Quorum sensing in Streptomyces coelicolor

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Chapter 1

General introduction

I. Bacterial Quorum Sensing

United we stand

Bacterial survival in the environment is challenging. To increase their survival capacities as unicellular organisms, bacteria employ mechanisms by which they can join forces with other members in a population. For this purpose bacteria communicate through the sending and sensing of chemical compounds that reflect an increase in cell density or changes in environmental or physiological conditions (Xavier *et al.*, 2003, Boyer *et al.*, 2009). This mechanism of bacterial communication is called Quorum Sensing (QS) (Fuqua *et al.*, 1994) and allows bacteria to socialise, coordinating or synchronising their behaviour to regulate diverse mechanisms that provide a benefit to the community, and thus act as multicellular organisms.

In a QS mechanism, bacteria produce and release small compounds to the extracellular environment in a specific phase of growth, or in response to specific environmental or physiological conditions (Winzer *et al.*, 2002). These compounds are signalling molecules that accumulate until they reach a certain threshold before they are perceived by a specific receptor protein creating a specific response. This response causes a coordinated behaviour in all members of the population, namely a coordinated change in gene expression (Figure 1). This mechanism allows bacteria to act as a big multicellular organism. Other extracellular compounds released by bacteria are not QS-signalling molecules, such as some toxic bacterial metabolites, because the response produced by them is limited to the mechanism needed to detoxify them (Winzer *et al.*, 2002). QS-signalling molecules comply with the above criteria and produce a cellular response that provides an advantage to the population.

A large variety of mechanisms in the bacterial cell are regulated by QS. These are generally mechanisms that are unproductive when carried out by a single cell, and become effective when carried out by a group. Examples are bioluminescence, virulence factor production, biofilm formation, secondary metabolites and antibiotic production, competence and sporulation (Figure 1) (reviewed in Fuqua *et al.* (2002), Bassler *et al.* (2006), Williams *et al.* (2007), Garg *et al.* (2014)). A comprehensive understanding of bacterial quorum sensing provides important and useful tools for the discovery and

development of new antimicrobial agents. QS itself has also become a target to avoid some of these bacterial responses, e.g. for antivirulence drugs or to prevent biofilm formation. These QS related antimicrobials differ from the traditional antibiotics that kill or inhibit the growth of the pathogens, because they do not affect growth, but just avoid the production of pathogenicity factors, such as virulence factors or the formation of biofilms, thus avoiding the selective pressure that causes the appearance of antibiotic resistance. In addition, QS is an important tool to discover new natural compounds with antibacterial activity by awakening cryptic biosynthetic gene clusters. QS also has interesting applications in synthetic biology (artificial QS networks have been engineered with different biotechnological and therapeutical application), as biosensors for pathogen diagnosis and cancer cell detection, and to prevent biofouling (reviewed in Choudhary *et al.* (2010) and Garg *et al.* (2014)).

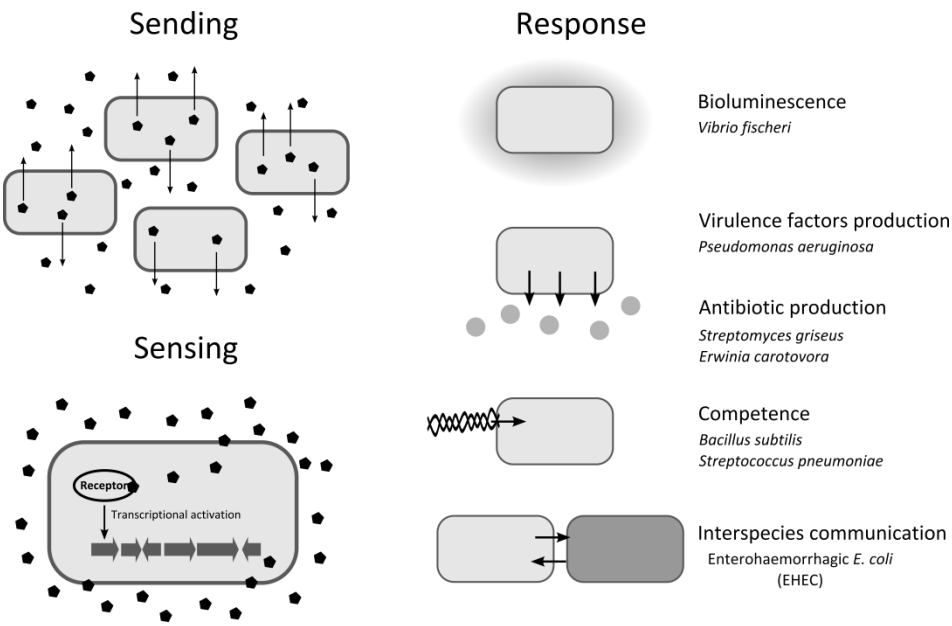

Figure 1. Schematic representation of bacterial Quorum Sensing. Sending: Bacteria produce and release small compounds to the extracellular environment where they accumulate. Sensing: When the concentration of these small compounds or signalling molecules reaches a certain level, specific receptor proteins “sense” their presence which provokes transcriptional activation of specific genes. This creates a specific

response in all members of the population. Response: Quorum Sensing regulates mechanisms such as bioluminescence, virulence factor production, antibiotic production, competence and interspecies communication.

Diversity of QS mechanisms in bacteria

Quorum sensing mechanisms are widely distributed in the bacterial world. Three main classes of bacterial signalling molecules exist; the acyl homoserine lactones (AHLs), characteristic of Gram-negative bacteria, small modified peptides in Gram-positive bacteria and AI-2 (autoinducer-2) found in both Gram-positive and negative bacteria that is thought to facilitate interspecies communication.

AHL systems are the most common in Gram-negative bacteria and the most studied. They control different mechanisms in very diverse Gram-negative bacteria such as bioluminescence in *Vibrio fischeri*, virulence factor production in *Pseudomonas aeruginosa*, virulence factors and antibiotic production in *Erwinia carotovora*, root nodulation in *Rhizobium leguminosarum* and conjugation for transfer of the Ti plasmid in *Agrobacterium tumefaciens* (Miller *et al.*, 2001, Henke *et al.*, 2004, Williams *et al.*, 2007, Atkinson *et al.*, 2009, Garg *et al.*, 2014). These molecules are synthesised by LuxI-type synthases and sensed by LuxR type specific receptors that upon binding of the signalling molecules, bind to the DNA and activate transcription of their target genes (Fuqua *et al.*, 2002, Li *et al.*, 2012).

Gram-positive bacteria predominantly communicate with small post-translationally processed peptides. These secreted peptides are recognised by membrane associated two component sensor histidine kinase systems. Signal transduction occurs by phosphorylation cascades of regulation that ultimately end up activating a transcriptional regulator responsible for regulation of target genes (Kleerebezem *et al.*, 1997, Sturme *et al.*, 2002). These autoinducer peptides (AIPs) can be linear and unmodified, such as the competence and sporulation factor (CSF) from *Bacillus subtilis* (Solomon *et al.*, 1995), the competence stimulatory peptide (CSP) from *Streptococcus pneumonia* (Håvarstein *et al.*, 1995), or class II antimicrobial peptides (AMP) of lactic acid bacteria (Nes *et al.*, 2000). Some AIPs present different modifications. Nisin, produced by *Lactococcus lactis* or subtilin produced by *B. subtillis*, which are class I AMP or lantibiotics, contain dehydrated amino acids and typical β-methyl-lanthionines (Willey *et al.*, 2007). Other modified

peptides contain cyclic lactones or thiolactone structures, such as AIP-1 from *Staphylococcus aureus* controlling the production of virulence factors in this human pathogen (Ji *et al.*, 1995). *Bacillus subtilis* produces the competence factor ComX, which is a modified peptide containing an isoprenylated tryptophan residue (Magnuson *et al.*, 1994) (reviewed in Kleerebezem *et al.* (1997), Sturme *et al.* (2002), Thoendel *et al.* (2010)).

A different group of molecules, the AI-2-type autoinducers are synthesised and recognised by both Gram-positive and negative bacteria, and are thought to be a universal signal that facilitates interspecies communication (Xavier *et al.*, 2003). Autoinducer-2 (AI-2) is a furanosyl borate diester found for the first time in the Gram-negative bioluminescent bacteria *Vibrio harveyi* (Bassler *et al.*, 1994, Chen *et al.*, 2002). Many other diverse Gram-negative and Gram-positive genera have been reported to produce the same or a related molecule (Surette *et al.*, 1999, Miller *et al.*, 2001). All AI-2-type compounds are derived from a common precursor, 4,5-dehydroxy-2,3 pentanedione (DPD), product of the LuxS enzyme (Xavier *et al.*, 2005). The AI-2 signalling system has been studied in *Vibrio harveyi*: the AI-2 receptor is LuxP, which activates a phosphorylation cascade that leads to the derepression of LuxR, activator of genes for bioluminescence (Bassler *et al.*, 1994, Surette *et al.*, 1999, Ng *et al.*, 2009). Besides controlling bioluminesce in *V. harveyi* this QS AI-2 signalling mechanism also controls virulence factor production in *V. cholera* (Miller *et al.*, 2002, Zhu *et al.*, 2002) and biofilm formation in the Gram-positive *Bacillus subtilis* (Lombardia *et al.*, 2006). Highly conserved LuxS homologues are widespread in different Gram-positive and Gram-negative genera. However, the LuxP receptor protein is only present in *Vibrio* species (Sun *et al.*, 2004). Other species might have a different yet unidentified receptor protein but it is also possible that these AI-2/LuxS systems are not involved in signalling and quorum sensing in non-*Vibrio* species. In fact, LuxS also has a role in the activated methyl cycle (AMC) for the detoxification of S-adenosyL methionine (SAM). Due to this a debate exists regarding whether LuxS and AI-2 have a signalling role in non-*Vibrio* organisms or whether AI-2 is an intermediate of the AMC and not a signal (Sun *et al.*, 2004, Turovskiy *et al.*, 2007, Williams *et al.*, 2007, Wang *et al.*, 2012).

An additional QS signalling molecule involved in interspecies communication, autoinducer-3 (AI-3), was found in EHEC (Enterohaemorrhagic *E. coli*). AI-3 is

produced by the intestinal flora and works as a signal activating transcription of virulence genes in EHEC (Sperandio *et al.*, 2003). This autoinducer can also interact with the epinefrin/norepinephrine hormonal system of mammals, which indicates that this compound is also involved in interkingdom communication between pathogenic bacteria and their hosts (Sperandio *et al.*, 2003).

