCCCTGACCCGAG	pSOK809	p9401-LP2
809-94012- R	CCTCTAGAGTCGACCTGCAAGAGGCTGT GGGGGTCCC	pSOK809	p9401-LP2
94012-LP-F	TGCAGGTCGACTCTAGAGGAT	<i>Streptomyces</i> sp. MP94-01 gDNA	p9401-LP2
94012-LP-R	GTGAACTCTCCTTCGATCGATG	<i>Streptomyces</i> sp. MP94-01 gDNA	p9401-LP2

809-9810-F	GGCGGCATGAGTGTGCAGGTCGACTCTA GAGGATCCGC	pSOK809	p9819-LP
809-9810-R	CGGTTGACGACCGTGAACTCTCCTTCGAT CGATGGACAGC	pSOK809	p9819-LP
9810-LP-F	CGAAGGAGAGTTCACGGTCGTCAACCGT ACAGAAGGGA	<i>Streptomyces</i> sp. MP98-10 gDNA	p9819-LP
9810-LP-R	GAGTCGACCTGCACACTCATGCCGCCCC GCA	<i>Streptomyces</i> sp. MP98-10 gDNA	p9819-LP
809Sven-F	CCTTGGCCACGACCTGCAGGTCGACTCT AGAGGATCCG	pSOK809	pSVEN-LP
809Sven-R	GGTCGGTACGGCAACGGTGAACTCTCCT TCGATCGATGGACAGC	pSOK809	pSVEN-LP
Sven-LP-F	AGGAGAGTTCACCGTTGCCGTACCGACC ACAGAGAGG	<i>S. venezuelae</i> ATCC 10712 gDNA	pSVEN-LP
Sven-LP-R	AGTCGACCTGCAGGTCGTGGCCAAGGTC GACCGC	<i>S. venezuelae</i> ATCC 10712 gDNA	pSVEN-LP
809-Snou-F	CCATCGACCTGTTGCTGCAGGTCGACTCT AGAGGATCCGCG	pSOK809	pSNOU-LP
809-Snou-R	CCGGTACGGAAGGTTCGGTGAACTCTCC TTCGATCGATGGACAGC	pSOK809	pSNOU-LP
Snou-LP-F	AGGAGAGTTCACCGAACCTTCCGTACCG GAACGCGT	<i>S. noursei</i> ATCC 11455 gDNA	pSNOU-LP
Snou-LP-R	AGTCGACCTGCAGCAACAGGTCGATGGC TTCTTGCCTGGT	<i>S. noursei</i> ATCC 11455 gDNA	pSNOU-LP



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Les peptides lasso des actinobactéries : diversité chimique et rôle écologique

Les peptides lasso sont des peptides bioactifs bactériens issus de la voie de biosynthèse ribosomale et subissant des modifications post-traductionnelles, caractérisés par une structure entrelacée dite en lasso. Ils possèdent un cycle macrolactame en position N-terminale, traversé par la queue C-terminale. Cette topologie de type rotaxane, maintenue par piégeage de la queue C-terminale dans le cycle via des acides aminés encombrant et/ou des ponts disulfure, confère à ces peptides une structure compacte et stable. Les actinobactéries recèlent la plus grande diversité et gamme d'activités biologiques parmi les peptides lasso (antibactériens, anti-VIH, antagonistes de récepteurs..), et l'exploration de génomes suggère une diversité encore plus grande, puisque certains clusters portent des gènes codant des enzymes de modifications post-traductionnelles jamais observées auparavant. Cependant, l'expression de ces peptides semble être rigoureusement contrôlée, rendant leur production en laboratoire difficile à partir de la bactérie productrice. Le rôle écologique et les mécanismes de régulation des peptides lasso ne sont pas très documentés. Leur compréhension permettrait d'améliorer la production et de mieux exploiter les activités biologiques des peptides lasso.

La première partie de mon travail de thèse porte sur la découverte de nouveaux peptides lasso chez les actinobactéries par expression des clusters de gènes correspondant dans un hôte hétérologue de *Streptomyces*. Pour cela, un système d'expression basé sur une voie de régulation impliquant une protéine SARP (*Streptomyces* antibiotic regulatory protein) a été développé. L'analyse de génomes d'une collection d'actinobactéries, en particulier d'un ensemble de souches isolées d'éponges, nous a permis de sélectionner six clusters qui ont été exprimés selon cette stratégie. Cette approche a permis la production de trois peptides lasso dotés de nouvelles caractéristiques, identifiés par spectrométrie de masse.

La seconde partie de mon travail de thèse porte sur le mécanisme de régulation et le rôle écologique des peptides lasso en prenant la svicéucine, produite par *Streptomyces sviceus*, comme modèle d'étude. La génération de souches inactivées pour la production de svicéucine a permis de révéler que l'expression de la svicéucine est contrôlée par un système de régulation codé à proximité des gènes de biosynthèse. La caractérisation de ces souches mutantes a révélé une sensibilité accrue à de fortes concentrations de KCl et également, de façon spécifique, à un générateur d'ion superoxyde, la phénazine methosulfate. Ces éléments suggèrent que la svicéucine pourrait jouer un rôle dans la résistance aux stress osmotique et oxydatif au cours du cycle de vie de *S. sviceus*.

Mots clés : Peptides lasso, expression hétérologue, ingénierie génétique, Streptomyces.

Lasso peptides from Actinobacteria: chemical diversity and ecologicole role

Lasso peptides are ribosomally synthesized and post-translationally modified peptides produced by bacteria, characterized by a remarkable mechanically-interlocked structure. The lasso topology, reminiscent to a rotaxane, consists in an N-terminal macrolactam ring threaded by a C-terminal tail. This compact and stable structure is stabilized by steric entrapping of the tail in the ring, through bulky amino acid(s) and/or disulphide bonds. Lasso peptides produced by *Actinobacteria* display the greatest chemical diversity and a range of biological activities (antibacterial, anti-HIV, receptor antagonist...), therefore are of high pharmaceutical interest. Genome mining revealed that *Actinobacteria* have enormous potential to biosynthesize novel lasso peptides, e.g. harbouring new post-translational modifications. However, the expression of these peptides is generally controlled by complex regulatory systems, making their production under laboratory conditions difficult. Understanding the ecological role and regulation mechanisms of lasso peptides would help to improve production and better exploit the biotechnological potential of these molecules.

The first part of my work deals with the identification of new lasso peptides from *Actinobacteria*, using heterologous expression in *Streptomyces* hosts. An expression system based on the *Streptomyces* antibiotic regulatory protein (SARP)-controlled element was developed, which opens the perspective to fully explore the chemical diversity of lasso peptides from *Actinobacteria*. Genome mining of a collection of *Actinobacteria*, in particular strains isolated from marine sponges, allowed us to select six clusters that were expressed using this strategy. This approach allowed the production of three lasso peptides with new characteristics, identified by mass spectrometry.

The second part of my work deals with the regulation mechanism and ecological role of lasso peptides using sviceucin, a lasso peptide produced by *Streptomyces sviceus*, as the model for study. It was demonstrated that sviceucin biosynthesis in *S. sviceus* is controlled differentially by two response regulators which are part of a two-component system. Mutant *S. sviceus* strains deficient in sviceucin production showed altered morphology under certain conditions and displayed increased sensitivity specifically towards phenazine methosulfate, a superoxide anion generator. These results suggest a link of sviceucin and oxidative stress response, which could lead to modulation of morphology in *S. sviceus*.

Key words: Lasso peptides, heterologous expression, genetic engineering, Streptomyces