Streptomyces venezuelae: a source of ECF-sigma factors for genetic switches and a model for antibiotic resistance studies

Dissertation zur Erlangung des Doktorgrades der naturwissenschaftlichen Dr. rer. nat. an der Fakultät für Biologie der Ludwig-Maximilians-Universität München

von

Dayane Cristiny Rodrigues Araújo

München, 2019

Erstgutachter: Prof. Dr. Thorsten Mascher
Zweitgutachter: Prof. Dr. Heinrich Jung
Datum der Abgabe: 02.10.2019
Datum der mündlichen Prüfung: 17.12.2019

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt wurde. Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die folgende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

Munich, 02 October 2019

Dayane Cristiny Rodrigues Araújo

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources. As well I declare that, I have not submitted a dissertation without success and not passed the oral exam. The present dissertation (neither the entire dissertation nor parts) has not been presented to another examination board.

Munich, 02 October 2019

Dayane Cristiny Rodrigues Araújo

Table of contents

TABLE OF CONTENTS

List of Figures

List of Tables

ABBREVIATIONS

Abbreviations

AMPs – antimicrobial peptides

αNTD – αN-terminal domain

AS – anti-σ factor

ATP – adenosine-5'-triphosphate

dsDNA – double strand DNA

ECF – extracytoplasmic function

EDTA – ethylene diamine triacetic acid

HK – histidine kinase

NCR – non-conserved region of σ

N-FLAG tag – N-terminus flag tag

PCR – polymerase chain reaction

RNAP – RNA polymerase

RR – response regulator

SDS – sodium dodecyl sulfate

LFH – long-flanking homology

LPS – lipopolysaccharide

MFS – major facilitator superfamily

MIC – minimum inhibitory concentration

MCS – multiple cloning sites

NCBI – National Center for Biotechnology Information

NTG – N-methil-N'-nitro-N-nitrosoguanidine

OD_{600} – optical density at a wavelength of 600 nm

TCS – two-component system

TM – transmembrane domain

ASD – anti-sigma domain

ABBREVIATIONS

σR – sigma region

ZASD – zinc binding anti-sigma domain

RIP – regulated intramembrane proteolysis

RPM – revolutions per minute

RLU – relative luminescence values

w/v – weight per volume

SUMMARY

Summary

The soil is a challenging habitat where microorganisms are exposed to adverse environmental conditions and where they compete for space and resources. Surviving in such situations requires bacteria to permanently monitor their environment and initiate the appropriate responses to counteract the deleterious effects of a given adverse condition. For this, bacteria have sophisticated systems to sense environmental changes and elaborate specialized responses in order to overcome conditions that could cause harm. These systems are i) one-component system (1CS), ii) two-component systems, and iii) extracytoplasmic function (ECF) σ factors. Streptomycetes are gram-positive soil bacteria with a complex life cycle, being antibiotic producers and also antibiotic resistant. Additionally, these bacteria present one of the highest repertoires of ECF σ factors making them the ideal model organism for this study. The aims of this study were to **i)** use of ECF σ factors of *S. venezuelae* to design and implement orthogonal switches in *B. subtilis* and **ii)** elucidate the mechanisms of resistance against antimicrobial peptides in *S. venezuelae*. The performance of the ECF19-based switch was tested in the presence of its anti-σ factor, upon variation in copy number of each constituent transcriptional unit, type of inducible promoter, ECF σ factor stability and under the effects of antisense transcription. This systematic characterization of the ECF19 switch has shown it is the best performing heterologous ECF-based switch so far described for *B. subtilis*. Its activity is nonetheless compromised by genetic perturbations caused by changes in the copy number, protein stability and antisense transcription. This study also demonstrates that *S. venezuelae* is highly resistant to the antimicrobial peptide bacitracin. By performing random mutagenesis followed by genome sequencing and RNAseq analysis, it was possible to identify the genes that might be involved in bacitracin resistance in *S. venezuelae*.

ZUSAMMENFASSUNG

Zusammenfassung

Der Erdboden ist ein herausforderndes Habitat, in dem Mikroorganismen widrigen Umwelteinflüssen ausgesetzt sind und zusätzlich um Raum und Ressourcen konkurrieren müssen. Um in solchen Situationen überleben zu können, sind die Bakterien permanent darauf angewiesen, ihre Umgebung zu überwachen. Bei gegebenen ungünstigen Bedingungen müssen entsprechende angemessene Reaktionen initiiert werden, damit sie diesen schädlichen Effekten entgegen wirken zu können. Für diese Aufgabe stellen Bakterien komplexe Systeme bereit, die die Umweltveränderungen wahrnehmen und spezialisierte Antworten auslösen, um Bedingungen zu überwinden, die Schäden verursachen können. Diese Systeme sind i) Einkomponentensysteme (1CS), ii) Zweikomponentensysteme (2CS) und iii) extracytoplasmic function (ECF)-Sigmafaktoren.

Streptomyceten sind grampositive Bodenbakterien mit komplexen Lebenszyklen, die Antibiotika produzieren aber auch selbst antibiotische Resistenzen aufweisen. Zusätzlich stellen diese Bakterien ein sehr breites Repertoire an ECF-Sigmafaktoren bereit, was sie zu einem optimalen Modellorganismus für diese Arbeit macht.

Das Ziel dieser Arbeit bestand darin, i) die Nutzung von ECF-Sigmafaktoren in S. venezuelae zu erläutern, darauf aufbauend orthogonale Schalter in B. subtilis zu konzipieren und zu implementieren. ii) Des Weiteren sollten die Mechanismen der Resistenz gegen antimikrobielle Peptide in S. venezuelae aufgeklärt werden.

Die Leistungsfähigkeit des, auf ECF19 basierenden, Schalters wurde unter verschiedenen Bedingungen getestet: in der Anwesenheit seines anti-Sigmafaktors, nach Veränderung der Kopiezahl jeder einzelnen Transkriptionseinheit, unter der Kontrolle unterschiedlicher Typen des induzierenden Promoters, auf Stabilität des ECF-Sigmafaktors und auf den Effekt der antisense-Transkription. Diese systematische Charakterisierung des ECF19-Schalters hat gezeigt, dass es sich dabei um einen heterologen ECF-Schalter mit der besten Leistung in B. subtilis handelt, die bisher beschrieben wurde. Die Aktivität des Schalters wird allerdings beeinträchtigt durch genetische Störungen, die durch Änderungen der Kopiezahl, der Proteinstabilität und antisense-Transkription hervorgerufen werden. Diese Arbeit hat weiterhin demonstriert, dass S. venezuelae hochresistent gegen das antimikrobielle Peptid, Bacitracin, ist. Aufgrund der Durchführung von zufälliger Mutagenese, gefolgt von Genomsequenzierung und RNAseq war es möglich, Gene zu identifizieren, die höchstwahrscheinlich in der Bacitracin-Resistenz in S. venezuelae eine wichtige Rolle spielen.

1. Introduction

1.1.Streptomyces venezuelae

The soil poses a highly competitive environment for bacterial growth, which is reflected in the production of a broad range of antimicrobial compounds by soil bacteria and, correspondingly, in a high prevalence of resistance against these antibiotics (Kieser *et al.*, 2000; D'Costa *et al.*, 2006). Streptomycetes are gram-positive soil bacteria which have complex life cycles and produce a range of secondary metabolites, most of which are biologically active and are often useful for medicine. This group of bacteria are considered as the most important producers of antibiotics and present a complex program of differentiation. *S. venezuelae* grows as branching vegetative mycelium that under adverse environmental conditions serves as a substrate for the formation of aerial hyphae. This morphological transition usually co-occurs with the production of secondary metabolites, such as antibiotics. Subsequently, aerial hyphae develop into chains of spores that will germinate initiating a new cycle (**Figure 1**) (Glazebrook *et al.*, 1990; Bibb *et al.*, 2012). *S. venezuelae* has recently become a new model organism for Streptomycetes and has been successfully used in transcriptional, proteomic and other analyses. In addition to being amenable to genetic manipulation, this bacterium has a relatively fast growth rate and requires a short period of culture for production of metabolites. Furthermore, *S. venezuelae* develops synchronously in liquid cultures and sporulates almost completely (> 90%), what is an advantage compared to the model organism *Streptomyces coelicolor* (Bibb *et al.*, 2012).

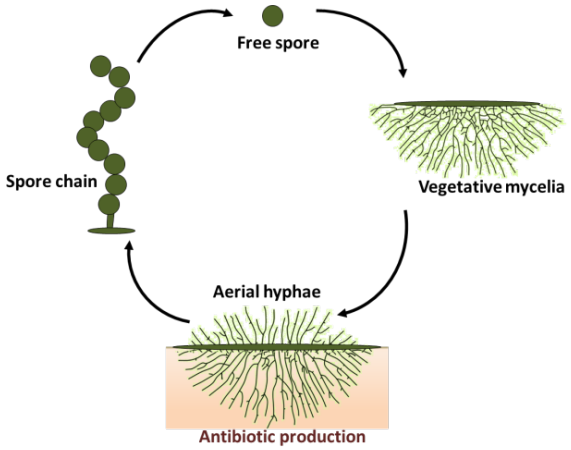


Figure 1. Life cycle of *Streptomyces venezuelae*

1.2.Antibiotic resistance in Streptomycetes

Streptomyces species produce numerous secondary metabolites including many clinically relevant antibiotics, which correspond to over 60 % of the known antibiotics (Le *et al.*, 2009). In consequence, self-protection resistance mechanisms are usually coregulated with the biosynthesis of antibiotics, and are expressed prior to or concomitantly with the presence of the antimicrobial molecules (reviewed in Mak, Xu and Nodwell, 2014). Thus, the biosynthesis of antibiotic is tightly regulated at many levels to ensure the exact time of production and to avoid potential toxicity for the producer organisms (Novakova *et al.*, 2010).

The soil is a competitive habit and the production of antimicrobial molecules is one of the most studied mechanisms of bacterial competition. In such context, not only mechanisms of resistance to self but also to antibiotics produced by neighbouring organisms are essential for survival (**Figure 2**).

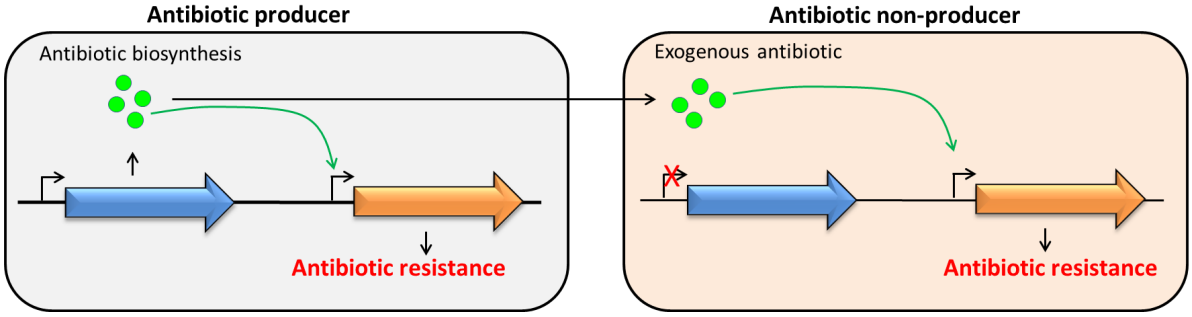


Figure 2. Representation of resistance determinants for antibiotic producing and antibiotic non-producing bacteria. Thick blue arrows represent biosynthetic genes, think orange arrow represent resitance genes, thin arrows represent promoters, and green cycles represent antimicrobial molecules (modified from Mak *et al.*, 2014).

Thus, it is not surprininsg that *Streptomyces* species are an important reservoir of antibiotic resistance genes in soil and can even carry resistance to clinical antibiotics (D'Costa *et al.*, 2006; Bhullar *et al.*, 2012; Schlatter and Kinkel, 2014). For example, *Streptomyces coelicolor* encodes resistance mechanism against vancomycin, however, it does not produce this antibiotic or any similar glycopeptide antibiotics (Hong *et al.*, 2004).

1.3.Signal transduction repertoire of *S. venezuelae*

Bacteria such as *Streptomyces* has complex mechanisms of signal transduction in order to deal with often-changing environmental conditions. One-component systems are the most abundant mechanisms of signal transduction in bacteria being characterised by the presence of both, the sensory domain and regulatory domain in a single polypeptide. The second most common mechanism of signal transduction are two-component systems that are composed of a sensor histidine kinase that is subject to autophosphorylation upon stimulus sensing, which further

activates its cognate response regulator through phosphate transfer (Ulrich, Koonin and Zhulin, 2005; Laub and Goulian, 2007). The third major mechanism by which bacteria sense and respond to external stimuli is through the extracytoplasmic function (ECF) σ factors that are are kept inactive by a negative regulator anti-σ factor in the absence of the inducing stimulus (Mascher, 2013) (**Figure 3**). A bacteria's lifestyle is correlated with the number of ECF σ factors it encodes: those facing challenging environments that have to survive diverse stress conditions, fluctuating nutrient availability, microbial competition and morphological differentiation encode a greater number of ECF σ factors than for example those intracellular pathogenic bacteria living in a more stable environment (Gruber and Gross, 2003; Staroń *et al.*, 2009; Jogler *et al.*, 2012; Mascher, 2013; Huang *et al.*, 2015; Paget, 2015).

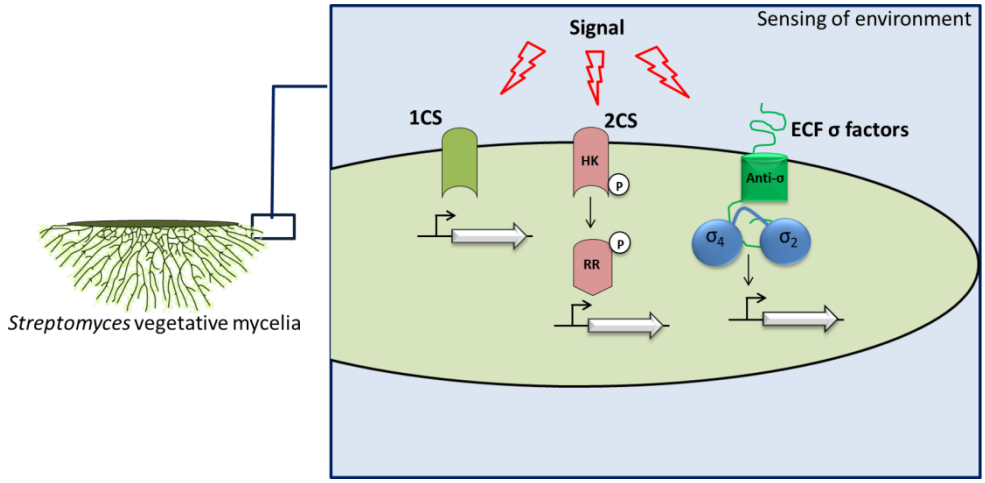


Figure 3. Representation of signal transduction mechanisms in bacteria. In the left is shown the *Streptomyces* vegetative mycelia. One-component system (1CS), two-component-system (2CS) and ECF σ factors sense the environmental stimulus (signal) and promp a specific response.

1.4.Extracytoplasmic function (ECF) σ factors

Bacteria express several genes whose products are required for normal growth but also for successfull adaptation to environmental changes. Hence, controling the production of these proteins, ensuring that they will be expressed at the right time and amount in order to sustain the bacteria's life cycle and allow adaptation to stress conditions is crucial (Gross *et al.*, 1998). Gene expression is primarily modulated at transcription initiation levels, which has as one central player the RNA polymerase (RNAP). The core bacterial RNAP is composed of five subunits: the large β and β' catalytic subunits, two identical α subunits and the small ω subunit. Alpha subunits consist of two independently folded domains joined by a flexible linker, being the α N-terminal domain (αNTD) responsible for the assembly of the β and β' subunits. The ω subunit seems to assist the folding of the β' subunit, acting as a chaperone (Blatter *et al.*, 1994;

Gourse, Ross and Gaal, 2000; Korzheva *et al.*, 2000; Hampsey and Reinberg, 2001; Browning and Busby, 2004; Österberg, Peso-Santos and Shingler, 2011). However, the core RNAP is not able to recognize promoters and initiate transcription by itself. A sixth dissociable subunit called σ factor is recruited by the core RNAP resulting in the formation of the RNAP holoenzyme allowing in this way the **i)** recognition of specific promoters, **ii)** correct RNAP holoenzyme positioning and **iii)** unwinding of the DNA-double strand downstream the transcript start site (**Figure 4**) (Gross *et al.*, 1998; Browning and Busby, 2004).

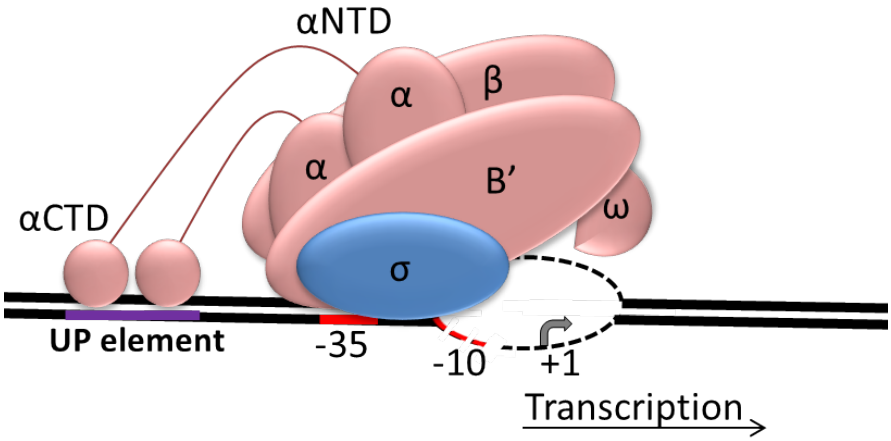


Figure 4. General representation of the transcription initiation complex. RNAP α, β, β' and ω subunits are showed in pink and the σ factor is showed in blue. σ factor recognition sequences (-35 and -10) are in red, up element is shown in purple and the transcription start site (+1) is represented by a grey arrow. The direction of transcription is indicated.

The most abundant σ factors belong to the σ^{70} family, which recognise specific sequences at positions -35 and -10 from the transcription start site (position +1). The σ^{70} family is divided in four groups: the group 1 comprises the essential housekeeping σ factors; group 2 their non-essential paralogues; group 3 contains σ factors involved in specific functions such as motility, heat shock resistance and sporulation; and, group 4 contains the extracytoplasmic function (ECF) σ factors (Wösten *et al.*, 1998; Gruber and Gross, 2003). Group 1 σ factors are composed of four conserved domains (σ_1, σ_2, σ_3 and σ_4) and an additional non-conserved region (NCR); the group 2 σ factors are closely related to those of group 1 but lack the region $\sigma_{1.1}$ of the σ_1 domain; the ones of group 3 lack the complete σ_1 domain and the NCR; and, group 4 σ factors contain only the σ_2 and σ_4 domains, which are sufficient for core RNAP binding and promoter recognition (Gross *et al.*, 1998; Helmann and Moran, 2002; Butcher, Mascher and Helmann, 2008; Mascher, 2013) (**Figure 5**). In the primary σ factors the domain σ_1 binds to a discriminator sequence located between the -10 region and the transcriptional start site (+1 region). Domain σ_3 recognizes one base upstream the -10 region called extended -10 (Barne *et al.*, 1997; Campbell *et al.*, 2002). The σ_4 domain binds to the -35 region and σ_2 domain binds

to the conserved -10 region. Initial binding of the RNAP holoenzyme to the promoter results in the formation of the closed complex, in which the DNA is double stranded. Unwinding of the dsDNA starts upstream the -10 region and results in the transition to the open complex, in which the RNAP holoenzyme interacts simultaneously with both single and double stranded DNA (Gross *et al.*, 1998; Campbell *et al.*, 2002). Shorty after initiation, the σ subunit is released from the RNAP holoenzyme, freeing the σ factor for association with other core RNAP and start a new round of transcription initiation (Murakami and Darst, 2003; Österberg, Peso-Santos and Shingler, 2011; Friedman and Gelles, 2012).

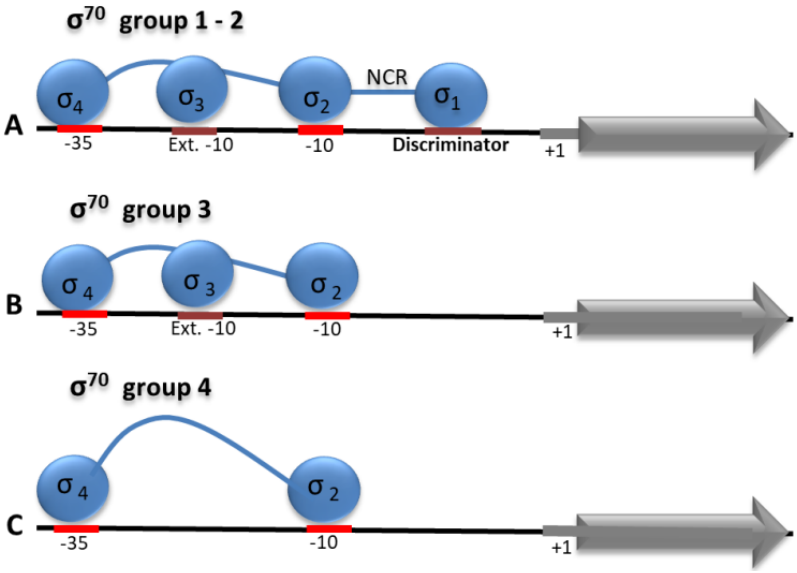


Figure 5. Domain architecture and target promoters of A) primary σ^{70}; B) σ^{70} group 3 of heat shock, flagellar and sporulation σ-factors; C) σ^{70} group 4 of alternative ECF-σ factors. The four σ domains (σ_1, σ_2, σ_3 and σ_4) are represented in blue. NCR stands for non-conserved region. Promoter regions are highlighted in light red. Extended -10 promoter region and discriminator are shown in dark red. Region + 1 in grey shows the transcription start site and grey arrows represent the coding sequence.

Besides being a component of the RNAP that is responsible for promoter specificity, as mentioded before, σ factors such as ECF σ factors also play essential roles in signal transduction (Sun *et al.*, 2017). The availability of ECF σ factors in the bacterial cell is generally modulated by a cognate anti-σ factor that sequesters the ECF σ factors, keeping them inactive in the absence of an inducing stimulus. In the presence of such stimulus, the anti-σ releases the ECF σ factor through proteolysis or conformational changes allowing in this way the recruitment of the ECF σ factor by the core RNAP. ECF σ factors are often co-transcribed with their cognate anti-σ factors and are subject of autoregulation. Thus, once the stimulus ceases, the transcriptional response mediated by ECF σ factors will be turned off by sequestration of the ECF σ factor by the anti-σ and the system is returned to its inactive state (Lonetto *et al.*, 1994; Hughes and Mathee, 1998; Reviewed in Paget and Helmann, 2003; Mascher, 2013; Reviewed

in Paget, 2015) (**Figure 6**). The ECF σ factor family is the most abundant and diverse family of σ factors. They have been classified in over 50 different subgroups in which some are phyla specific and others are widely distributed across bacteria (Staroń *et al.*, 2009; Rhodius *et al.*, 2013; Huang *et al.*, 2015).

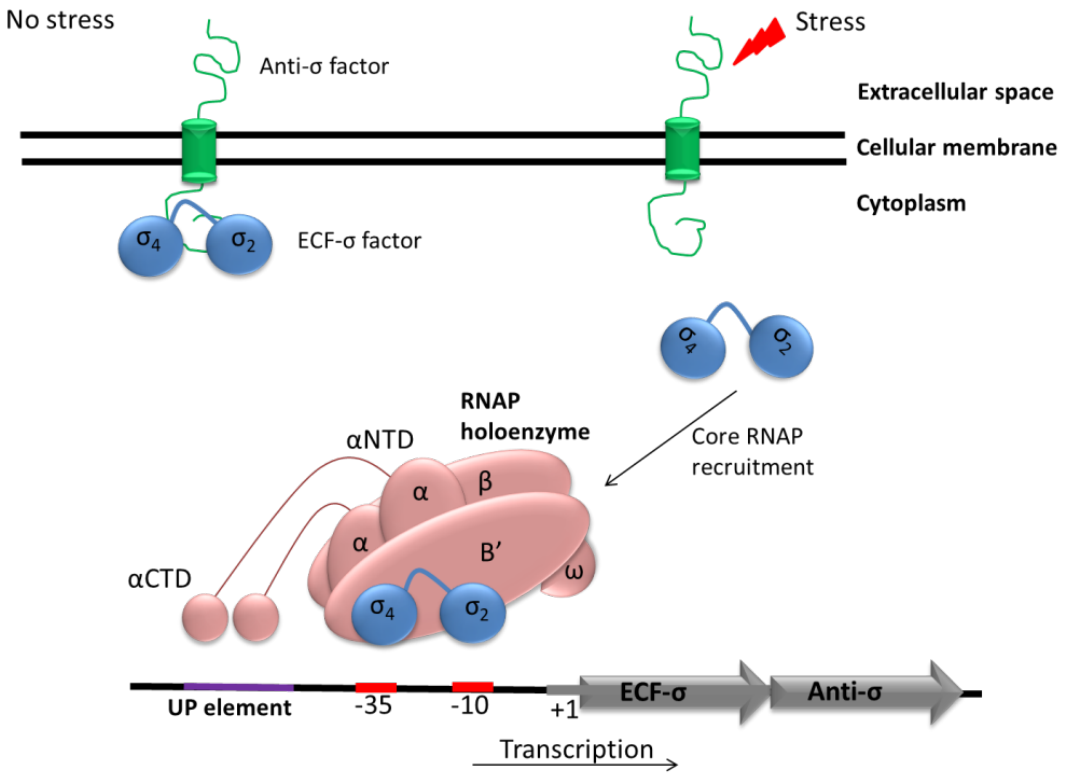


Figure 6. Hallmark characteristics of the signal transduction mediated by ECF σ factors. ECF σ factor domains are shown in blue; cognate anti-σ factor is shown in green. The RNAP core and its respective subunits are shown in pink. Promoter regions -10 and -35 are highlighted in red. Up element is shown is purple. Region +1 is shown in grey followed by grey arrows that represent the ECF-σ factor – anti-σ factor operon. (Figure adapted from Mascher 2013).

1.5.ECF σ factors as building parts for synthetic switches

Synthetic biology is focused on the rational engineering of biologically based (or inspired) systems. For this purpose, it is convinient to use standard parts such as promoters, terminators, ribosome binding sites, and gene coding sequences that can be put together in order to build synthetic circuits to introduce or modify biological functions (Serrano, 2007). Despite the use of well-characterized parts, the complexity of living systems can bring up unexpected behaviours as well as the loss of functionality of synthetic circuits when these are implemented in a different genetic context (Guet *et al.*, 2002; Morey *et al.*, 2012; Del Vecchio, Dy and Qian, 2016) (**Figure 7**).

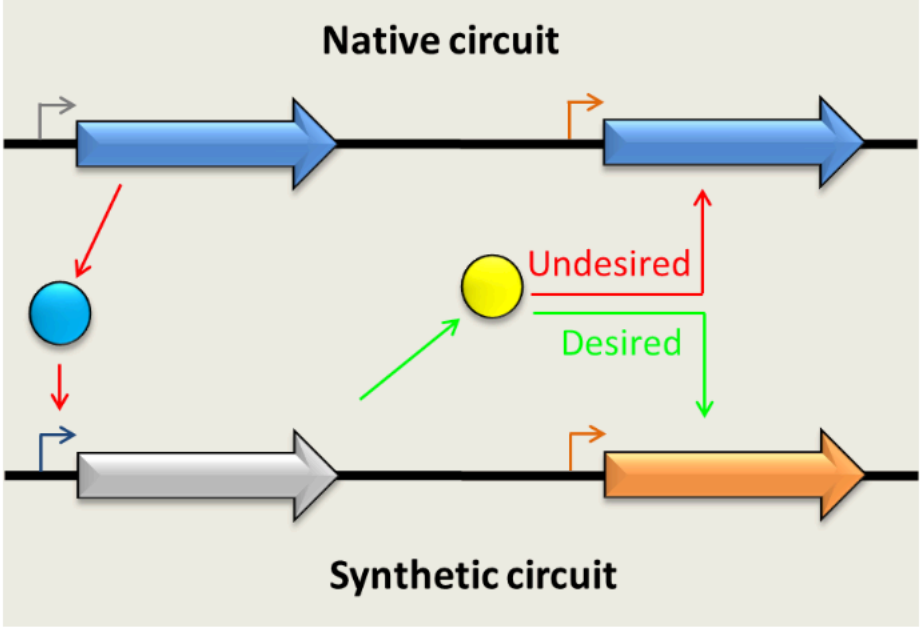


Figure 7. Representation of unexpected cross-interaction between a synthetic circuit with the host parts Thick arrows represent the genes, thin arrows represent promoters, circles represent gene products. Green long arrows represent desired behaviour and red long arrows represent undesired behaviour (adapted from Zhang, Tsoi and You, 2016).

Thus, the use of orthogonal parts (what means biological parts that are dissimilar to those occurring naturally) from different organisms can minimize the chances of cross-interactions with the native machinery. Until now, a variety of synthetic circuits were built using natural orthogonal parts such as dCas9, riboswitches and transcription factors (Stanton *et al.*, 2014; Gao and Elowitz, 2016; Westbrook and Lucks, 2017). These circuits are inspired by biological processes that are controlled by feedback loops, switching between OFF and ON states when in presence of a specific inducer (Clancy and Voigt, 2010). Rhodius and co-workers (2013) successfully implemented ECF-based switches in *E. coli* and a more recent work reported the use of ECF σ factors to build autonomous timers in *E. coli* and *B. subtilis* (Pinto *et al.*, 2018b). The minimal domain architecture of ECF σ factors and the fact that ECF promoters are highly conserved and can be easily found upstream the ECF coding genes (Staroń *et al.*, 2009), makes them actractive alternatives to classical transcription regulators in synthetic circuit design. Altogether, ECF σ factors have been shown to be modular, orthogonal, universal and scalable representing ideal building blocks for complex synthetic biology application.

The ability of *Streptomyces* to perceive and respond to diverse environmental challenges is crucial for its survival, what probably boosted the development of accurate mechanisms of signal transduction. Usually, secondary metabolism and differentiation in *Streptomyces* are controlled by ECF σ factors (Kormanec *et al.*, 2016). The classification of ECF σ factors made by Staron and co-workers (2009) showed that bacteria present an average of six ECF σ factors

per genome. However, *Streptomyces coelicolor*, for example, has a total of 51 ECF σ factors, which correspond to approximately 80% of the total number of σ factors in this organism (Paget, Mark SB; Hong, HJ; Bibb, 2002). A more recent phylum-specific analysis found over 2200 ECF σ factors in *Actinobacteria* genomes wherein *Streptomycetaceae* family was revealed as ECF-rich but also containing a large diversity of ECF σ factors. Those belong to twenty different ECF groups, of which eight are *Actinobacteria*-specific (Huang *et al.*, 2015). In accordance, *S. venezuelae* presents a wide repertoire of 47 ECF σ factors, belonging to over 30 groups and making this organism a good source of ECF σ factors for synthetic biology applications.

Bacillus subtilis is a soil gram-positive bacterium with a low GC content, which differentiates into spores to survive long periods of starvation. It is highly amenable to genetic manipulations, being extensively used as model organism for gram-positive bacteria (van Dijl and Hecker, 2013). *B. subtilis* possess only seven ECF σ factors (reviewed in Helmann, 2016) that mainly belong to different subgroups from those of *S. venezuelae* and implementation of ECF switches in *B. subtilis* has already been successful (Pinto *et al.*, 2018b). For these reasons, we argue that *B*. *subtilis* can as well be a good host for the implementation of *S. venezuelae* ECF σ factors.

1.6.Mechanisms of resistance to bacitracin

Bacitracin is a nonribosomally synthesized antimicrobial peptide (AMP), which is produced by soil bacteria such as *Bacillus licheniformis* (Nakano & Zuber 1990). Bacitracin targets the cell envelope by forming a complex with the lipid carrier undecaprenyl pyrophosphate (UPP), blocking the reclycing of UPP and consequentelly the biosynthesis of peptidoglycan precursors (Siewert, Gerhard; Strominger, 1967) (**Figure 8**) Gram-positive bacteria possess several mechanisms of resistance against cell wall antimicrobial peptide such as bacitracin. For example, the overproduction of undecaprenyl phosphate (UP) confers resistance to bacitracin in *Klebsiella* species by overcoming the sequestration of UPP by bacitracin (Sutherland, 1977). Studies in *Streptococcus mutans* showed two different mechanism of bacitracin resistance: one involving the synthesis of rhamnose-glucose polysachharide and other involving the mutans bacitracin resitance (mbr) genes, which seem to be related to glucan biosynthesis (Tsuda *et al.*, 2002).

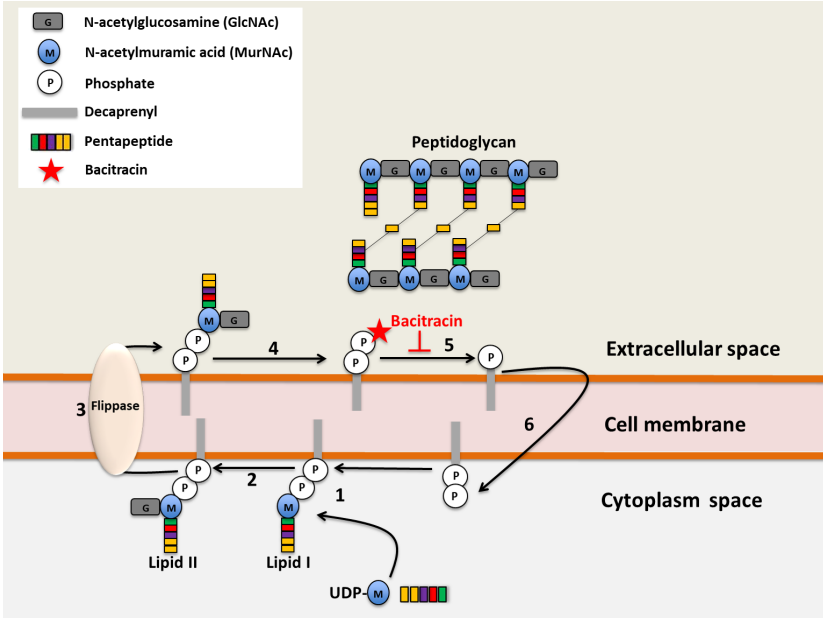


Figure 8. Lipid II cycle. Peptidoglycan subunits are synthetised in the cytoplasm where the UDP-activated precursor sugar N-acetylmuranic acid and pentapeptide (L-Ala, DiGlu, L-Lys, D-Ala, D-Ala) are assembled on the lipid carrier undecaprenyl phosphate (UPP) by the action of the enzymes MraY and Mur A-F, forming this way the the lipid I (1). Further, the addition of the UDP-N-acetylglucosamine by the enzyme MurG forms the lipid II (2). The lipid II is subsequently translocated by a flippase across the membrane towards the extracytoplasmic space (3). Then, the peptidoglycan subunit is transferred to a growing peptidoglycan chain by action of penicillin-binding proteins (4). The lipid carrier UPP is released (5) and recycled back to the cytoplasm (6), initiating a new cycle. UPP recycling is blocked by bacitracin (red star).