An exception to the utilization of small peptides for signalling in Gram-positive bacteria is found in the genus *Streptomyces*. These organisms mainly produce signalling molecules called γ-butyrolactones that have a similar structure to the AHLs produced by Gram-negative bacteria. These small compounds regulate morphological development and secondary metabolite production in these bacteria. A-factor was the first γ-butyrolactone described and the most studied (Horinouchi *et al.*, 2007). Many other γ-butyrolactones have been described in different species of *Streptomyces*. *Streptomyces* are one of the most important sources of natural antibiotics used in medicine and consequently they are the focus of very relevant lines of investigation for the discovery of new biologically active compounds. In this regard, γ-butyrolactone systems are a highly interesting topic of research since they have the potential for awakening cryptic biosynthetic gene clusters for the production of novel antibiotics. In addition, understanding the regulation of these γ-butyrolactone systems can be a useful tool to manipulate or engineer antibiotic synthesis pathways to overproduce commercially important antibiotics that are produced in very small amounts in nature.

II. Quorum Sensing in *Streptomyces*

The genus *Streptomyces*

Bacteria that live in the soil require exceptional means to survive in such a changeable environment. To overcome the obstacles that they encounter during their life cycle they have developed different mechanisms. *Streptomyces*, a genus of Gram-positive soil-dwelling bacteria, combat these problems by producing a large variety of metabolites, such as pigments for UV light protection, antifungals, antiparasitic agents or antibiotics, as well as extracellular xenobiotic degrading enzymes, to compete against other

organisms in the soil. They also produce other metabolites with important biotechnological applications such as anti-tumor agents, and immunosuppressant drugs, among others (Kieser *et al.*, 2000).

Antibiotic discovery has suffered a decline over the last decades characterised by a gap, or rather a void, in the detection of antibiotics with novel structures (Walsh, 2003, Silver, 2011). However, antibiotic resistant pathogenic bacteria keep emerging raising the need for detection of new antimicrobial compounds or other strategies to combat these pathogens. *Streptomyces* has gained increasing relevance in medicine and biotechnology in the last decades, for being the main source of antibiotics of natural origin used nowadays in medicine (Kieser *et al.*, 2000). Streptomycetes are the focus of many interesting and relevant research strategies due to their ability to produce a wide array of secondary metabolites and antimicrobial compounds, but also because they possess the genetic potential to provide additional novel antibiotics, judged from the abundance in putative secondary metabolite orphan gene clusters found in their genomes (Omura *et al.*, 2001, Bentley *et al.*, 2002, Ikeda *et al.*, 2003, Mochizuki *et al.*, 2003, Ohnishi *et al.*, 2008, Medema *et al.*, 2010, Olano *et al.*, 2014).

Regulation of secondary metabolism in streptomycetes is complex and many different mechanisms are involved. Antibiotic production is regulated by diverse environmental and physiological factors in *Streptomyces* such as nutrient limitation, the so called stringent response, regulatory proteins, and extracellular signals, such as γ-butyrolactones or other quorum sensing signalling molecules (PI factor, or butenolides are examples) (reviewed in Bibb (2005), van Wezel *et al*. (2011), Martin *et al.* (2012)). Understanding the function and regulation of quorum sensing signalling molecules and how these molecules influence antibiotic production is essential for the efficient manipulation of biosynthetic pathways to increase production of known antibiotics as well as to discover novel antimicrobial compounds.

Variety of signalling systems in *Streptomyces*

Antibiotic production and morphological differentiation in *Streptomyces* species is regulated by quorum sensing. They produce diverse signalling molecules that work as important regulatory factors for coordinating the production of secondary metabolites and sporulation. The most common

signalling molecules in *Streptomyces* are the γ-butyrolactones. However, several other signalling molecules have been reported to be produced in different *Streptomyces* species that regulate secondary metabolites production and sporulation in their hosts. Some of these signalling molecules are similar in structure to the 2,3 disubstituted γ-butyrolactones such as the butenolides and others present more diverse structures such as methylenomycin furans, PI-factor, diketopiperazines or the protein factor C (Figure 2).

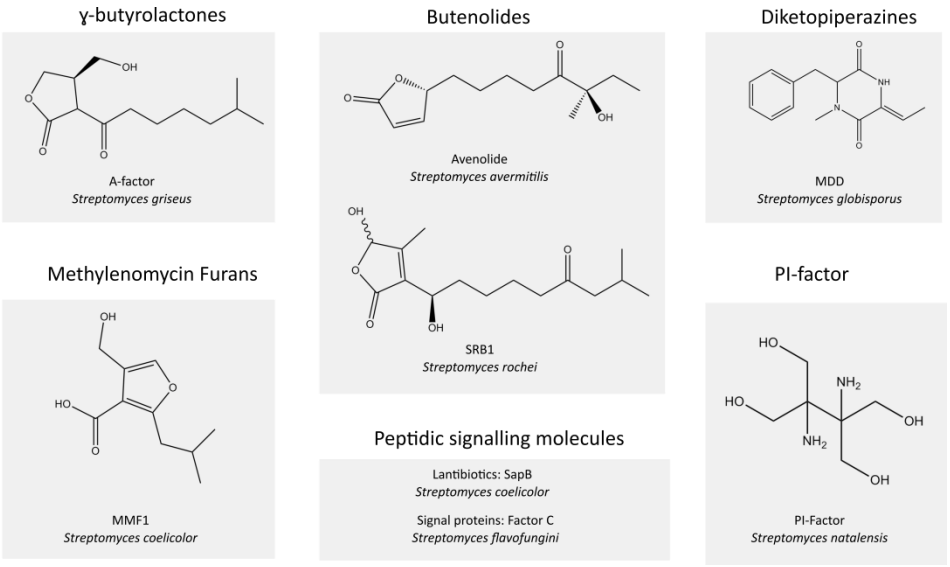

Figure 2. Signalling molecules produced by streptomycetes. The diverse structures of the small molecules, representatives of each class, are shown.

Butyrolactones

The first discovered and most known γ-butyrolactone is A-factor produced by *Streptomyces griseus* that induces secondary metabolite production and morphological differentiation in this bacterium (Horinouchi *et al.*, 2007). AfsA is the A-factor biosynthetic enzyme that synthesises A-factor in a growth phase-dependent manner (Kato *et al.*, 2007). The A-factor receptor protein ArpA is a transcriptional repressor that controls the expression of the transcriptional activator AdpA (Onaka *et al.*, 1995, Ohnishi *et al.*, 1999). Upon binding A-factor, ArpA loses its ability to bind to the promoter region of AdpA which results in activation of AdpA expression. AdpA exerts then its function activating the expression of a wide range of genes involved in antibiotic

production and morphological differentiation (Kato *et al.*, 2004, Akanuma *et al.*, 2009).

Many other *Streptomyces* species also produce different γ-butyrolactones that are involved in controlling secondary metabolism and morphological differentiation. *Streptomyces coelicolor*, the model organism of the genus, produces the γ-butyrolactones SCB1, SCB2 and SCB3 that regulate the production of antibiotics in this bacterium (Takano *et al.*, 2000, Hsiao *et al.*, 2009). These and other butyrolactone systems in other *Streptomyces* strains will be explained in more detail in the following section.

Butenolides

Avenolide is a 4-monosubstituted γ-butenolide that induces production of the anthelmintic avermectin in *Streptomyces avermitilis* (Figure 2) (Kitani *et al.*, 2011). The product of the gene *aco*, an acyl-coA oxidase, was reported to be required for avenolide synthesis. Three homologues of autoregulator receptors, AvaR1, AvaR2 and AvaR3 were found in the *S. avermitilis* chromosome in the vicinity of the *aco* gene. The gene encoding AvaR1 is flanked by the *aco* gene and the gene *cyp17* encoding a cytochrome P450 hydroxylase (Kitani *et al.*, 2011). Deletion of the homologues of these two genes in *Streptomyces fradiae* resulted in abolishment of the production of the antibiotic tylosin and of a yet unidentified signalling molecule: organic extracts of cultures of the deletion mutants of these genes failed to prevent the binding of TylP to its binding sequences as assessed by gel retardation assays, while extracts of the wild type strain prevented this binding (Bignell *et al.*, 2007). The *aco* and *cyp17* genes homologues in *S. fradiae* are also flanking the gene encoding for TylP, the putative signal molecule receptor protein. Although avenolide has only been found and characterised in *S. avermitilis*, the same genes and gene organization have also been found in *Streptomyces ghanaensis* and *Streptomyces griseoauranticus*, as well as in *S. fradiae*, suggesting that avenolide-type compounds are also being produced in these species (Kitani *et al.*, 2011, Mutenko *et al.*, 2014).

Avenolide-like butenolides have also been isolated from several marine *Streptomyces* species (Braun *et al.*, 1995, Mukku *et al.*, 2000, Cho *et al.*, 2001, Viegelmann *et al.*, 2014, Wang *et al.*, 2014a). Their signalling function has not been reported, but some have antibiotic activity (Braun *et al.*, 1995, Mukku *et al.*, 2000) and it has been suggested that they might be playing a role in

regulation of secondary metabolite production in these species (Viegelmann *et al.*, 2014).

Streptomyces rochei produces the 2,3 substituted γ-butenolides SRB1 and SRB2 (Figure 2). These butenolides induce the synthesis of the antibiotics lankacidin and lankamycin in this bacterium (Arakawa *et al.*, 2012). In this species an AfsA homologue SrrX is thought to be the key enzyme in the biosynthesis of SRB1 and SRB2. SrrA is the putative receptor protein of these signalling molecules. In the vicinity of the genes encoding for these proteins several other genes are located that encode enzymes for SRBs biosynthesis, regulatory proteins homologous to γ-butyrolactone receptors, *Streptomyces* antibiotic regulatory proteins (SARPs) and transcriptional activators that constitute a complex regulatory cascade assisting the SrrX/SrrA system in regulating the production of antibiotics in *S. rochei* (Arakawa *et al.*, 2007, Yamamoto *et al.*, 2008, Suzuki *et al.*, 2010).

Methylenomycin furans

In addition to γ-butyrolactones, *S. coelicolor* also produces the signalling molecules methylenomycin furans (MMFs) (Figure 2) that regulate production of the antibiotic methylenomycin. The product of the gene *mmfL*, located within the biosynthetic gene cluster of methylenomycin encoded on plasmid SCP1, shares 27% of amino acid identity with AfsA from *S. griseus* and was reported to be required for the biosynthesis of these signalling molecules in *S. coelicolor* (Corre *et al.*, 2008). The pathway for the biosynthesis of these signalling molecules has been proposed (Corre *et al.*, 2008, Corre *et al.*, 2010). Structurally related 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (AHFCAs) are putative intermediates of this biosynthetic pathway and have been isolated from different *Streptomyces* species. This suggests that other *Streptomyces* species are also producing these types of compounds (Corre *et al.*, 2008).

