Morphogenetic Signaling Molecules of the Streptomycetes

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1. Introduction

Selman Waksman's seminal discovery that streptomycin, a natural product produced by *Streptomyces griseus*,[1] could be used to treat tuberculosis set the stage for decades of subsequent discovery and research in the field of streptomycete biology. Initially, work focused narrowly on antibiotic production. What other secondary metabolites were produced by the actinomycetes? What regulates their biosynthesis? How could their production be maximized? In the quest to identify and analyze additional natural products, links between secondary metabolism and morphological differentiation became evident. This was most clearly demonstrated with two particular species, *S. griseus* and *Streptomyces coelicolor*. The discovery that the γ-butyrolactone A-factor controls both streptomycin synthesis and morphogenesis[2] was congruent with the observation that many developmental *S. coelicolor* mutants blocked in aerial hyphae formation also cannot produce antibiotics. However, despite decades of work by industrial and academic scientists, questions still remain regarding the interaction between secondary metabolism and morphological differentiation. The limits of our understanding are exemplified with the annotation of each actinomycete genome, which reveal the genetic potential for previously undiscovered secondary metabolite production. Further, elucidation of biosynthetic regulatory pathways often reveals complex integrative networks that extend and include morphogenesis regulation.[3]

The streptomycetes are soil dwelling Gram-positive bacteria that have evolved metabolic and morphological adaptations to enable their success in this harsh environment. However, recent advances in microbial ecology have led to a reevaluation of their contribution to soil microbial communities.[4] Prior to this decade, numerical assessments of microbial populations were based on culture-based studies. Indeed, the actinomycetes as a whole, and the streptomycetes in particular, are among the most easily cultivated prokaryotes from soil. By contrast, recent meta analysis of 16S rRNA gene libraries place their numeric contribution in any given soil habitat at no more than about 20%, with a mean close to 5%.[5] However, it is prudent to remember that individual streptomycetes may be present as spores, from which DNA may not be efficiently recovered. In addition, the syncitial morphology of these microbes results in the packaging of multiple chromosomes in a single filament, which may also impact metagenomic analysis. Thus, while culture-based surveys may overestimate their dominance in soil microbial communities, it is difficult to ascertain the accuracy of nonculture based methodologies.

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Growth in soil is optimized by the elongation of vegetative filaments (hyphae) that penetrate the substrate and secrete degradative enzymes. This enables the assimilation of otherwise recalcitrant macromolecules. Substrate hyphae are syncitial, with only an occasional septum; thus, each vegetative compartment possesses multiple genomes.[6] As these filaments grow, they branch and create a dense mat called a substrate mycelium. Vegetative growth ceases when these hyphae begin to differentiate into upwardly growing aerial hyphae. It is at this time that substrate hyphae begin to produce a variety of secondary metabolites, including those with antimicrobial activity. The development of aerial hyphae is sustained by lysis of a subset of the substrate hyphae. As aerial hyphal growth continues, septation develops at regularly spaced intervals. These compartments give rise to a chain of spores; each spore eventually pinches off and provides a means of dispersal for these otherwise nonmotile microbes.

Here, we focus on the life cycles of two genetically well-defined species, *S. coelicolor* and *S. griseus*, There are several important differences between these two model organisms, apart from the antibiotics they produce. While *S. coelicolor* spores are gray due to a type II polyketide spore pigment,[7] many strains of *S. griseus* produce spores that appear olive green or yellow as a result of a type III polyketide pigment.[8] *S. griseus* is able to sporulate in liquid, while *S. coelicolor* undergoes morphological differentiation only when grown on solid substrates. In both species, the development and analysis of morphological mutants has been key to gaining insight into genetic and structural events required for differentiation. Two classes of mutants are of particular importance. The bald (*bld*) mutants are unable to form fuzzy, white aerial hyhae under most condiditions, while the white (*whi*) mutants are blocked in the transition from aerial hyphae to pigmented spores.

For many years it was assumed that the sole trigger of morphological differentiation in the streptomycetes was nutrient deprivation. The classic 1983 study of Kendrick and Ensign[9] demonstrated that nutrient downshift could efficiently induce sporulation of *S. griseus* in liquid culture, supporting this notion. The link between secondary metabolism and morphogenesis was thought to be a consequence of shared regulatory mechanisms active after the commitment to morphological differentiation was made. However, the first global regulators of antibiotic production were reported in 1990;[10] mutation of these genes results in *S. coelicolor* strains that fail to produce any antibiotics but undergo wild-type sporulation.[11]