Additionally, the upregulation of the enzyme undecaprenyl-pyrophosphate phosphateses (UppP) confers resistance to bacitracin in *B. subtilis* and *Enterococcus faecalis* (Cao and Helmann, 2002; Shaaly *et al.*, 2013). Modifications on the cell wall charge such as alanylation or overproduction of teichoic acids were shown to confer resistance to bacitracin in *Staphylococcus aureus*, *Clostridium difficile* and *E. faecalis* (Peschel *et al.*, 1999; McBride and Sonenshein, 2011; Abranches *et al.*, 2013).

Another common mechanism that confers high-level resistance to bacitracin is the expression of ATP-binding cassette (ABC) transport system. For example, the bacitracin producing bacteria *Bacillus licheniformis* processes a self-immunity mechanism against bacitracin that is based in the expression of the BcrAB transport system (Podlesek, Herzog and Comino, 1997). The expression of such transporters is controlled by two-component systems, which are usually encoded in the neighbourhood of the ABC transporter (Joseph *et al.*, 2002; Dintner *et al.*, 2011). This type of tranporters is well exemplified by the BceRS-BcrAB system from *B. subtilis*, where the BceRS correspond to a regulatory component located upstream of the sensor domain and ABC transporter BceAB (Ohki *et al.*, 2003; Gebhard, 2012; Dintner *et al.*, 2014).

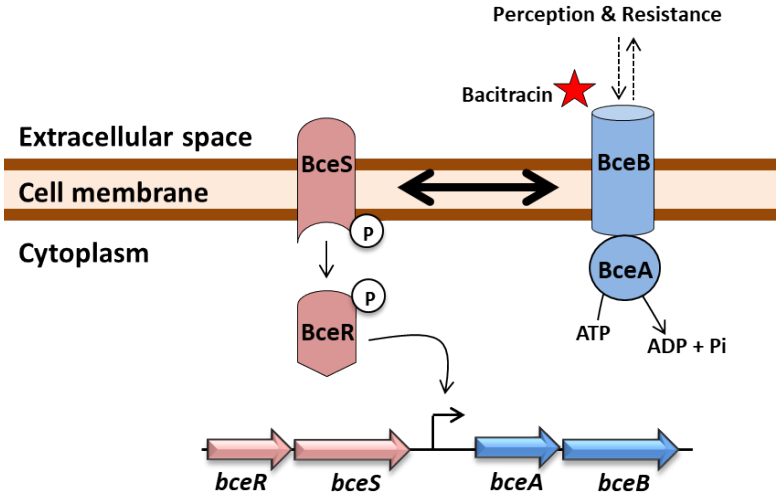


Figure 9. Depiction of the BceRS-BceAB bacitracin resistance mechanism *of Bacillus subtilis*. Genes and proteins representing the two-component system BceRS are shown in pink and transporter's permease and ATP-binding domain are showed in blue. The presence of the antimicrobial peptide (e.g. bacitracin) is indicated by a red star. The proposed interaction between BceB and BceS is shown by a thick double-headed arrow. The phospho-relay between BceS and BceR, ATP hydrolysis by BceA, and activation of the *bceA* promoter by BceR are shown by thin single-headed arrows (adapted from Dintner et al., 2014).

1.7.Scope of the thesis

1.7.1. Implementation of ECF-based switches in *B. subtilis*

ECF σ factors have been introduced as a valuable resource for implementing synthetic programs of gene expression (Rhodius *et al.*, 2013; Pinto *et al.*, 2018b). The conservation of the core RNA polymerase across bacteria and orthogonality of ECF σ factors could allow the implementation of ECF-based switches in a variety of species avoiding unwanted behaviours caused by host part interference. Thus, this thesis aimed to design and implement synthetic switches based in ECF σ factors. For this purpose, ECF σ factors from the high GC Actinobacteria *S. venezuelae* were codon optimized and implememted into the host cell *B. subtilis*. The activity of the ECF-based switches was analysed via luminescence measurements.

1.7.2. Antimicrobial peptide resistance in *Streptomyces venezuelae*

Resistance against antimicrobial peptides (AMPs) is well understood in Firmicutes (Draper *et al.*, 2008; Alkhatib *et al.*, 2012; Gebhard, 2012), but to date has not been investigated in detail in actinomycetes. Several members of the latter group have recently been found to also produce AMPs, and to contain immunity genes required for self-protection (Claesen and Bibb, 2010; Foulston and Bibb, 2010; Sherwood, Hesketh and Bibb, 2013). Yet little, if any, information is available on their resistance against AMPs produced by other bacteria. Considering the co-

occurrence of actinomycetes and firmicutes in the soil environment, it was hypothesised that actinomycetes should frequently be exposed to AMPs and thus should possess dedicated resistance mechanisms to defend themselves. Preliminary studies have shown that *S. venezuelae* is highly resistant to the AMP bacitracin, thus, this thesis also aimed at the discovery of bacitracin resistance mechanisms in *S. venezuelae*. For this, *S. venezuelae* was randomly mutagenized and screeneend for bacitracin-sensitive strains. Further genome sequencing and RNAseq analysis were performed.

2. Materials and Methods

2.1. Implementation of ECF σ factor-based switches in *B. subtilis*

2.1.1. Selection of ECF σ factors and codon optimization

S. venezuelae ATCC 10712 proteome was obtained from the Protein database of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/protein) and the protein sequences were identified and classified by using the ECF*finder* tool (http://ecf.g2l.bio.uni-goettingen.de:8080/ECFfinder/) (Staroń *et al.*, 2009) or by similarity analysis with those groups classified by Huang et al., 2015. A subset of ten ECF σ factors groups that were not present in *B. subtilis* 168 were selected for implementation into *B. subtilis* 168 (**Table 1**) and their promoters were predicted accordingly to specific putative target promoter motifs previously described (Staroń *et al.*, 2009; Gómez-Santos *et al.*, 2011; Jogler *et al.*, 2012; Huang *et al.*, 2015; Pinto and Mascher, 2016).

Table 1. Selected ECF σ factors of *Streptomyces venezueale*

ECF σ factor	Protein accession number	ECF group
Sven_4513	WP_041662789	ECF02
Sven_4870	WP_015036056	ECF12
Sven_4793	WP_015035979	ECF14
Sven_0063	WP_041661965	ECF17
Sven_0399	WP_041661965	ECF19
Sven_6501	WP_015037682	ECF20
Sven_3668	WP_041662652	ECF27
Sven_2914	WP_015034116	ECF38
Sven_3369	WP_015034570	ECF38
Sven_6611	WP_015037792	ECF38
Sven_3215	WP_015034416	ECF39
Sven_3278	WP_015034479	ECF39
Sven_3293	WP_015034494	ECF39
Sven_3759	WP_015034958	ECF39
Sven_4575	WP_015035762	ECF39
Sven_0015	WP_015031223	ECF51

In order to ensure the expression of the ECF σ factors from *S. venezuelae* in *B. subtilis*, the ECF σ factor-encoding genes were codon adjusted based on the codon usage frequency of both organisms, determined using a set of predicted highly expressed genes (Karlin *et al.*, 2001) (**Table 2**). An RBS and an N-terminal FLAG tag were added upstream the codon adjusted coding sequence. Appropriate restriction sites were incorporated at either end and forbidden restriction sites - correspondingly to the RFC10 BioBrick standard - were removed. These DNA fragments were chemically synthetized by GeneArt, Germany (Invitrogen, Thermo Fischer Scientific) and later cloned into selected vectors.

Table 2. Codon usage frequency of *S. venezuelae* and *B. subtilis*

	Streptomyces venezueale ATCC 10712			*Bacillus subtilis* 168		
Amino acid	**Codon**	**Count**	**Frequency**	**Codon**	**Count**	**Frequency**
Ala	GCC	734	62,0%	GCC	74	9,6%
	GCG	364	30,8%	GCG	176	22,9%
	GCT	69	5,8%	GCT	292	38,0%
	GCA	16	1,4%	GCA	226	29,4%
Arg	CGC	476	59,4%	CGC	198	31,5%
	CGT	240	30,0%	CGT	364	58,0%
	CGG	72	9,0%	CGG	5	0,8%
	CGA	7	0,9%	CGA	12	1,9%
	AGG	6	0,7%	AGG	1	0,2%
	AGA	0	0,0%	AGA	48	7,6%
Asn	AAC	301	99,3%	AAC	286	73,5%
	AAT	2	0,7%	AAT	103	26,5%
Asp	GAC	638	99,1%	GAC	255	44,3%
	GAT	6	0,9%	GAT	321	55,7%
Cys	TGC	42	85,7%	TGC	11	35,5%
	TGT	7	14,3%	TGT	20	64,5%
Gln	CAG	372	100,0%	CAG	129	34,7%
	CAA	0	0,0%	CAA	243	65,3%
Glu	GAG	805	96,5%	GAG	202	24,7%
	GAA	29	3,5%	GAA	615	75,3%
Gly	GGC	614	65,1%	GGC	225	27,1%
	GGT	295	31,3%	GGT	328	39,5%
	GGA	17	1,8%	GGA	249	30,0%
	GGG	17	1,8%	GGG	29	3,5%
His	CAC	186	99,5%	CAC	92	58,6%
	CAT	1	0,5%	CAT	65	41,4%
Ile	ATC	537	98,9%	ATC	376	60,3%
	ATT	6	1,1%	ATT	248	39,7%
Leu	CTG	417	49,9%	CTG	91	11,2%
	CTC	397	47,5%	CTC	39	4,8%

	CTT	20	2,4%	CTT	405	49,8%
	TTG	1	0,1%	TTG	83	10,2%
	CTA	0	0,0%	CTA	38	4,7%
	TTA	0	0,0%	TTA	158	19,4%
Lys	AAG	654	100,0%	AAG	126	16,7%
	AAA	0	0,0%	AAA	627	83,3%
Met	ATG	212	100,0%	ATG	245	95,7%
	ATA	0	0,0%	ATA	11	4,3%
Phe	TTC	285	99,3%	TTC	200	69,0%
	TTT	2	0,7%	TTT	90	31,0%
Pro	CCG	368	68,7%	CCG	110	28,4%
	CCC	159	29,7%	CCC	3	0,8%
	CCT	9	1,7%	CCT	167	43,2%
	CCA	0	0,0%	CCA	107	27,6%
Ser	TCC	210	47,4%	TCC	35	7,5%
	TCG	178	40,2%	TCG	5	1,1%
	AGC	49	11,1%	AGC	61	13,1%
	TCT	5	1,1%	TCT	212	45,5%
	AGT	1	0,2%	AGT	33	7,1%
	TCA	0	0,0%	TCA	120	25,8%
Thr	ACC	429	70,3%	ACC	16	3,0%
	ACG	172	28,2%	ACG	78	14,7%
	ACT	9	1,5%	ACT	186	35,2%
	ACA	0	0,0%	ACA	249	47,1%
Trp	TGG	64	100,0%	TGG	37	100%
Tyr	TAC	219	99,5%	TAC	146	61,3%
	TAT	1	0,5%	TAT	92	38,7%
Val	GTC	698	72,1%	GTC	106	11,9%
	GTG	212	21,9%	GTG	109	12,3%
	GTT	54	5,6%	GTT	383	43,1%
	GTA	4	0,4%	GTA	290	32,7%

ECF σ factor target promoters were obtained by annealing of complementary oligonucleotides (**Table 3**) generating the appropriate overhangs for EcoRI and SpeI mediated cloning. For each annealing reaction, a control reaction using only the forward oligonucleotide was made in parallel. The annealing of the oligonucleotides was confimed by electrophoresis and ethidium bromide staining.

Table 3. Oligonucleotides for promoter generation

Oligonucleotide name	Description	Sequence 5‘ – 3‘
TMP0053	P_{Sven_4513} Fwd	AATTCGCGGCCGCTTCTAGAGGCGCGCAGCTCGGCGGGGTCCTGCGGAACCGTGATC GCGTGGAGGTCGACGACCATGTA
	P_{Sven_4513}Rev	CTAGTACATGGTCGTCGACCTCCACGCGATCACGGTTCCGCAGGACCCCGCCGAGCT GCGCGCCTCTAGAAGCGGCCGCG
TMP0054	P_{Sven_4870} Fwd	AATTCGCGGCCGCTTCTAGAGGATCGTCTCGCTCCGCTGACCCACCGGGAATGTTGA GCGGGGGGCGACGGTTGTTCCGTGCTA
	P_{Sven_4870} Rev	CTAGTAGCACGGAACAACCGTCGCCCCCCGCTCAACATTCCCGGTGGGTCAGCGGAG CGAGACGATCCTCTAGAAGCGGCCGCG
TMP0055	P_{Sven_4793} Fwd	AATTCGCGGCCGCTTCTAGAGGATCGGGGAGGAGTGCTTCGGCGTCTTCTCAGGTCG GCGGGTGAGCCGAAATCCGTGATA
	P_{Sven_4793} Rev	CTAGTATCACGGATTTCGGCTCACCCGCCGACCTGAGAAGACGCCGAAGCACTCCTC CCCGATCCTCTAGAAGCGGCCGCG
TMP0056	P_{Sven_0063} Fwd	AATTCGCGGCCGCTTCTAGAGCCGGCTCCATGTGACCCGGCTCACATGAACCGACGG TGCAGGCGGCGCGTGTGGGGCACTA
	P_{Sven_0063}Rev	CTAGTAGTGCCCCACACGCGCCGCCTGCACCGTCGGTTCATGTGAGCCGGGTCACAT GGAGCCGGCTCTAGAAGCGGCCGCG
TMP0057	P_{Sven_6501} Fwd	AATTCGCGGCCGCTTCTAGAGGGCCGGTGGAACCGGGAGGAAACACCCATGCTGATC ACCGGCCTCGTCGCGCTCGGAGTCCTGTA
	P_{Sven_6501} Rev	CTAGTACAGGACTCCGAGCGCGACGAGGCCGGTGATCAGCATGGGTGTTTCCTCCCG GTTCCACCGGCCCTCTAGAAGCGGCCGCG
TMP0058	P_{Sven_3668} Fwd	AATTCGCGGCCGCTTCTAGAGCGGCATGGGTGCCGCAAGGCGGTCTCGGGAATGTAC GCCCCTTAGGATCCGTTGGGTGGGGTTA
	P_{Sven_3668} Rev	CTAGTAACCCCACCCAACGGATCCTAAGGGGCGTACATTCCCGAGACCGCCTTGCGG CACCCATGCCGCTCTAGAAGCGGCCGCG
TMP0059	P_{Sven_2914} Fwd	AATTCGCGGCCGCTTCTAGAGTCGTGGGGTGACACACGTCTGGTGGGTTGAAGGCGC CCTTCACCCCACCGTGTCCGTCTCTTCTA
	P_{Sven_2914} Rev	CTAGTAGAAGAGACGGACACGGTGGGGTGAAGGGCGCCTTCAACCCACCAGACGTG TGTCACCCCACGACTCTAGAAGCGGCCGCG
TMP0060	$P_{Sven_}$3369 Fwd	AATTCGCGGCCGCTTCTAGAGCACCGTCGAAAAGGGTGACGCACGCGTACAACCCTG CCGGGGGGAAGCGTGTCCAACATGCGTA
	P_{Sven_3369} Rev	CTAGTACGCATGTTGGACACGCTTCCCCCCGGCAGGGTTGTACGCGTGCGTCACCCTT TTCGACGGTGCTCTAGAAGCGGCCGCG
TMP0061	P_{Sven_6611} Fwd	AATTCGCGGCCGCTTCTAGAGCCCTTGGACCTTTGGCGACCCGCCTGGACAGCTCGAC GAGCGGCCGCTTAGGGTCGGGGTCCGTA
	P_{Sven_6611} Rev	CTAGTACGGACCCCGACCCTAAGCGGCCGCTCGTCGAGCTGTCCAGGCGGGTCGCCA AAGGTCCAAGGGCTCTAGAAGCGGCCGCG
TMP0062	P_{Sven_3215} Fwd	AATTCGCGGCCGCTTCTAGAGGACGGCTGCCCCGCACAGCCCCGTGACAACCGCTCC GTAGCGTCATCGACGACACGAGGTA
	P_{Sven_3215} Rev	CTAGTACCTCGTGTCGTCGATGACGCTACGGAGCGGTTGTCACGGGGCTGTGCGGGG CAGCCGTCCTCTAGAAGCGGCCGCG
TMP0063	P_{Sven_3278} Fwd	AATTCGCGGCCGCTTCTAGAGGGGCTGGCCCCGCCACACCACCCTCACACCCCTGAC GCCGACCGACTCCGACCCATCGCGATA
	P_{Sven_3278}Rev	CTAGTATCGCGATGGGTCGGAGTCGGTCGGCGTCAGGGGTGTGAGGGTGGTGTGGCG GGGCCAGCCCCTCTAGAAGCGGCCGCG
TMP0064	P_{Sven_3293} Fwd	AATTCGCGGCCGCTTCTAGAGCTGGCCGCGGGAGTCTGGCAGTCAGGCCTCGGACAG TTCATCGGCGGACTCGTCATCGTCGCCTA
	P_{Sven_3293} Rev	CTAGTAGGCGACGATGACGAGTCCGCCGATGAACTGTCCGAGGCCTGACTGCCAGAC TCCCGCGGCCAGCTCTAGAAGCGGCCGCG
TMP0065	P_{Sven_3759} Fwd	AATTCGCGGCCGCTTCTAGAGACCCTGAGGGTGTTCCCGGAGCGTCTCCACCCACAG GAGGTCGGGTCGTCCCCACCCCTTA
	P_{Sven_3759} Rev	CTAGTAAGGGGTGGGGACGACCCGACCTCCTGTGGGTGGAGACGCTCCGGGAACACC CTCAGGGTCTCTAGAAGCGGCCGCG
TMP0066	P_{Sven_4575} Fwd	AATTCGCGGCCGCTTCTAGAGGAGGACGTCGAGTTCACGGCAGCGGGCCACGATCTC GGCGGCGAAGATCCCCTCCGCCACGAATA
	P_{Sven_4575} Rev	CTAGTATTCGTGGCGGAGGGGATCTTCGCCGCCGAGATCGTGGCCCGCTGCCGTGAA CTCGACGTCCTCCTCTAGAAGCGGCCGCG
TMP0067	P_{Sven_0015} Fwd	AATTCGCGGCCGCTTCTAGAGGGAGCACCTGCCGGGCAGCGCTCTGCGGGACCTGCG GCGCTTCCAGTGGGACTTCGCCACTA
	P $_{Sven\_0015}$ Rev	CTAGTAGTGGCGAAGTCCCACTGGAAGCGCCGCAGGTCCCGCAGAGCGCTGCCCGGC AGGTGCTCCCTCTAGAAGCGGCCGCG
TMP0068	P_{Sven_0399} Fwd	AATTCGCGGCCGCTTCTAGAGGGGGGCGCCGGTGACGAAGAGCGCTCCCATCCGCAG GGCCGTCCGCTACGAAGAACGGGCGTA
	P_{Sven_0399} Rev	CTAGTACGCCCGTTCTTCGTAGCGGACGGCCCTGCGGATGGGAGCGCTCTTCGTCACC GGCGCCCCCCTCTAGAAGCGGCCGCG
TMP0102	P_{Sven_3185} Fwd	AATTCGCGGCCGCTTCTAGAGGAGTGCCGAAGGGTGCCGTCCGACCCGTAACTCTTT CGAGTGACCGTCGTTGAGAGTGCTA
	P_{Sven_3185} Rev	CTAGTAGCACTCTCAACGACGGTCACTCGAAAGAGTTACGGGTCGGACGGCACCCTT CGGCACTCCTCTAGAAGCGGCCGCG
TMP0053	P_{Sven_4513} Fwd	AATTCGCGGCCGCTTCTAGAGGCGCGCAGCTCGGCGGGGTCCTGCGGAACCGTGATC GCGTGGAGGTCGACGACCATGTA

	P_{Sven_4513}Rev	CTAGTACATGGTCGTCGACCTCCACGCGATCACGGTTCCGCAGGACCCCGCCGAGCT GCGCGCCTCTAGAAGCGGCCGCG
TMP0054	P_{Sven_4870} Fwd	AATTCGCGGCCGCTTCTAGAGGATCGTCTCGCTCCGCTGACCCACCGGGAATGTTGA GCGGGGGGCGACGGTTGTTCCGTGCTA
	P_{Sven_4870} Rev	CTAGTAGCACGGAACAACCGTCGCCCCCCGCTCAACATTCCCGGTGGGTCAGCGGAG CGAGACGATCCTCTAGAAGCGGCCGCG
TMP0055	P_{Sven_4793} Fwd	AATTCGCGGCCGCTTCTAGAGGATCGGGGAGGAGTGCTTCGGCGTCTTCTCAGGTCG GCGGGTGAGCCGAAATCCGTGATA
	P_{Sven_4793} Rev	CTAGTATCACGGATTTCGGCTCACCCGCCGACCTGAGAAGACGCCGAAGCACTCCTC CCCGATCCTCTAGAAGCGGCCGCG
TMP0056	P_{Sven_0063} Fwd	AATTCGCGGCCGCTTCTAGAGCCGGCTCCATGTGACCCGGCTCACATGAACCGACGG TGCAGGCGGCGCGTGTGGGGCACTA
	P_{Sven_0063}Rev	CTAGTAGTGCCCCACACGCGCCGCCTGCACCGTCGGTTCATGTGAGCCGGGTCACAT GGAGCCGGCTCTAGAAGCGGCCGCG
TMP0057	P_{Sven_6501} Fwd	AATTCGCGGCCGCTTCTAGAGGGCCGGTGGAACCGGGAGGAAACACCCATGCTGATC ACCGGCCTCGTCGCGCTCGGAGTCCTGTA
	P_{Sven_6501} Rev	CTAGTACAGGACTCCGAGCGCGACGAGGCCGGTGATCAGCATGGGTGTTTCCTCCCG GTTCCACCGGCCCTCTAGAAGCGGCCGCG
TMP0058	P_{Sven_3668} Fwd	AATTCGCGGCCGCTTCTAGAGCGGCATGGGTGCCGCAAGGCGGTCTCGGGAATGTAC GCCCCTTAGGATCCGTTGGGTGGGGTTA
	P_{Sven_3668} Rev	CTAGTAACCCCACCCAACGGATCCTAAGGGGCGTACATTCCCGAGACCGCCTTGCGG CACCCATGCCGCTCTAGAAGCGGCCGCG
TMP0059	P_{Sven_2914} Fwd	AATTCGCGGCCGCTTCTAGAGTCGTGGGGTGACACACGTCTGGTGGGTTGAAGGCGC CCTTCACCCCACCGTGTCCGTCTCTTCTA
	P_{Sven_2914} Rev	CTAGTAGAAGAGACGGACACGGTGGGGTGAAGGGCGCCTTCAACCCACCAGACGTG TGTCACCCCACGACTCTAGAAGCGGCCGCG
TMP0060	P_{Sven_3369} Fwd	AATTCGCGGCCGCTTCTAGAGCACCGTCGAAAAGGGTGACGCACGCGTACAACCCTG CCGGGGGGAAGCGTGTCCAACATGCGTA
	P_{Sven_3369} Rev	CTAGTACGCATGTTGGACACGCTTCCCCCCGGCAGGGTTGTACGCGTGCGTCACCCTT TTCGACGGTGCTCTAGAAGCGGCCGCG
TMP0061	P_{Sven_6611} Fwd	AATTCGCGGCCGCTTCTAGAGCCCTTGGACCTTTGGCGACCCGCCTGGACAGCTCGAC GAGCGGCCGCTTAGGGTCGGGGTCCGTA
	P_{Sven_6611} Rev	CTAGTACGGACCCCGACCCTAAGCGGCCGCTCGTCGAGCTGTCCAGGCGGGTCGCCA AAGGTCCAAGGGCTCTAGAAGCGGCCGCG
TMP0062	P_{Sven_3215} Fwd	AATTCGCGGCCGCTTCTAGAGGACGGCTGCCCCGCACAGCCCCGTGACAACCGCTCC GTAGCGTCATCGACGACACGAGGTA
	P_{Sven_3215} Rev	CTAGTACCTCGTGTCGTCGATGACGCTACGGAGCGGTTGTCACGGGGCTGTGCGGGG CAGCCGTCCTCTAGAAGCGGCCGCG
TMP0063	P_{Sven_3278} Fwd	AATTCGCGGCCGCTTCTAGAGGGGCTGGCCCCGCCACACCACCCTCACACCCCTGAC GCCGACCGACTCCGACCCATCGCGATA
	P_{Sven_3278}Rev	CTAGTATCGCGATGGGTCGGAGTCGGTCGGCGTCAGGGGTGTGAGGGTGGTGTGGCG GGGCCAGCCCCTCTAGAAGCGGCCGCG
TMP0064	P_{Sven_3293} Fwd	AATTCGCGGCCGCTTCTAGAGCTGGCCGCGGGAGTCTGGCAGTCAGGCCTCGGACAG TTCATCGGCGGACTCGTCATCGTCGCCTA
	P_{Sven_3293} Rev	CTAGTAGGCGACGATGACGAGTCCGCCGATGAACTGTCCGAGGCCTGACTGCCAGAC TCCCGCGGCCAGCTCTAGAAGCGGCCGCG
TMP0065	P_{Sven_3759} Fwd	AATTCGCGGCCGCTTCTAGAGACCCTGAGGGTGTTCCCGGAGCGTCTCCACCCACAG GAGGTCGGGTCGTCCCCACCCCTTA
	P_{Sven_3759} Rev	CTAGTAAGGGGTGGGGACGACCCGACCTCCTGTGGGTGGAGACGCTCCGGGAACACC CTCAGGGTCTCTAGAAGCGGCCGCG
TMP0066	P_{Sven_4575} Fwd	AATTCGCGGCCGCTTCTAGAGGAGGACGTCGAGTTCACGGCAGCGGGCCACGATCTC GGCGGCGAAGATCCCCTCCGCCACGAATA
	P_{Sven_4575} Rev	CTAGTATTCGTGGCGGAGGGGATCTTCGCCGCCGAGATCGTGGCCCGCTGCCGTGAA CTCGACGTCCTCCTCTAGAAGCGGCCGCG
TMP0067	P_{Sven_0015} Fwd	AATTCGCGGCCGCTTCTAGAGGGAGCACCTGCCGGGCAGCGCTCTGCGGGACCTGCG GCGCTTCCAGTGGGACTTCGCCACTA
	P $_{Sven\_0015}$ Rev	CTAGTAGTGGCGAAGTCCCACTGGAAGCGCCGCAGGTCCCGCAGAGCGCTGCCCGGC AGGTGCTCCCTCTAGAAGCGGCCGCG
TMP0068	P_{Sven_0399} Fwd	AATTCGCGGCCGCTTCTAGAGGGGGGCGCCGGTGACGAAGAGCGCTCCCATCCGCAG GGCCGTCCGCTACGAAGAACGGGCGTA
	P_{Sven_0399} Rev	CTAGTACGCCCGTTCTTCGTAGCGGACGGCCCTGCGGATGGGAGCGCTCTTCGTCACC GGCGCCCCCCTCTAGAAGCGGCCGCG

Putative -35 and -10 promoter elements are underlined.

2.1.2. Plasmid construction and transformations

Genes encoding the ECF σ factors were inserted into the vector pBS0E-*xylR*P_{xylA} or pBS2E-P_{xylA} via XbaI or EcoRI and SpeI restriction sites. The promoters where inserted into pBS3C*lux* via EcoRI and SpeI sites. pBS3Clux carries the *luxABCDE* operon that integrates into the *sacA*

locus on *B. subtilis*, which codes a non-essential phosphosucrase. Genes encoding the transcription factors CarD and RpbA were inserted into pBS4S-P_{xylA} via XbaI and PstI sites. Cognate anti-σ factor 19 was inserted into pBS0K-P_{liaI} or pBS1K-P_{liaI} via XbaI and PstI, or into pBS0E-*xylR*P_{xylA}-*SVEN_0399* via EcoRI and XbaI. The transformations with pBS0E-*xylR*P_{xylA} and pBS0-P_{liaI} derivatives, and integration of pBS2E-P_{xylA} and pBS3C*lux* into *B. subtilis* were confimed by colony PCR using oligonucleotides showed in **table 4**. The integration of pBS4S-P_{xyl} derivatives in the *thrC* locus was confirmed through threonine auxotropy (Sambrook, 2001; Radeck *et al.*, 2013) and that of pBS1K derivatives in the *amyE* locus by the inability to degrade starch.

Table 4. Additional oligonucleotides used in this study

Primer name	Description	Sequence 5' – 3'
TM0410	pBS0E check fwd	GTTTAAACAACAAACTAATAGGTGATG
TM2890	pBS0E check fwd	ATTACCGCCTTTGAGTGA
TM2262	pBS3Clux check fwd	GAGCGTAGCGAAAAATCC
TM2263	pBS3Clux check rev	GAAATGATGCTCCAGTAACC
TM2505	pBS3Clux sacA front check fwd	CTGATTGGCATGGCGATTGC
TM2506	pBS3Clux sacA front check rev	ACAGCTCCAGATCCTCTACG
TM2507	pBS3Clux sacA back check fwd	GTCGCTACCATTACCAGTTG
TM2508	pBS3Clux sacA back check rev	TCCAAACATTCCGGTGTTATC
TM3081	pBS2E check fwd	GGCAACCGAGCGTTCTG
TM3082	pBS2E check rev	CTGACAGCGTTTCGATCC
TM3224	pBS0K check fwd	CCGTTACACTAGAAAACCG
TM3225	pBS0K check rev	CTGTGGATAACCGTATTACC
TM4085	pBS2E LacA front check fwd	TGCTGCAAAAGAATTTTGTGTCCG
TM4086	pBS2E LacA front check rev	AGGACTCTCTAGCTTGAGGC
TM4087	pBS2E LacA back check fwd	CTGCAGAGATATCGATTTCAAGC
TM4088	pBS2E LacA back check rev	CTTTGCTTTTCATGATTTCATCCC
TM0137	Kan cassette fwd	CAGCGAACCATTTGAGGTGATAGG
TM0138	Kan cassette rev	CGATACAAATTCCTCGTAGGCGCTCGG
TM5144	*ydeB* up fragment fwd	CCGATTCCTGATATAATAAAGAAG
TM5145	*ydeB* up fragment rev	CCTATCACCTCAAATGGTTCGCTGGGTTAGATACATCCCAAAAG
TM5146	*ydeB* down fragment fwd	CGAGCGCCTACGAGGAATTTGTATCGGAAACATATCATCCACCTCC
TM5147	*ydeB* down fragment rev	CGCCATTACGTTATGTAATGGATTATAG
TM4621	pBS4S check fwd	CTGTGAGAAATCACCGATTG
TM4622	pBS4S check rev	TCCTGATCCAAACATGTAAG

5' end of joining primers used for the *ydeB* deletion are underlined.

General cloning procedures such as endonuclease restriction, ligation and PCR were performed with enzymes and buffers from New England Biolabs (NEB, Ipswich, MA, USA) according to

the manufacture's protocols. OneTaq® polymerase or Q5® polymerase were used for PCR amplifications. Plasmids were generated according to BioBrick standards. Plasmid preparations were obtained according to the manufacture's protocol with the HiYield Plasmid Mini-Kit (Süd-Laborbedarf GmbH (SLG), Gauting, Germany). *E. coli* competent cells were transformed according to OpenWetWare (*http://openwetware.org/wikii/TOP_chemically_competent_cells*). A list of the plasmids generated in this study is shown in **table 5**. *B. subtilis* transformations were carried out as described previously (Harwood and Cutting, 1990).

Table 5. List of plasmids used in this study.