PI-factor

PI-factor is another structurally different signalling molecule discovered in *Streptomyces natalensis* regulating the production of the antibiotic pimaricin (Figure 2). This diamino-hydroxymethyl-butanediol autoinducer was reported to restore pimaricin production in a pimaricin non-producing *S. natalensis* strain and increased production in the wild type by 33% (Recio *et al.*, 2004).

The enzymes responsible for the biosynthesis of this molecule have not yet been identified.

Diketopiperazines

In S*treptomyces globisporus*, the diketopiperazine MDD ((L)-N-methylphenylalanyl-dehydrobutyrine diketopiperazine) (Figure 2) regulates the production of the antitumor antibiotic landomycin E and sporulation. *S. globisporus* possess a butyrolactone receptor homologue with high nucleotide sequence identity to *arpA* of *S. griseus* (92%). MDD also induces sporulation, streptomycin production and A-factor production in a non-producing non-sporulating *S. griseus* mutant strain. Diketopiperazines (DKP) are biologically active compounds that are known to mimic the actions of other signalling molecules (such as AHLs in LuxR systems) (Holden *et al.*, 1999, Wang *et al.*, 2010, Tommonaro *et al.*, 2012). MDD is proposed to bind to ArpA in *S. griseus* mimicking the action of A-factor. This explains the biological effect of this compound in *S. griseus* (Matselyukh *et al.*, 2015) and suggests that these compounds are involved in interspecies communication between these strains.

Peptidic signalling molecules

Streptomyces species also produce lantibiotics, antimicrobial peptides that are necessary to enable morphological differentiation. The best known representative of these ribosomally synthesised small modified peptides is SapB from *S. coelicolor*, acting as a biological surfactant that facilitates the formation of aerial mycelium (Kodani *et al.*, 2004, Willey *et al.*, 2006, de Jong *et al.*, 2012). The SapB pre-peptide is encoded in the gene *ramS* and is modified by the enzyme RamC, with similarity to lantibiotic modifying enzymes, to yield the mature SapB (Kodani *et al.*, 2004). However, while other typical lantibiotics like nisin from *Lactococcus lactis* and subtilin from *Bacillus subtilis* show signalling roles (Willey *et al.*, 2007), these *Streptomyces* lantibiotics do not appear to have a signalling function, but they are rather dedicated to their mechanical role in aerial mycelium formation (Willey *et al.*, 2006).

Normally signalling molecules are small diffusible molecules or small secreted peptides. However, *Streptomyces albidoflavus* produces a 324 amino acid secreted protein with signalling functions. This protein called factor C is able to restore aerial mycelium formation, sporulation and A-factor production in a

sporulation deficient, A-factor non-producing *S. griseus* (Birko *et al.*, 2007, Kiss *et al.*, 2008).

Antibiotics as signalling molecules

It has become evident in the last decade that antibiotics, besides their role as antimicrobials, may have an additional function as signal molecules at sub-inhibitory concentrations. When they are present in sub-lethal amounts they provoke changes in gene expression that affect different processes and behaviours: β-lactam antibiotics such as imipenem and penicillin, and aminoglycoside antibiotics such as tobramycin, stimulate the formation of biofilms in human pathogens such as *Pseudomonas aeruginosa* or *Streptococcus pneumonia* (Bagge *et al.*, 2004, Hoffman *et al.*, 2005, Rogers *et al.*, 2007); the macrolide antibiotic azithromycin inhibits biofilm formation and decreases virulence by interfering with quorum sensing systems of *P. aeruginosa* (Nalca *et al.*, 2006); tetracyclines increase virulence in *P. aeruginosa* (Linares *et al.*, 2006); thiazolyl peptide antibiotics promote the formation of biofilms in *Bacillus subtilis* (Bleich *et al.*, 2015) (reviewed in Yim *et al.* (2007), Romero *et al.* (2011), Andersson *et al.* (2014). Antibiotics also have a role as signals in determining nutrient use in diverse isolates of *Streptomyces* species (Vaz Jauri *et al.*, 2013). Interestingly, several antibiotics were able to function as inducers to activate the expression of two cryptic gene clusters in the pathogenic bacteria *Burkholderia thailandensis* when present in low concentrations (Seyedsayamdost, 2014).

Thus, antibiotic activity observed in the laboratory might not reflect the real function of these compounds in nature (Bibb, 2005). In fact, it has been argued that the primary function of these compounds in nature is as signalling molecules, and that they only act as actual antibiotics when they reach a certain concentration in the medium (Davies *et al.*, 2006, Yim *et al.*, 2006).

In *Streptomyces*, there is also evidence of the function of antibiotics as intraspecific signals. Some homologues of γ-butyrolactone receptor proteins are in fact not able to bind the butyrolactone signalling molecules and therefore have been called pseudo-γ-butyrolactone receptors (Xu *et al.*, 2010b). ScbR2, from *Streptomyces coelicolor* and JadR2 from *Streptomyces venezuelae* are two of these pseudo-γ-butyrolactone receptors that were reported to bind antibiotics instead of binding γ-butyrolactones *in vitro*. ScbR2 binds the endogenous antibiotics actinorhodin and undecylprodigiosin which

prevents the binding of ScbR2 to the promoter of *cpkO* (Xu *et al.*, 2010b), encoding for the activator of the gene cluster for the synthesis of the antibiotic coelimycin (Takano *et al.*, 2005). ScbR2 was also reported to bind to the DNA in the promoter region of *redD*, encoding for the pathway specific activator of the Red biosynthetic gene cluster and *adpA* (Wang *et al.*, 2014b), encoding for a global transcriptional activator, that binds to the promoter regions of *redD* as well as *actII-ORF4*, encoding for the pathway specific activator of the Act biosynthetic gene cluster (Park *et al.*, 2009). ScbR2 binding to Act and Red presumably prevents ScbR2 binding to the *redD* and *adpA* promoters. This suggests that Act and Red are acting as signals to regulate their own production. In a similar way, JadR2 binds the endogenous antibiotics chloramphenicol and jadomycin thus preventing its binding to the promoter of *jadR1*, encoding for the activator of the jadomycin biosynthetic gene cluster (Xu *et al.*, 2010b). Interestingly, ScbR2 could also bind jadomycin, indicating that *S. coelicolor* can also respond to the presence of exogenous antibiotics which suggests that interspecies communication exists between these two strains (Wang *et al.*, 2014b). Homologues of ScbR2 and JadR2 have also been found in *S. coelicolor* (CprA and CprB) and *S. virginiae* (BarB) which are also not able to bind γ-butyrolactones (Sugiyama *et al.*, 1998, Kawachi *et al.*, 2000, Bhukya *et al.*, 2014). However, the specific ligands for these homologues have not been identified and it is not yet known whether these proteins can bind antibiotics.

Reinforced regulation or division of labour?

Different signalling molecules coexist in some *Streptomyces* species.

In *S. natalensis*, both PI-factor and a putative γ-butyrolactone system (identified by the presence of genes encoding for AfsA and ArpA homologues) regulate production of pimaricin (Recio *et al.*, 2004, Lee *et al.*, 2005, Lee *et al.*, 2008). Interestingly, *S. natalensis* is able to respond to A-factor, the γ-butyrolactone produced by *S. griseus*; addition of this compound restored the production of pimaricin in a pimaricin non-producing *S. natalensis* strain (Recio *et al.*, 2004). This indicates that, either PI-factor and the yet unidentified γ-butyrolactone are sharing the same receptor protein, or a cross-talk of receptors is taking place in *S. natalensis*. *S. ghanaensis*, producer of the antibiotic moenomycin, possesses genes homologous to γ-butyrolactone and γ-butenolide synthases (*afsA* and *aco* homologues, respectively) suggesting

that this strain is producing both signalling molecules (Mutenko *et al.*, 2014). The function of these genes or their protein products has not been characterised. However, an AfsA homologue from *S. lividans* was shown to be necessary for the successful heterologous expression of the moenomycin cluster from *S. ghanaensis*: the gene cluster heterologously expressed in a AfsA deficient strain of *S. lividans* failed to produce the antibiotic, while the antibiotic was produced when the same gene cluster was expressed in a *S. lividans* wild type strain. This suggests that the AfsA homologue is needed for the production of this antibiotic (Mutenko *et al.*, 2014).

In these examples both signalling systems seem to be regulating the same antibiotic production. Antibiotic production and sporulation occur in specific phases of growth and these processes must be tightly regulated. The presence of two different signalling systems regulating the same physiological process may constitute a reinforced regulation ensuring the correct timing and functioning of such processes.

In contrast, in *S. coelicolor*, different signalling molecules regulate production of different antibiotics. γ-Butyrolactones directly regulate the production of coelimycin and MMFs regulate production of methylenomycin. There seems to be division of labour in this species and every signalling molecule is dedicated to the regulation of the production of a specific antibiotic. However, in *S. coelicolor* the same γ-butyrolactones also regulate the production of the antibiotics actinorhodin and undecylprodigiosin, but in an indirect manner, since the butyrolactone receptor protein ScbR does not directly regulate the expression of the biosynthetic gene clusters for these antibiotics (Takano *et al.*, 2005). It is therefore possible that the presence of several signalling systems in a given species is not with the purpose of reinforcing the regulation. Maybe only one of the signalling systems present is dedicated to a specific process (antibiotic production or sporulation) and the other system is regulating these processes indirectly. Interconnection of signalling systems by cross-talk is also likely to occur to ensure a tighter regulation of these physiological processes.

Butyrolactone systems in *Streptomyces*

The most common and best known QS signalling molecules in *Streptomyces* are the γ-butyrolactones. These molecules are used to regulate secondary metabolite production and morphological differentiation in the same species,

but can also function as signals for interspecies communication to regulate these mechanisms in other species. γ-Butyrolactone systems are broadly distributed in streptomycetes. All these systems consist of a γ-butyrolactone synthase and a receptor protein that detects the accumulation of the signalling molecules and provokes a regulatory response that ends in antibiotic production and in some cases also in sporulation. However, many aspects of the synthesis and regulation of these systems are still not understood. Given the great genetic potential of streptomycetes to produce novel secondary metabolites with new structures (Omura *et al.*, 2001, Bentley *et al.*, 2002, Ikeda *et al.*, 2003, Mochizuki *et al.*, 2003, Ohnishi *et al.*, 2008, Medema *et al.*, 2010, Olano *et al.*, 2014), a detailed understanding of the intricate mechanism of antibiotic induction by these signalling molecules will provide a valuable tool to discover new compounds with antibiotic activities by awakening dormant antibiotic biosynthetic gene clusters (Scherlach *et al.*, 2009, Medema *et al.*, 2011) as well as to control the timing and increase production of commercially interesting antibiotics.