While the extracellular stimulation of both streptomycin production and morphological differentiation by A-factor in *S. griseus* had been well established,[12] the importance of extracellular communication in *S. coelicolor* did not become apparent until the early 1990s. First, it was reported that a small, hydrophobic spore associated protein (SapB) was partly responsible for the capacity of aerial hyphae-forming strains (i.e., wild-type and *whi* mutants) to rescue the morphological defect in *bld* mutants of *S. coelicolor* when grown in close proximity.[13] Shortly thereafter, it was discovered that, even though all *bld* mutants are blocked in SapB production, certain *bld* mutants could restore the ability of other *bld* mutants to undergo morphological differentiation (and produce SapB) when plated close to, but not touching, one another. It was deduced that a molecular signal or signals produced by the "donor" *bld* strain was capable of overcoming the effects of the genetic lesion in the "recipient" strain.[14]

Since these early studies, a variety of morphogenetic factors have been discovered that influence the rate and extent to which streptomycetes differentiate. Here, we will review the discovery and activity of these molecules, with an emphasis on recent literature. For the purposes of this review, we will refer to physiological differentiation as the production of secondary metabolites (including antibiotics and signaling molecules) as distinct from morphological differentiation or morphogenesis.

2. The γ-Butyrolactones

To date, 14 different 2,3 disubstituted γ-butyrolactones (GBLs) from seven *Streptomyces* spp. have been identified (Table 1). Like the more familiar acyl homoserine lactones of Gram-negative bacteria, these differ by the length of the fatty acid side chain as well as in stereochemistry. Despite the structural similarities between the GBLs, to date there has been only one reported case of interspecies communication mediated by a GBL.[15] Most govern antibiotic production at nanomolar concentrations and a few also regulate morphological differentiation. The general mechanism by which GBLs exert their cellular effects is conserved as they typically bind to intracellular receptors that function as repressors. In this way, GBL−receptor interaction modulates the expression of target genes. In this section, we focus on A-factor as the most highly studied of the GBLs. It is required for both secondary metabolite production and morphogenesis. The interested reader is further directed to a comprehensive review of A-factor written by the late Professor Horinouchi.[16] We then review several other GBLs, particularly those known to influence morphological differentiation.

2.1. A-factor and the AdpA Regulon

Long before homoserine lactones were discovered, the capacity of A-factor (A for autoregulatory) to influence streptomycin production and morphogenesis in *S. griseus* was discovered.[2] The structure of A-factor, 2-(6′-methylhep-

Table 1. γ-Butyrolactone Signaling Proteins Identified in *Streptomyces* spp.

species	GBL	biosynthetic enzyme	receptor	regulated antibiotic	morphogenesis regulated?	references
S. griseus	A-factor	AfsA	ArpA	Streptomycin	Yes	16
S. coelicolor	SCB1, 2, 3, Acl-1	ScbA	ScbR	Act, Red, kas?	No	59, 62, 63, 71
Streptomyces virginiae	VB-A to E	BarX	BarA, BarB	Virginiamycin	No	130
Streptomyces lavendulae	IM-2	FarX	FarA	Showdomycin Minimycin	No	131
Streptomyces viridochromogenes	Factor I			Anthracyclins	Yes	132
Streptomyces bikiniensis	3, un-named					68
Streptomyces cyaneofuscatus						
Streptomyces fradiae		TylP	TylS TylQ	Tylosin	No	69
Streptomyces venezuelae		JadW1	JadR2	Jadomycin B	Yes, conditional	133
Streptomyces natalensis		Orf2	SngR	Natamycin	Yes	60a
Streptomyces acidiscabies		SabA	SabS, SapR	Polyketide WS5995B	Yes, conditional	60b
Streptomyces rochei		SrrX	SrrA, SrrB, SrrC	Lankamycin, Lankacidin	Yes	70
Streptomyces pristinaespiralis			SpbR	Pristinamycin	Yes	67
Streptomyces tendae			TarA	Nikkomycin	No	134
Streptomyces clavuligerus			BrpC	Cephamycin, Clavulanic acid	No	135
Streptomyces ansochromogenes			SabR	Nikkomycin	Yes, conditional	136
Streptomyces ambofaciens			AlpZ	Alpomycin	No	137
Kitasatospora setae			KsbA	Bafilomycin	No	64

tanoyl)-3*R*-hydroxymethyl-4-butanolide is shown in Figure 1. It is now known that A-factor indirectly controls the expression of more than a dozen genes. The mechanism by which A-factor exerts its effect has been dissected in detail and provides a good example of the multiple layers of regulation that govern secondary metabolite production and morphogenesis in *S. griseus*. A-factor is thought to diffuse between filaments within the mycelium. This type of communication has been called hormonal because A-factor exerts its effects at nanomolar concentrations on hyphae in a single mycelium, which are presumably clonal.[16] However, A-factor also communicates with more distant, genetically distinct *S. griseus* hyphae, as long as they express the A-factor receptor, ArpA.[17] In addition to binding A-factor, ArpA is a transcriptional repressor whose sole known target is *adpA*. Site-directed mutagenesis studies demonstrate that the A-factor binding domains and the DNA binding domains of ArpA are independent.[18] When ArpA binds A-factor, its