Name	Plasmid features and Contruction	Source
pBS0E xylRPxylA	ori1030, amp^R, mls^R.	(Popp *et al.*, 2017)
pBS0EPxyl	ori1030, amp^R, mls^R	(Popp *et al.*, 2017)
pDA0E xylRPxyl-SVEN_4513	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4513*	This study
pBS2ERPxyl	Integration at *lacA*, amp^R, mls^R, *xylR*, P_{xylA}	(Popp et al., 2017)
pBS0EPliaI	ori1030, amp^R, mls^R	(Popp *et al.*, 2017)
pBS2PliaI	Integration at *lacA*, amp^R, mls^R, P_{liaI}	(Popp et al., 2017)
pBS3Clux	*lux*-reporter vector, integration at *sacA*, amp^R,cm^R.	(Radeck *et al.*, 2013)
pBS0K	origin of replication of pUB110, amp^R, kan^R	(Joseph *et al.*, 2001)
pBS1K	integration at amyE, amp^R , kan^R	(Popp et al., 2017)
pBS4S	integration at thrC, amp^R , $spec^R$	(Radeck et al., 2013)
pDA 0E xylRPxyl-SVEN_4870	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4870*	This study
pDA 0E xylRPxyl-SVEN_4793	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4793*	This study
pDA 0E xylRPxyl-SVEN_0063	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0063*	This study
pDA 0E xylRPxyl-SVEN_0399	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399*	This study
pDA 0E xylRPxyl-SVEN_6501	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_6501*	This study
pDA 0E xylRPxyl-SVEN_3668	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3668*	This study
pDA 0E xylRPxyl-SVEN_2914	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_2914*	This study
pDA 0E xylRPxyl-SVEN_3369	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3369*	This study
pDA 0E xylRPxyl-SVEN_6611	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_6611*	This study
pDA 0E xylRP_{xyl}-SVEN_3215	pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3215*	This study

pDA 0E xylRP$_{xyl}$-SVEN_3278	pBS0E *xylR* P$_{xylA}$ RBS FLAG *SVEN_3278*	This study
pDA 0E xylRPxyl-SVEN_3293	pBS0E *xylR* P$_{xylA}$ RBS FLAG *SVEN_3293*	This study
pDA 0E xylRPxyl-SVEN_3759	pBS0E *xylR* P$_{xylA}$ RBS FLAG *SVEN_3759*	This study
pDA 0E xylRPxyl-SVEN_4575	pBS0E *xylR* P$_{xylA}$ RBS FLAG *SVEN_4575*	This study
pDA 0E xylRPxyl SVEN_0015	pBS0E *xylR* P$_{xylA}$ RBS FLAG *SVEN_0015*	This study
pDA 0E xylRPxyl-SVEN_0399/SVEN_0398	pBS0E *xylR* P$_{xylA}$ RBS FLAG *SVEN_0399/SVEN_0398*	This study
pDA 0E xylRPxyl-SVEN 0399 SVEN 0398 (p. 1-98)	pBS0E *xylR* P$_{xylA}$ RBS FLAG *SVEN_0399/SVEN_0398 cyto*	This study
pDA 0ExylRPxyl-SVEN_0399 SVEN_0398 $^{109\ AAA>GGG}$	pBS0E *xylR*P$_{xyl}$-*SVEN_0399 SVEN_0398* $^{109\ AAA>GGG}$	This study
pDP0EPxyl03	pBS0E P$_{xylA}$ RBS FLAG *SVEN_0399*	This study
pDP2E-Pxyl24	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399*	This study
pDP2E-Pxyl50	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(LAA)}$*	This study
pDP2E-Pxyl47	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(AAV)}$*	This study
pDP2E-Pxyl51	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(LVA)}$*	This study
pDP2E-Pxyl60	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(ASV)}$*	This study
pDP2E-Pxyl61	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(LDD)}$*	This study
pDP2E-Pxyl39	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(LAD)}$*	This study
pDP2E-Pxyl62	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(HHA)}$*	This study
pDP2E-Pxyl38	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(ISV)}$*	This study
pDP2E-Pxyl63	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(ISS)}$*	This study
pDP2E-Pxyl48	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(DAG)}$*	This study
pDP2E-Pxyl49	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399$_{(DVS)}$*	This study
pDP2E-Pxyl73	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399* P$_{veg}$	This study
pDP2E-Pxyl74	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399* P$_{lepA}$	This study
pDP2E-Pxyl77	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399* P$_{liaG}$	This study
pDP2E-Pxyl78	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399* P$_{sigW}$	This study
pDP2E-Pxyl79	pBS2E P$_{xylA}$ RBS FLAG *SVEN_0399* P$_{J23101}$	This study
pDA2ExylRPxyl_ECF19	pBS2E *xylR* P$_{xylA}$ RBS FLAG *SVEN_0399*	This study

pDP2EPliaI12	pBS2E P_{liaI} RBS FLAG *SVEN_0399*	This study
pDA3Clux-PSVEN_0399	pBS3C $P_{SVEN\_0399(-60\ to\ +1)}$ *luxABCDE*	This study
pDP3Clux43	pBS3C $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
pDP3Clux44	pBS3C $P_{SVEN\_0399(+129\ to\ +71)}$ *luxABCDE*	This study
pDA3Clux-PSVEN_4513	pBS3C P_{SVEN_4513} *luxABCDE*	This study
pDA3Clux-PSVEN_4870	pBS3C P_{SVEN_4870} *luxABCDE*	This study
pDA3Clux-PSVEN_4793	pBS3C P_{SVEN_4793} *luxABCDE*	This study
pDA3Clux-PSVEN_0063	pBS3C P_{SVEN_0063} *luxABCDE*	This study
pDA3Clux-PSVEN_6501	pBS3C P_{SVEN_6501} *luxABCDE*	This study
pDA3Clux-PSVEN_3668	pBS3C P_{SVEN_3668} *luxABCDE*	This study
pDA3Clux-PSVEN_2914	pBS3C P_{SVEN_2914} *luxABCDE*	This study
pDA3Clux-PSVEN_3369	pBS3C P_{SVEN_3369} *luxABCDE*	This study
pDA3Clux-PSVEN_6611	pBS3C P_{SVEN_6611} *luxABCDE*	This study
pDA3Clux-PSVEN_3215	pBS3C P_{SVEN_3215} *luxABCDE*	This study
pDA3Clux-PSVEN_3278	pBS3C P_{SVEN_3278} *luxABCDE*	This study
pDA3Clux-PSVEN_3293	pBS3C P_{SVEN_3293} *luxABCDE*	This study
pDA3Clux-PSVEN_3759	pBS3C P_{SVEN_3759} *luxABCDE*	This study
pDA3Clux-PSVEN_4575	pBS3C P_{SVEN_4575} *luxABCDE*	This study
pDA3Clux-PSVEN_0015	pBS3C P_{SVEN_0015} *luxABCDE*	This study
pDA3Clux-PSVEN_3204	pBS3C P_{SVEN_3204} *luxABCDE*	This study
pDA3Clux-PSVEN_3658	pBS3C P_{SVEN_33658} *luxABCDE*	This study
pDA3Clux-PSVEN_5424I	pBS3C $P_{SVEN\_5424\ distal}$ *luxABCDE*	This study
pDA3Clux-PSVEN_5424II	pBS3C $P_{SVEN\_5424\ proximal}$ *luxABCDE*	This study
pDA3Clux-PSVEN_5424I PSVEN_5424II	pBS3C $P_{SVEN\_5424\ distal\ +\ proximal\ promoters}$*luxABCDE*	This study
pDA3Clux-SVEN_3966	pBS3C P_{SVEN_3966} *luxABCDE*	This study
pDA 0KPliaI-AS19	pBS0K P_{liaI} RBS *SVEN_0398*	This study
pDP0K-lux03	pBS0K $P_{SVEN\_0399(-60\ to\ +1)}$ luxABCDE	This study

pDA 1KPliaI-AS19	pBS1K P_{liaI} RBS *SVEN_0398*	This study
pDA 4SPxyl-Sven_3966	pBS4S P_{xyl} RBS *Sven_3966*	This study
pDA 4SPxyl-Sven_1012	pBS4S P_{xyl} RBS *Sven_1012*	This study

2.1.3. Long-Flanking Homology PCR

Oligonucleotide encompassing 24 nucleotides were designed about 1 kb upstream of the *ydeB* gene (up forward) and another oligonucleotide with 20 nucleotides (up reverse) long was designed downstream of the start of the *ydeB* gene having 6 nucleotides overlapping the coding sequence. A region corresponding to one end of the kanamycin cassette was added to the 5` end of this oligonucleotide sequence. Further, twenty base pairs oligonucleotide was designed upstream the gene *ydeB* (down forward) with 7 nucleotides overlapping the coding sequence. At the 5` end of this oligonucleotide was added the sequence corresponding to the other end of the kanamycin cassette. The downstream reverse oligonucleotide was designed with 28 bp about 1 kb downstream of the gene *ydeB*.

The up and down fragments were amplified from *B. subitilis* 168 chromosomal DNA though PCR reactions using Phusion Polymerase (NEB, Ipswich, MA, USA). PCR reactions were purified by PCR purification HiYield Plasmid Mini-Kit, eluted in 35 µl of elution buffer (Süd-Laborbedarf GmbH (SLG), Gauting, Germany) and the concentration determined by using Nanodrop and 1.5 % agarose gel. One hundrend ng of both up and down fragments were joined by PCR using Phusion Polymerase and the up forward and down reverse primers. The PCR product was checked by 1.5 % agarose gel and purified using a purification kit (Hi-Yield clean up, Süd-Laborbedarf GmbH (SLG), Gauting Germany). 15 x 1.0^{-9} of the purified product was used to perform standard *Bacillus subtilis* transformation procedure and the obtained colonies were verified by colony PCR.

2.1.4. Strains and media

E. coli strains DH5α, DH10β or XL1-Blue were used for cloning. *E. coli* and routinely grown in Luria-Bertani (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) at 37°C with agitation. Ampicillin (100 µl/ml) was added for selection and maintenance of plasmid in *E. coli*. *B. subtilis* strains were grown in LB medium supplemented with the relevant antibiotics for selection: chloramphenicol (5 µg/ml), kanamycin (1 µg/ml), erythromycin (1 µg/ml) and lincomycin (25 µg/ml), or spectomycin (100 µg/ml). Solid media additionally contained 1.5% (w/v) agar. Report assays were performed using LB medium or MOPS-based

chemically defined medium with succinate and glutamate (MCSE) (Radeck *et al.*, 2013). All the strains used in this study are listed in **table 6.**

Table 6. Strains used in this study

Strain	Genotype	Reference
E. coli		
DH5α	*E. coli* F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ⁻	Laboratory stock
DH10β	*E. coli* F⁻ *endA1 deoR*⁺ *recA1 galE15 galK16 nupG rpsL Δ(lac)X74* φ80*lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC)* Str^R λ⁻	Laboratory stock
XL1-Blue	*E. coli endA1 gyrA96*(nal^R) *thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB*⁺ *lacI*^{q} *Δ(lacZ)M15] hsdR17*(r_K^- m_K^+)	Laboratory stock
Bacillus subtilis		
168	*B. subtilis trpC2*	Laboratory stock
TMB3447	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4513*	This study
TMB3448	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4870*	This study
TMB3449	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4793*	This study
TMB3450	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0063*	This study
TMB3451	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399*	This study
TMB3452	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_6501*	This study
TMB3453	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3668*	This study
TMB3454	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_2914*	This study
TMB3455	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3369*	This study
TMB3456	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3215*	This study
TMB3457	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3278*	This study
TMB3458	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3293*	This study
TMB3459	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_3759*	This study
TMB3460	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4575*	This study
TMB3461	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0015*	This study
TMB3462	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_6611*	This study
TMB4002	168 *sacA*::*cat* P_{SVEN_0399} *luxABCDE*	This study
TMB3516	168 *sacA*::*cat* P_{SVEN_4513} *luxABCDE* pBS0E*xylR* P_{xylA} *SVEN_4513*	This study
TMB3517	168 *sacA*::*cat* P_{SVEN_4870} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_4870*	This study
TMB3518	168 *sacA*::*cat* P_{SVEN_4793} *luxABCDE* pBS0E*xylR* P_{xylA} *SVEN_4793*	This study
TMB3519	168 *sacA*::*cat* P_{SVEN_0063} *luxABCDE* pBS0E*xylR* P_{xylA} *SVEN_0063*	This study
TMB3520	168 *sacA*::*cat* P_{SVEN_0399} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_0399*	This study

TMB3521	168 *sacA*::*cat* P_{SVEN_6501} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_6501*	This study
TMB3522	168 *sacA*::*cat* P_{SVEN_3668} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_3668*	This study
TMB3523	168 *sacA*::*cat* P_{SVEN_2914} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_2914*	This study
TMB3524	168 *sacA*::*cat* P_{SVEN_3369} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_3369*	This study
TMB3525	168 *sacA*::*cat* P_{SVEN_6611} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_6611*	This study
TMB3526	168 *sacA*::*cat* P_{SVEN_3215} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_3215*	This study
TMB3527	168 *sacA*::*cat* P_{SVEN_3278} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_3278*	This study
TMB3528	168 *sacA*::*cat* P_{SVEN_3293} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_3293*	This study
TMB3529	168 *sacA*::*cat* P_{SVEN_3759} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_3759*	This study
TMB3530	168 *sacA*::*cat* P_{SVEN_4575} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_4575*	This study
TMB3531	168 *sacA*::*cat* P_{SVEN_0015} *luxABCDE* pBS0E *xylR* P_{xylA} *SVEN_0015*	This study
TMB3661	168 *ydeB*::*kan*	This study
TMB5782	168 *ydeB*::*kan sacA*::*cat* P_{SVEN_4870} *luxABCDE* pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4870 thrC*::*spec* P_{xylA} RBS FLAG *SVEN_3966*	This study
TMB5783	168 *ydeB*::*kan sacA*::*cat* P_{SVEN_4870} *luxABCDE* pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_4870 thrC::spec* P_{xylA} RBS FLAG *SVEN_1012*	This study
TMB3451	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399*	This study
TMB3520	168 *sacA*::*cat* P_{SVEN_0399} *luxABCDE* pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399*	This study
TBM3655	168 *sacA*::*cat* $P_{SVEN\_0399\ (-30\ to\ +1)}$ *luxABCDE lacA*::*erm* P_{xylA} RBS FLAG *SVEN_0399* pBS0K P_{liaI} RBS *SVEN_0398*	This study
TMB3656	168 *sacA*::*cat* $P_{SVEN\_0399\ (-30\ to\ +1)}$ *luxABCDE lacA*::*erm xylR* P_{xylA} RBS FLAG *SVEN_0399 amyE*::*kan* P_{liaI} RBS *SVEN_0398*	This study
TMB3790	168 *sacA*::*cat* P_{SVEN_0399} *luxABCDE lacA*::*erm* pBS2E P_{xylA} RBS FLAG *SVEN_0399*	This study
TMB3901	168 *sacA*::*cat* P_{SVEN_0399} *luxABCDE* pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399 SVEN_0398*	This study
TMB3902	168 *sacA*::*cat* $P_{SVEN\_0399\ (-30\ to\ +1)}$ *luxABCDE* pBS0E *xylR* P_{xylA} SVEN_0398 $_{(p.1\text{-}98)}$ SVEN_0399	This study
TMB5449	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399 sacA*::*cat* P_{SVEN_3204} *luxABCDE*	This study
TMB5450	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399 sacA*::*cat* P_{SVEN_3658} *luxABCDE*	This study
TMB5451	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399 sacA*::*cat* $P_{SVEN\_5424\ distal}$ *luxABCDE*	This study
TMB5452	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399 sacA*::*cat* $P_{SVEN\_5424\ proximal}$ *luxABCDs*	This study
TMB5453	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN 0399 sacA*::*cat* $P_{SVEN\_542\ distal\ +\ proximal}$*luxABCDE*	This study
TMB5454	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399 sacA*::*cat* P_{SVEN_3966} *luxABCDE*	This study
TMB5455	168 *sacA*::*cat* P_{SVEN_0399} *luxABCDE* pBS0E *xylR* P_{xylA} RBS FLAG *SVEN_0399 + SVEN_0398* $^{109\ AAA>GGG}$	This study
TMB5457	168 pBS0E *xylR* P_{xylA} RBS FLAG *SVEN 0399 sacA*::*cat* *luxABCDE*	This study
TMB3854	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399 sacA*::*cat* P_{SVEN_0399} *luxABCDE*	This study
TMB5432	168 *sacA*::*cat* P_{SVEN_0399} *luxABCDE* pDP0EP_{xyl03}	This study
TMB5433	168 *lacA*::*erm* P_{xylA} RBS FLAG SVEN_0399 pDP0K-lux03	This study
TMB5434	168 pDP0EPxyl03 pDP0K-lux03	This study
TMB5443	168 *lacA*::*erm* P_{liaI} RBS FLAG *SVEN 0399 sacA*::*cat* P_{SVEN_0399} *luxABCDE*	This study

TMB5495	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399*$_{(ISV)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5496	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399*$_{(LAD)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TBM5501	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399*$_{(AAV)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5502	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399*$_{(DAG)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5503	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN_0399*$_{(DVS)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5504	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN_0399*$_{(LAA)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5505	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN_0399*$_{(LVA)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TBM5592	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN_0399*$_{(ASV)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5593	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN_0399*$_{(LDD)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5594	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399*$_{(HHA)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5595	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN_0399*$_{(ISS)}$ *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5603	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399 sacA*::cat $P_{SVEN\_0399(-35\ to\ +1)}$ *luxABCDE*	This study
TMB5604	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399 sacA*::*cat* $P_{SVEN\_0399(-129\ to\ +71)}$ *luxABCDE*	This study
TMB5718	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399* P_{veg} *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5719	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399* P_{lepA} *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5735	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN_0399* P_{liaG} *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5736	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399* P_{sigW} *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study
TMB5752	168 *lacA*::*erm* P_{xylA} RBS FLAG *SVEN 0399* P_{J23101} *sacA*::*cat* $P_{SVEN\_0399(-30\ to\ +1)}$ *luxABCDE*	This study

2.1.5. Luciferase assays

Luciferase activities of the strains carrying derivatives of the pBS3C*lux* reporter vector were measured using a Synergy™ 2 multi-mode microplate reader from BioTek® (Winooski, VT, USA) under control of the software Gen5™. Day cultures were inoculated with overnight cultures diluted 1:165 and incubated at 37 °C under agitation (200 rpm). Cultures were diluted to an OD_{600} 0.05 in a volume of 100 µl per well in 96-well plates (black wells, clear bottom; Greiner Bio-one, Frickenhausen, Germany) that were incubated at 37 °C with agitation. OD_{600} and luminescence were measured every 5 minutes for one-hour (OD_{600} ~0.1) and then 2 µl of the inducer to final concentration of 0.5 % (w/v) xylose or 20 µg/µl of bacitracin were added. Cultures were incubated at 37°C at 200 rpm and the OD_{600} and luminescence were monitored every 5 minutes for 16 hours. Optical density (OD_{600}) and relative luminescence values (RLU) were corrected by subtracting the negative controls (not inoculated medium). Thereafter, RLU/OD600 values were calculated for individual measurements and for each condition the mean and standard deviation of RLU/OD600 values were determined from three biological triplicates.

2.1.6. *In silico* analysis of ECF19

The ECF19 regulon was predicted *in silico* using the Virtual Footprint tool on the prokaryotic database of gene regulation (PRODORIC) (http://www.prodoric.de) (Münch *et al.*, 2003). ECF19 bipartite sequence motifs were searched for. The genome scan parameters for the promoter positions were set to be less than 350 nucleotides upstream of the start codon of the gene. The space between the most downstream residue of the -35 motif and the most upstream residue of the -10 motif was set as minimum 16 nucleotide and maximum 20 residues. After genome scanning and filtering, the predicted promoters were manually analysed. Genome organization and synteny of the ECF19 across *Streptomycetes* was obtained using the webtool SyntTax (Oberto, 2013).

Protein sequences of ECF19 (Sven_0399) and anti-σ 19 (Sven_0398) of *S. venezuelae* were retrieved from StrepDB - the *Streptomyces* Annotation Server. SigK and RskA protein sequences of *Mycobacterium tuberculosis* were retrieved from NCBI database under the accession numbers CCP43176.1 and CCP43175.1, respectively. Sequence alignment and homology structure modelling of ECF19 and anti-σ 19 of *S. venezuelae* were obtained using the online tool Phyre2 (Kelley *et al.*, 2015). Subsequently, the resulting structure models were superposed using the software Pymol (Schrödinger, 2015).

2.2. High level of Bacitracin resistance in *S. venezueale*

2.2.1. Bacterial strain, growth conditions and antibiotics

The bacterial strains used in this study are listed in **table 7**. *S. venezuelae* and *S. coelicolor* were cultivated in MYM medium (STUTTARD, 1982) with 50% tap water and supplemented with 200 µl of trace elements element solution (Kieser *et al.*, 2000). *Bacillus* antibiotic producer species where grown in Luria-Bertani (LB) broth medium at 37 °C overnight.

S. venezuelae and *S. coelicolor* spores were harvested from MYM medium with 4 ml of 20% glycerol using sterile cotton pads to scrub the lawns of *Streptomyces* on the plates. The suspensions were collected using a 2 ml syringe and stored in 1.5 ml sterile tubes at -20 °C (Bush *et al.*, 2013). In antibiotic stress treatment experiments, it was used commercially available bacitracin (Sigma, Germany), vancomycin (Sigma, Gemany), ramoplanin (Sigma, Germany), lysozyme (AppliChem, Germany), daptomycin (Sigma, Germany), D-cycloserine (Biochemika, Germany), ampicillin (Roth, Germany), penicillin G (Flukaanalytica, Germany), fosfomycin (Sigma, Germany) and spectinomycin (Sigma, Germany).

Table 7. Strains used in this study

Strain	Description	Source/Reference
Streptomycetes		
S. coelicolor A3(2)	Wild-type strain	Laboratory stock
S. venezuelae ATCC 10712	Wild-type strain	John Innes Centre, UK
Mut1-Mut6	Bacitracin-sensitive mutants of *S. venezuelae* ATCC10712 derived by random chemical mutagenesis	This study
Bacilli		
B. subtilis ATCC 6633	Wild-type strain; producer of subtilin	Laboratory stock
B. subtilis NCIB 3610	Wild-type strain	Laboratory stock
B. subtilis 168	Laboratory wild-type strain	Laboratory stock
B. licheniformis DSM13	Wild-type strain	Laboratory stock
B. licheniformis ATCC 10716	Wild-type strain; producer of bacitracin	Laboratory stock
B9	Natural *Bacillus* isolate; putative peptide antibiotic producer	Nithya *et al.*, 2012
C2	Natural *Bacillus* isolate; putative peptide antibiotic producer	Nithya *et al.*, 2012
EC1	Natural *Bacillus* isolate; putative peptide antibiotic producer	Nithya *et al.*, 2012
ME1	Natural *Bacillus* isolate; putative peptide antibiotic producer	Nithya *et al.*, 2012
N12	Natural *Bacillus* isolate; putative bacitracin producer Nithya *et al.*, 2012	

2.2.2. Chemical random mutagenesis and screening

S. venezuelae was mutagenized with 2.5 mg/ml NTG (N-methyl-N'-nitro-N-nitrosoguanidine) (TCI, Germany) diluted in 2.5 ml TM buffer (50 mM Tris Cl, pH 7.5, 10 mM Magnesium Sulfate) pH 9 and then divided into two different 2 ml tubes with 1.2 ml each to get a final concentration of 1 mg/ml NTG. It was added 0.625 ml of the spore suspension (3.1×10^{10} spores/ml) in each tube containing NTG plus TM buffer and incubated for 2 hours at 30 °C. The spores were washed with TM buffer, pelleted by centrifugation and then resuspended in 20% glycerol (Delić, Hopwood and Friend, 1970; Kieser *et al.*, 2000). Then 10^2 spores/ml from the mutagenized spore suspension were plated in MYM media for 4 days at 30 °C to get single colonies. The single colonies were picked up to a master plate using a 48 spots grid. Each master plate was replicated using a 48-pin replicator in two new MYM agar plates, one with 100 µg/ml bacitracin and other with no bacitracin, respectively, and incubated for 24 hours at 30 °C.

2.2.3. Determination of bacitracin susceptibility

Streptomyces species were tested for resistance against different concentrations of bacitracin by disc diffusion agar method. The zones of inhibition were measured after incubation at 30 °C for

24 h. The minimum inhibitory concentration (MIC) was determined by resazurin microtiter assay plate method (Sarker, Nahar and Kumarasamy, 2007). Resazurin is an oxidation-reduction indicator that changes color from blue to pink to indicate reduction and consequently cell viability. Resazurin sodium salt powder (Sigma, Germany) was prepared at 0.01% (w/v) in distilled water and sterilized by filtration. Ninety-six well plates (Sarstedt, Germany) were used for the MIC assays. Into each well was added a mix solution containing MYM media, resazurin (0.001%) and different concentrations of bacitracin. Subsequently, 10 µl of spore suspension containing 10^4 spores/ml was added in each well. As controls, a well contained no bacteria and other no antibiotics. The microtiter plates were incubated for 24 hours at 30 °C. The MIC was determined as the lowest concentration that prevented the color change, i.e., in which cells were no longer metabolically active (Vidal-Aroca *et al.*, 2009; Silva and Ferreira, 2013).

2.2.4. Determination of sensitivity spectra against cell wall active antibiotics

Determination of the sensitivity to cell wall antibiotics was made by inoculating $5x10^5$ spores/ml of the *Streptomyces* strains into MYM soft agar, which were plated in petri dishes and left to dry for 30 min at room temperature. Six millimeter discs were soaked with 20 µl of nine cell wall antibiotics: bacitracin (100 µg), vancomycin (40 µg), ramoplanin (40 µg), lysozyme (40 µg), daptomycin (40 µg), D-cycloserine (40 µg), ampicillin (40 µg), penicillin G (40 µg) and fosfomycin (40 µg). Spectinomycin (40 µg) inhibits protein synthesis and was used here as a control. The soaked discs were subsequently placed into the MYM soft agar containing the *Streptomyces* strains. The plates were incubated at 30 °C for 24 h. The zone of inhibition was measured to determine the sensitivity against each antibiotic.

2.2.5. Interaction between *Streptomyces* and different *Bacillus* spp. antibiotic producer strains

All antibiotic producer strains were grown in 4 ml LB medium at 37 °C for 24 h. An amount of $5x10^5$ spores/ml of each *Streptomyces* strain were spread on MYM agar plates using a glass spreader and the plates were dried for 30 min. Five microliters of the overnight culture of each antibiotic producer strains were spotted onto the plates containing the *Streptomyces* strains and incubated at 30 °C for 24 h for posterior zone of inhibition measurement.

2.2.6. *S. venezuelae* genomic DNA extraction

The DNA isolation method is a modification of the salting out procedure described previously (Pospiech and Neumann, 1995; Kieser *et al.*, 2000). *Streptomyces* strains were grown in liquid MYM media at 30 °C for 18 h and cultures were harvested as 10 ml aliquots and subsequently washed once with 10% sucrose. Pellets were resuspended in 2 ml SET buffer (75 mM NaCl, 25 mM EDTA pH 8, 20 mM Tris pH 7.5). *Streptomyces* was incubated with 2 µl RNase (10 mg/ml) and 40 µl lysozyme (15 mg/ml) for 60 min at 37 °C, where a drop of 10 % SDS was added every 10 minutes. Subsequently, 56 µl proteinase K (20 mg/ml) and 240 µl of 10% SDS were added to the *Streptomyces* suspension and incubated at 55 °C for 2 hours, inverting occasionally. Following, 800 µl of 5 M NaCl was added, mixed gently by hand and allowed to cool at room temperature. 2 ml of chloroform were added, mixed gently at room temperature for 30 minutes and separated by centrifugation at 6000 rpm for 15 minutes. The aqueous phase was transferred with a cut-off 5 ml tip into a new tube. The nucleic acids in the aqueous phase were precipitated by adding 0.6 volumes of isopropanol, and collected by centrifugation at 8000 rpm at 4°C for 10 min. The pellet was washed with 70% ethanol and dried. The dry pellet was resuspended in 500 µl TE (10 mM Tris pH 8.0, 1 mM EDTA) solution and stored at 4°C. The DNA yield was calculated from the absorbance at 260 nm for clean DNA samples (A_{260}/A_{280} between 1.8 and 2.0). A sample of DNA was loaded onto a 1% agarose gel for visualization and quality control.

2.2.7. Genome sequencing of bacitracin-sensitive *S. venezuelae*

The genome sequence for *S. venezuelae* ATCC 10712 is available on NCBI database (accession number NC_018750). For the mutants obtained through NTG treatment, library construction and sequencing were performed by the genomic service unit of the Ludwig-Maximilians Universität (LMU) München. To obtain an accurate quantification of the DNA library the DNA was quantified using Qubit dsDNA BR Assay system, a fluorometric based method specific for duplex DNA combined with use of Bioanalyzer DNA HS Chip from Aligent Technology 2100 Bioanalyzer. Fifty nanograms were used for the first step, tagmentation and the samples were mixed to 20 µl volume with ultra-pure water. Libraries were sequenced as recommended by MiSeq Manual (Illumina, Inc.).

To generate consensus sequences, the genome of *S. venezuelae* wild-type strain from our laboratory stock was assembled by comparison to the reference strain *S. venezuelae* ATCC 10712 (GenBank accession no. NC_018750) checking this way for any changes that may have

occurred. Further, each *S. venezuelae* mutant strain was assembly by comparison to our reference using CLC genomic workbench (CLC, bio, Qiagen).

2.2.8. Antibiotic stress condition and harvest procedure

Streptomyces strains ($5x10^5$ spores/ml) were grown in metal spring-containing 100 ml flasks filled with 20 ml liquid MYM medium. Cells were grown at 30 °C, under heavy shaking and when the exponential phase was reached, 10 ml of the cultures were transferred to two new metal spring-containing flasks with or without 40 µg/ml bacitracin and incubated for 20 minutes.

10 ml cultures were transferred using a glass pipette to precooled (-20 °C) centrifuge tubes containing 20 ml glycerol: 0.85% NaCl saline solution (3:2 volumes). The centrifuge tubes were placed in an ice-cold block and then centrifuged at -20 °C for 30 min. The tubes were placed back in the ice-cold blocks and the supernatant was removed followed by pellet resuspension in 1 ml glycerol: 0.85% NaCl saline solution (3:2 volumes) and transferred to microcentrifuge tubes. The tubes were cooled in liquid nitrogen and stored at -80 °C until RNA extraction (Villas-Bôas and Bruheim, 2007).

2.2.9. RNA isolation, library construction and RNA-seq analysis

The frozen *Streptomyces* cells were centrifuged at 10.000 *g* and 4 °C for 10 min and the supernatant discarded. Pellets were resuspended in 1 ml RNA plus (MPBio, France) and transferred for a bead-beater tube containing glass beads (MPBio, France). Cells were homogenized using a beat-beater for 3 cycles at 6.5 rpm for 30 seconds, resting on ice in between each cycle. It was added 1/5 volumes chloroform (Roth, Germany) to the homogenized samples in RNA plus, thoroughly mixed by vortexing and centrifuged at 10.000 *g* and 4 °C for 20 minutes. The supernatants were transferred to a new sterile tube, 2.5 volumes of 100% ethyl ethanol (EtOH) were added and thoroughly mixed by vortexing. The samples were stored at -20 °C for 12 h. The samples were then centrifuged at 10.000 *g*, 4 °C for 20 min. The pellets were washed twice with 500 µl of 80% ethanol (diluted with RNA free water). The supernatants were completely removed; the pellets were air-dried for 5 min and dissolved in 100 µl of RNase free water. Starting with the crude nucleic acids extract obtained in the previous steps, the RNA purification was performed using a Zymo column purification Direct-zol RNA MiniPrep kit (Zymo Research, Germany) following manufacturer's instructions (Zhang *et al.*, 2013). Samples were subjected to DNase treatment using DNA-free DNA removal kit (Ambion, Germany). RNAs were quantified using a bioanalyzer (Agilent 2100, Santa Clara, CA, USA)

and the quality was checked by electrophoresis in a 1.0 % agarose gel. The rRNA was removed using the Ribo-Zero™ Magnetic Kit for gram-positive bacteria (Epicentre, Germany). Three different samples were used for RNAseq. The RNA library was constructed using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Inc.) modified to select for larger sized RNA inserts, by spending less time fragmenting in the beginning and longer elongation during cDNA synthesis. Samples were sequenced by MiSeq (Illumina, Inc.). Sequence reads were imported into CLC Genomics Workbench 9.5. After mapping against the reference, genes with an adjusted P value under 0.05 and fold change above two were considered as being differentially expressed.

3. Results

3.1. ECF σ factor-based switches

3.1.1. Implementation of *S. venezuelae* ECF σ factor-based switches in *B. subtilis*

Sixteen ECF σ factors from *S. venezuelae* were codon adjusted, commercially synthetized and placed under control of the inducible promoter P_{xylA}. An additional copy of the gene encoding the P_{xylA} repressor XylR was also added given that it has been reported that additional copies of the XylR operator reduce its effectiveness on each given site (Gärtner, Geissendörfer and Hillen, 1988). Additionally, an N-terminal FLAG-tag was added to the ECF-σ factor (**Figure 10A).** The nature and location of this tag had been reported not to interfere with the ECF σ factor activity (Dufour, Landick and Donohue, 2008; Wecke *et al.*, 2012; Gangaiah *et al.*, 2014; Toyoda *et al.*, 2015; Mao *et al.*, 2017).

In order to check the activation of the ECF target promoters, a reporter system based on the bacterial luciferase operon *luxABCDE* was used (Radeck *et al.*, 2013). The promoters were inserted upstream of the *luxABCDE* operon (**Figure 10B**) and thereafter integrated into the *B. subtilis sacA* locus, which encodes a non-essential phosphosucrase involved in sucrose utilization (Lepesant *et al.*, 1975). The output of the ECF σ factor based switches was monitored through luminescence measurements.

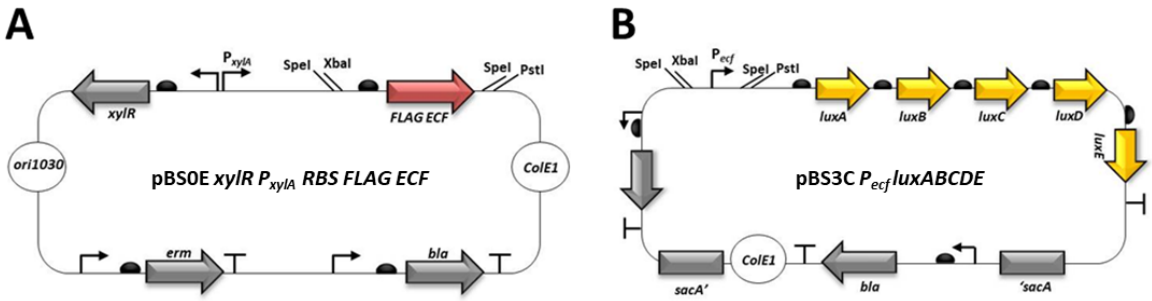


Figure 10. Vector maps showing plasmids carrying the ECF σ factors (A) and the ECFs promoters followed by the reporter *luxABCDE* (B). Rectangles represent regions that are necessary for homologous recombination into *B. subtilis* 168 chromosome. Circles represent origins of replication. Thick arrows represent open reading frames. 'T' represents terminators. Half circles represent ribosome binding sites. Thin arrows represent promoters. Oblique lines represent restriction sites.

The ECF σ factor switches were built as depicted in **Figure 11A**. No growth differences was observed between strains harboring the ECF-σ factor-based switches and the wild type strain, suggesting that in these conditions, the ECF-based switches were not toxic to *B. subtilis* (**Figure 11B)**. This data is **i**n accordance to similar work in *E. coli* and *B. subtilis* where ECF-σ factor-based switches, in general, did not affect the growth rate of the host (Rhodius *et al.*, 2013; Pinto *et al.*, 2018a).