γ-Butyrolactones are hormone-like small compounds that act in nanomolar concentrations by binding to their cytoplasmic receptor proteins that are usually transcriptional repressors, thereby preventing their binding to their DNA targets and thus provoking a regulatory cascade leading to antibiotic production (Bibb, 2005, Takano, 2006). It has been estimated that about 60% of *Streptomyces* species can produce some type of γ-butyrolactone (Yamada, 1999). To date, 14 different γ-butyrolactone structures have been identified. They all share a 2,3 disubstituted butyrolactone skeleton and differ in the reduction state of carbon 6 (C6), in the stereochemistry of C2 and 3 or in the length and branching of the side chain (Figure 3) (Khokhlov, 1980, Gräfe *et al.*, 1982, Gräfe *et al.*, 1983, Mori, 1983, Yamada *et al.*, 1987, Kondo *et al.*, 1989, Sato *et al.*, 1989, Mizuno *et al.*, 1994, Takano *et al.*, 2000, Hsiao *et al.*, 2009, Zou *et al.*, 2014). All the γ-butyrolactone described to date can be classified in one of the following types, based on their structural differences; A-factor type from *S. griseus* containing a keto group at carbon 6 (Khokhlov, 1980, Mori, 1983), IM-2 type from *S. lavendulae*, with a hydroxyl group at C6 with an R configuration (Sato *et al.*, 1989, Mizuno *et al.*, 1994), and VB type, or Virginiae Butanolides, from *S. virginiae* and *S. antibioticus*, with a hydroxyl group at C6 with an S configuration (Yamada *et al.*, 1987, Kondo *et al.*, 1989) (Figure 3). Table 1 shows a list of γ-butyrolactone systems in several *Streptomyces*

species, indicating the type of γ-butyrolactone produced, the genes involved and the function of these systems in their hosts.

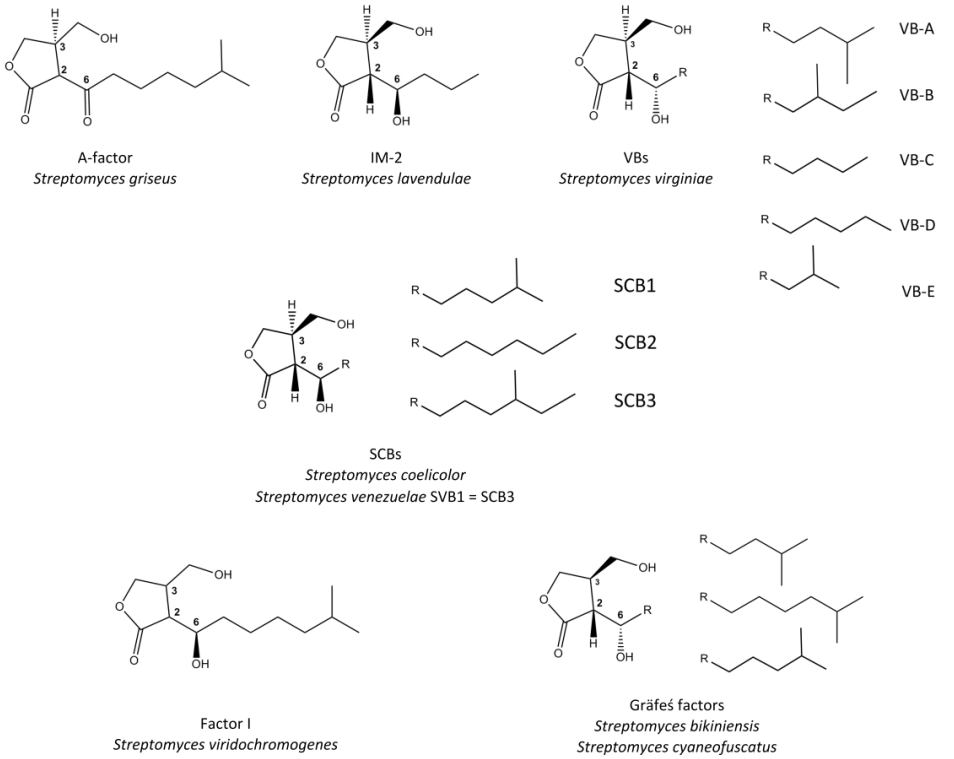

Figure 3. The γ-butyrolactones of *Streptomyces*. The structures of the fifteen γ-butyrolactones known to date to be produced by *Streptomyces* species are shown. The three structures shown on the top represent the three butyrolactone structural types: A-factor, IM-2 and VBs.

Butyrolactone synthesis

Synthesis of γ-butyrolactones is growth phase-dependent. These signalling molecules are synthesised in transition phase of growth in response to still unknown mechanisms, and exert their regulatory function during this phase of growth. The biosynthetic pathway of these molecules has been extensively studied in *Streptomyces griseus*, producer of the best known γ-butyrolactone A-factor that induces secondary metabolite production and sporulation in *S. griseus* (Figure 3). The A-factor biosynthetic pathway has been described in detail and some of the biosynthetic enzymes have been characterised (Kato *et*

al., 2007). AfsA is the key enzyme for A-factor synthesis, and was reported to catalyse the condensation of dihydroxyacetone phosphate (DHAP) with a fatty acid derivative to produce a fatty acid ester that is non-enzymatically converted to a butenolide phosphate by an intramolecular aldol condensation. This compound is reduced by the oxidoreductase BprA and then a phosphatase removes the phosphate group to give A-factor. Although this is the major enzymatic pathway in these bacteria, A-factor can also be synthesised through an alternative route, which corresponds to the main biosynthetic route described by Sakuda, 1993 for the biosynthesis of VBs in the high VB producer *S. antibioticus* (Sakuda *et al.*, 1993, Kato *et al.*, 2007). In this route, the fatty acid ester formed after the first condensation step, is first dephosphorylated and then reduced in several steps to yield A-factor. In *S. antibioticus*, as well as in *S. virginiae*, A-factor type compounds are further reduced by a specific ketoreductase. This ketoreductase has been characterised in *S. virginiae* (BarS1) and *S. antibioticus* (Shikura *et al.*, 1999, Shikura *et al.*, 1999, Shikura *et al.*, 2002).

Homologues of AfsA are found in several *Streptomyces* species (Table 1). Some of these AfsA homologues have been experimentally proven to participate in the synthesis of γ-butyrolactones by deleting their corresponding genes from the chromosome and resulting in loss of γ-butyrolactone production: ScbA, in *S. coelicolor*, producer of SCBs (*Streptomyces coelicolor* butyrolactones) (Takano *et al.*, 2001); BarX, in *S. virginiae*, producer of VBs (virginiae butanolides) (Lee *et al.*, 2010); FarX, in *S. lavendulae*, producer of IM-2 (Kitani *et al.*, 2010), JadW1 in *S. venezuelae* that produces SVB1, with identical structure to SCB3 from *S. coelicolor* (Figure 3) (Zou *et al.*, 2014), ScgA in *S. chattanoogensis* (Du *et al.*, 2011b) and SagA in *S. aureofaciens* (Mingyar *et al.*, 2015) producers of yet unidentified signalling molecules. Furthermore, all these mutant strains were affected in antibiotic production indicating the role of these homologues in the regulation of secondary metabolism in these bacteria. As AfsA, these homologues are thought to catalyse the first condensation step of a fatty acid derivative and a glycerol derivative in the biosynthesis of these signalling molecules.

Table 1. γ-Butyrolactone systems in *Streptomyces* species. GBLs, γ-butyrolactones; S, butyrolactone synthase; R, butyrolactone receptor; pseudoR, pseudo butyrolactone receptor; -, unidentified. Function indicates which antibiotic synthesis pathway is regulated by the butyrolactone system and whether it affects sporulation.

	GBLs	S	R	pseudoR	Function	source
S. coelicolor	SCB1, SCB2, SCB3	ScbA	ScbR	ScbR2	actinorhodin, undecylprodigiosin, coelimycin	1
S. griseus	A-factor	AfsA	ArpA	-	streptomycin, grixazone, polyketide and sporulation	2
S. virginiae	VB-A to VB-E	BarX	BarA	BarB	virginiamycin	3
S. lavendulae	IM-2	FarX	FarA	FarR2	D-cycloserine, nucleoside antibiotics and indigoidine	4
S. venezuelae	SVB1 = SCB3	JadW1	JadR3	JadR2	jadomycin B	5
S. fradiae	-	-	TylP	TylQ	tylosin	6
S.chattanoogensis	-	ScgA	ScgR	-	pimaricin and sporulation	7
S. clavuligerus	-	-	Brp	-	clavulanic acid and cephamycin	8
S.viridochromogenes *S.cyaneofuscatus* *S.bikiniensis*	Gräfe´s Factors	-	-	-	anthracycline and sporulation in *S. griseus*	9

1- (Takano *et al*, 2000, Takano *et al*, 2001, Takano *et al*, 2005, Hsiao *et al*, 2007, Hsiao *et al*, 2009) **2**- (Hara *et al*, 1982, Miyake *et al*, 1990, Ando *et al*, 1997, Kato *et al*, 2007, Akanuma *et al*, 2009) **3**- (Yamada *et al*, 1987, Kondo *et al*, 1989, Kinoshita *et al*, 1997, Kawachi *et al*, 2000, Lee *et al*, 2010) **4**- (Sato *et al*, 1989, Ruengjitchatchawalya *et al*, 1995, Waki *et al*, 1997, Kitani *et al*, 1999, Kitani *et al*, 2008, Kitani *et al*, 2010, Kurniawan *et al*, 2014) **5**- (Yang *et al*, 1995, Yang *et al*, 2001, Wang *et al*, 2003, Zou *et al*, 2014) **6**- (Stratigopoulos *et al*, 2002, Bignell *et al*, 2007) **7**- (Du *et al*, 2011b) **8**- (Santamarta *et al*, 2005) **9**- (Gräfe *et al*, 1982)

Butyrolactone receptor proteins

The γ-butyrolactone receptor proteins are TetR transcriptional repressors that bind to the DNA in specific target regions and generally function as autoregulators controlling their own gene expression (Cuthbertson *et al.*, 2013). Based on the crystal structure of CprB, the only crystal structure of a γ-butyrolactone receptor protein that has been solved so far (Natsume *et al.*, 2004, Bhukya *et al.*, 2014), it is known that these proteins suffer a change in conformation upon binding of their ligands (the signalling molecules). This change in conformation prevents these proteins from binding to the DNA in their specific target regions. The binding of the signalling molecules thus results in activation of expression of the target genes that these receptors were repressing. The ArpA A-factor receptor protein in *S. griseus* has been extensively studied (Miyake *et al.*, 1990, Onaka *et al.*, 1997, Onaka *et al.*, 1997, Ohnishi *et al.*, 1999, Kato *et al.*, 2004). Homologues of ArpA were found in numerous *Streptomyces* species (for a list of homologues see Takano *et al.* (2005)); ScbR in *S. coelicolor* (Takano *et al.*, 2001), BarA in *S. virginiae* (Kinoshita *et al.*, 1997), FarA in *S. lavendulae* (Waki *et al.*, 1997), are just examples and some of the best characterised (Table 1). ArpA is an exception within the γ-butyrolactone receptors since it is not an autoregulator.