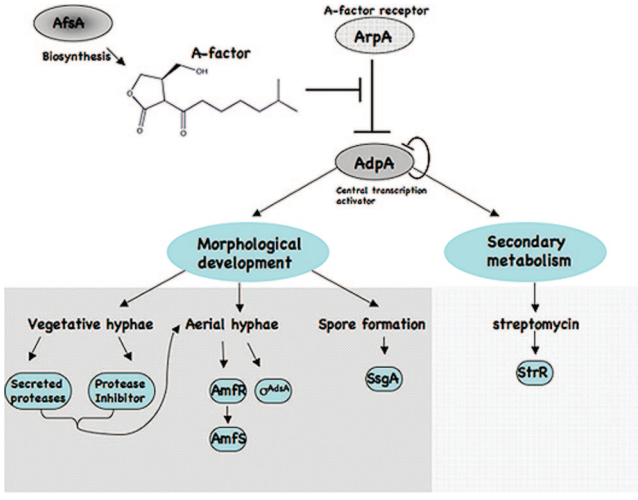


Figure 1. The *S. griseus* A-factor regulon. A-factor synthesis is catalyzed in part by AfsA, and upon accumulation, the GBL binds to its intracellular receptor ArpA, which in the absence of A-factor serves to repress expression of the transcriptional activator AdpA. AdpA in turn activates transcription of genes encoding secreted proteases and a protease inhibitor, which are thought to be involved in the cannibalization of vegetative hyphae; genes whose products are important in raising aerial hyphae, and in sporulation. In addition, AdpA directly activates *strR* transcription leading to the production of streptomycin. A-factor expression of AdpA has also been implicated in the production of a yellow pigment (grixazone) and a another polyketide (not shown).[16]

capacity to bind DNA is lost and *adpA* is expressed. AdpA then activates the transcription of a suite of genes, some of which are also regulators (Figure 1). The amplification of the A-factor signal does not continue unabated, however, as AdpA also represses its own structural gene, thereby forming a negative feedback loop.[19]

A-factor governs streptomycin production through AdpA-dependent activation of *strR* transcription. StrR is the pathway specific activator of the streptomycin biosynthetic gene cluster.[20] A-factor governs morphological differentiation via AdpA activation of at least three genes: an extracytoplasmic function (ECF) sigma factor (*adsA*), a regulatory gene involved in aerial hyphae formation (*amfR*), and a gene required for septation during sporulation (*ssgA*). The first of these genes, *adsA*, was discovered to be A-factor dependent by screening for DNA fragments that bound to recombinant AdpA.[21] In the same study, it was shown that, although an *adsA* deletion mutant fails to produce aerial hyphae, displaying a bald phenotype, it continues to produce wild-type levels of streptomycin. This was the first member of the AdpA regulon that was found to exclusively affect morphogenesis. At about the same time, the *adsA* ortholog in *S. coelicolor*, called *bldN*, was reported.[22] Like the *adsA* null mutant, *bldN* strains remain in the vegetative state; however, unlike *adsA*, there is no evidence that its expression is under the control of the *S. coelicolor* AdpA ortholog BldH/AdpA$_{Sc}$.

AdpA regulation of *amfR* expression represents the first step in a multilevel series of events that ultimately results in the secretion of the surface active peptide AmfS. On the basis of amino acid sequence homology with SapB,[23] it is deduced that AmfS facilitates the upward growth of aerial hyphae[24] by reducing the surface tension at the colony−air interface.[25] There are two AdpA binding sites upstream of *amfR* (at approximately −200 and −60), and AdpA binding has been shown to be responsible for transient upregulation of *amfR* immediately before and during the onset of aerial hyphae production.[26] *amfR* encodes a response regulator (whose signal kinase is unknown; indeed it may not require phosphorylation for activity[27]), that once activated, in turn activates transcription of a convergently transcribed gene cluster that includes *amfTSBA*. Again, homology with the gene cluster responsible for SapB biosynthesis[28] suggests that *amfT* encodes a lantibiotic modification enzyme that dehydrates and cyclizes the *amfS* translational product. Modified AmfS is then exported, presumably by the ABC transporters AmfBA; leader cleavage must occur either during or after

export. AmfR binding activates transcription of *amfTSBA*, while the DNA binding protein BldD represses its expression.[29] Interestingly, while *amfR* and *amfTSBA* are orthologous to *S. coelicolor ramR* and *ramCSAB*, respectively (as discussed in section 8), *ramR* transcripton does not appear to be dependent on GBL signaling or BldD,[30] although it is nonetheless governed by AdpA.[31]