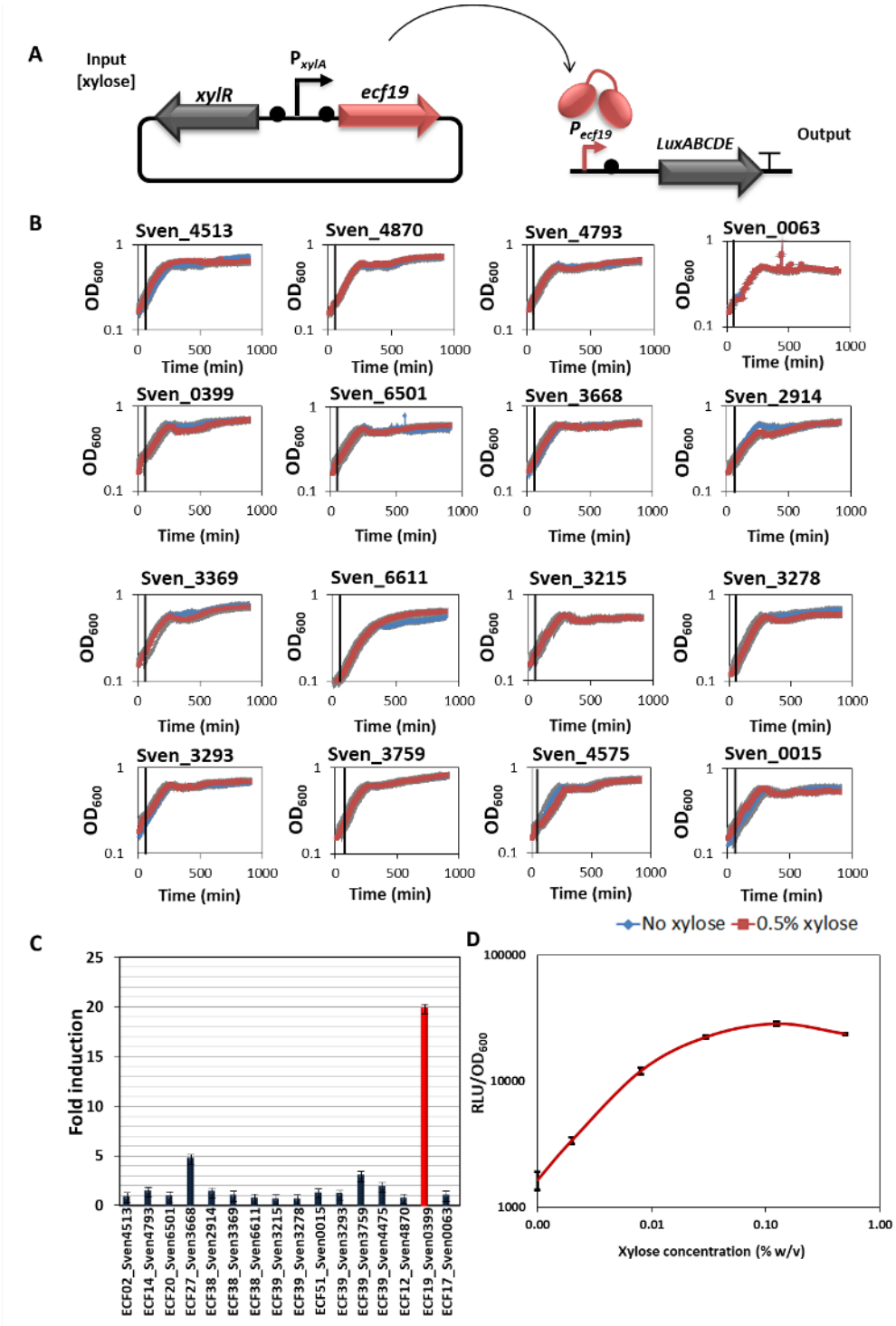


Figure 11. Activity of ECF- based switches build from *S. venezuelae* native ECF σ factors. (A) Representation of the ECF σ 19 switch. Expression of the ECF σ factors was induced by xylose. The activity of the corresponding ECF σ factor target promoter was monitored via luminescence measurements. Thick arrows represent open reading frames. Half circles represent ribosome binding sites. Thin arrows represent promoters. Plasmid is represented as rounded rectangle. (B) Growth curves of the strains containing each ECF-based switch. Blue lines show the growth without induction of the ECF-based switches and red lines show growth of cultures in which the expression of the ECF-based switches was induced (0.5% xylose) after 60 minutes (vertical black line). (C) Fold induction of each switch. The output of each switch was assessed from the relative luminescence units (RLU) normalized by the optical density measured at 600 nm (OD_{600nm}). (D) Dose-response curve of ECF (Sven_0399)-based switch in which the switch output is represented through relative luminescence units (RLU) normalized by the optical density measured at 600 nm (OD_{600}) achieved 90 min after the addition of the inducer. The graph presents the measurements performed with the *B. subtilis* strain harbouring the switch

pictured in figure 11A. Final concentrations of xylose used for induction of P_{xylA} were 0, 0.002, 0.008, 0.03, 0.125 or 0.5 % (w/v). Vertical bars represent standard deviations calculated from three independent experiments.

Here, only one out of 16 ECF-based switches were active while the remaining ones showed no activity (**Figure 11C**). The active ECF-switch belongs to the group ECF19 (Sven_0399) and showed about twenty-fold increase of promoter activity within 5 min after addition of 0.5% xylose (w/v). The concentration of inducer required to turn ON the Sven_0399-based switch was below 0.002% xylose (**Figure 11 D**), that was lower than what was needed to turn ON others ECF-based switches successfully implemented in *B. subtilis* (Pinto et al., 2019).

3.2 Characterisation of ECF19-based switch

3.2.1 ECF19 alignment and homology structure modelling

The ECF σ factor K (sigK) is a well-studied example of ECF19 in Mycobacteria being involved in controlling the expression of the antigen proteins Mbt70 and Mbt83, which are virulence determinants (Charlet *et al.*, 2005; Saïd-Salim *et al.*, 2006). It has been demonstrated that the intracellular levels of SigK in *M. tuberculosis* are regulated by a membrane-associated anti-σ factor named RskA. Under inducing stimuli the anti-σ RskA is subject to regulated intramembrane proteolysis (RIP), which involves three steps: first, a site-1 protease (S1P) cleaves the RskA ectodomain; then a site-2 protease (S2P) cleaves the transmembrane helice, releasing the $RskA_{cyto}$/SigK complex from the membrane; finally, the complete dissociation of the ECF-σ factor and the AS is achieved in the cytoplasm through selective degradation of the AS cytosolic part (Makinoshima and Glickman, 2005; Urban, 2009; Sklar *et al.*, 2010; Shukla *et al.*, 2014). Structural studies of SigK-RskA interaction in *M. tuberculosis* showed two cysteines (Cys133 and Cys183) forming a disulphide bridge in the σ_4 domain of SigK which acts as a sensor in order to induce the dissociation of SigK from the cytoplasmic part of its negative regulator RskA. Multiple sequence alignments have shown that these disulphide-forming cysteines are conserved in 70% of the homologs from different species of which 27 belong to *Streptomyces* spp. (Shukla *et al.*, 2014).

Sequence alignment (**Figure 12**) and structure comparison (**Figure 13**) of the ECF19-AS19 complex of *M. tuberculosis* (PDB entry 4NQW) and *S. venezuelae* showed that ECF19 of S. venezuelae also has the disulphide-forming cysteines as previously reported for Mycobacteria SigK by Shukla and co-workers (2014).

Figure 12. Sequence alignment of the ECF19 (A) and AS19 (B) of *S. venezuelae* with SigK and RskA of *M. tuberculosis* (PDB entry 4NQW), respectively. Identical residues are highlighted with a grey background, green helices represent α-helices, pink boxes show the conserved disulphide-forming cysteines, red background indicates insertions relative to template and Orange background indicates deletions relative to template. Sequence alignment was obtained using the online tool Phyre2 (Kelley *et al.*, 2015).

The conserved disulphide-forming cysteines Cys133 and Cys183 in SigK of *M. tuberculosis* align to Cys186 and Cys216 in ECF19 of *S. venezuelae* (**Figure 12A**, **Figure 13A**). In like manner, superposition of the anti-σ factor structures show high homology between *S. venezuelae* AS19 and RskA of *M. tuberculosis* (**Figure 12B**, **Figure 13B**). Additionally, the homology between the *S. venezuelae* ECF19-AS19 complex to the template corresponding to the crystal structure of *M. tuberculosis* SigK-RskA interaction (PDB entry 4NQW) is extensive (**Figure 13C**). Shukla and co-workers (2014) suggested that the effect of redox stimuli on SigK-RskA interaction is most likely conserved across other ECF σ factors presenting disulphide-forming cysteines.

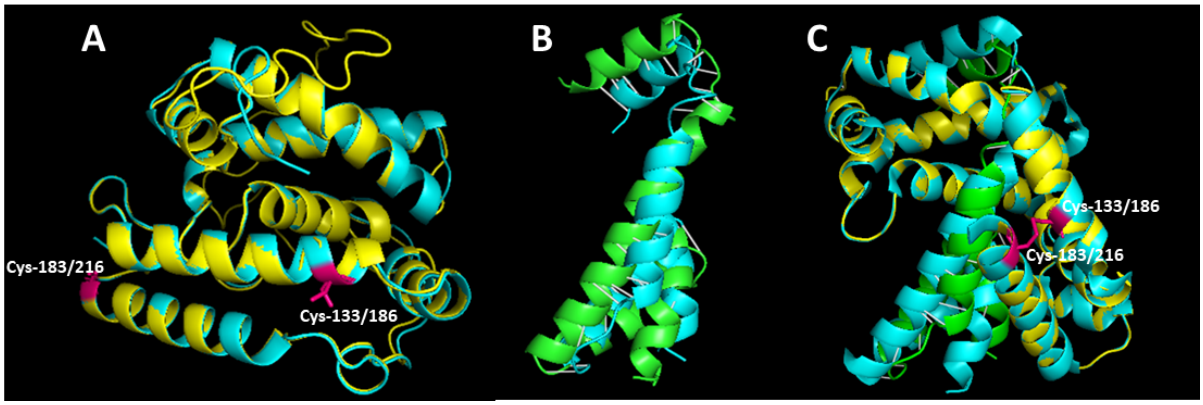


Figure 13. Homology structure modelling analyses. (A) Superposition of *S. venezuelae* ECF19 (yellow) on *M. tuberculosis* SigK (blue). (B) Superposition of *S. venezuelae* AS19 (green) on *M. tuberculosis* RskA (blue). (C) Superposition of both *S. venezuelae* ECF19 (yellow) and its AS19 (green) on the SigK-RskA complex (PBD entry 4NQW; blue). The conserved disulphide-forming cysteines in the ECF σ factor are shown in pink. White lines show the distance between correspondent amino acids. Structure models were obtained using the online tool Phyre2 (Kelley *et al.*, 2015) and superposition was performed on pyMol (Schrödinger, L. 2015).

3.2.2 Genomic context of ECF19

In *M. tuberculosis*, the ECF19 SigK regulates the expression of genes encoding the antigenic proteins Mbt83 and Mbt70 (Saïd-Salim *et al.*, 2006). Veyrier and co-workers (2008) showed that in some *Actinobacteria* these genes can be localized in the same or different loci than the *sigK*. Here, the genome organization and synteny of the ECF19 from *S. venezuelae* was compared with that of other actinobacterial species previously analysed by Veyrier. In *Streptomyces* genomes, they are usually located upstream conserved genes that encode oxidoreductases, fasciclins (homologous of MPT83 antigen protein) and cyclopropane-fatty-acyl-phospholipid synthases (**Figure 14**), which have been reported as part of the ECF19 regulon in *Mycobacteria*.

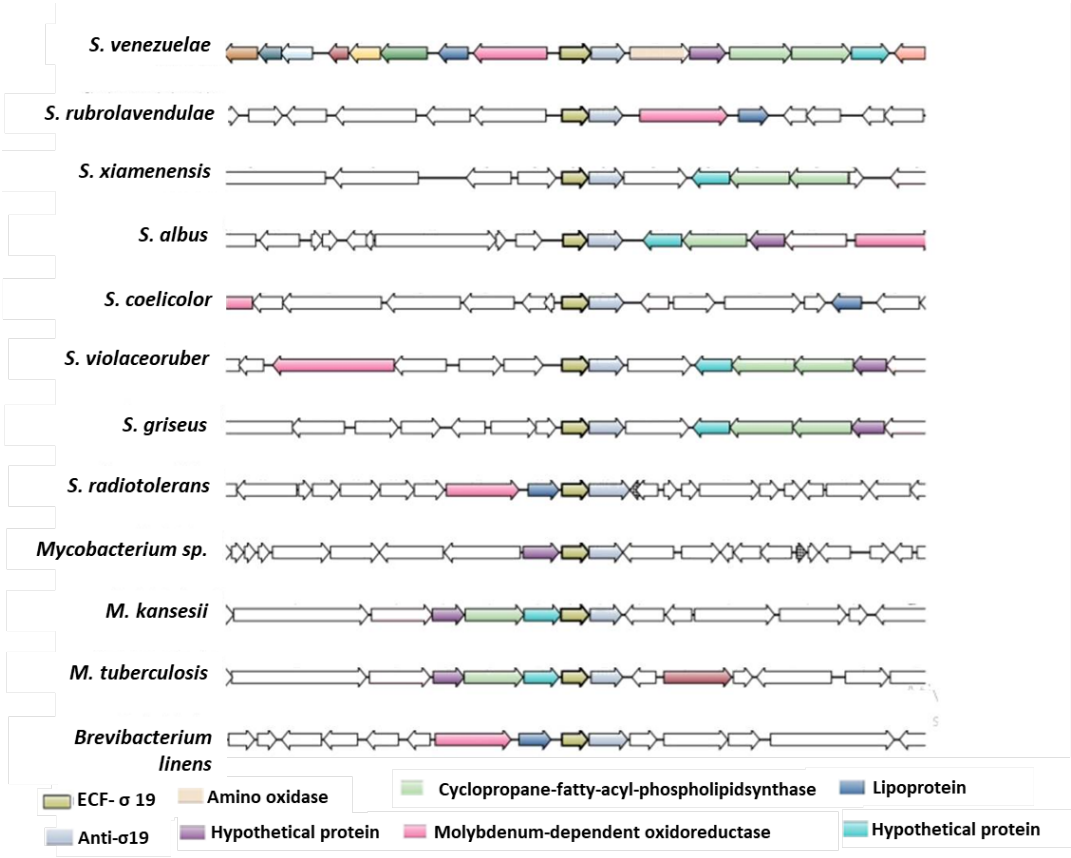


Figure 14. Genomic context and synteny of ECF19 in Actinobacteria obtained by using the web tool SyntTax (Oberto, 2013). Homologous genes are represented with the same colours along the different species. Bit scores threshold ≥20%.

3.2.3 Prediction of the ECF19 regulon in *S. venezuelae*

In bacteria, the formation of the complex DNA-RNAP is initiated though the recognition of specific promoter DNA by the σ subunit of the RNAP (Saecker, Record and Dehaseth, 2011; Feklístov *et al.*, 2014). Regulon studies on SigK of Mycobacteria (Veyrier, Saïd-Salim and Behr, 2008) and the ECF σ factor classification made by Staroń and collaborators (2009) identified the promoter sequence motif for ECF19, which is rich in CCGATCC in the -35 region and GAA in the -10 region (**Figure 15**).

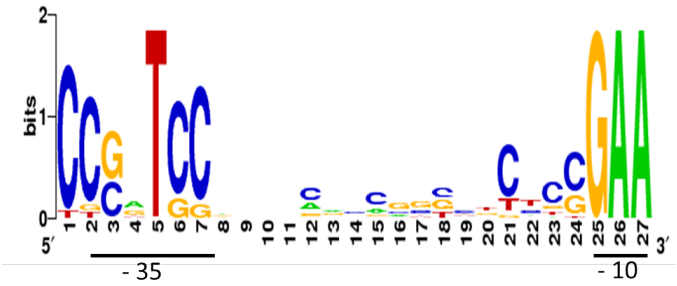


Figure 15. Weblogo of ECF19 target promoters illustrating the degree of sequence conservation (Staroń *et al.*, 2009).

Here, the P_{sven_0399} sequence motif was used to screen the *S. venezuelae* genome for the ECF19 putative target promoters using the Virtual Footprint tool PRODORIC (Münch *et al.*, 2003). Thirteen putative promoters were identified (**Table 8**). These predicted ECF19 target promoters had a spacer of 18 and 20 bp between -10 and -35 motifs and were located between 47 to 339 bp from the start codon. In order to confirm some of the promoters predicted by PRODORIC, the transcription start sites (TSS) of *S. venezueale* in different phases of growth were checked (Bush *et al.*, 2016) and eight of the ECF19 predicted promoters showed some basal level of transcription initiation in these conditions. The promoter sequences confirmed by TSS data are shown in bold in **table 8**.

Table 8. ECF19 putative target promoters

Start (genome coordinates)	End (genome coordinates)	Promoter Sequence (putative -10 and -35 elements are underlined)	-10/-35 spacer length (bp)	-35/start codon distance (bp)	Downstream gene	Annotations
381263	3812988	CCTTCCCCGTTCGTGAAGAACCGCCGAA	19	122	*sven_0354*	Hypothetical protein - SecY superfamily
426462	426487	CATCCGCAGGGCCGTCCGCTACGAAGAA	20	89	*sven_0399*	RNA polymerase sigma-70 factor ECF19
359637	359661	CCGTTCCGGGCCCATTGTGTGCGCCGAA	18	93	*sven_0333*	Hypothetical protein
3217396	**3217421**	**TCGTCCGGAATGCGGACGTCCGGATCGAA**	**20**	**76**	***sven_2943***	**Putative two-component system sensor kinase**
3329847	**3329872**	**CCGTCCGGAGGTGACCATTGCCGACCGAA**	**20**	**65**	***sven_3043***	**Pyruvate formate-lyase**
3512289	**3512314**	**CCGTCCGCCATCCGGACGAGCTGTCCAGA**	**18**	**59**	***Sven3204***	**Dihydroxy-acid dehydratase**
3974201	**3974225**	**TCGTCCACCTACGCAGCGGTGCATCGAA**	**20**	**252**	***sven_3658***	**Protein YidD / membrane insertase activity**
1069418	1069441	CCGTCCTACCGTCCCGCCATGACCGAA	20	47	*sven_0940*	Signal peptidase I
4771902	4771926	CCGTCCGGTGCACAGATGGCGCTTCGAA	18	68	*sven_4431*	Putative secreted protein
7979786	7979813	CCCTCCAGCCCTGGACGTGTGACCCGAA	20	256	*sven_7255*	Methylenetetrahydrofolate dehydrogenase (NADP+)
4212275	4212298	CCATCCGACACCCCGACGAAGTACGAA	19	236	*sven_3873*	NADH dehydrogenase
4307352	**4307380**	**CCGTCCGCGCAGCCGCTCCGACCTGCGAA**	**19**	**140**	***sven_3966***	**CarD transcriptional regulator**
5874550	**5874573**	**TCGTCCGCATACCCGAACATCATCGAA**	**19**	**101**	***sven_5417***	**putative two-component system sensor kinase**
5880880	**5880905**	**CCATTCTCATGGAAGCCCGGCCCTTGGAA**	**18**	**118**	***sven_5424I***	**RecA protein**
5880997	**5881020**	**CGATCGACTCAAGCAAACCGGGTGGAA**	**18**	**3**	***sven_5424I***	**RecA protein**
8027115	8027138	CCGTCCGCTACACTGAGGGACTGCGAA	19	218	*sven_7296*	Putative integral membrane protein

In order to confirm the predicted ECF19 target promoters, they were implemented in the ECF19-based switch (**Figure 16A**). As before, implementation of most of the target promoters was not toxic to *B. subtilis*, except for the proximal promoter of Sven_5424 which resulted in growth defect 250 minutes after induction. However, none of switches with the predicted ECF19 target promoters showed activity (**Figure 16B**).

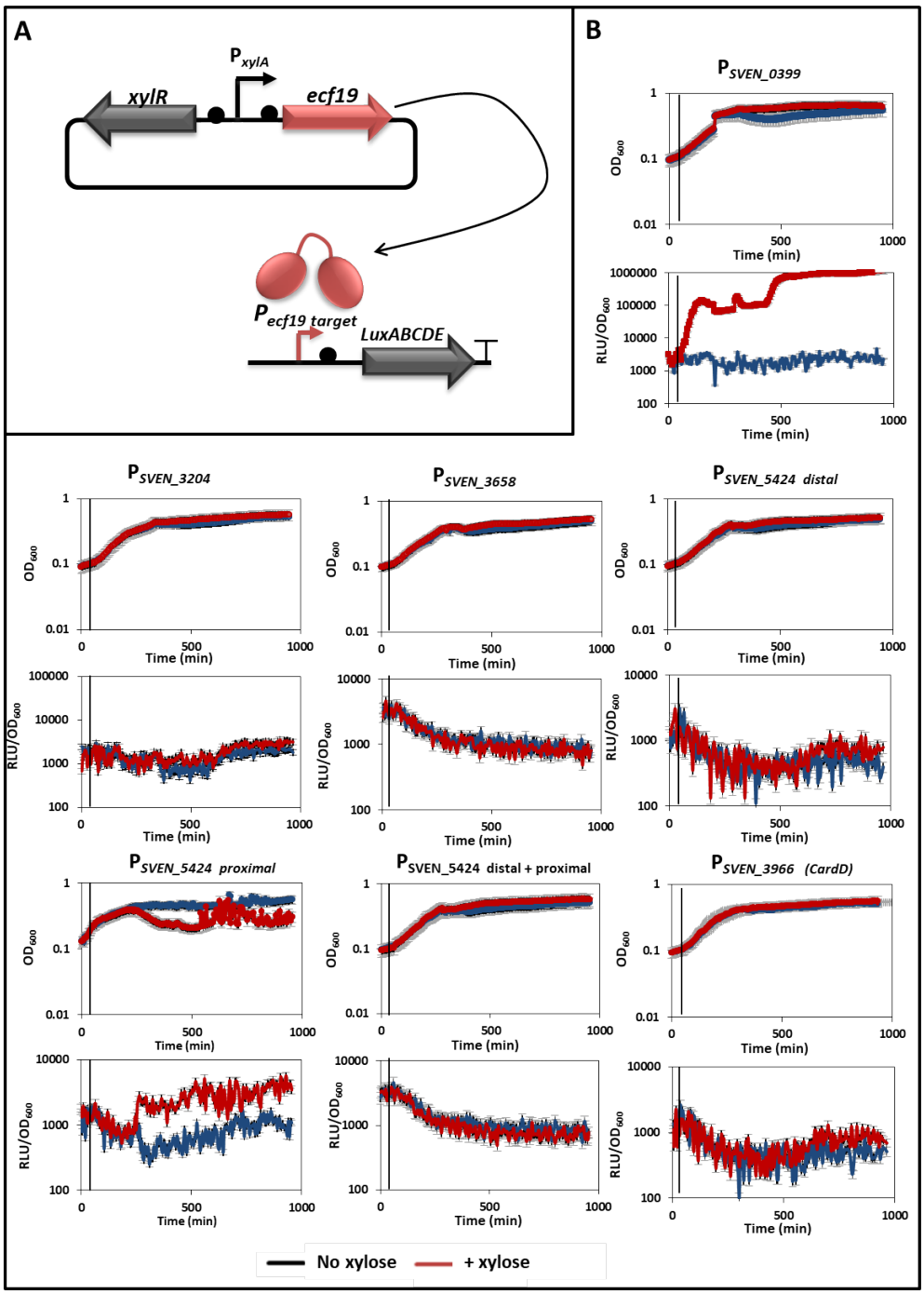


Figure 16. ECF19 target promoters (A) Genetic design of the ECF19-based switch. Thick arrows represent open reading frames. Half circles represent ribosome binding sites. Thin arrows represent promoters. Plasmid is represented as rounded rectangle. (B) Growth curves assays (left) and luminescence outputs (right) of the ECF19-based switch build using different putative ECF19 target promoters. Luminescence output is represented through relative luminescence units (RLU) normalized by the optical density measured at 600 nm (OD600nm). Vertical back line crossing the graphs indicates the time of addition of the inducer.

3.3 Modulation of the ECF19-based switch behaviour

3.3.1 Anti-σ factor 19 implementation

Previous studies in SigK of *M. tuberculosis* showed that ECF σ factor 19 activity is regulated by its antagonist anti-σ factor 19 (Makinoshima and Glickman, 2005; Urban, 2009; Sklar *et al.*, 2010; Shukla *et al.*, 2014). In order to test whether the AS19 would affect the activity of the ECF19-based switch in *B. subtilis*, the AS19 gene of *S. venezuelae* was codon adjusted and later integrated into the *amyE* (amylase) locus of *B. subtilis* or placed into a replicative vector, in both cases under control of the bacitracin-inducible promoter P_{liaI} (Mascher *et al.*, 2004; Toymentseva *et al.*, 2012; Radeck *et al.*, 2013) (**Figure 17A**). The P_{liaI} promoter, which is induced by bacitracin, has been well characterized elsewhere as a sensitive and strong promoter with low basal level of expression that is induced in a concentration-dependent manner (Mascher *et al.*, 2004; Toymentseva *et al.*, 2012; Radeck *et al.*, 2013). Expression of the ECF σ factor 19 was induced by the addition of xylose and after one hour the expression of the anti-σ factor 19 was induced by the addition of bacitracin.

It has been previously reported that the implementation of anti-σ factors could be toxic to *E. coli* (Rhodius *et al.*, 2013). Nevertheless, in our system the expression of the AS19 caused no visible growth defect in the host strain (**Figure 17B**). In these conditions AS19 did not interfere with the ECF19 activity (**Figure 17C**) in contrast to studies in *E. coli,* where a high fraction of anti-σ factors repressed the activity of their cognate ECFs (Rhodius *et al.*, 2013).

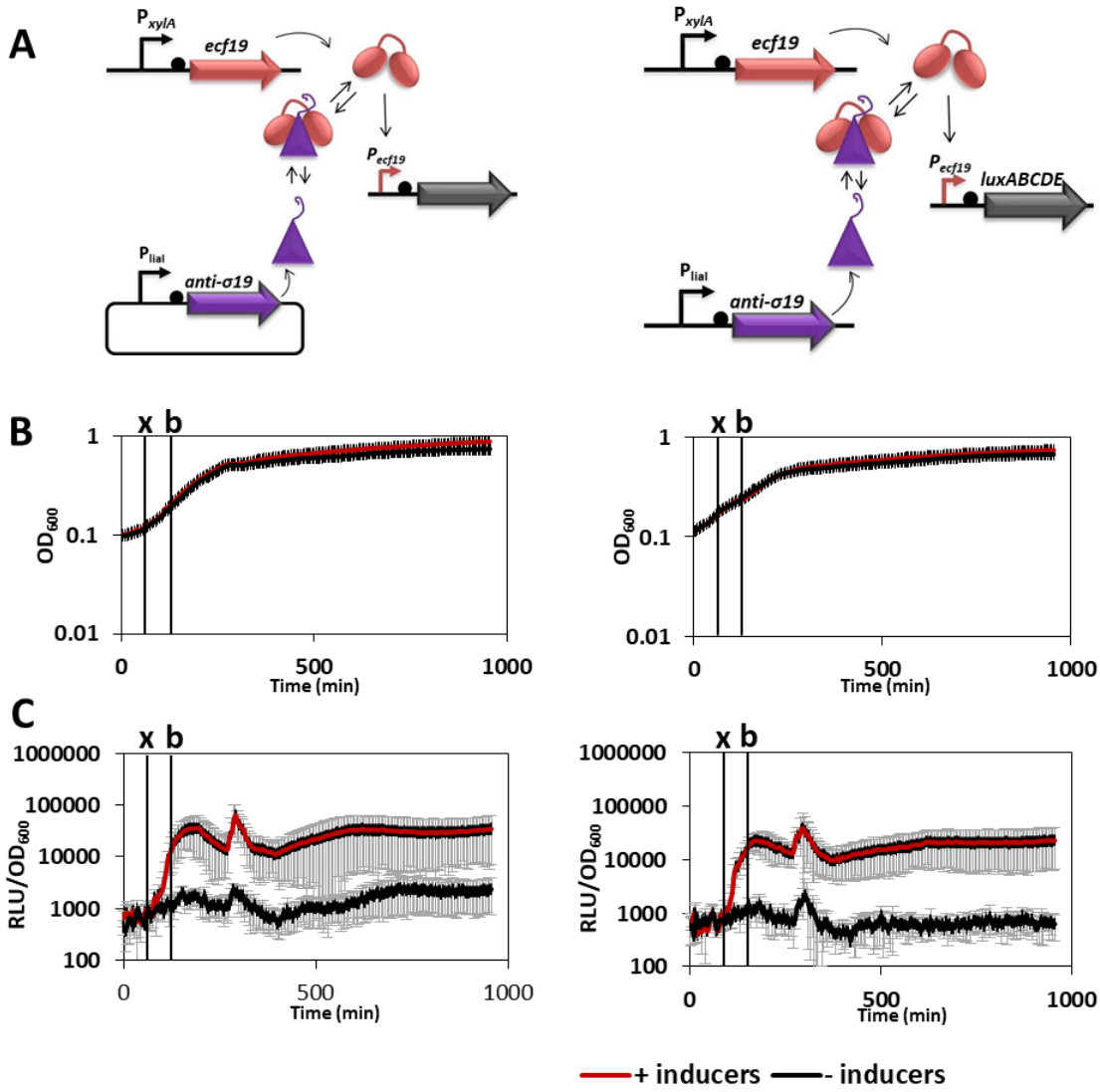


Figure 17. Anti-σ19 implementation. (A) Genetic depiction of the ECF σ factor 19 switch plus anti-σ factor 19. The ECF σ factor 19 is integrated in the *amyE* locus of *B. subtilis* under control of the promoter P_{xylA} and the anti-σ factor 19 in a replicative vector, whose expression is induced by bacitracin through the promoter P_{liaI}. Thick arrows represent open reading frames. Half circles represent ribosome binding sites. Thin arrows represent promoters. Plasmid is represented as rounded rectangle. (B) Growth curve and (C) luminescence output of the respective strains are shown. Luminescence output is represented through relative luminescence units (RLU) normalized by the optical density measured at 600 nm (OD_{600}). Black lines show the cultures without induction, red lines show cultures under the first induction with xylose (0.5%) after 60 minutes of growth (vertical black line marked with "x") and the second induction with bacitracin (20 ug/ul) 60 minutes after the first induction (vertical black line marked with "b"). Grey vertical error bars represent standard deviations calculated from three independent experiments.

Additionally, the ECF19 was implemented together with the full-length AS19 or only its cytoplasmic part in a replicative vector both under control of a single P_{xylA} promoter, as showed in **Figure 18A**. As observed previously, *B. subtilis* showed no growth defect when carrying the anti-σ factor (**Figure 18B**). The presence of the AS19, encoded in the same operon as ECF19, was able to decrease the switch's activity by 2.7-fold when compared to the strain lacking the AS19. Further, the expression of only the cytoplasmic part of the AS19 resulted in a decrease

in activity of 9-fold when compared to the strain lacking the AS19 (**Figure 18C** and **Figure 18D**).

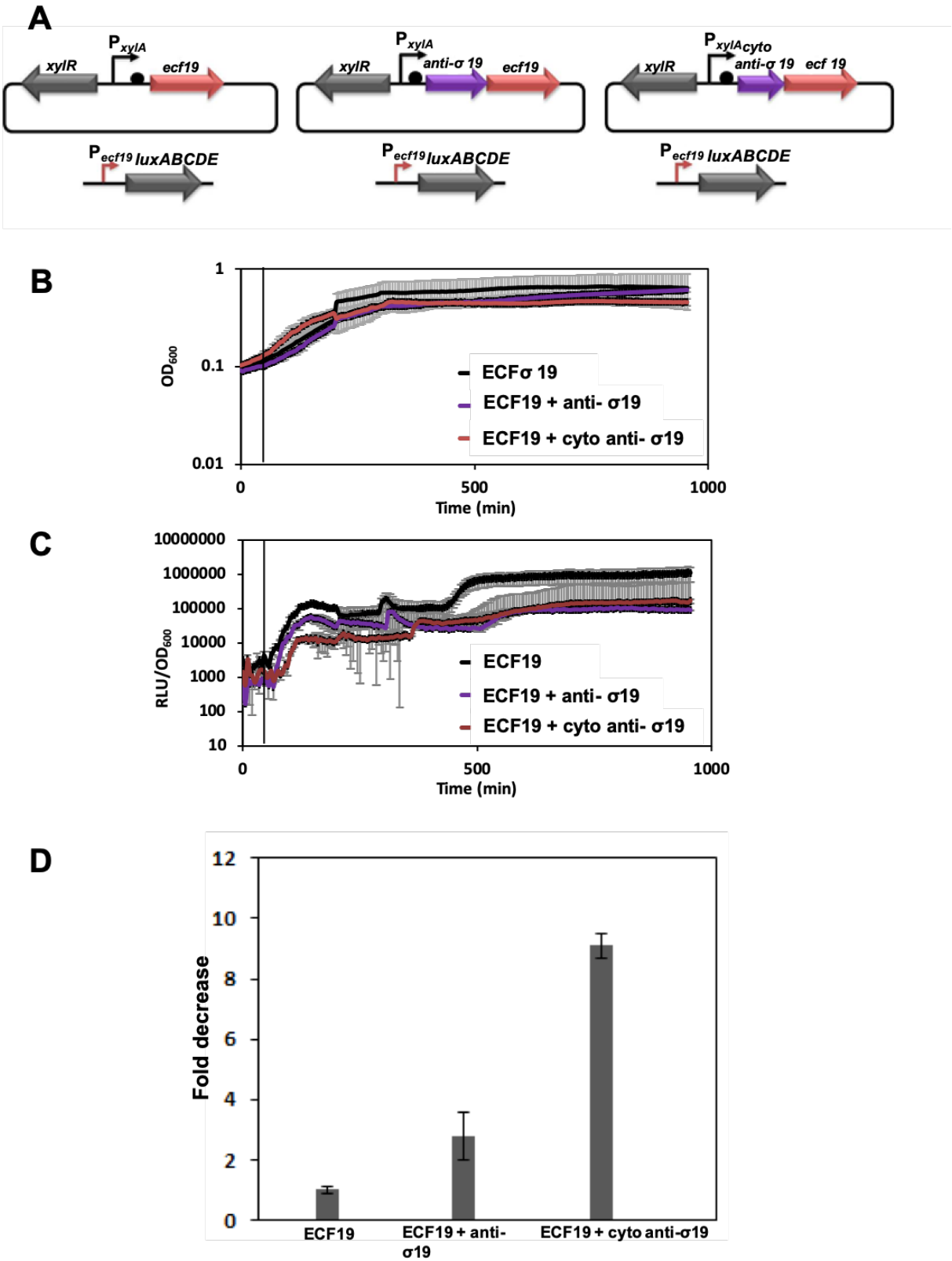


Figure 18. AS19 implementation (A) Genetic depiction of the anti-σ factor 19 implemented in the same operon than ECF-σ factor 19. Thick arrows represent open reading frames. Half circles represent ribosome binding sites. Thin arrows represent promoters. Plasmids are represented as rounded rectangles. (B) Growth curves assays. (C) Luminescence output represented through relative luminescence units (RLU) normalized by the

optical density measured at 600 nm (OD600nm). (D) Fold decrease of ECF19 activity when in presence of anti-σ 19. Vertical black line crossing the graphs in panels A and B indicate the time of addition of the inducer. Grey vertical error bars represent standard deviations calculated from three independent experiments.

3.3.2 Variations of the inducible promoters

The ECF19 switch was built under control of the P_{xylA} promoter plus an additional copy of *XylR* repressor (**Figure 19A, left**). P_{xylA} is induced by xylose in a concentration-dependent manner and it is controlled by the *XylR* repressor in *B. subtilis* (Gärtner, Geissendörfer and Hillen, 1988; Jeong *et al.*, 2015). Thus, in order to access variations in the switch behaviour, the *xylR* repressor coding gene was removed (**Figure 19A, middle**), which caused no growth defect (**Figure 19B**) and consequently improved the performance of the ECF19 switch (**Figure 19C, middle**). The fact that the presence of the additional copy of *xylR* causes a reduction in the overall activity of the switch was unexpected to us. However, a thorough inspection of the available literature revealed that XylR by itself mediated xylose-independent glucose-depended repression of P_{xylA} (Kraus *et al.*, 1994; Radeck *et al.*, 2013). Our hypothesis is that here, the additional copy of *xylR* intensified the glucose-dependent repression of XylR over P_{xylA}, decreased the dynamic range and maximal output of the ECF19 switch as already observed for switches based in ECF σ factors from other groups other than ECF19 (Pinto *et al.*, 2019).

Next, the evaluation of the behaviour of ECF19-based switch under control of the P_{liaI} promoter was performed (**Figure 19A, right**). Regarding the viability of the cells, there was no growth defect when ECF19 was under control of P_{liaI} (**Figure 19B, right**).

Here, P_{liaI} drove the expression of ECF19 in a concentration-dependent manner, showing a lower basal level and a sigmoid dose-response curve (**Figure 19D, right**), characteristic of P_{liaI} itself (Radeck et al., 2013; Pinto *et al.*, 2019). Also, worth mentioning is that the P_{liaI} driven switch showed a transitory activation that contrasts to the stable activation seen in the P_{xylA} driven switch (Figure 19). This characteristic behaviour of ECF-based switches under control of P_{xylA} and P_{liaI} has been also described for other ECF-based switches (Pinto *et al.*, unpublished).