Regulation of antibiotic production

Antibiotic biosynthetic gene clusters are often regulated by several regulatory proteins that hierarchically constitute a cascade of regulation that ends in antibiotic production. Some of these regulatory proteins are normally γ-butyrolactone receptor homologues that work as signal transduction systems. Interestingly, only one of these receptor homologues is a real γ-butyrolactone receptor that is able to bind the signalling molecules while the other is generally a pseudo-γ-butyrolactone receptor that is not able to bind γ-butyrolactones but can bind antibiotics instead (Xu *et al.*, 2010b) (Table 1). For example in *S. fradiae* five proteins regulate the biosynthetic gene cluster for the production of the antibiotic tylosin. TylP and TylQ are two γ-butyrolactone receptor homologues but only TylP is thought to be the actual receptor of a yet unidentified γ-butyrolactone (Bignell *et al.*, 2007). TylP forms a regulatory cascade together with the other 4 regulators, TylQ (also a repressor), TylR, TylS and TylU (transcriptional activators). TylR is a global activator of the tylosin gene cluster (Cundliffe, 2008). In *S. venezuelae* four proteins regulate the

biosynthetic gene cluster of the antibiotic jadomycin B. Two of them, JadR2 and JadR3, have homology to γ-butyrolactone receptors. JadR3 was reported to be the actual γ-butyrolactone receptor in this species which binds the γ-butyrolactone SVB1 (Figure 3). JadR3 represses itself and JadR2, a pseudo-γ-butyrolactone receptor, as well as JadW1, the SVB1 synthetase, and activates JadR1, an atypical response regulator that is an activator of the jadomycin biosynthetic gene cluster (Yang *et al.*, 2001, Zou *et al.*, 2014). An additional regulator JadR* located within the biosynthetic gene cluster for the production of jadomycin represses JadR1 transcription as well as transcription of other key genes in the biosynthetic gene cluster (Zhang *et al.*, 2013). In *S. coelicolor*, four regulators control the expression of the CPK biosynthetic gene cluster for the production of the antibiotic coelimycin (Pawlik *et al.*, 2007); ScbR, its homologue ScbR2 (sharing 32% of amino acid identity with ScbR) and the SARP regulators CpkO and CpkN. ScbR, the γ-butyrolactone receptor, regulates expression of the SARP protein CpkO, activator of the CPK gene cluster (Takano *et al.*, 2005). ScbR2, encoded within the CPK gene cluster, and described as a pseudo butyrolactone receptor due to its inability to bind γ-butyrolactones (Xu *et al.*, 2010b), represses expression of CpkO and is able to bind the antibiotics actinorhodin and undecylprodigiosin providing an additional signalling mechanism to control the tight expression of antibiotic production (Xu *et al.*, 2010b, Wang *et al.*, 2011).

In *S. griseus* the regulatory cascade that leads to antibiotic production is led by ArpA through the transcriptional activator AdpA. ArpA, the A-factor receptor is a repressor of AdpA which works as a global transcriptional activator that binds to the promoter region of several genes encoding for proteins involved in secondary metabolism and sporulation, such as *strR*, activator of the streptomycin gene cluster (Horinouchi *et al.*, 2007). A similar scenario occurs in *S. clavuligerus*. The butyrolactone receptor Brp is able to bind to the promoter of AdpA repressing its transcription (Lopez-Garcia *et al.*, 2010). AdpA activates transcription of *ccaR*, encoding an activator of genes in the cephamycin C-clavulanic acid biosynthesis cluster (Santamarta *et al.*, 2002). In *S. coelicolor*, AdpA directly controls the transcription of *redD* and *actII-ORF4* pathway specific regulators of the Act and Red biosynthetic clusters (Park *et al.*, 2009). AdpA is not directly regulated by ScbR (Takano *et al.*, 2003), as in the case of its corresponding homologues in *S. griseus* and *S. clavuligerus*, but indirectly, since ScbR2, the pseudo-γ-butyrolactone receptor encoded within the CPK

cluster, whose expression is directly regulated by ScbR, was reported to bind to its promoter region repressing its expression (Wang *et al.*, 2014b). In other *Streptomyces* species the γ-butyrolactone receptor directly controls the expression of activators of gene clusters for the biosynthesis of their target antibiotics instead of *via* an AdpA-like global activator. This is the case for *S. fradiae* (TylP controls the expression of TylR, activator of the tylosin biosynthetic cluster) (Bignell *et al.*, 2007), *S. virginiae* (BarA controls the expression of BarB which in turn controls the expression of VmsR, activator of the virginiamycin biosynthetic gene cluster) (Matsuno *et al.*, 2004) and *S. venezuelae*, (JadR1, directly controlled by JadR3, activates the jadomycin biosynthetic gene cluster) (Zou *et al.*, 2014). Also in *S. coelicolor*, where AdpA appears to act as an intermediate between the SCB system and the production of Act and Red, ScbR is also able to directly control the expression of the CPK cluster for the production of the antibiotic coelimycin by activating expression of *cpkO* (Takano *et al.*, 2005). Although the regulatory cascade leading to antibiotic production has not been described in other *Streptomyces* species, AdpA-like proteins have been found in some of them. In *S. chattanoogensis*, AdpA indirectly regulates the production of the antibiotic pimaricin (Du *et al.*, 2011a). In *S. ansochromogenes*, this protein regulates production of nikkomycin by controlling expression of *sanG*, encoding for an activator of the nikkomycin gene cluster (Pan *et al.*, 2009). AdpA in *S. ghanaensis* binds to the promoter region of key genes for moenomycin biosynthesis thus controlling the production of this antibiotic (Makitrynskyy *et al.*, 2013). In *S. lividans*, as well as in *S. griseus*, members of the AdpA regulon have been identified by genome and proteome-scale strategies (Akanuma *et al.*, 2009, Hara *et al.*, 2009, Guyet *et al.*, 2014). The function of AdpA in regulating antibiotic production in these organisms is clear and it seems likely that the AdpA regulatory cascade is directly or indirectly dependent on butyrolactone signalling systems.

III. *Streptomyces coelicolor* butyrolactone system

Streptomyces coelicolor A3(2) is the model organism of the genus *Streptomyces* and the one studied in most detail genetically. It is known to produce at least 5 different antibiotics; the blue pigmented polyketide antibiotic actinorhodin (Act) (Wright *et al.*, 1976b), the red pigmented tripyrrole non-diffusible antibiotic undecylprodigiosin (Red) (Rudd *et al.*, 1980), the Calcium Dependent Antibiotic or CDA (Hopwood *et al.*, 1983), the plasmid encoded cyclopentanoid antibiotic methylenomycin (Wright *et al.*, 1976a), and the recently discovered CPK polyketide antibiotic coelimycin A and yellow pigment coelimycin P1, which is the actual end product of the CPK biosynthetic pathway (Gomez-Escribano *et al.*, 2012, Challis, 2014). The genome sequence of *S. coelicolor* M145 (a wild type strain lacking both the SCP1 and SCP2 plasmids), published in 2002, revealed the presence of many more putative secondary metabolite biosynthetic gene clusters which indicates that this species has the potential to produce additional bioactive compounds (Bentley *et al.*, 2002).

The γ-butyrolactones play an important role in regulation of antibiotic production in *S. coelicolor*. The γ-butyrolactones in *S. coelicolor* (SCBs) directly regulate the production of coelimycin (Takano *et al.*, 2005, Takano, 2006) and indirectly affect the production of actinorhodin (Act) and undecylprodigiosin (Red) (Takano *et al.*, 2000, Takano *et al.*, 2001). Three SCBs (*Streptomyces coelicolor* Butyrolactones) have been characterised so far (SCB1, SCB2 and SCB3) and more are expected to be produced by this species (Takano *et al.*, 2000, Hsiao *et al.*, 2009). Their structures have been elucidated (Takano *et al.*, 2000, Hsiao *et al.*, 2009) and they all share an IM-2 configuration and differ in the length and branching of the side chain (Figure 3).

The key proteins involved in this SCB system have been identified. ScbA is the key enzyme involved in the biosynthesis of SCBs (Hsiao *et al.*, 2007). ScbR is the SCB receptor protein that directly controls the expression of the CPK gene cluster for the production of the antibiotic coelimycin (Takano *et al.*, 2005). In this section, the role of these proteins in the SCB system (SCB synthesis, receptor recognition, role in antibiotic production and regulation) will be described, as well as new evidence that contributes to a better understanding of such a complex signalling mechanism.

Synthesis

While the A-factor and the VB biosynthetic pathway have been described in detail in *S. griseus* and *S. virginiae*, little is known about the SCB biosynthetic pathway in *S. coelicolor*. In this PhD thesis we have identified new *S. coelicolor* enzymes involved in SCB biosynthesis and present new data that provides novel insights into the SCBs biosynthetic pathway.