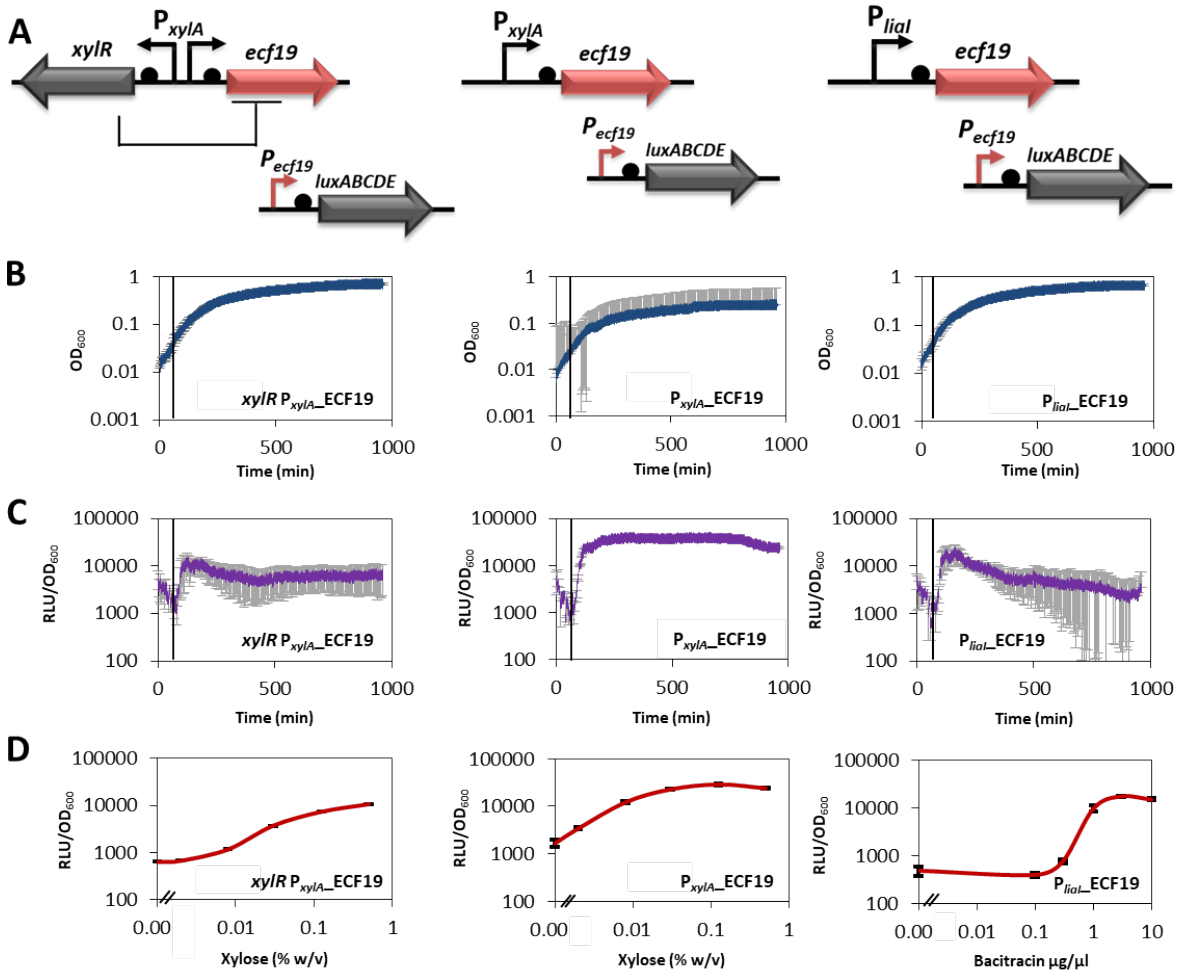


Figure 19. ECF19-based switch control by different inducible promoters. (A) Genetic design of the generated ECF-based switches. Thick arrows represent open reading frames. Circles represent ribosome binding sites. Thin arrows represent promoters. An arrow with a 'T' end represents repression. (B) Growth curves of the strains carrying the ECF19-based switch under control of different inducible promoters. (C) Output curves (luminescence over time) generated after induction with 0.5% xylose for strains carrying P_{xylA} promoter and 10 µg/ml bacitracin for strains carrying P_{liaI} promoter. (D) Dose-response curves using the luminescence output value, represented through relative luminescence units (RLU) normalized by the optical density measured at 600 nm (OD_{600}), obtained 90 min after the addition of the inducer to the exponentially growing culture. Each graph represents the measurements performed with the *B. subtilis* strain harbouring the switches presented in A. Final concentrations of xylose used for induction of P_{xylA} were 0, 0.002, 0.008, 0.03, 0.125 or 0.5 % (w/v) while final concentration of bacitracin used for induction of P_{liaI} were 0, 0.1, 0.3, 1, 3 or 10 µg/ml. Vertical black line crossing the graphs indicates the time of addition of the inducer. Grey vertical error bars represent standard deviations calculated from three independent experiments.

3.3.3 Variations of copy number

Plasmid copy number strongly affects the behaviour of genetic circuits (Loinger and Biham, 2009). The robustness of the ECF19-based switch was tested by placing its transcriptional units in four distinct genetic designs (**Figure 20A**) in order to independently or simultaneously increase the number of copies of the *ecf19* transcription unit or the reporter transcription unit.

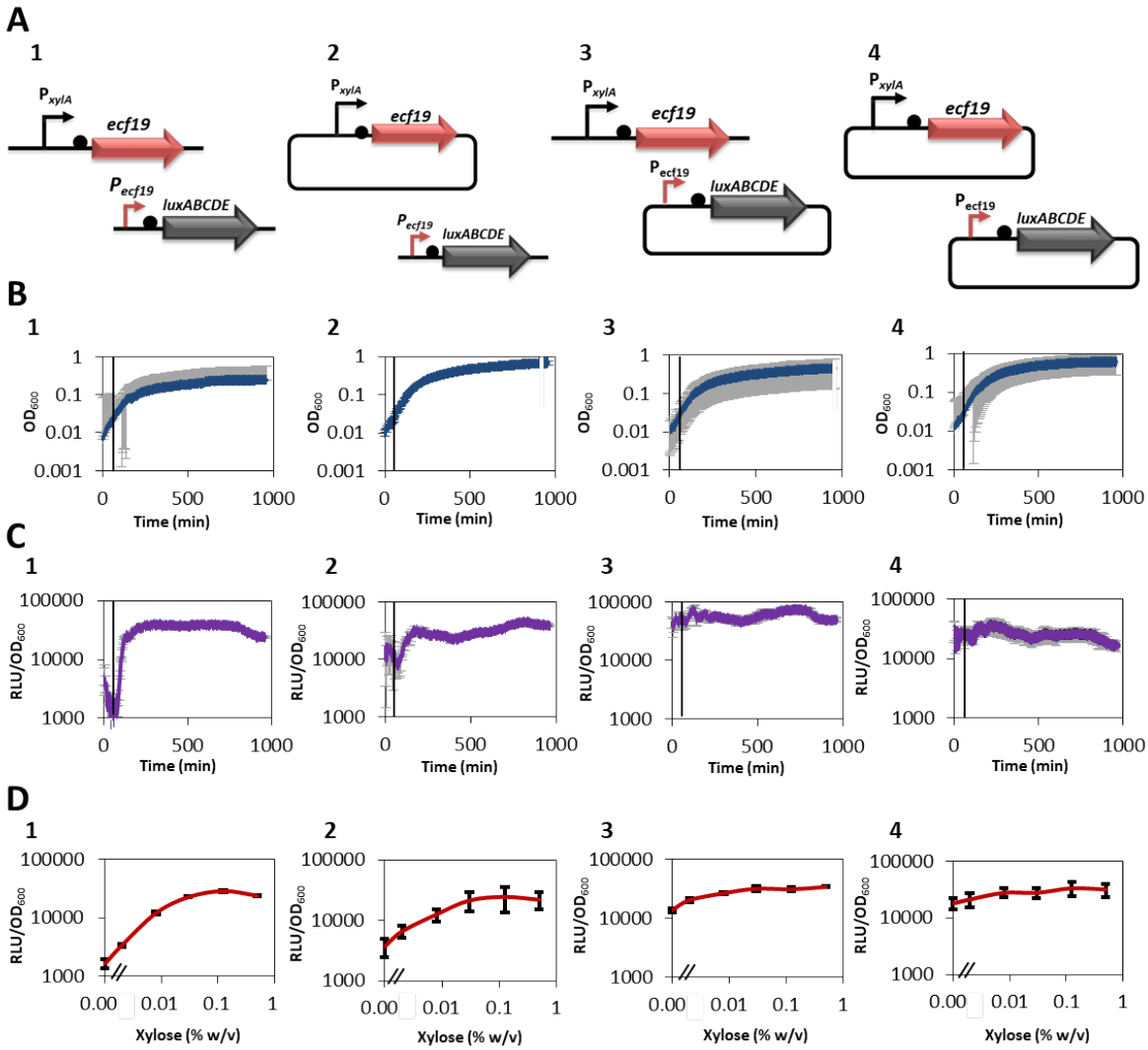


Figure 20. Changes in the copy number of the transcriptional units that constitute the ECF19 switch. (A) Genetic design of the ECF19-based switch by changing the copy number of each transcriptional module. Thick arrows represent open reading frames. Half circles represent ribosome binding sites. Thin arrows represent promoters. Plasmids are represented as rounded rectangles. (B) Growth curves of the strains carrying each of the four different genetic designs depicted in panel A after induction with 0.5% xylose. (C) Output curves (luminescence over time) generated after induction with 0.5% xylose. (D) Dose-response curves using the luminescence output value which is represented through relative luminescence units (RLU) normalised by the optical density measured at 600 nm (OD600nm), obtained 90 min after addition of inducer to the exponentially growing culture. Each graph (1-4) represents the measurements performed with the *B. subtilis* strain harbouring the switch presented in A1-4. Final concentrations of xylose used for dose-response assays were 0, 0.002, 0.008, 0.03, 0.125 or 0.5 % (w/v). Vertical back line crossing the graphs indicates the time of addition of the inducer. Grey vertical error bars represent standard deviations calculated from three independent experiments.

None of the variations in copy numbers in any of the transcriptional units caused growth defects in the strains carrying these distinct ECF19-based switches (**Figure 20B**). Firstly, our ECF19-based switch was implemented in the *B. subtilis* chromosome, presenting only one copy of each transcriptional unit: P_{xylA}-*ecf19* and the reporter gene P_{ecf19}-*luxABCDE* (**Figure 20A.1**). Further, the unit P_{xylA}-*ecf19* was placed in a replicative plasmid in order to increase its copy number in

relation to the reporter cassette P_{ecf19}-*luxABCDE*. Multiple copies of *ecf19* caused an increase in the basal levels of the switch together with a decrease in the maximum output, which results in a decrease in the overall performance of the switch (**Figure 20C.2** and **Figure 20D.2**). In contrast, the ECF19-based switch designed to have multiple copies of the reporter transcriptional unit presented higher basal level that rendered the P_{ecf19} constitutively active (**Figure 20C.3 and 4** and **Figure 20D.3 and 4**). Our data shows that the ECF19-based switch is stable to changes in the *ecf19* transcriptional units but that it is in contrast sensitive to changes in the unit carrying the reporter.

3.3.4 Variations of ECF19 protein stability

In prokaryotes, the SsrA tmRNA marks aberrant proteins for degradation by the addition of a peptide (SsrA) tag. These proteins are then degraded in the cytoplasm by the ClpXP and ClpXA proteases. These peptide tags are conserved in Gram-positive bacteria and it has been already shown that the nature of the last three terminal residues controls the level of protein stability (Wiegert and Schumann, 2001; Ahlawat and Morrison, 2009; Tao and Biswas, 2015). This feature has been previously used to manipulate the protein levels in synthetic circuit in *E. coli* (Stricker *et al.*, 2008) and to study and induce SsrA-mediated mechanism in *B. subtilis* (Wiegert and Schumann, 2001; Griffith and Grossman, 2008). In order to access the stability of the ECF19-based switch, eleven variations of SsrA tag were added to the 3' end of *ecf19* (**Figure 21A**). All the ECF19_SsrA variants were built using the least frequently used codon as observed for the native SsrA tmRNA in *B. subtilis* (Pinto *et al.*, 2019).

Contrary to other studies where the increase in proteases recruitment caused by the overexpression of the tagged proteins interfered with growth rate (Andersen *et al.*, 1998), none of the strains carrying the SsrA tagged ECF19 showed any growth defect (**Figure 21B**). The ECF19-based switches tagged with the native *B. subtilis* SsrA_LAA as well as the variant SsrA_LVA were inactive. The SsrA_LDD tag was the only switch showing the same output curves and dose-response behaviour than the switch without any tag.

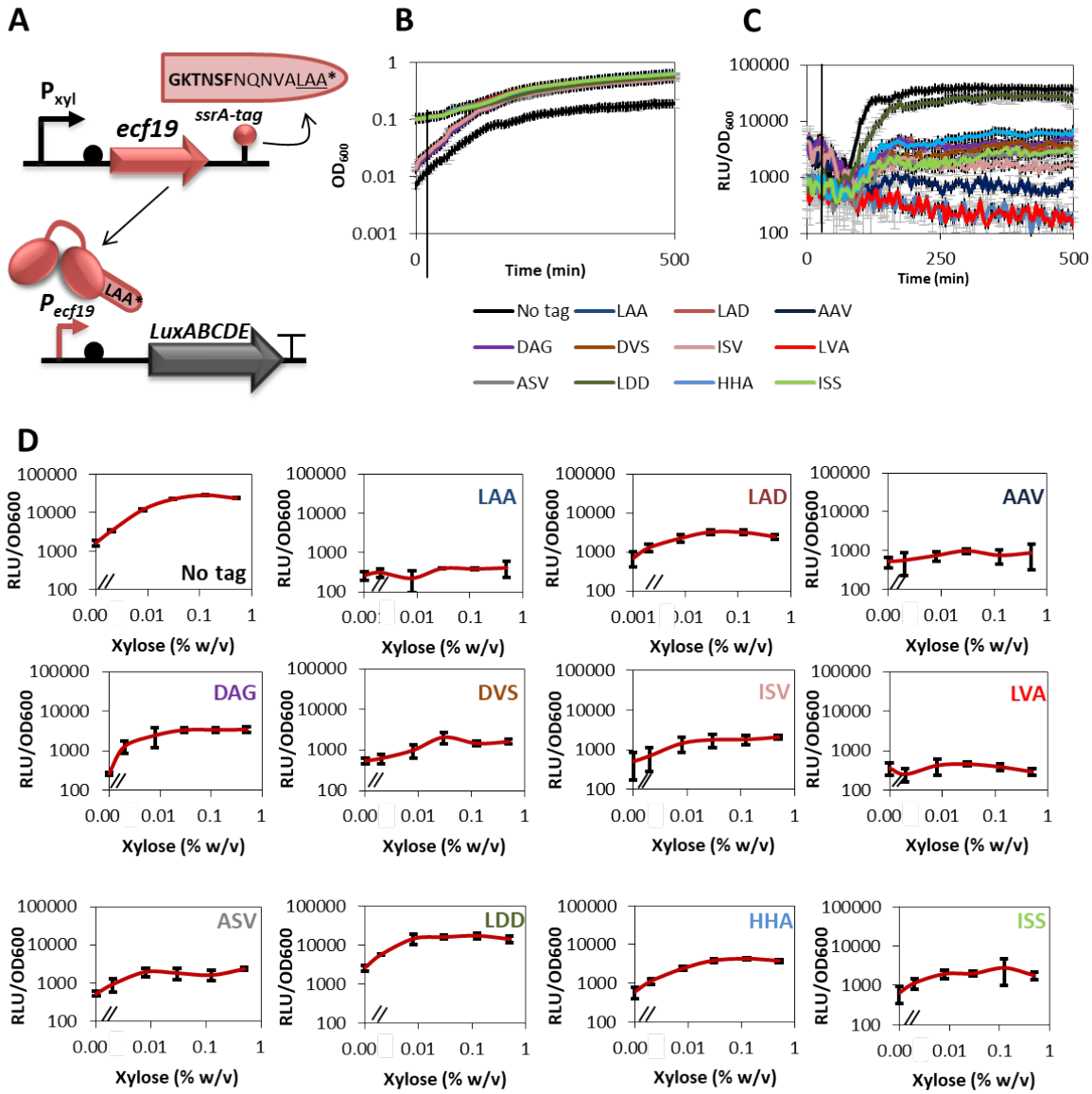


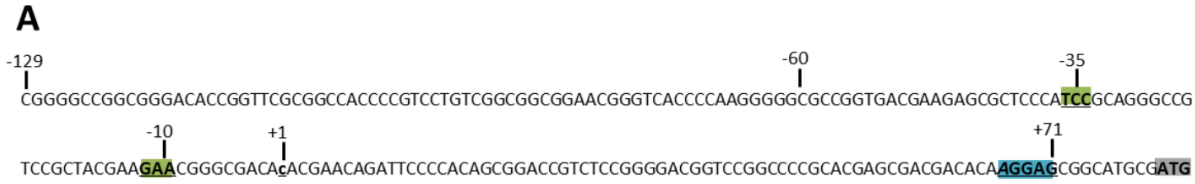
Figure 21. Stability of the ECF19 with fused SsrA tags. (A) Genetic design of the variants of ECF19-based switch. Thick arrows represent open reading frames. Half circles represent ribosome binding sites. Thin arrows represent promoters. The 'lollypop' shape indicates the relative positions of the SsrA tag. On the right side the native sequence of the SsrA tag is shown in a pink box. The variable section of the tag is underlined. (B) Growth curves of the strains carrying the ECF19 tagged with different SsrA and induced with 0.5% xylose. (C) Output curves (luminescence over time) generated by induction with 0.5% xylose. (D) Dose-response curves using the luminescence output value which is represented through relative luminescence units (RLU) normalised by the optical density measured at 600 nm (OD_{600nm}), obtained 90 min after addition of inducer to the exponentially growing culture. The top of each graph has a representation of the ECF19 with the SsrA variant implemented. Vertical black lines crossing the graphs indicate the time of addition of the inducer. Grey vertical error bars represent standard deviations calculated from three independent experiments.

In general, the remaining tagged switches maintained the switch activation, however, they showed significant decrease in the maximum output curves to over 10-fold. Additionally, the time dynamics of most of the switches showed a delay in activation when compared to the non-tagged ECF19 (**Figure 21C** and **Figure 21D**).

3.3.5 Variations of the *ecf19* promoter size

The σ^{70} family of σ factors recognize distinct promoters based on consensus sequences at the -35 and -10 regions upstream the transcription start site (Rosenberg and Court, 1979; Harley and Reynolds, 1987). Additionally, an A+T rich element upstream the -35 region increases the transcription by interaction with α subunits of the RNA polymerase and it has been shown that modifications of this UP element can result in altered promoter activity (Estrem *et al.*, 1998; Holátko *et al.*, 2012; Rhodius *et al.*, 2013).

Details of the *ecf19* promoter region are depicted in **Figure 22A**. Primarily, P_{ecf19} was implemented from -60 to +1. Subsequently, two different versions of P_{ecf19} were created: a shorter version from -35 to +1 and a longer version from -129 to +70, which extends until the RBS sequence (**Figure 22B**). None of these modifications in the P_{ecf19} interfered with the *B. subtilis* growth (**Figure 22C**). Nevertheless, these changes led to altered behaviour of the ECF19-based switch, where the promoter lacking the 29 nucleotides upstream the -35 region (UP element) showed a decrease in the output curve during the luminescence over time measurements. Additionally, the dose-response assays showed a differential behaviour related to the concentration range of inducer (**Figure 22D**) where the minimum size promoter (-35 to +1) showed the same dynamics but lower output while the longer promoter covering from -129 position to +71 was not active (**Figure 22E**). We hypothesize that the reason why the longer promoter, containing all the necessary promoter elements, is not active is due to its high GC content that can present as a challenge to the *B. subtilis* RNA polymerase and lead to ineffective transcription initiation.



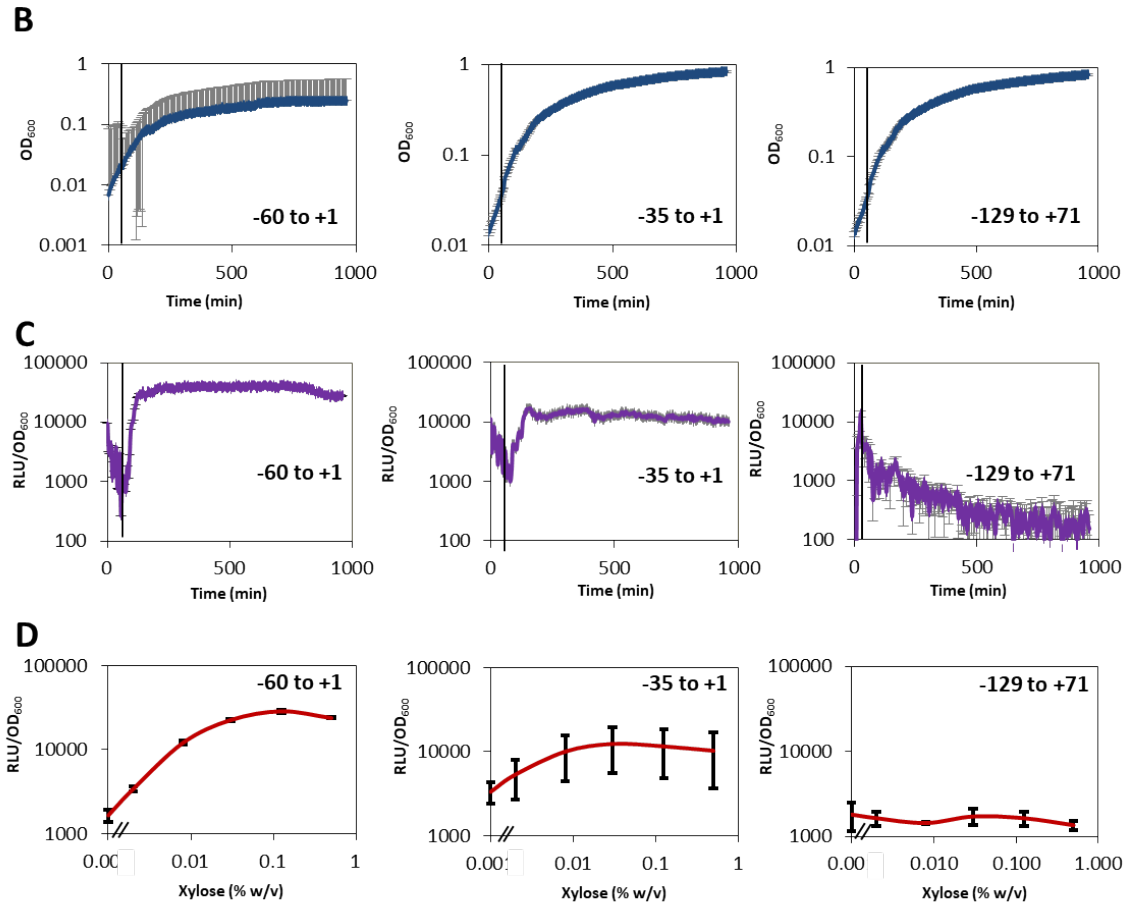


Figure 22. ECF19-based switch upon variation of the promoter size. (A) ECF19 promoter sequence with the relevant positions marked above the sequence. Start codon is shown in a grey box. Promoter -35 and -10 elements are shown in green boxes while +1 is underlined and showed by the use of lowercase character. The ribosome binding (RBS) site sequence is shown in a blue box. (B) Growth curves of the strains carrying the ECF19-based switches with different promoter sizes induced by 0.5% xylose measured in an optical density of 600 nm (C) Output curves (luminescence over time) generated after induction with 0.5% xylose. (D) Dose-response curves drawn using the luminescence output value, represented through relative luminescence units (RLU) normalized by the optical density measured at 600 nm (OD_{600}), achieved 90 min after the addition of the inducer to the exponentially growing culture. Start and end positions of the promoter variants are labelled inside each graph. Final concentrations of xylose used for induction of P_{xylA} were 0, 0.002, 0.008, 0.03, 0.125 or 0.5 % (w/v). Vertical black line crossing the graphs indicates the time of addition of the inducer. Grey vertical error bars represent standard deviations calculated from three independent experiments.

3.3.6 Regulation of ECF19 switch by anti-sense transcription

In order to check the effect of antisense transcription, five promoters with different strengths were added in the antisense orientation of the *ecf19* gene (**Figure 23A**): **(i)** the weakest promoter P_{J23101}, an *E. coli* σ^A-dependent promoter generated as part of a combinatorial library of constitutive promoters for the iGEM competition (Anderson promoter collection, 2006); followed by **(ii)** P_{liaG} and **(iii)** P_{lepA}, which are *B. subtilis* σ^A-dependent promoters involved in the envelope stress response system and, translation elongation and heme biosynthesis, respectively (Jordan *et al.*, 2006; Michna *et al.*, 2016); **(iv)** P_{sigW}, a *B. subtilis* σ^W-dependent

promoter, which is a target promoter of the ECF-σ factor σ^W (Helmann and Moran, 2002); and the strongest promoter **(v)** P_{veg}, another σ^A-dependent promoter which regulates genes involved in biofilm formation in *B. subtilis* (Michna *et al.*, 2016).

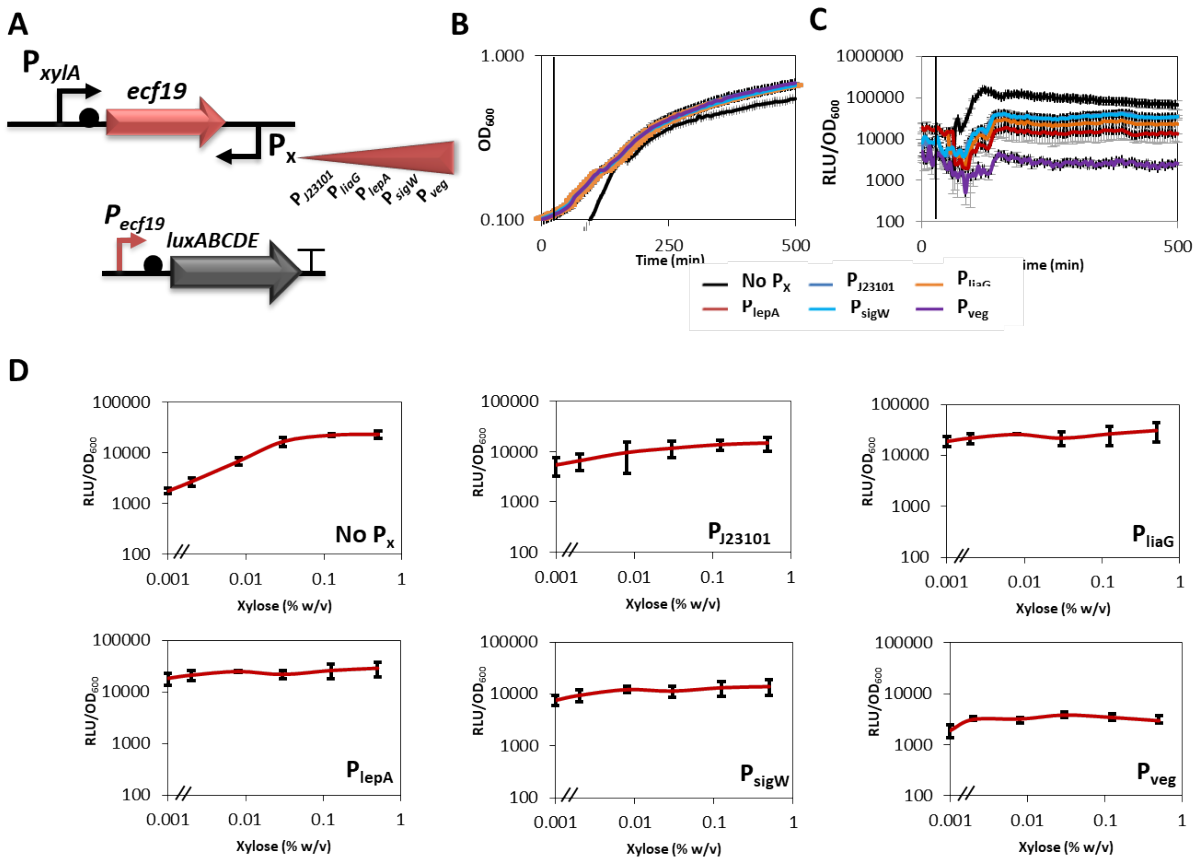


Figure 23. Influence of antisense transcription in the switch behaviour. (A) Genetic design of the ECF19-based switches. Thick arrows represent open reading frames. 'T' represents terminators. Half circles represent ribosome binding sites. Thin arrows represent promoters. The relative strength of the antisense promoter (P_x) is shown in the right side of P_x. (B) Growth curves (optical density of 600 nm) of the strains carrying the ECF19-based switches with different promoter sizes, after induction with 0.5% xylose. (C) Output curves (luminescence over time) generated after induction with 0.5% xylose. (D) Dose-response curves obtained by using the luminescence output value, represented through relative luminescence units (RLU) normalized by the optical density measured at 600 nm (OD_{600}) obtained 90 min after induction. Each graph represents the measurements performed with each *B. subtilis* strain harbouring the ECF19-based switch variants shown in A and each graph is labelled with the antisense promoter used. Final concentrations of xylose used for induction of P_{xylA} were 0, 0.002, 0.008, 0.03, 0.125 or 0.5 % (w/v). Vertical black line crossing the graphs indicates the time of addition of the inducer. Grey vertical error bars represent standard deviations calculated from three independent experiments.

None of the switches carrying the antisense promoters containing switches caused any defect in growth (**Figure 23B**). Nevertheless, P_{veg} caused a drastic decrease in the output curve over time (~10-fold) and a 45 minutes delay for activation. Nevertheless, the other promoters showed a slightly decrease in the maximal achieved output (**Figure 23C**). The dose-response curves showed that the antisense transcription caused changes in the threshold of activation which

decreased drastically in all the switches carrying antisense promoters. Additionally, a reduced basal level of expression of the switches carrying the P_{J23101}, P_{sigW} and P_{veg} was observed (**Figure 23D**).

3.3.7 Implementation of Actinobacteria transcription factors

The high number of inactive switches prompted us to investigate reasons for this, given that this observation was not in agreement with what has been previously observed in *E. coli* (Rhodius et al., 2013). Hence, we turned our attention to additional transcription factors that might be necessary for the activity of *S. venezuelae* ECF σ factors. *Actinobacteria* require two additional transcription factors for initiation - CarD and Rpb. CarD activates transcription in a σ-independent way, where the RNA interaction domain (RID) interacts with the β1-lobe in the β subunit of RNA polymerase and the C-terminal domain interacts with the promoter DNA, being essential to stabilize the open complex and prevent transcription bubble collapse (Bae *et al.*, 2015; Lee and Borukhov, 2016). RpbA is known to interact with the σ_2 domain and a non-coding region of group 1 and certain group 2 σ factors. It contacts DNA (phosphate backbone) upstream of -10 element (-13 / -14 positions) and could be working on the stabilization of the open complex (Tabib-Salazar *et al.*, 2013; Hubin *et al.*, 2015).

B. subtilis has a CarD homolog (YdeB) with 33% identity but no RpbA homolog. We then set out to investigate if the lack of any of these additional transcription factors was the reason behind the inactivity of the *S. venezuelae* ECF σ factors implemented in *B. subtilis*. The *B. subtilis ydeB* gene was replaced by a kanamycin resistance cassette through the long flank homology PCR method. Genes coding for CarD or RpbA were integrated into the *thrC* locus in one of the previously inactive switches (Sven_4870) (**Figure 24A).** The presence of CarD caused no growth defect in *B. subtilis* (**Figure 24B, left**) while RbpA caused a decreased exponential growth rate, even when in non-inducing conditions (**Figure 24B, right**).

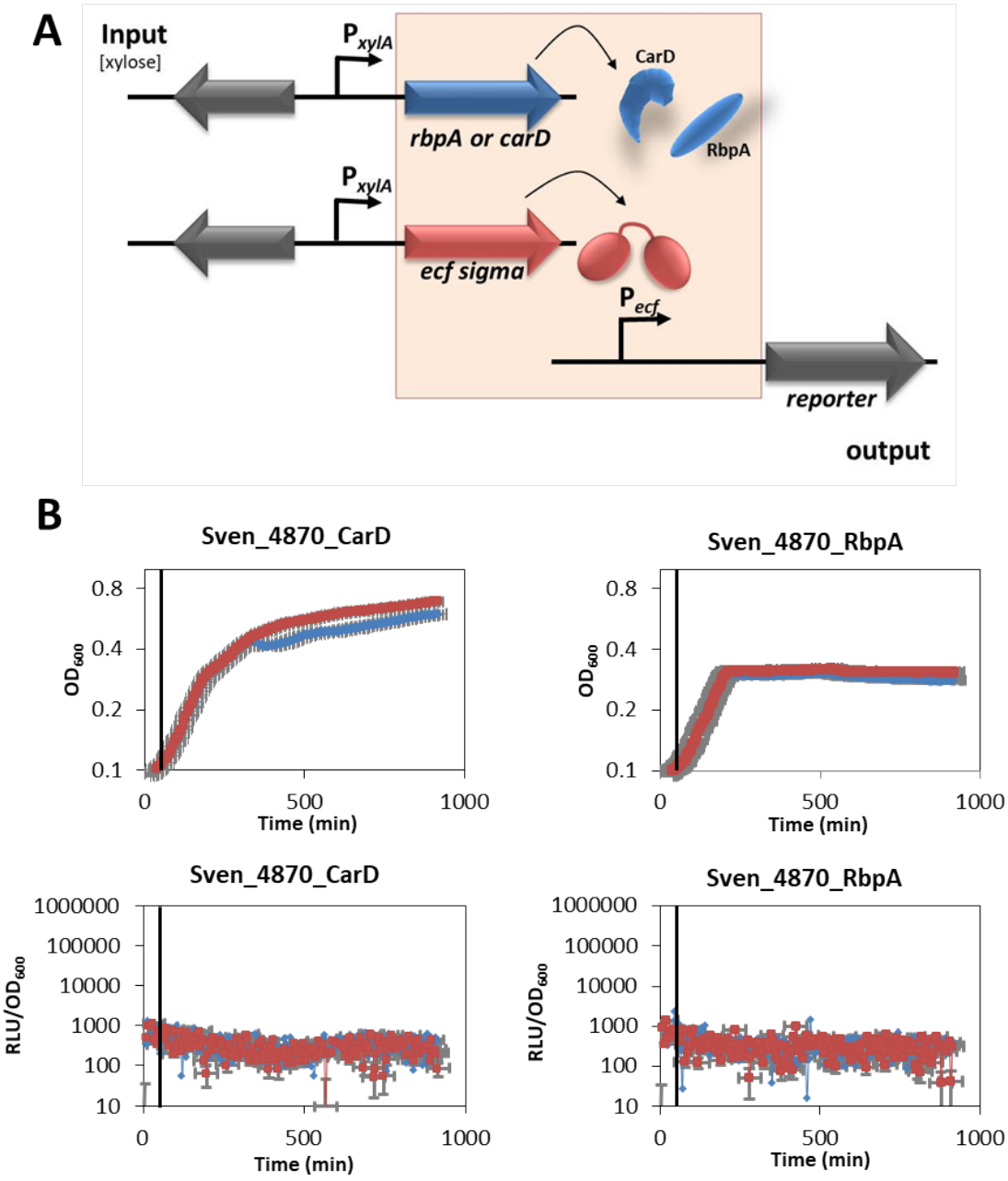


Figure 24. Activity of the ECF sigma factors Sven_4870 in the presence of transcription factors CarD or RbpA. (A) ECF-σ factor and CarD or RbpA expression was induced by xylose through the promoter P_{xylA}. The activity of P_{Sven_4870} was monitored through luminescence measurements. Thick arrows represent open reading frames. Half circles represent ribosome binding sites. Thin arrows represent promoters (B) Growth curve (left) and luminescence (right) of the strains containing Sven_4870 plus CarD or RbpA. Blue lines show the cultures without induction and red lines show cultures under induction (0.5% xylose).

3.4 High level bacitracin resistance in *S. venezuelae*

3.4.1 *Streptomyces venezuelae* is highly resistant against bacitracin

The co-existence of antibiotic-producing bacteria in the soil can lead to the development of antibiotic resistance mechanisms (Surette and Wright, 2017). A study testing a variety of soil bacteria against 24 drugs showed that 80% of these bacteria were multidrug resistant (Walsh and Duffy, 2013). Additionally, resistance mechanisms found in soil bacteria has been also found in clinical isolates (Peterson and Kaur, 2018). The facts mentioned above enforces the need for discovery of the mechanisms of resistance against antibiotic in bacteria such *Streptomyces*.

To gain an overview of the sensitivity spectra of *Streptomyces* sp. against cell wall active antibiotics, a previous study compared the inhibition of eight species from our laboratory collection by nine cell wall active antibiotics from different chemical classes. *S. venezuelae* displayed a striking resistance to bacitracin in this disk diffusion assay. Comparison of the inhibition zones of *S. coelicolor* and *S. venezuelae* revealed large differences in sensitivity, with about 50-fold more bacitracin required to inhibit *S. venezuelae* to a similar degree as *S. coelicolor*.

Determination of the minimal inhibitory concentration (MIC) of an antibiotic using the classical broth dilution technique is not feasible with filamentous bacteria such as streptomycetes. We therefore adopted the method developed by Sarker and colleagues (2007) that uses resazurin as an indicator of growth, which produced robust results for both species of *Streptomyces*. These quantitative assays confirmed the striking difference in bacitracin MIC between *S. coelicolor* (5 µg/ml) and *S. venezuelae* (128 µg/ml) (**Table 8**). Importantly, this high-level resistance of *S. venezuelae* was specific to the peptide bacitracin, as it displayed similar sensitivity against the glycosylated peptides vancomycin and ramoplanin, and an increased sensitivity against β-lactams compared to *S. coelicolor* (**Figure 25**).

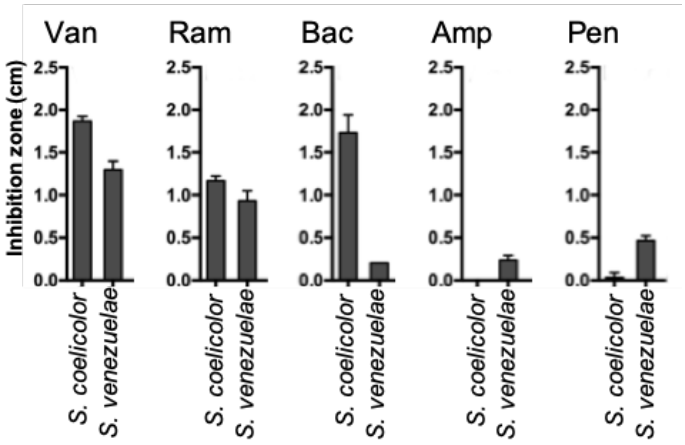


Figure 25. Sensitivity of *S. coelicolor and S. venezueale* to cell wall active antibiotics. Disc diffusion assays on lawns of both *Streptomyces* species. Antibiotics were added on filter discs, pre-soaked with 40 µg vancomycin (Van), ramoplanin (Ram), ampicillin (Amp) or penicillin G (Pen), or with 100 µg bacitracin (Bac). Growth inhibition was scored after 24 h incubation. No inhibition was observed for daptomycin, D-cycloserine, fosfomycin or nisin, thus these data were not included in the figure.

3.4.2 Random chemical mutagenesis generated bacitracin-sensitive derivatives of *S. venezuelae*

The striking observation of the high-level resistance of *S. venezuelae* against bacitracin, which was not seen in most of the other streptomycetes previous analyzed, stimulated our interest in elucidating the molecular mechanisms for this striking AMP resistance.

To identify the genes required for bacitracin resistance, spore suspensions of *S. venezuelae* were randomly mutagenized with the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The spores were then germinated and plated under sub-inhibitory concentration of bacitracin (100 µg/ml). Approximately 20.000 clones were screened for the inability to grown in presence of bacitracin. All candidate clones identified as sensitive in this initial screen were characterized with respect to growth rate, and only clones that displayed similar growth to the wild type were chosen for further analysis to ensure sensitivity was not due to grossly altered growth behavior.

The candidate clones were then analysed by MIC assays using the resazurin indicator method described above, resulting in a final set of six bacitracin sensitive mutants of *S. venezuelae*, designated Mut1 through Mut6 (**Table 9**).

Table 9. Bacitracin sensitivity of *S. venezuelae*, its derived mutants and S. coelicolor.

Strain	Zone of inhibition[a]	MIC[b] (µg/ml)
S. venezuelae wild-type	2.7 ± 0.6 mm	128
Mut1	3.3 ± 0.6 mm^{ns}	50
Mut2	3.2 ± 0.3 mm^{ns}	64
Mut3	2.7 ± 0.6 mm^{ns}	64
Mut4	9.8 ± 1.4 mm **	10
Mut5	2.7 ± 0.6 mm^{ns}	64
Mut6	3.2 ± 0.3 mm^{ns}	50-64
S. coelicolor	15.3 ± 0.6 mm	5

[a] Data were derived from disc diffusion assays and are shown as mean ± standard deviation of three independent experiments; ** $p < 0.005$; ns, not significant from an unpaired t-test between each mutant and wild-type *S. venezuelae*; *S. coelicolor* is shown for comparison and no t-test was performed. [b]Minimal inhibitory concentration determined from broth-dilution assays using resazurin as growth indicator; assays were performed in three independent replicates; where results differed between replicates, a range of concentrations is given.

Five of these mutants displayed only a slight decrease in resistance of approximately two-fold, whereas the MIC of Mut4 was strongly reduced from 128 µg/ml to 10 µg/ml. It should be noted that the two-fold change in bacitracin resistance of mutants 1, 2, 3, 5 and 6 also did not result in significant changes in inhibition zones (**Table 9**).

3.4.3 *Streptomyces venezuelae* sensitivity spectra against cell wall active antibiotics

To gain a better understanding of antimicrobial peptide resistance in *S. venezuelae* and its mutants, they were tested for sensitivity against other cell wall active antibiotics. The bacitracin-sensitive strains showed no increased sensitivity to the other cell wall antibiotics tested here. For Mut4, disc diffusion assays reflected the significant decrease in bacitracin resistance, but no changes were observed for the remaining antibiotics, suggesting that this mutant had specifically lost its resistance against bacitracin and not acquired a defect causing overall antibiotic sensitivity (**Figure 26**).

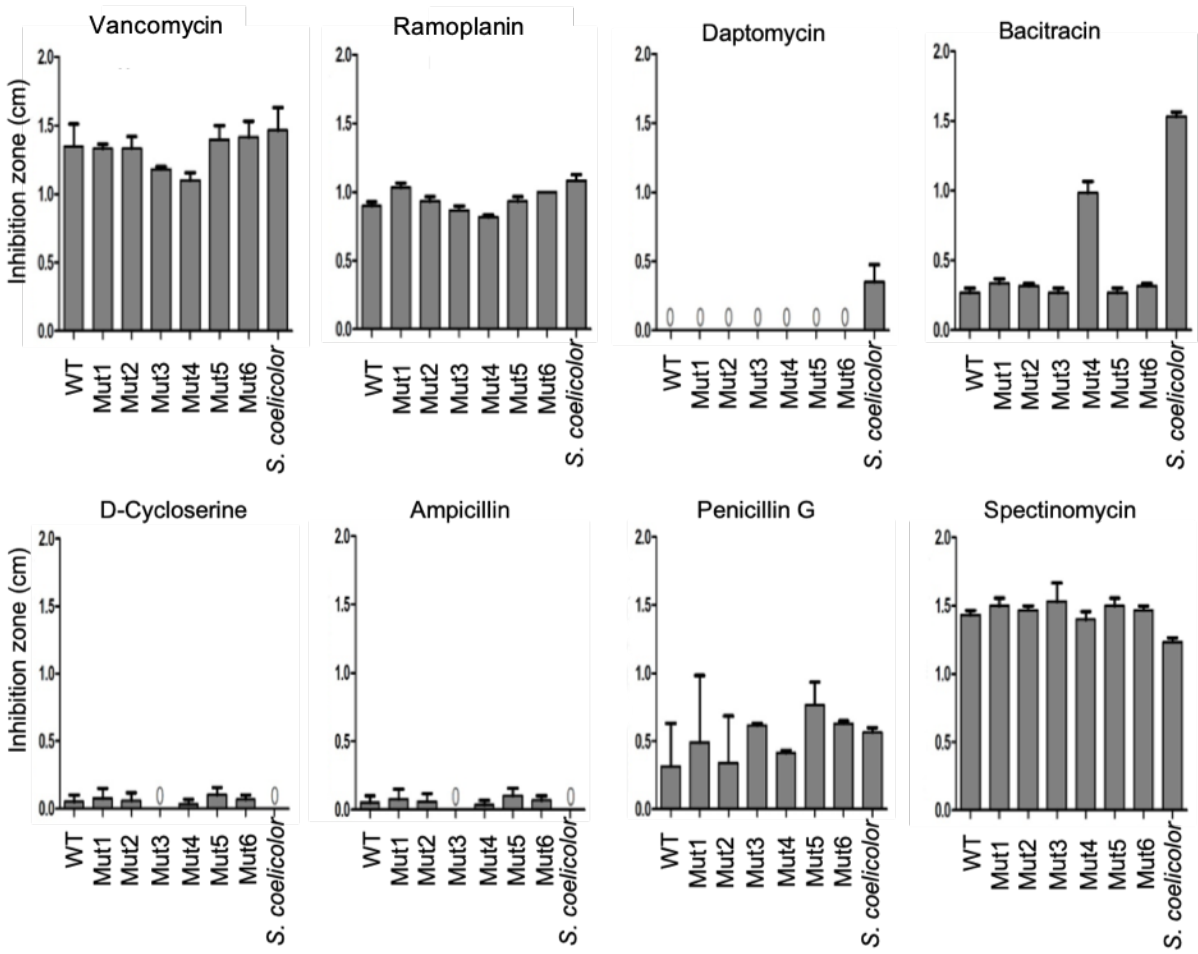


Figure 26. Sensitivity of *S. venezuelae* to cell wall active antibiotics. Disc diffusion assays on lawns of different *Streptomyces* strains. Antibiotics were added on filter discs pre-soaked with 40 µg vancomycin, ramoplanin, lysozyme, daptomycin, D-cycloserine, ampicillin, penicillin G, fosfomycin or spectinomycin, or with 100 µg bacitracin. Growth inhibition was scored after 24 h incubation. *S. coelicolor* is used for comparison. Spectinomycin is a protein synthesis inhibitor and it was used as a control. Results are shown as inhibition zone diameters and presented as the mean ± standard deviation of at least three independent experiments. 0, no growth inhibition was observed. No inhibition was observed for lysozyme and fosfomycin, thus these data were not included in the figure.

3.4.4 *S. venezuelae* resistance against growth-inhibition by antimicrobial peptide producers

Many AMPs, including bacitracin, are produced by bacteria belonging to the phylum Firmicutes, which frequently share the soil habitat with actinobacteria. To test if these producers could inhibit the growth of streptomycetes, a spot-on-lawn assays was performed, were suspensions of ten different *Bacillus* sp. strains were spotted onto lawns of either *S. coelicolor*or or *S. venezuelae* (**Figure 27A**). *B. subtilis* wild-type W168 did not inhibit any of the streptomycetes strains while the undomesticated wild type *B. subtilis* NCIB3610 strain and the subtilin-producer ATCC6633 (Klein, Kaletta and Entian, 1993) were able to clearly inhibit the growth of *S. coelicolor*, but caused no or only minor inhibition of *S. venezuelae* and its mutants. The bacitracin-producer *B. licheniformis* ATCC10716 again inhibited *S. coelicolor*, but not *S.*

venezuelae wild-type, further confirming the bacitracin-resistance of the latter but unexpectedly, it did not inhibit the *S. venezuelae* bacitracin-sensitive mutants. The second *B. licheniformis* strain, DSM13, only produced minor inhibition zones on either streptomycete. The remaining strains had previously been isolated as putative producers of peptide antibiotics (Nithya and Halami, 2012). Of these, EC1 and B9 caused marked growth inhibition of *S. coelicolor*, but only slightly inhibited *S. venezuelae* and its mutants. However, some of the bacitracin-sensitive mutants presented an increased growth inhibition when in contact with the strains NCIB3610 and B9 (**Figure 27B**).

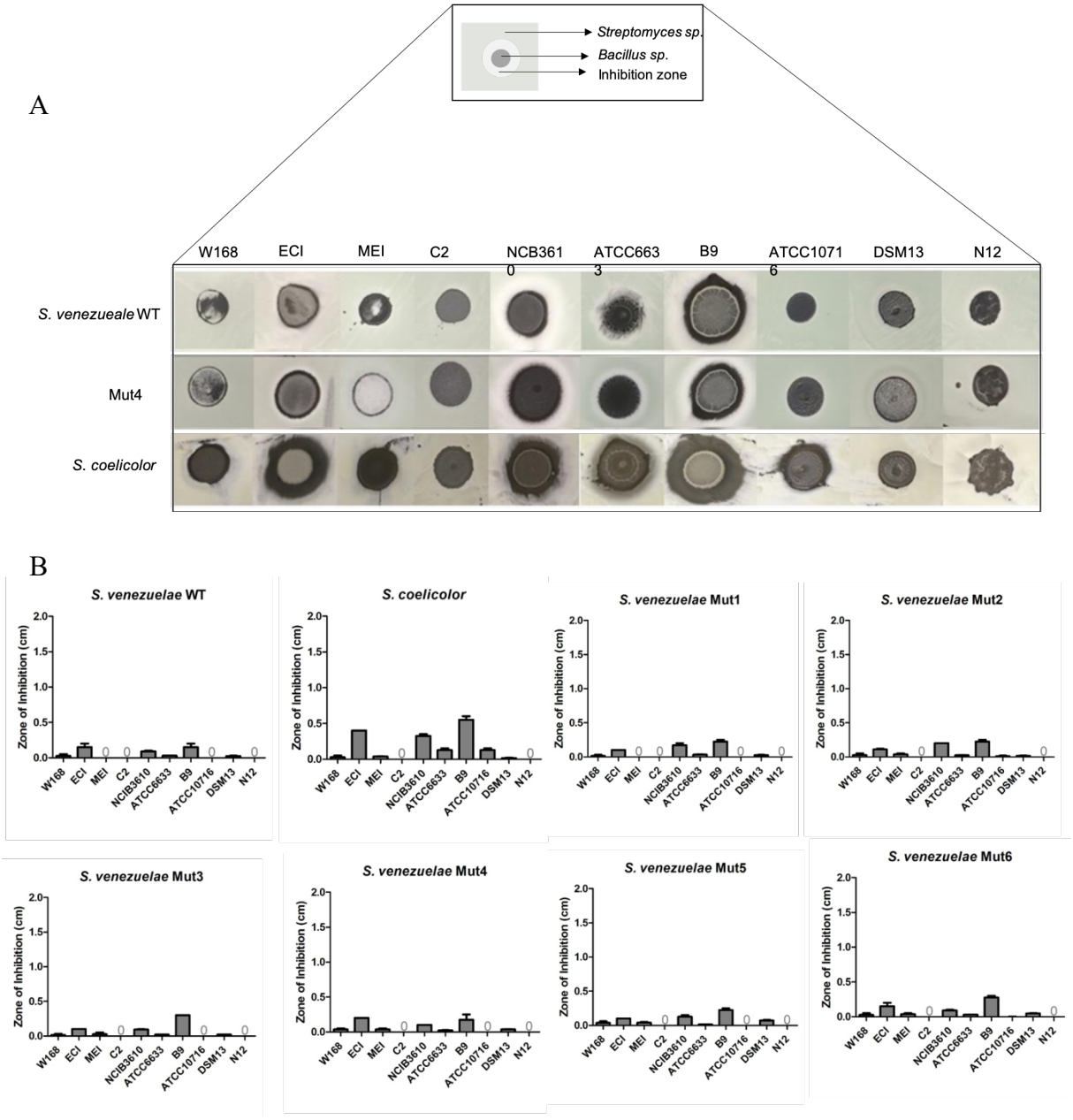


Figure 27. Inhibition of *S. coelicolor* and *S. venezuelae* growth by *Bacillus* sp. Experiments were performed as spot-on-lawn assays where 5 µl overnight culture of a *Bacillus* sp. was spotted onto a lawn of the respective streptomycete. (A) Representative results for *S. venezuelae*, *S. venezuelae* bacitracin-sensitive mutant 4 and *S. coelicolor* strains are shown. (B) Results shown as inhibition zone diameters. The *Streptomyces* isolates are named in the top of each graph. Growth inhibition was scored as the diameter of the clear zone, corrected for

the diameter of the *Bacillus* colony after 24 h incubation. Results are shown as inhibition zone diameters and presented as the mean ± standard deviation of three independent experiments. 0, no growth inhibition was observed.

3.4.5 Identification of mutations associated with bacitracin resistance in *S. venezuealae*

Since the acquired bacitracin sensitivity of the *S. venezuelae* mutants remained stable and these strains showed to be insensitive to other cell wall antibiotics, we expected to identify the genomic alterations responsible for bacitracin resistance in *S. venezuelae* by performing genome sequencing of these strains. Point mutations, insertions and deletions (InDels) of each strain were identified relative to the reference genome *S. venezuelae* ATCC 10712 deposited in the NCBI under reference sequence NC_018750.1. Our laboratory wild-type strain was also sequenced as a control. The genome sequencing results showed that only the bacitracin-sensitive mutant 4 presented non-synonymous mutations. Eleven point mutations were identified, of which eight were predicted as deleterious by using the online tool Protein Variation Effect Analyzer (PROVEAN) (http://provean.jcvi.org/seq_submit.php). In addition, three one base pair deletions were also identified (**Table 10**).

Table 10. Mutations in *S. venezuealae* mutant 4 identified by Illumina sequencing.

Type of mutation	Position (chromosome coordinates)	Mutation	Site/Amino acid change	ORF	Annotation/Function
	1860147	gtg>atg	V to M	*Sven_1662*	ATP-dependent RNA helicase
	2370594	gcc>gtc	A to V*	*Sven_2202*	Proposed lipid II flipase Mur J
	2573383	ggg>gcg	G to A	*Sven_2380*	Ribonuclease G
	4315815	ggc>gac	G to D*	*Sven_3974*	SN-glycerol-3-phosphate transport ATP-binding protein UgpC
SNPs	4826299	gcc>acc	A to T*	*Sven_4475*	Hypothetical protein
	5307481	gcc>gtc	A to V	*Sven_4932*	Hypothetical protein
	5319931	acc>gtc	T to I*	*Sven_4947*	Putative methyltransferase
	5502767	ggc>gac	G to D*	*Sven_5105*	Hypothetical protein
	6454375	cgg>gac	R to Q*	*Sven_5927*	DNA topoisomerase IB
	7250596	gcg>acg	A to T*	*Sven_6632*	Putative glycoside hydrolase
	7408742	gcg>acg	A to T*	*Sven_6766*	Serine phosphatase RsbU, regulation of sigma subunit
INDELS	655584	one bp deletion		*Sven_0570*	Putative ABC transporter (ATPase)
	3460069	one bp deletion		*Sven_3164*	Putative Oxidoreductase
	6868775	one bp deletion		*Sven_6295*	Branched-chain amino acid transport ATPase (Liv-transporter)

*Amino acid variants predicted as deleterious

A point mutation in a gene coding for a proposed lipid II flippase MurJ (*Sven_2202*) led to substitution of alanine to valine at position 528. MurJ flippase is involved in peptidoglycan

biosynthesis by transporting the lipid II-anchored peptidoglycan precursors from the cytoplasm to the extracellular space during the process of peptidoglycan biosynthesis (Sham *et al.*, 2014). Another amino acid substitution (Glycine to Aspartic acid) at the position 312 was identified in the SN-glycerol-3-phosphate importer UgpC subunit (*Sven_3974*), member of the CUT 1 subfamily of ABC importers involved in uptake of SN-glycerol-3-phosphate (G3P) and G3P diesters that are components of glycerophospholips, the main constituent of biological membranes. This importer showed to be active under phosphate starvation conditions in *E. coli* (Schneider, 2001; Wuttge *et al.*, 2012).

Another gene with a predicted deleterious point mutation encodes a hypothetical protein (*Sven_4475*) with a metallopeptidase domain that is similar to the L-Ala-D-Glu endopeptidase of *B. subtilis,* which has functions in cell wall organization, cell cycle and sporulation (Horsburgh, Atrih and Foster, 2003). The gene coding a hypothetical protein with a histidine kinase domain (*Sven_5105*) which forms an operon with a gene coding a DNA-binding response regulator from LuxR family also presented a point mutation predicted as deleterious. A topoisomerase IB (*Sven_5927*) coding gene presented a point mutation resulting in an arginine in the place of a glutamine at the amino acid position 128. Topoisomerases are responsible for relaxing positively and/or negatively supercoiled DNA, what is vital for replication, transcription and recombination processes (Forterre *et al.*, 2007).

Additionally, the gene *Sven_6632* coding for a putative glycoside hydrolase family 3 presented a point mutation that resulted in a substitution of an alanine for threonine at the position 576. Glycosyl hydrolases are a widespread group of enzymes involved in carbohydrate metabolic process and the family 3 of glycoside hydrolases comprises enzymes like beta-glucosidase, N-acetyl beta-glucosaminidase, beta-xylosidase, glucan beta-1,3-gluconase, and exo-1,3- 1,4 glucanase (Davies and Henrissat, 1995; Henrissat *et al.*, 1995). A gene coding for a positive regulator of σ^B (*Sven_6766*), a general stress response σ-factor in Gram positive bacteria (Delumeau *et al.*, 2004a), presented a point mutation that resulted in an substitution of glycine to aspartic acid at position 389. Also, one base pair deletion was identified in the genes coding for a putative ABC-transporter (*Sven_0570*), a putative oxidoreductase (*Sven_3164*) and a Branched-chain amino acid transport ATPase (Liv-transporter) (*Sven_6295*).

3.4.6 Differentially expressed genes in *S. venezuelae* in the presence of bacitracin

In order to obtain the transcriptome of *S. venezuelae* wild type in the presence of bacitracin, RNAseq analysis was performed. We found 151 genes as upregulated in presence of bacitracin. Genes in putative operons or clustered together are highlighted in shades of grey (**Table 11**).

Table 11. Upregulated genes in *S. venezuelae* treated with bacitracin (Fold change ≥5, P ≤0.005).

Gene	Fold change	Function/annotation
SVEN_0054	12.21	Hypothetical protein
SVEN_0055		Dimethylallyltransferase
SVEN_0057	12.77	Hypothetical protein
SVEN_0059		ABC-type multidrug transport system
SVEN_0109	5.59	Mannan endo-1,4-beta-mannosidase precursor
SVEN_0124	11.92	Choline or glycine betaine ABC transporter
SVEN_0230	18.06	RNA polymerase sigma factor SigB
SVEN_0232	13.36	SAM-dependent methyltransferases
SVEN_0333	43.92	Hypothetical protein
SVEN_0372	125.11	Putative two-component system sensory histidine kinase
SVEN_0373		Putative membrane protein
SVEN_0456	9.53	Vancomycin B-type resistance protein VanW
SVEN_0457		Beta-lactamase class C
SVEN_0460	98.35	Membrane protein, putative
SVEN_0890	13.62	Putative integral membrane protein
		Undecaprenyl-diphosphatase
SVEN_1073	20.23	Hypothetical protein
SVEN_1181	21.07	Transcriptional regulator, GntR family domain
SVEN_1216	5.11	Glycine betaine ABC transport system
SVEN_1300	39.01	Hypothetical protein
SVEN_1373	21.76	Putative DHA2-subfamily multidrug transporter
SVEN_1676	7.56	Glutamate synthase
SVEN_1688	7.67	Putative membrane protein
SVEN_1715	5.77	ABC-type multidrug transport system
SVEN_1719	7.08	Hypothetical protein
SVEN_1720	5.82	Putative membrane protein
SVEN_1818	5.29	Large membrane protein
SVEN_1833	66.55	Integral membrane protein
SVEN_1834	61.59	Putative integral membrane protein
SVEN_1836	27.45	Endo alpha-1,4 polygalactosaminidase
SVEN_1879	8.39	Putative reductase
SVEN_1937	22.21	Putative transmembrane efflux protein
SVEN_1938		Putative membrane protein
SVEN_1939		TetR family regulator (Multi drug resistance)
SVEN_2048	21.03	Hypothetical protein
SVEN_2050	6.04	Antibiotic biosynthesis monooxygenase
SVEN_2300	5.51	Undecaprenyl pyrophosphate synthetase
SVEN_2301		Repair protein RecO
SVEN_2351	7.15	Hypothetical protein DUF194, DegV family
SVEN_2382	22.92	Integral membrane protein
SVEN_2393	16.89	Rod shape-determining protein MreB
SVEN_2394	8.73	Two-component sensor kinase
SVEN_2418	150.2	Hypothetical protein
SVEN_2419		Hypothetical protein
SVEN_2436	6.7	4-alpha-glucanotransferase (amylomaltase)

SVEN_2447	15.82	Secreted protein
SVEN_2448	13.46	Amino acid ABC transporter solute-binding protein
SVEN_2449	16.96	Hypothetical protein
SVEN_2450	12.95	Serine or threonine protein kinase
SVEN_2451	6.83	Serine or threonine protein phosphatase
SVEN_2452	8.87	Conserved hypothetical protein SC6D10.11
SVEN_2459	38.24	Hypothetical protein
SVEN_2559	52.09	Hypothetical protein
SVEN_2560	13.79	Butyryl-CoA dehydrogenase
SVEN_2561	24.85	acyl-CoA thioesterase II
SVEN_2611	6.86	Chitin binding protein
SVEN_2651	6.79	L-Proline or Glycine betaine transporter ProP
SVEN_2732	6.13	Serine or threonine protein kinase
SVEN_2790	26.72	Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily A1
SVEN_2812	11.67	Hypothetical protein
SVEN_2815	6.78	Glycosyl transferase, family 2
SVEN_2817	10.24	Hypothetical protein
SVEN_3050	6.21	Glycosyl transferase, group 2 family protein
SVEN_3076	9.87	D-alanyl-D-alanine carboxypeptidase
SVEN_3135	7.39	Cytosine deaminase
SVEN_3248	8.47	Putative membrane protein (Abhydrolase super family)
SVEN_3279	17.71	Hypothetical protein
SVEN_3299	5.12	Hypothetical protein
SVEN_3398	13	Putative ABC transporter ATP-binding protein
SVEN_3399	15.03	Putative ABC transporter membrane protein
SVEN_3434	150.2	Undecaprenyl- phosphategalactosephosphotransferase
SVEN_3437	41.02	Putative integral membrane protein
SVEN_3483	10.57	Hypothetical protein - RNA polymerase sigma-70 factor
SVEN_3484	6.55	Erythropoiesis-stimulating protein (response regulator - LuxR family)
SVEN_3532	9.74	Lyase VOC domain
SVEN_3534	6.4	ABC-type multidrug transport system, ATPase component
SVEN_3630	5.62	Cell division protein FtsW (lipid II flipase)
SVEN_3631	5.16	Cell division protein FtsI
SVEN_3703	361.49	Putative membrane spanning protein
SVEN_3704	113.15	Transcriptional regulator, IclR family
SVEN_3705	60.03	putative regulator (Streptomyces sporulation and cell division protein, SsgA)
SVEN_3706	10.48	Putative membrane protein
SVEN_3751	5.25	Macro domain, possibly ADP-ribose binding module
SVEN_3774	8.45	putative glycosyl transferase /
SVEN_3791	6.13	Hypothetical protein
SVEN_3840	8.66	HP with META domain-containing protein
SVEN_3857	55.87	Possible diacylglycerol kinase, catalytic region
SVEN_3858	31.94	Integral membrane protein
SVEN_3859	21.85	Putative RNA polymerase ECF sigma factor
SVEN_3860	22.85	Sporulation associated protein
SVEN_3921	23.27	Putative secreted protein
SVEN_3948	8.41	Putative membrane protein
SVEN_4108	7.64	Oxirreductase

SVEN_4111	5.43	Hypothetical protein
SVEN_4181	5.08	Hypothetical protein with DMT (drug/metabolite transporter) family
SVEN_4183	5.22	A-factor biosynthesis protein AfsA
SVEN_4229	6.91	RNA polymerase ECF-subfamily sigma factor
SVEN_4231	9.56	Putative cyclohex-1-ene-1-carboxylate:CoA ligase
SVEN_4270	22.67	NADH-ubiquinone oxidoreductase chain F
SVEN_4277	5.88	NADH-ubiquinone oxidoreductase chain M
SVEN_4285	16.03	Di-or tripeptide transporter
SVEN_4321	12.41	Hypothetical protein from Barstar_like super family
SVEN_4375	8.29	PIG-L family deacetylase
SVEN_4542	18.05	Putative lipoprotein murein L,D-transpeptidase
SVEN_4571	59.55	Integral membrane/VanZ family protein (antibiotic teicoplanin resistance)
SVEN_4582	187.36	Hypothetical protein
SVEN_4606	12.5	Putative lipoprotein
SVEN_4647	6.4	Hypothetical protein
SVEN_4740	15.08	Threonine synthase
SVEN_4743	5.3	Transcriptional regulator, GntR
SVEN_4750	19.71	Putative membrane protein GH18 (glycosyl hydrolase, family 18)
SVEN_4751	5.79	Hypothetical protein from Fiu superfamily
SVEN_4832	6.51	Hypothetical protein - ATPase superfamily
SVEN_4848	7.91	DUF124 domain-containing protein
SVEN_4849	6.02	DUF124 domain-containing protein
SVEN_4954	11.49	Endoglycoceramidase
SVEN_4972		ABC transporter ATP-binding site
SVEN_4973	9.59	Hypothetical protein
SVEN_4974		RNA polymerase ECF-subfamily sigma factor
SVEN_4976	9.5	Hypothetical protein
SVEN_5030	7.49	Putative two-component system sensor kinase
SVEN_5090	7.01	Putative MerR-family transcriptional regulator
SVEN_5181	13.4	Putative integral membrane protein
SVEN_5224	10.63	Putative lipoprotein
SVEN_5225	17.47	Hypothetical protein with a phosphatase 2C domain-containing protein
SVEN_5226	34.97	Hypothetical protein with Von Willebrand factor type A (vWA) domain
SVEN_5227	12.85	Hypothetical protein from DNA_pol3_gamma3 super family
SVEN_5476	24.65	Siderophore synthetase small component, acetyltransferase
SVEN_5505	9.25	Putative membrane protein
SVEN_5770	5.02	hypothetical protein from BlaI/MecI/CopY family transcriptional regulator
SVEN_5771		Integral membrane protein
SVEN_5813	14.43	N-acetylmuramoyl-L-alanine amidase
SVEN_5834	9.58	Hypothetical membrane protein
SVEN_5898	16.13	Glycogen debranching enzyme
SVEN_6021	8.67	Cold shock domain-containing protein
SVEN_6023	7.23	Major facilitator superfamily permease
SVEN_6392	28.93	Hypothetical protein
SVEN_6402	11.39	Putative regulatory protein
SVEN_6403	6.76	Fer4_19 and zf-CDGSH domain-containing protein
SVEN_6423	8.61	Oxidoreductase
SVEN_6669	12.53	Peptidase

SVEN_6845	5.87	Hypothetical protein
SVEN_6884	12.08	FAD-dependent NAD(P)-disulphide oxidoreductase
SVEN_6950	47.37	Acetyl-coenzyme A carboxyl transferase alpha or beta chain
SVEN_7021	13.04	Hypothetical protein from Golgi phosphoprotein 3 (GPP34) family
SVEN_7040	8.84	Branched-chain amino acid aminotransferase
SVEN_7041	8.75	Putative ligase or carboxylase protein
SVEN_7043	6.21	Major facilitator superfamily MFS_1 protein
SVEN_7044	6.66	Periplasmic_Binding_Protein_Type_2 super family
SVEN_7178	9.88	Secreted protein
SVEN_7261	7.41	Hypothetical protein from Abhydrolase super family
SVEN_7336	5.48	D-alanyl-D-alanine carboxypeptidase
SVEN_7342	25.01	Transcriptional regulator, AraC family
SVEN_7346	10.62	Hypothetical protein
SVEN_7347		Transcriptional regulator, PadR family

Putative operons are highlighted in grey.

4 Concluding discussion

4.1 S. venezueale ECF σ factor 19 can be successfully implemented into *B. subtilis*

Gene expression is the core of this engineering process and the bacterial ECF σ factors showed to be a potential source of regulatory parts for synthetic biology due to their orthogonality, modularity and scalability. The use of ECF σ factors to build synthetic circuits has been previously described (Chen and Silver, 2012; Shin and Noireaux, 2012; Rhodius *et al.*, 2013; Pinto *et al.*, 2018a, 2019). Nevertheless, heterologous regulatory parts have been rarely extrapolated beyond the taxonomic borders. A study with the same rational design used here showed that ECF σ factors from *Bacillus licheniformis* and *Bacillus cereus* were successfully implemented in *B. subtilis*, with no need of further manipulation (Pinto et al., 2018; Pinto et al., 2019). This indicates that *B. subtilis* is more likely to accept foreign parts from closely related species. The unsuccessful implementation of most the *S. venezuelae* ECF σ factors, and the surprisingly activity of ECF 19 in *B. subtilis* observed in this study reinforces the need for further investigation on how synthetic switches can interfere with the host cell -physiology.

4.2 Anti-σ factor AS19 modulates the output of the ECF19 based switch

The level of ECF19 in the cell is negatively regulated by the membrane associated AS19 which forms a complex with the ECF19 preventing its interaction with the RNA polymerase (Shukla *et al.*, 2014). Studies in *M. tuberculosis* reported that the ECF19 SigK is released through multiple mechanisms involving the cleavage of the ectodomain of the AS RskA by a site-1 protease which triggers the site-2 protease Rip1 releasing the ECF19-AS19$_{cyto}$ complex from the membrane (Makinoshima and Glickman, 2005; Urban, 2009; Sklar *et al.*, 2010; Shukla *et al.*, 2014).