ScbA is the key enzyme for biosynthesis of the SCBs (Hsiao *et al.*, 2007). Other genes in the vicinity of *scbA* encode proteins that are also involved in the biosynthetic pathway (Chapter 3); ScbB, a protein homologous to BarS1 in *S. virginiae*, as well as ScbC, a homologue of BprA from *S. griseus*. ScbB and ScbC, as well as ScbA, are required for SCB biosynthesis in *S. coelicolor* (Figure 4). The presence of these proteins in *S. coelicolor*, as well as the presence of the SCB precursors 6-dehydro-SCBs in extracts of the wild type strain *S. coelicolor* M145 detected by LC/MS analysis in this work (Chapter 3) suggests that one or both of the pathways described in *S. griseus* and *S. virginiae* are also occurring in *S. coelicolor*.

ScbA, the SCBs key biosynthetic enzyme

ScbA is a homologue of AfsA and the key enzyme in the biosynthesis of SCBs compounds (Hsiao *et al.*, 2007). ScbA shares 66% amino acid identity with AfsA and thus is thought to catalyse the first condensation step in the biosynthesis of SCBs (Hsiao *et al.*, 2007) (Figure 4B). This enzyme is required for SCB biosynthesis since deletion of its gene from the chromosome of *S. coelicolor* (mutant strain M751) resulted in inability to produce SCBs, overproduction of Act and Red and abolishment of production of the antibiotic coelimycin (Takano *et al.*, 2001, Gottelt *et al.*, 2010).

ScbA contains two AfsA repeats that are thought to contain the active sites of the protein, based on their significant homology to the region around the active sites of the fatty acid synthases FabA and FabZ (Hsiao *et al.*, 2007). Conserved residues E78, E240 and R243, in the putative active site of ScbA, were confirmed to be essential for the activity of the enzyme in SCBs production. The corresponding residues for E78 and E240 in the active sites of FabA and FabZ (a glutamate and an aspartate) are also conserved and were reported to be catalytic residues in these enzymes (Leesong *et al.*, 1996, Kimber *et al.*, 2004, Kostrewa *et al.*, 2005). Point mutations were introduced in

the *scbA* gene in its original locus in the chromosome of the wild type strain *S. coelicolor* M145 to mutate conserved residues E78, E240, R243 (Hsiao, 2009) as well as R81 (Chapter 4) in the putative active site of ScbA. This resulted in inability of these mutant strains to produce SCBs and differences in antibiotic production compared to the wild type strain (Hsiao, 2009) which indicates that these residues in the active site are essential for the activity of ScbA. An additional highly conserved residue, R174, located in the putative loop region connecting the AfsA repeats, was also mutated resulting in abolishment of SCB production and also affected antibiotic production of the mutant strain confirming that R174 is also essential for ScbA activity (Chapter 4). These results indicate that irregular structural protein motifs such as loops are of great importance for the activity of ScbA in SCB synthesis.

ScbB and ScbC, essential reductases for SCB biosynthesis

Besides ScbA, two other proteins are also necessary for the synthesis of SCBs in *S. coelicolor*. ScbB and ScbC are encoded adjacent to the *scbA* and *scbR* genes, together constituting a gene cluster (Figure 4A and Table 1). *scbB* (*sco6264*) encodes a short chain alcohol dehydrogenase and *scbC* (*sco6267*) encodes a dehydrogenase. Deletion of these genes from the chromosome of *S. coelicolor* M145 resulted in abolishment of SCB production which shows that they are essential for SCB biosynthesis (Chapter 3).

ScbB shares 32% amino acid identity with BarS1 from *S. virginiae* which was reported to catalyse the reduction of 6-dehydro-VBs into the VBs in the last reduction step in the butyrolactone biosynthetic pathway (Shikura *et al.*, 2002). In *S. coelicolor*, ScbB was able to catalyse *in vitro* the corresponding reduction using chemically synthesised 6-dehydro-SCBs as substrates to yield SCBs (Chapter 3), suggesting that this enzyme is the one responsible for the last reduction step in the SCB biosynthetic pathway (Figure 4B). Homologues of ScbB and BarS1 have been found in several other *Streptomyces* strains: ScgX in *S. chattanoogensis*, and JadW3 in *S. venezuelae*. These proteins are thought to carry out the same reactions reported for their homologues in *S. coelicolor* and *S. virginiae*.

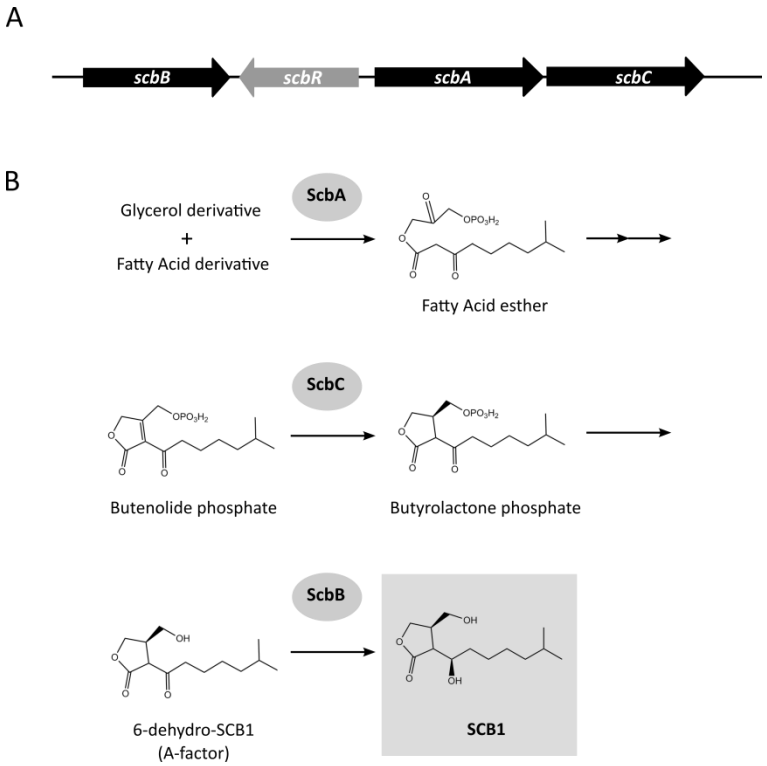

Figure 4. A) Gene organisation of the SCB gene cluster in *Streptomyces coelicolor*. The genes encoding the enzymes required for SCB biosynthesis (ScbA, ScbB and ScbC) are organised in a cluster, together with *scbR*, encoding the SCB receptor protein. The *scbA* and *scbC* genes are transcribed in an operon. **B) Proposed biosynthetic route for SCBs.** The predicted route is based on the biosynthetic pathways described for A-factor in *S. griseus* (Kato *et al.*, 2007) and for VBs in *S. virginiae* (Sakuda *et al.*, 1993). Only the proposed route for SCB1 is shown, as an example.

ScbC shares 76% amino acid identity with BprA reductase from *S. griseus* (Kato *et al.*, 2007). Thus, it is likely to carry out the reduction of the butenolide phosphate intermediate of the pathway to yield the intermediate compound that will be subsequently dephosphorylated to yield A-factor (Figure 4B). A deletion mutant of *scbC*, however, showed no accumulation of the butenolide phosphate as assessed by LC/MS analysis of ethyl acetate extracts of this mutant strain. In fact, none of the predicted intermediates of the pathway, based on the pathways described *in S. griseus* and *S. virginiae* (Sakuda *et al.*, 1993, Kato *et al.*, 2007), were detected in these extracts or in extracts of the *scbB* gene deletion mutant (Chapter 3). Accumulation of intermediates may be detrimental for the activity of the different enzymes in the pathway and

therefore these intermediates may be redirected to other pathways. It is also possible that a different sequence of enzymatic steps is occurring in *S. coelicolor* and therefore other intermediates are being accumulated. Further experimentation will be needed to identify the intermediates of the SCB biosynthetic pathway in *S. coelicolor*.

The *scbA* and *scbC* genes are transcribed together in an operon (Chapter 3) (Figure 4A). This agrees well with the similar antibiotic phenotype observed for the deletion mutant strains of these two genes (Chapter 3). This co-transcription of *scbA* and *scbC* is presumably ensuring that both enzymes are produced in similar amounts to maintain the correct flux through the pathway and allowing for an efficient biosynthesis of SCBs.

Role of ScbR in antibiotic production

The gene coding for ScbA lies adjacent and divergently oriented from *scbR* (Figure 4A). ScbR is the SCB receptor protein and the central element of the SCB system. It is an autorepressor: it binds to its own promoter region in the so-called site R, repressing expression of its own gene (Takano *et al.*, 2001). SCBs prevent binding of ScbR to the DNA, by changing its conformation and thus relieving this repression, allowing transcription of the *scbR* gene.

In addition to repressing the transcription of its own gene, ScbR also controls the expression of the CPK gene cluster for the biosynthesis of the coelimycin antibiotic and yellow pigment coelimycin P1 (Figure 5) (Takano *et al.*, 2005, Gomez-Escribano *et al.*, 2012). It represses the transcription of *cpkO* coding for a SARP (*Streptomyces* Antibiotic Regulatory Protein) activator of the cluster by binding to site OA in the promoter region of this gene. SCBs activate the expression of this gene and consequently expression of the cluster by preventing ScbR binding to site OA, relieving the repression (Figure 5). ScbR has also been reported to bind to an additional site in the promoter of *cpkO*, called site OB although the role of ScbR through binding to this site is not yet known (Takano *et al.*, 2005). An additional binding site for ScbR was found *in silico* in the promoter of the gene *sco6268* (*orfB*), encoding a putative histidine kinase. Binding of ScbR to this site has been experimentally proven (Bunet, 2006) but neither the function of this binding activity nor the function of this protein is known.

ScbR2 is a homologue of ScbR sharing 32% identity. Interestingly, the gene encoding this protein is located within the CPK gene cluster, which is directly controlled by ScbR (Takano *et al.*, 2005). ScbR2 is a repressor of the CPK cluster and its absence provokes an overproduction of coelimycin (Gottelt *et al.*, 2010). Given its inability to bind γ-butyrolactones, ScbR2 has been described as a pseudo-γ-butyrolactone receptor (Xu *et al.*, 2010b). In addition, ScbR2 binds to the promoter region of *scbA* blocking its transcription presumably in the last stages of growth. ScbR2 also binds to the promoter of *cpkO* repressing its transcription, and exerting another level of regulation in the expression of this gene cluster (Wang *et al.*, 2011) (Figure 5).

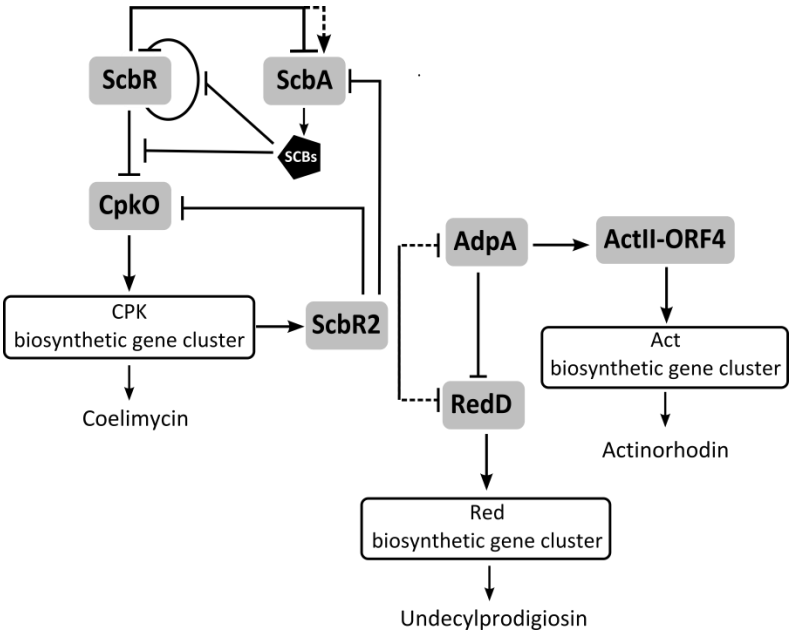

Figure 5. Scheme showing the role of ScbR in antibiotic production in *S. coelicolor*. Arrows indicate activation, arrows with a line at the end indicate repression. Dashed lines indicate possible functions. ScbR controls the production of coelimycin directly and the production of Act and Red indirectly.

Besides ScbR and ScbR2, another three γ-butyrolactone receptor proteins exist in *S. coelicolor*: CprA, CprB (both sharing 32% of amino acid identity with ScbR), and SCO6323 (sharing 29% amino acid identity). CprA and CprB regulate production of the antibiotics actinorhodin and undecylprodigiosin (Onaka *et al.*, 1998, Takano, 2006). CprB is an autoregulator that binds to its own

promoter region. It also binds to the promoter region of *cprA* and it is likely controlling the expression of this gene. CprB was also shown to bind to the promoter region of *scbR* and *cpkO* which suggests that this regulator is also controlling the SCB system and the expression of the coelimycin gene cluster (Bhukya *et al.*, 2014). However, CprA and CprB do not respond to the signalling molecules since they are not able to bind γ-butyrolactones (Sugiyama *et al.*, 1998, Bhukya *et al.*, 2014). Their precise function in the SCB system and in regulation of antibiotic production remains to be elucidated. The function of SCO6323 and whether this regulator can bind butyrolactones is still unknown.

While ScbR directly regulates the expression of the CPK antibiotic biosynthetic gene cluster by repressing *cpkO*, the SCB system is also involved in the indirect regulation of Act and Red synthesis in *S. coelicolor* (Figure 5): overproduction of Act and Red was observed in a *scbA* deletion mutant while a *scbR* deletion mutant showed a delay in Red production. Yet, ScbR does not bind to the promoter regions of the pathway specific regulators for Act and Red production (Takano *et al.*, 2001). Interestingly, in a recent report, ScbR2 was found to be able to bind to the promoter region of *redD*, as well as to the promoter region of *adpA* (Wang *et al.*, 2014b). AdpA is a global regulator for antibiotic biosynthesis and morphological development in *S. griseus* (Ohnishi *et al.*, 2005, Akanuma *et al.*, 2009). In *S. coelicolor*, AdpA has been reported to bind to the promoter region of *redD*, as well as to the promoter region of *actII-ORF4* (Park *et al.*, 2009). Furthermore, an *adpA* deletion mutant showed overexpression of Red and abolishment of Act production (Takano *et al.*, 2003), which suggests a role in activating *actII-ORF4* expression while repressing transcription of *redD*. ScbR is not able to bind to the promoter of AdpA in *S. coelicolor* (Takano *et al.*, 2003). It is thus possible that ScbR is exerting its regulatory role through ScbR2, and that AdpA is the missing link by which the SCB system regulates the production of Act and Red (Figure 5).

S. coelicolor A3(2) M145 and M600 are two wild type strains that show different antibiotic production profiles. They both lack the plasmids SCP1 and SCP2 and differ genetically in that M600 possesses two long terminal inverted repeats at its chromosomal ends that result in 1005 duplicated genes compared to M145 (Weaver *et al.*, 2004). Phenotypically, M600 is delayed in Act and Red synthesis compared to M145 (Gottelt *et al.*, 2012). Interestingly, another form of ScbR exists in M600, with a single amino acid substitution.

However, this alternative form of ScbR was not responsible for the differences in antibiotic phenotype between these two strains (Gottelt *et al.*, 2012). Quantitative Reverse Transcription-PCR (qRT-PCR) analyses performed on RNA from both strains in different stages of growth, revealed that the expression profiles of *scbR* in both strains were different: levels of *scbR* transcript were detected only in basal levels in the M600 strain while they were induced in transition phase of growth in M145. The differential expression of *scbR* in both strains is likely to be responsible for the different phenotypes observed (Chapter 5).

Regulation of the SCB system

The SCB system is regulated by a complex mechanism. An intricate network of interconnected factors is involved constituting different levels of regulation. Growth phase determinants, transcriptional regulators and overlapping promoters seem to participate in the regulation providing several layers of regulatory complexity. The whole regulatory circuit of this system is still not fully understood. In this work, we have gained novel insights and uncovered additional factors that are necessary to ensure the strict regulation of this signalling system.

The SCB system is tightly regulated at the transcriptional level (Takano *et al.* (2001) and Chapter 2). ScbR, the SCB receptor protein, is an autoregulator that represses transcription of its own *scbR* gene by binding to a site in the DNA upstream of its promoter region. The repression of ScbR over its own promoter is prevented by the SCBs. An additional ScbR binding site (site A) was found in the promoter region of *scbA* by gel retardation assays (Takano *et al.*, 2001). ScbR has a growth phase-dependent dual role in controlling *scbA* transcription via this site A (Figure 6): it represses *scbA* transcription in early stages of growth and induces its expression in transition phase probably aided by another protein (Chapter 2). The exact mechanism by which *scbA* expression is induced is still unknown. ScbA also seems to be involved in regulating its own expression, since a *scbA* deletion mutant showed a remarkable reduction in transcription of this gene, assessed by targeting the untranslated region (UTR) of *scbA*, still present in the deletion mutant (Takano *et al.*, 2001). Both ScbR and ScbA seem to be involved in the activation of *scbA* transcription, and therefore a ScbA-ScbR interaction is likely to be responsible (Takano *et al.*, 2001). Furthermore, several other proteins bind to the promoter region of

scbA/scbR that could also have a role in activating *scbA* expression. NdgR, a regulator of nitrogen source-dependent growth (Yang *et al.*, 2009), and DasR, involved in the metabolism of N-acetyl glucosamine (Rigali *et al.*, 2008, van Wezel *et al.*, 2011), were reported to bind to this *scbA/scbR* promoter region (Figure 6). This indicates a connection between nitrogen and carbon metabolism and the SCB system and therefore a link between primary and secondary metabolism. Induction of *scbA* expression coincides in time with the transition from primary to secondary metabolism (Nieselt *et al.*, 2010). It thus appears likely that these proteins are involved in activating the expression of *scbA*.

Regulation of the SCB system is a complex growth phase-dependent mechanism. In exponential phase of growth, basal levels of ScbR exist which are sufficient to bind to the DNA in site R, blocking its own transcription. Expression of *scbA* is activated in transition phase of growth, and γ-butyrolactones are synthesized. These signalling molecules bind to their receptor protein ScbR which provokes conformational changes on this protein that prevent its DNA binding ability and resulting in transcription of *scbR*. In the same way, ScbR blocks transcription of *cpkO* in exponential phase of growth in the absence of γ-butyrolactones. In transition phase, expression of *cpkO* is allowed due to the presence of γ-butyrolactones, which leads to the expression of the CPK cluster (Figure 6).

The SCB system is controlled by a complex regulatory circuit where ScbA and ScbR mutually regulate their gene transcription (Takano *et al.* (2001) and Chapter 2 of this thesis). Besides this mutual expression regulation between ScbA and ScbR, additional factors are involved in maintaining the tight control of this system. The *scbA* and *scbR* genes lie adjacent and divergently oriented in the chromosome (Figure 6). It is noteworthy that their transcripts overlap by 53 bp and these overlapping transcripts constitute an antisense RNA (asRNA) (Moody *et al.*, 2013). This asRNA also seems to constitute another factor aiding in the regulation of expression of these genes in a still unknown way. In fact, this asRNA has been reported to affect the bistability of the SCB system genetic switch in a mathematical model developed by Chatterjee et al. (Chatterjee *et al.*, 2011). Their model suggests that this asRNA is essential to maintain the bistability of the system. It is possible that this *scbA/scbR* cis-antisense RNA is regulating the amount of *scbA* and *scbR* transcripts produced. Antisense RNAs

(asRNAs) have several regulatory functions in transcription and in some cases can affect protein translation (reviewed in Storz *et al.* (2004), Thomason *et al.* (2010), Lybecker *et al.* (2014)).

Additional proteins have been reported to bind to the *scbA/scbR* promoter region that may be also affecting the transcription of *scbA* and *scbR* (Figure 6). (1) CprB is a homologue of ScbR that regulates production of Act and Red production. It binds to the promoter region of *scbR* which suggests a role in regulating the SCB system (Bhukya *et al.*, 2014). It also binds to its own promoter region, thus likely regulating its own expression. This protein is however not able to bind γ-butyrolactones (Sugiyama *et al.*, 1998, Bhukya *et al.*, 2014). (2) ScbR2 is a pseudo-γ-butyrolactone receptor and transcriptional repressor of the CPK gene cluster that also binds to a DNA region overlapping with site A in the promoter region of *scbA* repressing *scbA* expression (Wang *et al.*, 2011). ScbR2 transcription starts in late exponential phase and therefore this protein is thought to have a role in shutting down the expression of *scbA* in stationary phase of growth, once the SCBs have carried out their signalling function (Gottelt *et al.*, 2010, Xu *et al.*, 2010b). (3) SlbR binds to a DNA region in the vicinity of site R and is thought to activate expression of *scbR* since *scbR* transcript levels were slightly reduced in a deletion mutant of *slbR*. This protein represses antibiotic production and sporulation and is also able to interact with SCB1 (Yang *et al.*, 2008, Yang *et al.*, 2012). (4) The SCO3201 protein binds to a DNA site overlapping site A and it enhances *scbA* expression when overexpressed, although deletion of the *sco3201* gene from the chromosome did not yield any phenotypical differences compared to the wild type, which indicates that this protein is not essential for activation of *scbA* expression and instead seems to work as a mimic of ScbR (Xu *et al.*, 2010a).