Here, the activity the ECF19-based switch was not affected by the co-expression of its full length cognate anti-σ factor (**Figure 17**). This observation was somehow surprising, since ASs successfully regulated the activity of their cognate ECF in *E. coli* ECF-based switches without the necessity of further manipulations (Rhodius *et al.*, 2013). Next, only the cytoplasmic part of the AS19 was implemented into the ECF19-based switch, and this caused a decrease in output up to 9-fold (**Figure 18**). However, this downregulation was observed only when the AS19$_{cyto}$ was placed in the same operon than ECF19.

The reason why the $AS19_{cyto}$ was able to regulate the ECF19 only when placed in the same operon is unclear. However, one can speculate that this organization can enable coordinated expression and regulation. In fact, studies of sporulation proteins under control of SigF in *B. subtilis* showed that operon organisation together with translational coupling are important to keep the ratio between protein concentrations which is altered when co-regulation is not present (Zaslaver *et al.*, 2004; Hazkani-Covo and Graur, 2005; Price *et al.*, 2005; Iber, 2006; Price, Arkin and Alm, 2006).

4.3 Robustness of the ECF19 based genetic switch

Promoters are modular units and the variety of well-known promoters offers many choices for obtaining circuits with ample expression ranges (Kelly *et al.*, 2009). Choosing the type of promoter is crucial for the design of a synthetic switch. Thus, the activation and stability of the ECF19-based switch under control of two naturally occurring inducible promoters was compared. The used promoters were: **(i)** P_{xylA} which is induced by xylose and repressed by XylR and **(ii)** P_{liaI} which is induced by bacitracin. The activity of these promoters has been already evaluated (Radeck *et al.*, 2013), and while the induction mediated by P_{xylA} had a constant output that of P_{liaI} is only transient. Here we have shown that the ECF19 switch has an output curve (luminescence over time) that follows the same shape of that of the inducible promoter controlling the ECF expression (**Figure 19**).

Changes in the copy number of genes and promoters can strongly affect the behaviour of genetic circuits by switching gene expression to or from an oscillatory state, particularly the ones involving feedback as the genetic switches (Atkinson *et al.*, 2003; Ingolia and Murray, 2007; Mileyko, Joh and Weitz, 2008). Parts of synthetic circuits are often inserted in plasmids due the easier manipulation and the stronger signal obtained but on the other hand, these circuits are subject of fluctuations and noise in gene expression. Genes encoded on the chromosome have a copy number that correlates to that of the chromosome and are more stable to fluctuations in the expression level (Loinger and Biham, 2009).

Genetic circuits can be also controlled at a post-translational level. The SsrA tag system has been used to create variants of GFP reporter protein with different half-lives in *E. coli* and *Pseudomonas putida* (Andersen *et al.*, 1998; Kim *et al.*, 2000; Singh *et al.*, 2000; Bohn, Binet and Bouloc, 2001), and also to modify the rate of protein degradation in a dual-feedback oscillator circuit in order to decrease the protein lifetime and improve the temporal responsiveness of the transcriptional factors (Stricker *et al.*, 2008). Additionally, five σ factors

of *B. subtilis* have been tagged with SsrA in order to create a set of degradable factors in an *E. coli* cell-free expression toolbox (Shin and Noireaux, 2012).

Taking this in consideration, the stability of the response of the ECF19-based switch to ECF SsrA tagging was investigated. Eleven SsrA tag variants, reported to confer different protein stabilities, were tested. With exception of three switches (one carrying the native *B. subtilis* SsrA-tag (LAA), the variant LVA and the variant LDD), all the others SsrA variants led to alterations in the time of activation and maximum output of the switch. Unlike, similar studies using different groups of ECF-based switches showed to be more robust in the presence of SsrA-tagging keeping this way their dynamics and output unchanged (Pinto *et al.*, 2019), thus, it is important to evaluate the effect of SsrA-tagging in each individual protein of interest.

Several promoter elements have important roles because they are recognition or interaction sites for the RNAP and additional transcription factors. The UP element is an AT-rich sequence upstream the -35 motif of a given promoter, often located within the -40 to -55 regions, and responsible for thr interaction with the α C-terminal domain of the RNAP (Perez-Martin, Rojo and de Lorenzo, 1994; Meng *et al.*, 2001; Pátek *et al.*, 2003). Here, truncations performed in the P_{ecf19} showed that the removal of this UP element (-60 to -36 region) caused a decrease in the dynamic range and maximum output curve of the ECF19-based switch. This result is in accordance with studies where the presence of an UP element enhanced expression from ECF promoters (Rhodius *et al.*, 2013) and those that highlighted its important role in the regulation of transcription initiation in *E. coli* and *Mycobacterium tuberculosis* by increasing transcription rate to up three-fold (Zuo and Steitz, 2015; Hubin *et al.*, 2017).

Antisense transcription occurs when two promoters are oriented in the opposite direction of a gene generating this way antisense RNAs (Georg and Hess, 2011). Antisense transcription has been used to evaluate the robustness of synthetic switches before and for some cases it has been showed to be cryptic, inducing changes in the host gene expression (Gorochowski et al., 2017). The behaviour of ECF 19 based switch was influenced by antisense transcription and similar results has been observed for ECF based switches from other ECF groups but ECF19. However, once the effect of this transcriptional interference is known, it is possible to avoid unwanted behaviour by adding antisense terminators (Pinto *et al.*, 2019).

4.4 Bacitracin resistance mechanisms in *S. venezuelae*

The most striking observation in this study is the high-level resistance of *S. venezuelae* against bacitracin and against inhibition by *Bacillus* species. We were therefore interested in elucidating the molecular mechanisms for this marked AMP resistance. Because bacitracin

resistance is well understood in low-GC Gram-positive bacteria, we first analysed the genome sequence of *S. venezuelae* ATCC10712. The genome contains a single gene annotated as undecaprenyl pyrophosphate (UPP) phosphatase, which is responsible for dephosphorylation of the lipid carrier UPP during cell wall biosynthesis. Because UPP is the cellular target of bacitracin (Siewert, Gerhard; Strominger, 1967), UPP phosphatases can often impart some protection against this antibiotic (Podlesek *et al.*, 2000; Cao *et al.*, 2002; Shaaly *et al.*, 2013). However, because dephosphorylation of UPP is essential for the biosynthesis of peptidoglycan (El Ghachi *et al.*, 2005), such a gene is found in all available Streptomyces genomes and can thus no explain the unique bacitracin resistance of *S. venezuelae*.

A second common mechanism for AMP resistance is the Dlt-system, which modified teichoic acids to restrict access of the AMP to the cell membrane (Peschel *et al.*, 1999; Saar-Dover *et al.*, 2012; Revilla-Guarinos *et al.*, 2014), but this system is not present in actinobacteria. The third main mechanism of AMP resistance in Gram-positive bacteria is through ATP-dependent transport systems (Gebhard, 2012; Revilla-Guarinos *et al.*, 2014). It has been previously shown that BceAB-like systems, which are the primary bacitracin-resistance determinant in *B. subtilis* (Rietkötter, Hoyer and Mascher, 2008), are restricted to the Firmicutes and not found in actinobacteria (Dintner *et al.*, 2011). Self-protection in bacitracin producer strains is accomplished by the transporter BcrAB (Neumüller, Konz and Marahiel, 2001), and a homologous transporter mediates high-level bacitracin resistance in *Enterococcus faecalis* (Manson *et al.*, 2004), however, no homologous genes to the transport permease BceB could be identified in the genome of *S. venezuelae*.

To identify the genes required for bacitracin resistance, we randomly mutagenised spore suspensions of *S. venezuelae*. A strong bacitracin sensitive mutant was found; and precise mutations present in Mut4 were identified. The isolation of a single highly sensitive mutant, that moreover specifically lost its resistance to bacitracin but no other cell wall active antibiotics, points towards the existence of a defined molecular determinant of resistance.

Taking in consideration the number and distinct cellular process of the mutant genes found in *S. venezuelae* Mut4 (**Table 10**), functional analysis to evaluate the effect of each mutation separated will be necessary in the future. However, the transcriptomic analysis of the bacitracin stimulon in *S. venez*uelae together with literature mining could shine a light on the genes that could most likely be involved in bacitracin resistance in *S. venezuelae*.

Our transcriptomics results showed that over 100 genes were more than 5-fold upregulated in the presence bacitracin. The list of genes in **table 11** shows that these genes are involved in diverse biological process. Previous studies in *S. coelicolor* showed that the presence of

bacitracin upregulated over 1000 genes, in which only the minority changed the expression in response to bacitracin uniquely. Most of the genes were affected similarly by vancomycin or moenomycin (Hesketh et al., 2011).

When we looked at the genes that were upregulated in the presence of bacitracin in the study made by Hesketh and co-workers (2011), we could identify common genes present in our transcriptomic data. Alike to *S. coelicolor*, the bacitracin stimulon in *S. venezuelae* showed upregulation of genes involved in the extracellular peptidoglycan biosynthesis and cell wall remodeling and division (**Figure 28**). The bacitracin stimulon in *S. venezueale* also showed upregulation of genes involved in the cytoplasmic process of peptidoglycan precursor biosynthesis while these genes were described as repressed in the presence of bacitracin in *S. coelicolor* (Hesketh et al., 2011). In addition to peptidoglycan biosynthesis, cell wall remodeling is an important process to maintain shape and differentiation.

Sigma factors were the major response found in the bacitracin stimulon in *S. coelicolor* (Hesketh et al., 2011), where 33 upregulated genes were identified as being part of the SigB regulon. Our data showed that for *S. venezuelae*, the presence of bacitracin led to the upregulation of 5 sigma factors, including SigB. SigB is described as involved in osmoprotection and differentiation in *S. coelicolor* (Huang *et al.*, 1999; Shaaly *et al.*, 2013).

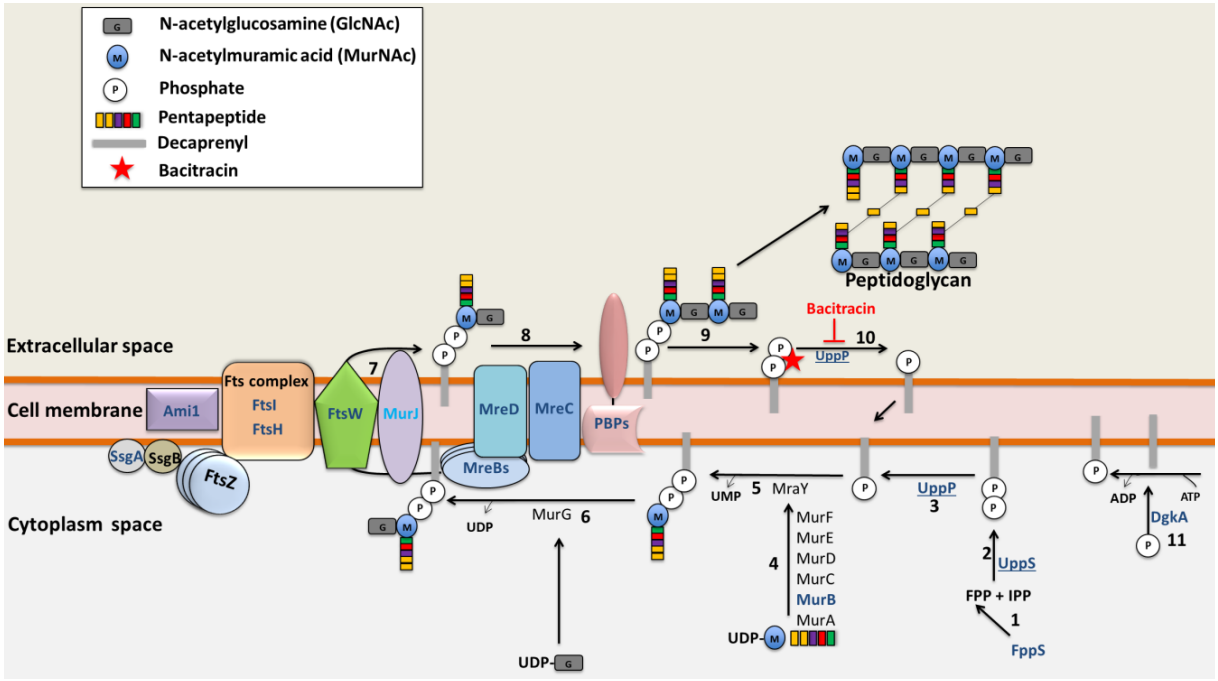


Figure 28. Peptidoglycan biosynthesis scheme. Peptidoglycan biosynthesis requires the lipid carrier undecaprenylphosphate (UP). In the cytoplasm, farnesyl pyrophophate (FPP) is synthetized by the FPP synthetase (1). Then, undecaprenyl pyrophosphate (UPP) through sequential condensation of FPP and isopentenyl pyrophosphate (IPP) by is synthetised by the action of UPP synthase (UppS) (2). UPP is dephosphorylated to UP by undecaprenyl pyrophosphate phosphatase (UppP) (3). UDP-N-acetyl muramic acid pentapeptide (UDP-MurNAc-pentapepitide) is formed by the enzymes MurA-F (4). MraY catalases the reaction of UP with UDP-MurNAc-pentapepitide to form lipid I (5). Then lipid I reacts with UDP-N-acetyl glucosamine (UDP-GlcNAc) to form lipid II by action of MurG (6). Lipid II is translocated to the extracellular space by a lipid II flippase (7). The disaccharide pentapeptide is incorporated to the growing peptidoglycan chain by penicillin binding proteins (PBPs) (8) and the UPP is released to be recycled by UppP (9). However, the binding of bacitracin to the UPP complex inhibits the UPP recycling (10). Gram-positive bacteria present free undecaprenyl which is phosphorylated to UP by the action of DgkA (11). Division (Fts complex, Ssgs and Ami1) and elongation (Mre complex) proteins are shown. In dark blue are the products of genes that were upregulated in presence of bacitracin; in underlined dark blue are gene products found very close to upregulated genes; in light blue represents is the mutated MurJ found in the bacitracin-sensitive strain of *S. venezuelae*. Bacitracin is represented by a red star and bacitracin action is shown in red.

Our study showed that the presence of bacitracin caused an upregulation of up to 125-fold of a putative operon coding a putative membrane protein (Sven_0373) and the homologous of the regulators of SigB: two-component system sensory histidine kinase RsbK (Sven_0372), serine phosphatase RsbU (Sven_0371), anti-σ^B factor antagonist RsbV (anti anti-σ factor) (Sven_0370) and anti-σ factor RsbW (*Sven_0370*). Rsb proteins are usually found in an operon upstream the coding gene for σ^B (Lee *et al.*, 2004). Studies in *B. subtilis*, *L. monocytogenes* and *S. coelicolor* showed that RsbU activity is triggered by a multiprotein signaling stressosome (RsbR-RsbS-RsbT) (Wise and Price, 1995; Hecker, Pané-Farré and Uwe, 2007). Despite of the conservation of RsbU, RsbV and RsbW, the components of the stressosome (RsbR-RsbS-RsbT) are not present in *S. venezueale*. However, the regulation of RsbV, RsbW and σ^B in *B. cereus* involves a multisensory histidine kinase RsbK and a RsbY phosphatase (RsbU homologous) (Marles-Wright and Lewis, 2010). *S. venezuelae* also present a gene coding for a sensory

histidine kinase upstream the *rsbU-rsbV-rsbW* genes and in addition to this, it presents two additional SigB regulators coding homologous for the osmotic stress adaptation proteins OsaC and OsaB, which are located upstream the *sigB* gene (**Figure 29A**). OsaC and OsaB were described as regulatory proteins for post-osmotic stress modulation of σ^B activity in *S. coelicolor* (De Been *et al.*, 2011). Here, our transcriptomic data showed the possible involvement of SigB and σ^B regulators of *S. venezuelae* in the response to bacitracin (**Figure 29B**). Similar findings were reported for *S. coelicolor*, which had over 30 genes belonging to the SigB regulon induced in presence of bacitracin (Martínez *et al.*, 2009).

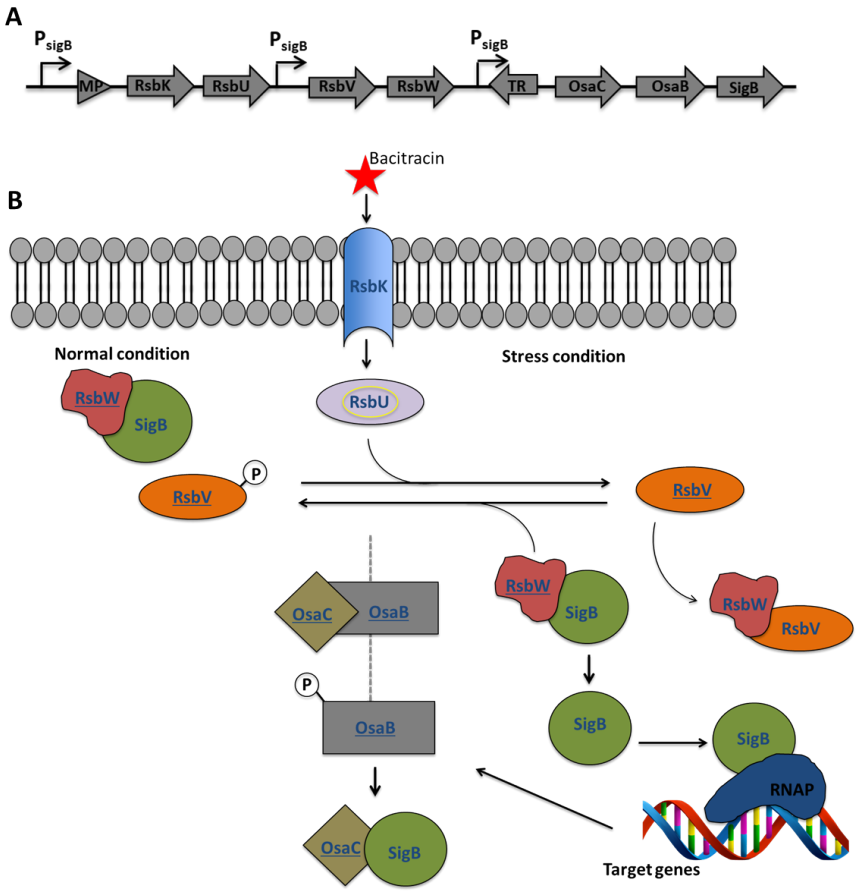


Figure 29. Modulation of σ^B activitiy. (A) Operon structure of *sigB* and *sigB* regulators genes in *S. venezuelae*. Thick arrows represent open reading frames. Thin arrows indicate putative *sigB* target promoters predicted using the online tool PRODORIC (http://prodoric.tu-bs.de/vfp/). (B) Under normal conditions the phosphorylated RsbV is inactive being unable to bind RsbW thereby σ^B is sequestered by the anti-σ factor RsbW, preventing the induction of the SigB regulon. Under stress condition (e.g., bacitracin), the sensory histidine kinase (HK) RsbK transmits the signal to RsbU, which de-phosphorylates RsbV allowing its binding to the anti-σ factor RsbW, releasing RsbW from SigB. RsbW phosphorylate RsbV to keep its inactivity. Once SigB binds RNA polymerase transcribing the σ^B regulon, the OsaC phosphorylate its antagonist OsaB releasing OsaC for binding to σ^B what negatively regulates the expression of *sigB*. In dark blue are the products of genes that were upregulated in presence of bacitracin; in underlined dark blue are gene products in the same operon or very close to upregulated genes; yellow cycle indicates mutated RsbU found in the bacitracin-sensitive strain of *S. venezuelae*. Bacitracin is represented by a red star. Modified from Martínez et al., 2009.

S. venezuelae encodes 14 genes annotated as RsbU but only two of them is found surrounded by or nearby other Rsb coding genes (*sven_0371* and *sven_0375*). The other RsbU coding genes are located close to genes related to drug efflux, antibiotic biosynthesis, and light and oxygen stress responses. Here, we found a point mutation in a gene coding for a RsbU regulator (*sven_6766*) in the Mut4 bacitracin-sensitive strain of *S. venezuelae*. The *sven_6766* gene is placed in an operon along with a gene involved in the biosynthesis of mitomycin antibiotics. Although σ^B is known as responsible for general stress response, it has been reported that it responds to cell wall-acting antibiotics like vancomycin and bacitracin in *B. subtilis* and *Listeria monocytogenes* (Hesketh *et al.*, 2011). Further, transcriptional studies in *S. colelicolor* also showed the involvement of σ^B in the response to antibiotics that target the cell envelope (Delumeau *et al.*, 2004a; Hecker, Pané-Farré and Uwe, 2007; Shin, Brody and Price, 2010). Taking in consideration the upregulation of the *rsb* operon in *S. venezuelae* treated with bacitracin in addition to the point mutation found in one of the *rsbU* genes of the bacitracin-sensitive mutant of *S. venezuelae*, it could be likely that RsbU and consequently σ^B is involved in the bacitracin response in *S. venezuelae*.

In this study, the bacitracin-sensitive strain of *S. venezuelae* presented one base pair deletion in the ABC leucine-isoleucine-valine (Liv) transporter (Sven_6295), which is the second gene in an operon composed of four liv-transporters (*livF-livM*). Liv transporters can be involved in the transport of a variety of compounds such sugars, ions, peptides and more complex molecules (Hesketh *et al.*, 2011). Besides, one base pair deletion was also found in a gene coding a putative ABC transporter (*Sven_0570*). This putative ABC transport system does not share any common domain with the known bacitracin efflux systems previously described; it was not found as upregulated in presence of bacitracin; and it does not present a neighboring two-component system. Despite the lack of similarities with the ABC-transporters involved in bacitracin resistance reported until date, the permease component of this putative ABC transporter presents a YadH domain which is annotated as an ABC-type multidrug transport system. Considering that, further studies will be needed to reveal if these ABC transporters are somehow involved in bacitracin resistance.

5 Conclusion and Outlook

Here, we showed that *B. subtilis* has a low acceptance for ECF σ factors derived from *S. venezuele*. The reason why only the ECF19-switch was active is not clear. However, the characterization of the ECF19-based switch showed that alterations in different parts of the switch do not improve the switch performance and the presence of antisense transcription and the addition of ssrA-tag can negatively affect the switch behavior. Further research work is necessary in order to understand the differences between the transcription machinery across bacteria in order to optimize the use of heterologous ECF σ factors based switches in non-related organisms.

S. venezuelae showed to be resistant to bacitracin and the random mutagenesis generated one strong bacitracin-sensitive mutant (Mut4), which presented mutation in a number of genes involved in different cellular processes. In the presence of bacitracin, *S. venezuleae* upregulated over 100 genes with part of them being known for being involved in antibiotic response, eg. cell wall biosynthesis and remodeling genes. This fact is not surprisingly since bacitracin targets the lipid II recycling pathway, which is essential for peptidoglycan biosynthesis. We could not correlate the mutations found in the bacitracin-sensitive Mut4 and the bacitracin stimulon results from the transcriptomic data of *S. venezuelae* in the presence of bacitracin. Based on our results, it is hard to infer that there is a clear relationship between the two data. However, the point mutation found in the positive regulator of σ^B RsbU in the bacitracin-sensitive Mut4 in addition to the upregulation of genes involved in the SigB regulation found in our transcriptome analysis could indicate the possible involvement of the SigB regulon in the response to bacitracin. Generation of bacitracin-sensitive strains of *S. venezuelae* and accessing the transcriptomic profile of the wild type strain in presence of bacitracin did not elucidate the mechanism of resistance to bacitracin in *S. venezuelae*. Thus, functional studies involving deletion and complementation of the putative genes involved in bacitracin resistance are essential. The differences in antimicrobial production by *Bacillus* sp. and in resistance against these compounds of Streptomyces sp. are likely to play an important role in determining the composition of bacterial soil communities. Identification of the molecular determinant of the observed high-level bacitracin resistance of *S. venezuelae* will further contribute to our understanding of antibiotic resistance and its role in natural bacterial populations. The isolation of a single highly sensitive mutant, that moreover specifically lost its resistance to bacitracin but no other cell wall active antibiotics, will be crucial to the following studies on bacitracin resistance in *S. venezueale*.

6 References

Abranches J, Tijerina P, Avilés-Reyes A, Gaca AO, Kajfasz JK (2013) The Cell Wall-Targeting Antibiotic Stimulon of *Enterococcus faecalis*. *PLoS ONE* 8(6):e64875. doi:10.1371/journal.pone.0064875.

Ahlawat S and Morrison DA (2009) ClpXP degrades SsrA-tagged proteins in *Streptococcus pneumoniae*. *Journal of Bacteriology*, 191(8):2894-2898. doi:10.1128/JB.01715-08.

Alkhatib Z, Abts A, Mavaro A, Schmitt L, Smits SHJ (2012). Lantibiotics: How do producers become self-protected?. *Journal of Biotechnology*, 159(3):145-154. doi:10.1016/j.jbiotec.2012.01.032.

Andersen JB, Sternberg C, Poulsen LK, Bjørn SP, Givskov M, Molin S (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Applied and Environmental Microbiology*, 64(6):2240-2246. doi:10.1145/2107596.2107598.

Atkinson MR, Savageau MA, Myers JT, Ninfa AJ (2003). Development of genetic circuitry exhibiting toggle switch or oscillatory behavior in *Escherichia coli*. *Cell*, 113(5):597-607. doi:10.1016/S0092-8674(03)00346-5.

Bae B, Feklistiv A, Lass-Napiorkowska A, Landick R, Darst SA (2015). Structure of a bacterial RNA polymerase holoenzyme open promoter complex. *eLife*, 4:e08504. doi:10.7554/eLife.08504.

Barne KA, Bown JA, Busby SJW, Minchin SD (1997) Region 2.5 of the *Escherichia coli* RNA polymerase σ70 subunit is responsible for the recognition of the "extended -10" motif at promoters. *EMBO Journal*, 16(13):4034-4040. doi:10.1093/emboj/16.13.4034.

De Been M, Francke C, Siezen RJ, Abee, T (2011) Novel sigmaB regulation modules of Gram-positive bacteria involve the use of complex hybrid histidine kinases. *Microbiology*, 157(1):3-12. doi:10.1099/mic.0.045740-0.

Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD (2012) Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS ONE,* 4(7):e34953. doi:10.1371/journal.pone.0034953.

Bibb MJ, Domonkos A, Chandra G, Buttner MJ (2012) Expression of the chaplin and rodlin hydrophobic sheath proteins in *Streptomyces venezuelae* is controlled by σBldN and a cognate anti-sigma factor, RsbN. *Molecular Microbiology*, 84(6):1033-1049. doi:10.1111/j.1365-2958.2012.08070.x.

Blatter EE, Ross W, Tang H, Gourse RL, Ebright RH (1994) Domain organization of RNA polymerase α subunit: C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. *Cell*, 78(5):889-896. doi:10.1016/S0092-8674(94)90682-3.

Bohn C, Binet E and Bouloc P (2001) Screening for stabilization of proteins with a trans-translation signature in *Escherichia coli* selects for inactivation of the ClpXP protease. *Molecular Genetics and Genomics*, 266(5):827-831. doi:10.1007/s00438-001-0601-1.

Browning DF and Busby SJ (2004) The regulation of bacterial transcription initiation. *Nature Reviews Microbiology*, 2(1):57-65. doi:10.1038/nrmicro787.

Bush MJ, Bibb MJ, Chandra G, Findlay KC, Buttner MJ (2013) Genes required for aerial growth, cell division, and chromosome segregation are targets of WhiA before sporulation in *Streptomyces venezuelae*. *mBio*, 4(5):e006684-13 doi:10.1128/mBio.00684-13.

Butcher BG, Mascher T and Helmann JD (2008) Environmental sensing and the role of extracytoplasmic function sigma factors. In *Bacterial Physiology*, 15:233-261. doi:10.1007/978-3-540-74921-9_8.

REFERENCES

Campbell EA, Muzzin O, Chlenov M, Sun JL, Olson CA, Weinman O, Trester-Zedlitz ML, Darst SA (2002). Structure of the bacterial RNA polymerase promoter specificity σ subunit. *Molecular Cell*, 9(3):527-539. doi:10.1016/S1097-2765(02)00470-7.

Cao M, Wang T, Ye R, Helmann JD (2002) Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σ(W) and σ(M) regulons, *Molecular Microbiology*, 45(5):1267-1276. doi:10.1046/j.1365-2958.2002.03050.x.

Cao M and Helmann JD (2002) Regulation of the Bacillus subtilis *bcrC* bacitracin resistance gene by two extracytoplasmic function σ factors. *Journal of Bacteriology*, 184(22):6123-6129. doi:10.1128/JB.184.22.6123-6129.2002.

Charlet D, Mostowy S. Alexander D, Sit L, Wiker HG, Behr MA (2005) Reduced expression of antigenic proteins MPB70 and MPB83 in *Mycobacterium bovis* BCG strains due to a start codon mutation in *sigK*. *Molecular Microbiology*, 56(5):1302-1313. doi:10.1111/j.1365-2958.2005.04618.x.

Chen AH and Silver PA (2012) Designing biological compartmentalization. *Trends in Cell Biology*, 22(12):662-670. doi:10.1016/j.tcb.2012.07.002.

Claesen J. and Bibb M. (2010) Genome mining and genetic analysis of cypemycin biosynthesis reveal an unusual class of post-translationally modified peptides. *Proceedings of the National Academy of Sciences*, 107(37):16297-16302. doi:10.1073/pnas.1008608107.

Clancy K and Voigt C A (2010) Programming cells: Towards an automated 'Genetic Compiler'. *Current Opinion in Biotechnology*, 21(4):572-581. doi:10.1016/j.copbio.2010.07.005.

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006) Sampling the antibiotic resistome. *Science*, 311(5759):374-377. doi:10.1126/science.1120800.

Davies G and Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. *Structure*, 9(3):853-859. doi:10.1016/S0969-2126(01)00220-9.

Delić V, Hopwood DA and Friend EJ (1970) Mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) in *Streptomyces coelicolor*. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 9(2):167-182. doi:10.1016/0027-5107(70)90055-2.

Delumeau O, Dutta S, Brigulla M, Kuhnke G, Hardwick SW, Völker U, Yudkin MD, Lewis RJ (2004a) Functional and structural characterization of RsbU, a stress signaling protein phosphatase 2C. *Journal of Biological Chemistry*, 279(39):40927-40937. doi:10.1074/jbc.M405464200.

van Dijl JM and Hecker M (2013) *Bacillus subtilis*: From soil bacterium to super-secreting cell factory. *Microbial Cell Factories*, 12:3. doi:10.1186/1475-2859-12-3.

Dintner S, Staron A, Berchtold E, Petri T, Mascher T, Gebhard S (2011) Coevolution of ABC transporters and two-component regulatory systems as resistance modules against antimicrobial peptides in Firmicutes bacteria. *Journal of Bacteriology*, 193(15):3851-3862. doi:10.1128/JB.05175-11.

Dintner S, Staron A, Berchtold E, Petri T, Mascher T, Gebhard S (2014) A sensory complex consisting of an ATP-binding cassette transporter and a two-component regulatory system controls bacitracin resistance in *Bacillus subtilis*. *Journal of Biological Chemistry*, 193(15):3851-3862. doi:10.1074/jbc.M114.596221.

Dufour YS, Landick R and Donohue TJ (2008) Organization and Evolution of the Biological Response to Singlet Oxygen Stress. *Journal of Molecular Biology*, 3:(383):713-730. doi:10.1016/j.jmb.2008.08.017.

Estrem ST, Gaal T, Ross W, Gourse RL (1998) Identification of an UP element consensus sequence for bacterial promoters. *Proceedings of the National Academy of Sciences*, 95(17):9761-9766. doi:10.1073/pnas.95.17.9761.

Feklístov A, Sharon BD, Darst SA, Gross CA (2014) Bacterial Sigma Factors: A Historical,

Structural, and Genomic Perspective. *Annual Review of Microbiology*, 68:357-376. doi:10.1146/annurev-micro-092412-155737.
Forterre P, Gribaldo S, Gadelle D, Serr MC (2007) Origin and evolution of DNA topoisomerases. *Biochimie*, 89(4):427-446. doi:10.1016/j.biochi.2006.12.009.
Foulston L C and Bibb M J (2010) Microbisporicin gene cluster reveals unusual features of lantibiotic biosynthesis in actinomycetes. *Proceedings of the National Academy of Sciences*, 107(30):13461-13466. doi:10.1073/pnas.1008285107.
Friedman L J and Gelles J (2012) Mechanism of transcription initiation at an activator-dependent promoter defined by single-molecule observation. *Cell*, 148(4):679-689. doi:10.1016/j.cell.2012.01.018.
Gangaiah D, Zhang X, Baker B, Fortney KR, Liu Y, Munson RS Jr, Spinola SM (2014) *Haemophilus ducreyi* RpoE and CpxRA appear To play distinct yet complementary roles in regulation of envelope-related functions. *Journal of Bacteriology*, 196(23):4012-4025. doi:10.1128/JB.02034-14.
Gao X J and Elowitz MB (2016) Synthetic biology: Precision timing in a cell. *Nature*, 538(7626):462-463. doi:10.1038/nature19478.
Gärtner D, Geissendörfer M and Hillen W (1988) Expression of the *Bacillus subtilis xyl* operon is repressed at the level of transcription and is induced by xylose. *Journal of Bacteriology*, 170(7):3102-3109. doi:10.1128/jb.170.7.3102-3109.1988.
Gebhard S (2012) ABC transporters of antimicrobial peptides in Firmicutes bacteria - phylogeny, function and regulation. *Molecular Microbiology*, 86(6):1295-1317. doi:10.1111/mmi.12078.
Georg J and Hess WR (2011) cis-Antisense RNA, Another Level of Gene Regulation in Bacteria. *Microbiology and Molecular Biology Reviews*, 75(2):286-300 doi:10.1128/MMBR.00032-10.
El Ghachi M, Derbise A, Bouhss A, Mengin-Lecreulx D (2005) Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli*. *Journal of Biological Chemistry*, 208(19):18689-18695. doi:10.1074/jbc.M412277200.
Glazebrook MA, Doull JL, Stuttard C, Vining LC (1990) Sporulation of *Streptomyces venezuelae* in submerged cultures. *Journal of General Microbiology*, 136(3):581-588. doi:10.1099/00221287-136-3-581.
Gómez-Santos N, Pérez J, Sánchez-Sutil MC, Moraleda-Muñoz A, Muñoz-Dorado J (2011) CorE from *Myxococcus xanthus* is a Copper-Dependent RNA polymerase sigma factor. *PLoS Genetics*, 7(6):e1002106. doi:10.1371/journal.pgen.1002106.
Gourse RL, Ross W and Gaal T (2000) UPs and downs in bacterial transcription initiation: The role of the alpha subunit of RNA polymerase in promoter recognition. *Molecular Microbiology*, 37(4):687-695. doi:10.1046/j.1365-2958.2000.01972.x.
Griffith KL and Grossman AD (2008) Inducible protein degradation in *Bacillus subtilis* using heterologous peptide tags and adaptor proteins to target substrates to the protease ClpXP. *Molecular Microbiology*, 70(4):1012-1025. doi:10.1111/j.1365-2958.2008.06467.x.
Gross CA, Chan C, Dombroski A, Gruber T, Sharp M, Tupy J, Young B (1998) The functional and regulatory roles of sigma factors in transcription. In *Cold Spring Harbor Symposia on Quantitative Biology*, 63:141-155. doi:10.1101/sqb.1998.63.141.
Gruber TM and Gross CA (2003) Multiple Sigma Subunits and the Partitioning of Bacterial Transcription Space. *Annual Review of Microbiology*, 57:441-466. doi:10.1146/annurev.micro.57.030502.090913.
Guet CC, Elowitz MB, Hsing W, Leibler S (2002) Combinatorial synthesis of genetic networks.