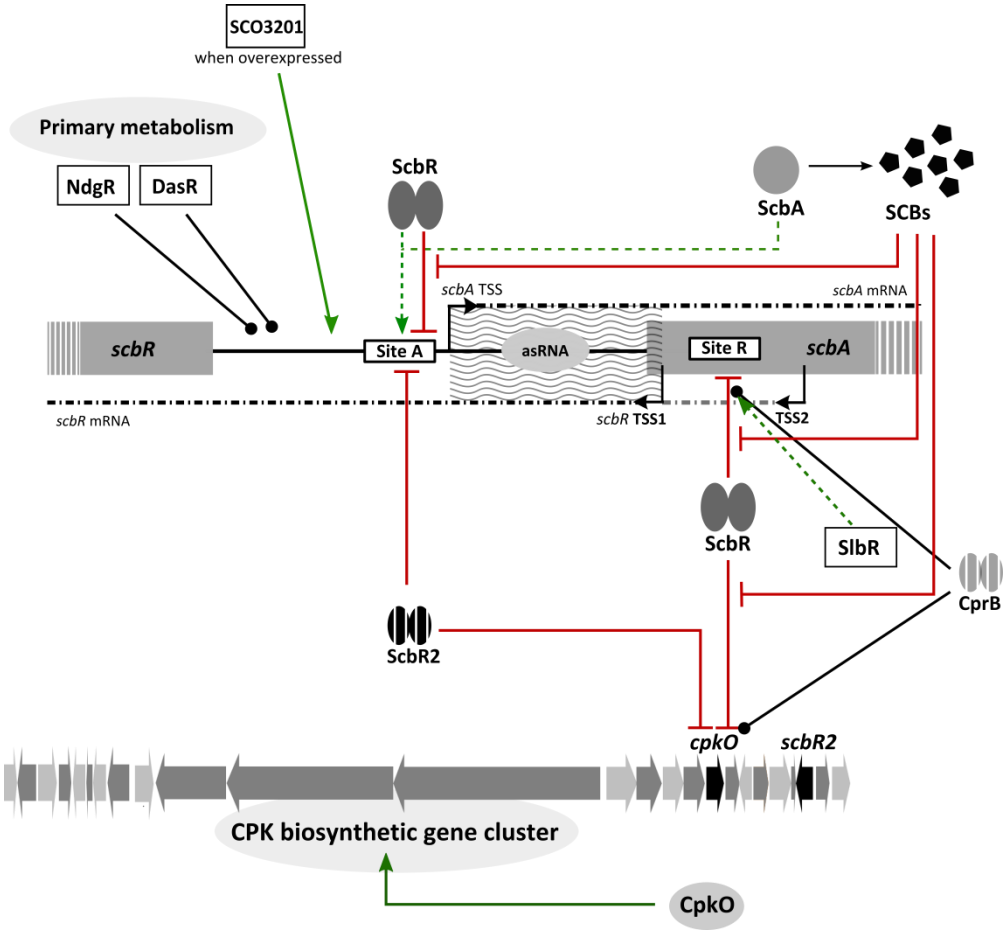

Figure 6. Schematic model of the regulatory mechanism of the SCB system. Red arrows with a line at the end indicate repression. Green arrows indicate activation; dashed green arrows indicate possible activation, but not experimentally confirmed. Black lines with a dot indicate unknown effect. Different regulatory levels are governing the functioning of the SCB system; i) Endogenous factors, ScbA, ScbR and the SCBs. ScbR represses the expression of itself binding to site R, of *scbA* binding to site A and expression of *cpkO*. ScbR and ScbA are both possibly activating the expression of *scbA*. ScbA synthesises SCBs that prevent ScbR repression at its DNA targets. Activation of the expression of the CPK cluster provides sufficient ScbR2 to repress *scbA* transcription presumably shutting down the action of the SCB system in stationary phase of growth. ii) Other transcription factors. Proteins NdgR and DasR bind to the *scbA/R* promoter region connecting primary metabolism with the SCB system. CprB binds to the promoter region of *scbR*, as well as to the promoter region of *cpkO*. SlbR is thought to activate *scbR* transcription. SCO3201, when overexpressed, activates *scbA* expression. iii) An antisense RNA (asRNA), depicted as wavy lines in the overlapping transcripts of *scbA* and *scbR*, is thought to modulate the expression of these genes. iv) A second transcription start site (TSS2) for *scbR* (TSSs are depicted as bent arrows). Transcription from TSS2 yields a longer transcript which results in a longer overlapping region between *scbA* and *scbR* transcripts.

An additional transcription start site for *scbR* (TSS2) has been identified that maps at 207 bp from the ScbR start codon and at 83 bp upstream from the previously identified TSS (TSS1) (Takano *et al.*, 2001) (Figure 6). Different expression profiles for *scbR* were detected from TSS2 in the wild type strains M145 and M600. Similar to the *scbR* expression profile form TSS1, the *scbR* transcript in M600 is produced at a basal level while in M145 *scbR* transcription is induced in transition phase (Chapter 5). The different phenotypes of these two wild type strains thus may be caused at least partly by the different expression of *scbR* from both TSSs. An additional regulatory factor that is present in the M145 wild type strain but absent in M600 is likely to be responsible for these differences in *scbR* expression. Furthermore, transcription from TSS2 yields a longer transcript for *scbR* and thus a longer overlapping region between *scbA* and *scbR* transcripts. It was reported that the longer an asRNA is, the more effective is its regulatory effect (Tatout *et al.*, 1998, Rasmussen *et al.*, 2007). The *scbA/scbR* asRNA is longer when scbR is expressed from TSS2 and thus, this asRNA is presumably more effective in regulating the transcription of these genes. This TSS2 constitutes another regulatory factor for the SCB system that may provide a more efficient regulation for the expression of these genes and adds to its complexity.

Transcriptional regulation is a complicated mechanism and in most cases several levels of regulation are taking place to achieve a tight and efficient control of the gene expression. The SCB system of *S. coelicolor* is a good example of this since it involves different regulatory mechanisms and levels of regulation to establish the necessary strict control of its members and thus ensure the right timing for antibiotic production.

IV. Scope of this thesis

Streptomyces species are a remarkable rich source of antibiotics. Signalling molecules such as γ-butyrolactones are an important factor for regulation of antibiotic production in these bacteria. Understanding how these signalling systems work and are regulated is essential for the subsequent rational engineering to enhance the production of commercially interesting antibiotics, and for the discovery of new antimicrobial compounds. Previous research on the *Streptomyces coelicolor* γ-butyrolactone (SCB) signalling system controlling antibiotic production in this bacterium showed the large complexity of this system and its relevance for antibiotic production. The great potential of this system for the engineering of antibiotic biosynthetic pathways and awakening of cryptic antibiotic gene clusters shows the need to further unravel its complex regulatory circuits and to explore in more depth its functional mechanism. The work described in this thesis constitutes an important contribution to this purpose. A comprehensive study of this system and the proteins involved is presented that provides new insights into the biosynthesis of the signalling molecules, its regulation, as well as into their regulatory effects and aids in a better understanding of this complex communication system and its involvement in antibiotic production.

In **Chapter 2**, we report the growth phase-dependent dual role of the SCB receptor protein ScbR in controlling the expression of *scbA*, encoding the key enzyme for SCBs synthesis: ScbR represses *scbA* transcription in exponential phase of growth, and is also required to induce transcription of *scbA* in transition phase. This tight regulation ensures the correct timing of production and functioning of the signalling molecules. Furthermore, we have shown that a *scbR* deletion mutant is able to produce SCBs, which indicates that ScbR is not essential for SCB production. Instead ScbR appears to modulate the SCB amount that is produced. Our data provides new information that allows a better understanding of the complexity of the SCB system.

Chapter 3 describes the identification and characterization of two new enzymes, ScbB and ScbC. Both reductases were found to be essential for the biosynthesis of SCBs in *S. coelicolor*. We have shown that ScbB is a ketoreductase that catalyses the reduction of the keto-group in chemically synthesized 6-dehydro SCBs to yield SCBs, which is thought to be the last step

in the biosynthetic pathway. In addition, gene expression analyses showed that *scbC* is in an operon with *scbA* and that ScbA as well as ScbR, the SCB receptor protein, are involved in regulation of *scbC* transcription. Deletion of the *scbB* and *scbC* genes from the chromosome of *S. coelicolor* resulted in abolishment of coelimycin production and in early production of actinorhodin and undecylprodigiosin compared to the wild type which shows the involvement of these enzymes and of this signalling system in regulating antibiotic production. Our data provides important insights into the biosynthesis of SCBs in *S. coelicolor*.

In **Chapter 4** we describe the identification of key conserved residues, R81 and R174 that are essential for the activity of ScbA, the key γ-butyrolactone biosynthetic enzyme. Residue R81 is located in the N-terminal AfsA repeat, in the putative active site and may determine the difference in enzyme reaction specificity between fatty acid synthases and AfsA homologues. Residue R174 is located in a region connecting both AfsA repeats in ScbA. Mutations R81K and R174H were introduced in *scbA* in its original locus in the chromosome and resulted in loss of SCB production; these amino acids thus are essential residues in ScbA protein functionality. The presence of an essential residue in a putative loop shows the importance of such an irregular structural motif in the activity of the protein. Our work provides novel insights into the complex structure-function relationship of ScbA and related AfsA proteins.

Chapter 5 describes the identification of an additional transcription start site (TSS2) for *scbR*, encoding the γ-butyrolactone receptor. We show that transcription is taking place from this additional site and have mapped its 5´end at 83 bp from the previously identified TSS1, yielding a longer *scbR* transcript. Transcription was induced in the wild type strain M145 in transition phase of growth but only basal levels were detected in strain M600 (that is delayed in antibiotic production compared to M145) along growth from both the original transcription start site (TSS1) and TSS2. Our data suggests that TSS2 plays a role in regulation of *scbR* transcription and thereby in regulation of antibiotic production in *S. coelicolor*. The presence of this secondary start site for *scbR* constitutes an additional regulatory factor demonstrating the complexity of regulation of this system. Our data provides new information allowing a better understanding of regulation of antibiotic synthesis in these bacteria.

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