Science, 296(5572):1466-1470. doi:10.1126/science.1067407.
Hampsey M and Reinberg D (2001) RNA Polymerase II Holoenzyme and Transcription Factors. *In*: Encyclopedia of Life Sciences, London, UK: Nature Publishhing Group, 1-7. doi:10.1002/9780470015902.a0003301.pub2.
Harley CB, and Reynolds RP (1987) Analysis of *E. coli* promoter sequences. *Nucleic Acids Research*, 15(5):2343-2361. doi:10.1093/nar/15.5.2343.
Harwood CR, Cutting SM (1990) Molecular Biological Methods for Bacillus. Chichester: John Wiley.
Hazkani-Covo E and Graur D (2005) Evolutionary conservation of bacterial operons: Does transcriptional connectivity matter? *Genetica*, 124(2-3):145-166. doi:10.1007/s10709-005-0950-5.
Hecker M, Pané-Farré J and Uwe V (2007) SigB-Dependent General Stress Response in *Bacillus subtilis* and Related Gram-Positive Bacteria. *Annual Review of Microbiology*, 61:215-236. doi:10.1146/annurev.micro.61.080706.093445.
Helmann JD (2016) *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors and defense of the cell envelope. *Current Opinion in Microbiology*, 30:122-132. doi:10.1016/j.mib.2016.02.002.
Helmann JD and Moran CP (2002) RNA Polymerase and Sigma Factors. *In Bacillus subtilis and Its Closest Relatives from Genes to Cells*:527-535. Edited by Sonenshein AL, Hoch JA, Losick R. Washington, DC: American Society for Microbiology. doi: 10.1128/9781555817992.
Henrissat B, Callebaut I, Fabrega S, Lehn P, Mornon JP, Davies G (1995) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. *Proceedings of the National Academy of Sciences*, 92(15):7090-7094. doi:10.1073/pnas.92.15.7090.
Hesketh A, Hill C, Mokhtar J, Novotna G, Tran N, Bibb M, Hong HJ (2011) Genome-wide dynamics of a bacterial response to antibiotics that target the cell envelope. *BMC Genomics*, 12:226. doi:10.1186/1471-2164-12-226.
Draper LA, Ross RP, Hill C, Cotter PD (2008) Lantibiotic Immunity. *Current Protein & Peptide Science*, 9(1):39-49. doi:10.2174/138920308783565750.
Holátko J, Silar R, Rabatinová A, Sanderová H, Halada P, Nešvera J, Krásny L, Pátek M (2012) Construction of in vitro transcription system for *Corynebacterium glutamicum* and its use in the recognition of promoters of different classes. *Applied Microbiology and Biotechnology*, 96(2):521-529. doi:10.1007/s00253-012-4336-1.
Hong HJ, Hutchings MI, Neu JM, Wright GD, Paget MS, Buttner MJ (2004) Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. *Molecular Microbiology*, 52(4):1107-1121. doi:10.1111/j.1365-2958.2004.04032.x.
Horsburgh GJ, Atrih A and Foster SJ (2003) Characterization of LytH, a differentiation-associated peptidoglycan hydrolase of *Bacillus subtilis* involved in endospore cortex maturation. *Journal of Bacteriology*, 185(12):3813-3820. doi:10.1128/JB.185.13.3813-3820.2003.
Huang X, Gaballa A, Cao M, Helmann JD (1999) Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function σ factor, σ^W. *Molecular Microbiology*, 31(1):361-371. doi:10.1046/j.1365-2958.1999.01180.x.
Huang X, Pinto D, Fritz, G, Mascher T (2015) Environmental sensing in Actinobacteria: A comprehensive survey on the signaling capacity of this phylum. *Journal of Bacteriology*, 197(15):2517-2535. doi:10.1128/JB.00176-15.
Hubin EA, Tabib-Salazar A, Humphrey LJ, Flack JE, Olinares PD, Darst SA, Campbell EA, Paget

MS (2015) Structural, functional, and genetic analyses of the actinobacterial transcription factor RbpA. *Proceedings of the National Academy of Sciences*, 23(112)7171-7176. doi:10.1073/pnas.1504942112.
Hubin EA, Lilic M, Darst, SA, Campbell EA (2017) Structural insights into the mycobacteria transcription initiation complex from analysis of X-ray crystal structures. *Nature Communications*, 8:16072. doi: 10.1038/ncomms16072.
Hughes KT and Mathee K (1998) THE ANTI-SIGMA FACTORS. *Annual Review of Microbiology*, 53:231-286. doi:10.1146/annurev.micro.52.1.231.
Iber D (2006) A quantitative study of the benefits of co-regulation using the spoIIA operon as an example. *Molecular Systems Biology*, 2:43. doi:10.1038/msb4100084.
Ingolia NT and Murray AW (2007) Positive-Feedback Loops as a Flexible Biological Module. *Current Biology*, 17(8):668-677. doi:10.1016/j.cub.2007.03.016.
Jeong DE, Park SH, Pan JG, Kim EJ, Choi SK (2015) Genome engineering using a synthetic gene circuit in *Bacillus subtilis*. *Nucleic Acids Research*, 43(6):e42. doi:10.1093/nar/gku1380.
Jogler C, Waldmann J, Huang X, Jogler M, Glöckner FO, Mascher T, Kolter R (2012). Identification of proteins likely to be involved in morphogenesis, cell division, and signal transduction in planctomycetes by comparative genomics. *Journal of Bacteriology*, 194(23):6419-6430. doi:10.1128/JB.01325-12.
Jordan S, Junker A, Helmann JD, Mascher T (2006). Regulation of LiaRS-dependent gene expression in *Bacillus subtilis*: Identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. *Journal of Bacteriology*, 188(14):5153-5166. doi:10.1128/JB.00310-06.
Joseph P, Fantino JR, Herbaud ML, Denizot F (2001) Rapid orientated cloning in a shuttle vector allowing modulated gene expression in *Bacillus subtilis*. *FEMS Microbiology Letters*, 205(1):91-97. doi:10.1016/S0378-1097(01)00449-9.
Joseph P, Fichant G, Quentin Y, Denizot F (2002) Regulatory relationship of two-component and ABC transport systems and clustering of their genes in the *Bacillus*/*Clostridium* group, suggest a functional link between them. *Journal of Molecular Microbiology Biotechnol*ogy, 4(5):503-513.
Karlin S, Mrázek J, Campbell A, Kaiser D (2001). Characterizations of highly expressed genes of four fast-growing bacteria. *Journal of Bacteriology*, 183(17):5025-5040. doi:10.1128/JB.183.17.5025-5040.2001.
Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, 10(6):845-858. doi:10.1038/nprot.2015.053.
Kelly JR, Rubin AJ, Davis JH, Ajo-Franklin CM, Cumbers J, Czar MJ, Mora K, Glieberman AL, Monie DD, Endy D (2009) Measuring the activity of BioBrick promoters using an in vivo reference standard. *Journal of Biological Engineering*, 3:4. doi:10.1186/1754-1611-3-4.
Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) *Practical Streptomyces Genetics*. *The John Innes Foundation, Norwich*. doi:10.4016/28481.01.
Kim YI, Burton RE, Burton BM, Sauer RT, Baker TA (2000) Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. *Molecular Cell*, 5(4):639-648. doi:10.1016/S1097-2765(00)80243-9.
Klein C, Kaletta C and Entian KD (1993) Biosynthesis of the lantibiotic subtilin is regulated by a histidine kinase/response regulator system. *Applied and Environmental Microbiology*, 149(3):209-224. doi:10.1016/j.jconrel.2010.09.023.
Kormanec J, Sevcikova B, Novakova R, Dagmar H, Bronislava R, Mingyar E (2016) The complex roles and regulation of stress response σ factors in *Streptomyces coelicolor*. In

Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria, pp. 328-343. doi: 10.1002/9781119004813.ch29.
Korzheva N, Mustaev A, Kozlov M, Malhotra A, Nikiforov V, Goldfarb A, Darst SA (2000) A structural model of transcription elongation. *Science*, 289(5479):619-625. doi:10.1126/science.289.5479.619.
Kraus A, Hueck C, Gärtner D, Hillen W (1994) Catabolite repression of the *Bacillus subtilis xyl* operon involves a cis element functional in the context of an unrelated sequence, and glucose exerts additional xylR-dependent repression. *Journal of Bacteriology*, 176(6):1738-1745. doi: 10.1128/jb.176.6.1738-1745.1994.
Laub MT and Goulian M (2007) Specificity in Two-Component Signal Transduction Pathways. *Annual Review of Genetics*, 41:121-145. doi:10.1146/annurev.genet.41.042007.170548.
Le TB, Fiedler HP, den Hengst CD, Ahn SK, Maxwell A, Buttner MJ (2009) Coupling of the biosynthesis and export of the DNA gyrase inhibitor simocyclinone in *Streptomyces* antibioticus. *Molecular Microbiology*, 72(6):1462-1474. doi:10.1111/j.1365-2958.2009.06735.x.
Lee EJ, Cho YH, Kim HS, Roe JH (2004) Identification of sigmaB-dependent promoters using consensus-directed search of *Streptomyces coelicolor* genome. *Journal of Microbiology (Seoul, Repub. Korea)*, 42(2):147-151.
Lee J and Borukhov S (2016) Bacterial RNA Polymerase-DNA Interaction—The Driving Force of Gene Expression and the Target for Drug Action. *Frontiers in Molecular Biosciences*, 3:73. doi:10.3389/fmolb.2016.00073.
Lepesant JA, Billault A, Kejzlarová-Lepesant J, Pascal M, Kunst F, Dedonder R (1975) Identification of the structural gene for sucrase in *Bacillus subtilis* Marburg. *Biochimie*, 56(11-12):1465-1470. doi:10.1016/S0300-9084(75)80268-9.
Loinger A and Biham O (2009) Analysis of genetic toggle switch systems encoded on plasmids. *Physical Review Letters*, 103(6):068104. doi:10.1103/PhysRevLett.103.068104.
Lonetto MA, Brown KL, Rudd KE, Buttner MJ (1994) Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. *Proceedings of the National Academy of Sciences of the United States of America*, 91(16):7573-7577. doi:10.1073/PNAS.91.16.7573.
Mak S, Xu Y and Nodwell JR (2014) The expression of antibiotic resistance genes in antibiotic-producing bacteria. *Molecular Microbiology*, 93(3):391-402. doi:10.1111/mmi.12689.
Makinoshima H and Glickman MS (2005) Regulation of *Mycobacterium tuberculosis* cell envelope composition and virulence by intramembrane proteolysis. *Nature*, 436(7049):406-409. doi:10.1038/nature03713.
Manson JM, Keis S, Smith JM, Cook GM (2004) Acquired bacitracin resistance in *Enterococcus faecalis* is mediated by an ABC transporter and a novel regulatory protein, BcrR. *Antimicrobial Agents and Chemotherapy*, 48(10):3743-3748. doi:10.1128/AAC.48.10.3743-3748.2004.
Mao XM, Sun N, Zheng Y, Li YQ (2017) Development of Series of Affinity Tags in *Streptomyces. Scientific Reports*, 7(1):6854. doi:10.1038/s41598-017-07377-4.
Marles-Wright J and Lewis RJ (2010) The stressosome: molecular architecture of a signalling hub. *Biochemical Society Transactions*, 38(4):928-933. doi:10.1042/BST0380928.
Martínez LF, Bishop A, Parkes L, Del Sol R, Salerno P, Sevcikova B, Mazurakova V, Kormanec J, Dyson P (2009) Osmoregulation in *Streptomyces coelicolor*: Modulation of SigB activity by OsaC. *Molecular Microbiology*, 71(5):1250-1262. doi:10.1111/j.1365-2958.2009.06599.x.
Mascher T, Zimmer SL, Smith TA, Helmann JD (2004) Antibiotic-inducible promoter regulated

by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. *Antimicrobial Agents and Chemotherapy*, 48(8):2888-2896. doi:10.1128/AAC.48.8.2888-2896.2004.
Mascher, T (2013) Signaling diversity and evolution of extracytoplasmic function (ECF) σ factors. *Current Opinion in Microbiology*, 16(2):148-155. doi:10.1016/j.mib.2013.02.001.
McBride SM and Sonenshein AL (2011) The *dlt* operon confers resistance to cationic antimicrobial peptides in *Clostridium difficile*. *Microbiology*,157(5):1457-1465. doi:10.1099/mic.0.045997-0.
Meng W, Belyaeva T, Savery NJ, Busby SJ, Ross WE, Gaal T, Gourse RL, Thomas MS (2001) UP element-dependent transcription at the *Escherichia coli rrnB* P1 promoter: positional requirements and role of the RNA polymerase alpha subunit linker. *Nucleic Acids Research*, 29(20):4166-4178. doi:10.1093/nar/29.20.4166.
Michna RH, Zhu B, Mäder U, Stülke J (2016) SubtiWiki 2.0 - An integrated database for the model organism *Bacillus subtilis*. *Nucleic Acids Research*, 44(D1):D654-62 doi:10.1093/nar/gkv1006.
Mileyko Y, Joh RI and Weitz JS (2008) Small-scale copy number variation and large-scale changes in gene expression. *Proceedings of the National Academy of Sciences*, 105(43):16659-16664. doi:10.1073/pnas.0806239105.
Morey KJ, Antunes MS, Barrow MJ, Solorzano FA, Havens KL, Smith JJ, Medford J (2012) Crosstalk between endogenous and synthetic components - synthetic signaling meets endogenous components. *Biotechnology Journal*, 7(7):846-855. doi:10.1002/biot.201100487.
Münch R, Hiller K, Barg H, Heldt D, Linz S, Wingender E, Jahn D (2003) PRODORIC: Prokaryotic database of gene regulation. *Nucleic Acids Research*, 31(1):266-269. doi:10.1093/nar/gkg037.
Murakami KS and Darst SA (2003) Bacterial RNA polymerases: The wholo story. *Current Opinion in Structural Biology*, 13(1):31-39. doi:10.1016/S0959-440X(02)00005-2.
Neumüller AM, Konz D and Marahiel MA (2001) The two-component regulatory system BacRS is associated with bacitracin “self-resistance” of *Bacillus licheniformis* ATCC 10716. *European Journal of Biochemistry*, 268(11):3180-3189. doi:10.1046/j.1432-1327.2001.02203.x.
Nithya V and Halami PM (2012) Antibacterial Peptides, Probiotic Properties and Biopreservative Efficacy of Native *Bacillus* Species Isolated from Different Food Sources. *Probiotics and Antimicrobial Proteins*, 4(4):279-290. doi:10.1007/s12602-012-9115-x.
Novakova R, Odnogova Z, Kutas P, Feckova L, Kormanec J (2010) Identification and characterization of an indigoidine-like gene for a blue pigment biosynthesis in *Streptomyces aureofaciens* CCM 3239. *Folia Microbiologica*, 55(2):119-125. doi:10.1007/s12223-010-0018-5.
Ohki R, Giyanto, Tateno K, Masuyama W, Moriya S, Kobayashi K, Ogasawara N (2003) The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*. *Molecular Microbiology*, 49(4):1135-1144. doi:10.1046/j.1365-2958.2003.03653.x.
Österberg S, del Peso-Santos T and Shingler V (2011) Regulation of Alternative Sigma Factor Use. *Annual Review of Microbiology*, 65:37-55. doi:10.1146/annurev.micro.112408.134219.
Paget MS, Hong HJ, Bibb MJ (2002) The ECF sigma factors of *Streptomyces coelicolor A3(2)*. in *SGM Symposium 6.* Cambridge, UK: Cambridge University Press. ISBN 0 521 81388 3, pp. 105–125.
Paget, MS (2015) Bacterial sigma factors and anti-sigma factors: Structure, function and

distribution. *Biomolecules*, 5(3):1245-1265. doi:10.3390/biom5031245.
Paget MS and Helmann JD (2003) The sigma70 family of sigma factors. *Genome Biology*, 4(1):203. doi:10.1186/gb-2003-4-1-203.
Pátek M, Nesvera J, Guyonvarch A, Reyes O, Leblon G (2003) Promoters of *Corynebacterium glutamicum*. *Journal of Biotechnology*, 104(1-3):311-323. doi:10.1016/S0168-1656(03)00155-X.
Perez-Martin J, Rojo F and de Lorenzo V (1994) Promoters responsive to DNA bending: a common theme in prokaryotic gene expression. *Microbiological Reviews*, 58(2):268-290.
Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Götz F (1999) Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *Journal of Biological Chemistry*, 274(13):8405-8410. doi:10.1074/jbc.274.13.8405.
Peterson E and Kaur P (2018) Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Frontiers in Microbiology*, 9:2928. doi:10.3389/fmicb.2018.02928.
Pinto D, Vecchione S, Wu H, Mauri M, Mascher T, Fritz G (2018a) Engineering orthogonal synthetic timer circuits based on extracytoplasmic function factors. *Nucleic Acids Research*, 46(14):7450-7464. doi:10.1093/nar/gky614.
Pinto D, Dürr F, Froriep F, Araújo D, Liu Q, Mascher T (2019) Extracytoplasmic function σ factors can be implemented as robust heterologous genetic switches in *Bacillus subtilis*. *iScience*, 13:380-390. doi:10.1016/j.isci.2019.03.001.
Pinto D and Mascher T (2016) The ECF Classification: A Phylogenetic Reflection of the Regulatory Diversity in the Extracytoplasmic Function σ Factor Protein Family. In *Stress and Environmental Regulation of Gene Expression and Adaptation in Bacteria*. Hoboken John Wiley & Sons, Inc64-96. doi:10.1002/9781119004813.ch7.
Podlesek Z, Comino A, Herzog-Velikonja B, Grabnar M (2000) The role of the bacitracin ABC transporter in bacitracin resistance and collateral detergent sensitivity. *FEMS Microbiology Letters*, 188(1):103-106. doi:10.1016/S0378-1097(00)00218-4.
Podlesek Z, Herzog B and Comino A (1997) Amplification of bacitracin transporter genes in the bacitracin producing *Bacillus licheniformis*. *FEMS Microbiology Letters*, 157(1):201-205. doi:10.1016/S0378-1097(97)00477-1.
Popp PF, Dotzler M, Radeck J, Bartels J, Mascher T (2017) The *Bacillus* BioBrick Box 2.0: Expanding the genetic toolbox for the standardized work with *Bacillus subtilis*. *Scientific Reports*, 7(1):15058. doi:10.1038/s41598-017-15107-z.
Pospiech A and Neumann B (1995) A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends in Genetics*, 11(6):217-218. doi:10.1016/S0168-9525(00)89052-6.
Price MN, Huang KH, Arkin AP, Alm EJ (2005) Operon formation is driven by co-regulation and not by horizontal gene transfer. *Genome Research*, 15(6):809-819. doi:10.1101/gr.3368805.
Price MN, Arkin AP and Alm EJ (2006) The life-cycle of operons. *PLoS Genetics*, 2(6):e96. doi:10.1371/journal.pgen.0020096.
Radeck J, Kraft K, Bartels J, Cikovic T, Dürr F, Emenegger J, Kelterborn S, Sauer C, Fritz G, Gebhard S, Mascher T (2013) The *Bacillus* BioBrick Box: Generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*. *Journal of Biological Engineering*, 7(1):29. doi:10.1186/1754-1611-7-29.
Revilla-Guarinos A, Gebhard S, Mascher T, Zúñiga M (2014) Defence against antimicrobial peptides: Different strategies in Firmicutes. *Environmental Microbiology*, 16(5):1225-1237. doi:10.1111/1462-2920.12400.
Rhodius VA, Segall-Shapiro TH, Sharon BD, Ghodasara A, Orlova E, Tabakh H, Burkhardt DH,

REFERENCES

Clancy K, Peterson TC, Gross CA, Voigt C. (2013) Design of orthogonal genetic switches based on a crosstalk map of σs, anti-σs, and promoters. *Molecular Systems Biology*, 9:702. doi:10.1038/msb.2013.58.

Rietkötter E, Hoyer D and Mascher T (2008) Bacitracin sensing in *Bacillus subtilis*. *Molecular Microbiology*, 68(3):768-785. doi:10.1111/j.1365-2958.2008.06194.x.

Rosenberg M and Court D (1979) Regulatory sequences involved in the promotion and termination of RNA transcription. *Annual review of genetics*, 13:319-353. doi:10.1146/annurev.ge.13.120179.001535.

Saar-Dover R, Bitler A, Nezer R, Shmuel-Galia L, Firon A, Shimoni E, Trieu-Cuot P, Shai Y (2012) D-Alanylation of Lipoteichoic Acids Confers Resistance to Cationic Peptides in Group B *Streptococcus* by Increasing the Cell Wall Density. *PLoS Pathogens*, 8(9):e1002891. doi:10.1371/journal.ppat.1002891.

Saecker RM, Record MT and Dehaseth PL (2011) Mechanism of bacterial transcription initiation: RNA polymerase - Promoter binding, isomerization to initiation-competent open complexes, and initiation of RNA synthesis. *Journal of Molecular Biology*, 412(5):754-771. doi:10.1016/j.jmb.2011.01.018.

Saïd-Salim B, Mostowy S, Kristof AS, Behr MA (2006) Mutations in *Mycobacterium tuberculosis* Rv0444c, the gene encoding anti-SigK, explain high level expression of MPB70 and MPB83 in *Mycobacterium bovis*. *Molecular Microbiology*, 62(5):1251-1263. doi:10.1111/j.1365-2958.2006.05455.x.

Sambrook JR and Russell DW (2001) Molecular cloning: a laboratory manual.*Cold Spring Harbor, N. Y: Cold Spring Harbor Laboratory*.

Sarker SD, Nahar L and Kumarasamy Y (2007) Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods*, 42(4):321-324. doi:10.1016/j.ymeth.2007.01.006.

Schlatter DC and Kinkel LL (2014) Global biogeography of *Streptomyces* antibiotic inhibition, resistance, and resource use. *FEMS Microbiology Ecology*, 88(2):386-397. doi:10.1111/1574-6941.12307.

Schneider E (2001) ABC transporters catalyzing carbohydrate uptake. *Research in Microbiology*, 152(3-4):303-310. doi:10.1016/S0923-2508(01)01201-3.

Schrödinger L (2015) The PyMOL molecular graphics system, version 1.8. *https://www.pymol.org/citing*. doi:mmi1753 [pii].

Serrano L (2007) Synthetic biology: promises and challenges. *Molecular Systems Biology*, 3:158. doi:10.1038/msb4100202.

Shaaly A, Kalamorz F, Gebhard S, Cook GM (2013) Undecaprenyl pyrophosphate phosphatase confers low-level resistance to bacitracin in *Enterococcus faecalis*. *Journal of Antimicrobial Chemotherapy*, 68(7):1583-1593. doi:10.1093/jac/dkt048.

Sham LT, Butler EK, Lebar MD, Kahne D, Bernhardt TG, Ruiz N (2014) MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. *Science*, 345(6193):220-222. doi:10.1126/science.1254522.

Sherwood EJ, Hesketh AR and Bibb, MJ (2013) Cloning and analysis of the planosporicin lantibiotic biosynthetic gene cluster of *Planomonospora alba*, *Journal of Bacteriology*, 195(10):2309-2321. doi:10.1128/JB.02291-12.

Shin JH, Brody MS and Price CW (2010) Physical and antibiotic stresses require activation of the RsbU phosphatase to induce the general stress response in *Listeria monocytogenes*. *Microbiology*, 156(9):2660-2669. doi:10.1099/mic.0.041202-0.

Shin J and Noireaux V (2012) An *E. coli* cell-free expression toolbox: Application to synthetic

gene circuits and artificial cells. *ACS Synthetic Biology*, 1(1):29-41. doi: 10.1021/sb200016s.
Shukla J, Gupta R, Thakur KG, Gokhale R, Gopal (2014) Structural basis for the redox sensitivity of the *Mycobacterium tuberculosis* SigK-RskA σ-anti-σ complex. *Acta Crystallographica Section D: Biological Crystallography*, 70(Pt4):1026-1036. doi:10.1107/S1399004714000121.
Siewert G and Strominger JL (1967) Bacitracin: An inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in biosynthesis of the peptidoglycan of bacterial cell walls. *Proceedings of the National Academy of Sciences of the United States of America*, 57(3):767-773. doi:10.1073/pnas.57.3.767.
Silva I and Ferreira H (2013) Drug Sensitivity Assay of *Xanthomonas citri subsp. citri* Using REMA Plate Method. *BIO-PROTOCOL*, 16(3):e861. doi: 10.21769/BioProtoc.861.
Singh SK, Grimaud R, Hoskins JR, Wickner S, Maurizi MR (2000) Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP. *Proceedings of the National Academy of Sciences*, 116(97):8898-8903. doi:10.1073/pnas.97.16.8898.
Sklar JG, Makinoshima H, Schneider JS, Glickman MS (2010) *M. tuberculosis* intramembrane protease Rip1 controls transcription through three anti-sigma factor substrates. *Molecular Microbiology*, 77(3):605-617. doi:10.1111/j.1365-2958.2010.07232.x.
Stanton BC, Nielsen AA, Tamsir A, Clancy K, Peterson T, Voigt CA (2014) Genomic mining of prokaryotic repressors for orthogonal logic gates. *Nature Chemical Biology*, 10(2):99-105. doi:10.1038/nchembio.1411.
Staroń A, Sofia HJ, Dietrich S, Ulrich LE, Liesegang H, Mascher T (2009) The third pillar of bacterial signal transduction: Classification of the extracytoplasmic function (ECF) σ factor protein family. *Molecular Microbiology*, 74(3):557-581. doi:10.1111/j.1365-2958.2009.06870.x.
Stricker J, Cookson S, Bennett MR, Mather WH, Tsimring LS, Hasty J (2008) A fast, robust and tunable synthetic gene oscillator. *Nature*, 456(7221):516-519. doi:10.1038/nature07389.
Stuttard C (1982) Temperate Phages of *Streptomyces venezuelae*: Lysogeny and Host Specificity Shown by Phages SV1 and SV2. *Microbiology*, 155:1219-123.
Sun D, Liu C, Zhu J, Liu W (2017) Connecting metabolic pathways: Sigma factors in *Streptomyces* spp. *Frontiers in Microbiology*, 8:2546. doi:10.3389/fmicb.2017.02546.
Surette MD and Wright GD (2017) Lessons from the Environmental Antibiotic Resistome. *Annual Review of Microbiology*, 71:309-329. doi:10.1146/annurev-micro-090816-093420.
Sutherland IW (1977) Bacterial Exopolysaccharides - Their Nature and Production. In *Surface Carbohydrates of the Prokaryotic Cell*. Academic Press, London New York San Francisco, pp27-96.
Tabib-Salazar A, Liu B, Doughty P, Lewis RA, Ghosh S, Parsy ML, Simpson PJ, O'Dwyer, Matthews SJ, Paget MS (2013) The actinobacterial transcription factor RbpA binds to the principal sigma subunit of RNA polymerase. *Nucleic Acids Research*, 41(11):5679-5691. doi:10.1093/nar/gkt277.
Tao L and Biswas I (2015) Degradation of SsrA-tagged proteins in streptococci. *Microbiology (United Kingdom)*, 161(Pt 4):884-894. doi:10.1099/mic.0.000048.
Toymentseva AA, Schrecke K, Sharipova MR, Mascher T (2012) The LIKE system, a novel protein expression toolbox for *Bacillus subtilis* based on the *liaI* promoter. *Microbial Cell Factories*, 11:143. doi:10.1186/1475-2859-11-143.
Toyoda K, Teramoto H, Yukawa H, Inui M (2015) Expanding the regulatory network governed by the extracytoplasmic function sigma factor σH in *Corynebacterium glutamicum*. *Journal of Bacteriology*, 197(3):483-496. doi:10.1128/JB.02248-14.
Tsuda H*, Yamashita Y, Shibata Y, Nakano Y, Koga T* (2002) Genes involved in bacitracin

resistance in *Streptococcus mutans*. *Antimicrobial Agents and Chemotherapy*, 46(12):3756-3764. doi:10.1128/AAC.46.12.3756-3764.2002.
Ulrich LE, Koonin EV and Zhulin IB (2005) One-component systems dominate signal transduction in prokaryotes. *Trends in Microbiology*, 13(2):52-56. doi:10.1016/j.tim.2004.12.006.
Urban S (2009) Making the cut: Central roles of intramembrane proteolysis in pathogenic microorganisms. *Nature Reviews Microbiology*, 7(6):411-423. doi:10.1038/nrmicro2130.
Del Vecchio D, Dy AJ and Qian Y (2016) Control theory meets synthetic biology. *Journal of the Royal Society Interface*, 13(120). doi:10.1098/rsif.2016.0380.
Veyrier F, Saïd-Salim B and Behr MA (2008) Evolution of the mycobacterial SigK regulon. *Journal of Bacteriology*, 190(6):1891-1899. doi:10.1128/JB.01452-07.
Vidal-Aroca F, Meng A, Minz T, Page MG, Dreier J (2009) Use of resazurin to detect mefloquine as an efflux-pump inhibitor in *Pseudomonas aeruginosa* and *Escherichia coli*. *Journal of Microbiological Methods*, 79(2):232-237. doi:10.1016/j.mimet.2009.09.021.
Villas-Bôas SG and Bruheim P (2007) Cold glycerol-saline: The promising quenching solution for accurate intracellular metabolite analysis of microbial cells. *Analytical Biochemistry*, 370(1):87-97. doi:10.1016/j.ab.2007.06.028.
Walsh F and Duffy B (2013) The Culturable Soil Antibiotic Resistome: A Community of Multi-Drug Resistant Bacteria. *PLoS ONE*, 8(6):e65567. doi:10.1371/journal.pone.0065567.
Wecke T, Halang P, Staron A, Dufour YS, Donohue TJ, Mascher T (2012) Extracytoplasmic function σ factors of the widely distributed group ECF41 contain a fused regulatory domain. *MicrobiologyOpen*, 1(2):194-213. doi:10.1002/mbo3.22.
Westbrook AM and Lucks JB (2017) Achieving large dynamic range control of gene expression with a compact RNA transcription-translation regulator. *Nucleic Acids Research*,45(9):5614-5624. doi:10.1093/nar/gkx215.
Wiegert T and Schumann W (2001) SsrA-mediated tagging in *Bacillus subtilis*. *Journal of Bacteriology*, 183(13)3885-3889. doi:10.1128/JB.183.13.3885-3889.2001.
Wise AA and Price CW (1995) Four additional genes in the *sigB* operon of *Bacillus subtilis* that control activity of the general stress factor sigma B in response to environmental signals. *Journal of Bacteriology*, 177(1):123-133.
Wösten MM (1998) Eubacterial sigma-factors. *FEMS microbiology reviews*, 22(3):127-150. doi:10.1111/j.1574-6976.1998.tb00364.x.
Wuttge S, Bommer M, Jäger F, Martins BM, Jacob S, Licht A, Scheffel F, Dobbek H, Schneider E (2012) Determinants of substrate specificity and biochemical properties of the sn-glycerol-3-phosphate ATP binding cassette transporter (UgpB-AEC2) of *Escherichia coli*. *Molecular Microbiology*, 86(4):908-920. doi:10.1111/mmi.12025.
Zaslaver A, Mayo AE, Rosenberg R, Bashkin P, Sberro H, Tsalyuk M, Surette MG, Alon U (2004) Just-in-time transcription program in metabolic pathways. *Nature Genetics*, 36(5):486-491. doi:10.1038/ng1348.
Zhang C, Tsoi R and You L (2016) Addressing biological uncertainties in engineering gene circuits. *Integrative Biology (United Kingdom)*, 8(4):456-464. doi:10.1039/c5ib00275c.
Zhang H, Finiguerra M, Dam HG, Yousong H, Xu Donghui, Liu G, Lin S (2013) An improved method for achieving high-quality RNA for copepod transcriptomic studies. *Journal of Experimental Marine Biology and Ecology*, 446:57-66. doi:10.1016/j.jembe.2013.04.021.
Zuo Y and Steitz TA (2015) Crystal structures of the *E.coli* transcription initiation complexes with a complete bubble. *Molecular Cell*,58(3):534-540. doi:10.1016/j.molcel.2015.03.010.

Curriculum Vitae

Acknowledgments

I will start with a big thanks to Professor Dr. Thorsten Mascher for accepting my application and opening the doors of his lab to me. Thank you for all the support during all these years.

Thanks to the Brazilian Federal Agency CAPES for funding my PhD for 4 years (2013 to 2017).

Thanks to Professor Dr. Susanne Gebhard for guiding me during my first years and for making me passionate about my project.

Thanks to Dr. Daniela Pinto for being there, always with the right answer or a great idea (you should know you are a genius). Thank you for the patience and for bringing my native language back to my working life. Thank you for being more than a supervisor, thank you for being a friend.

Thanks to Professor Dr. Heinrich Jung who accepted to be the second revisor of this work and all the other committee members that accepted my invitation.

Thanks to Qiang for all the help and all the fun! Thank you for being so calm and pass this peace to everyone around. Thank you for being a good friend. You and Daniela made the weekends in the lab sound not bad at all.

Thanks to Qian for bring all the sweeteness that a person can have to our lab lives in Dresden and thank you to Karen for all nice talks and fun. Thank you for bringing me to the gym girls!

Thanks to Chong and Sonja for making the lab 007 the best one! Thank you for helping me to settle in the lab and in Munich. You two will always be in my heart girls.

Thanks to Bruno, Ana and Gustavo for bringing home to Munich and for being by my side in all moments. O ‘My place’ sempre trará as melhores lembranças da vida na Deutschland.

Thanks to the Annett and Susanne for supporting everyone in the lab.

Thanks to Diana, Julia, Jara, Franzi, Phillip, Nina, Daniela 2, Szolt, Ainhoa, Martin, Helge, Didi, Caro and all the people that were part of AG Mascher at some point.

Thanks to all the colleagues from AG Bramkamp and AG Jung.

Dziękuję Mateusz for all the support and understanding. I wouldn’t have made it without you. Thank you for bringing light to the darkest days.

Obrigada Euter por fazer parte do inicio dessa jornada e por ter abraçado muitos dos meus sonhos como se fossem seus.

Obrigada a todos os meus amigos no Brasil por não deixar a distância nos distanciar.

Obrigada a toda minha família. Obrigada pai e mãe por sempre me incentivarem e por enteder minhas escolhas. Eu devo tudo a vocês.

Obrigada | Thank you | Danke Schön | 谢谢 |Dziękuję | Grazie | Gracias