Initially, it was thought that A-factor and AdpA controlled only the transition from substrate to aerial hyphae formation, so it was a surprise to discover that AdpA also activates transcription of *ssgA*.[32] The protein SsgA is needed for normal spore formation in both *S. griseus* and *S. coelicolor* and its localization is dynamic during aerial hyphae and spore development. Studies of SsgA in *S. coelicolor* reveal that it localizes at the tips of nascent aerial hyphae. As the hyphae grow, SsgA becomes distributed at regular intervals where it appears to mark the sites of future septum formation. Redistribution of SsgA continues as sporulation proceeds, and foci are found at each end of the mature spore. Interestingly, prior to germination, SsgA appears to mark the site of germ tube emergence.[33] It is hypothesized that SsgA functions as a chaperonin-like protein that marks sites on the cell wall requiring remodeling during septation and germination. To date, it is not known if SsgA interacts with proteins involved in altering the cell wall, such as peptidoglycan autolysins or biosynthetic enzymes.

The AdpA regulon also includes several proteins that contribute to the extracellular proteome of *S. griseus*. One protein that is conserved among the streptomycetes is SSI (*Streptomyces* subtilisin inhibitor),[34] a dimer of two identical subunits that strongly inhibits alkaline serine proteases.[35] At least six SSI target proteases (two in the trypsin family,[36] three chymotrypsin-like,[37] an one zinc metalloendopeptidase[38]) have been identified. These proteases are also part of the AdpA regulon (Figure 1). Unlike an *adpA* null strain, which is blocked in aerial hyphae formation,[39] deletion of any of these genes does not generate a developmental defect. Nonetheless, *S. griseus* formation of aerial hyphae is delayed when purified SSI is added exogenously to growing colonies.[35] While the role of these proteins in cellular physiology is not well understood, it has been suggested that they may be important in degrading and recycling proteins in the substrate hyphae, thereby supporting the growth of aerial filaments.[40] Such a model predicts that proteolytic activity is blocked during vegetative growth by the activity of a protease inhibitor. This has been demonstrated for at least one species, *Streptomyces exfoliatus*, which produces a trypsin inhibitor called leupeptin. Leupeptin itself is subject to developmentally regulated degradation, assuring the timely activation of its target proteases. Indeed, when leupeptin is added to colonies of *S. exfoliatus*, aerial hyphae formation is delayed.[41] Nucleases are also implicated in the cannibalization of substrate hyphae. For instance, *Streptomyces antibioticus* secretes two nucleases that hydrolyze DNA within substrate hyphae; in their absence, differentiation is strongly inhibited. Their importance and precise timing of activity are further demonstrated by the fact that one of these nucleases is a cryptic peptide released by proteolysis of a precursor protein; thus, the extracellular protease responsible for its release is also required for aerial hyphae formation.[42]

2.1.1. A-factor Biosynthesis

Early attempts to develop a *S. griseus* streptomycin production strain were hindered by unstable A-factor biosynthesis. The reason for this phenomenon was suggested when the putative gene for the A-factor biosynthetic enzyme, *afsA*, was mapped to one end of the linear *S. griseus* chromosome.[43] *Streptomyces* spp. have linear chromosomes with a high G + C (70−73%) content. Analysis of the sequenced genomes has identified the recombinogenetic tendencies of the genome ends,[44] thereby accounting for the frequent loss of the capacity to produce streptomycin.[43] It is therefore perhaps not surprising that *afsA* was not unequivocally identified as the gene encoding the key enzyme needed for A-factor biosynthesis until 2007.[45]

Kato et al.[45] used structural modeling of the *afsA* enzyme product to hypothesize that, like β-hydroxyacyl carrier protein (ACP) dehydratase, AfsA could most likely accept the acyl chain of acyl-ACP. This led to the prediction that AfsA catalyzes the condensation of a C3 compound with a C10 fatty acid derivative that possesses a β-ketoacyl chain, laying the foundation for the elucidation of the A-factor biosynthetic pathway (Figure 2). Interestingly, the AfsA catalyzed reaction, β-ketoacyl transfer from 8-methyl-3-oxononanoyl-dihydroxyacetone phosphate (DHAP) to produce an 8-methyl-3-oxononanoyl-DHAP ester, represents a novel type of acyl transfer.[45] Intramolecular aldol condensation results in the nonenzymatic conversion of the fatty acid ester into a butenolide phosphate. A reductase, BprA, encoded by a gene immediately downstream of *afsA*, then reduces the butenolide phosphate, which is then dephosphorylated to produce A-factor.

An alternate pathway for A-factor synthesis is also noted.[45] Here, the product of AfsA, the 8-methyl-3-oxononanoyl-DHAP ester, is first dephosphorylated. This is followed by aldol condensation and reduction by an unknown enzyme. While the identity of the phosphatase(s) and the alternate reductase remain unknown, they are not specific to *S. griseus*. This is demonstrated by the heterologous expression of *afsA* in *Escherichia coli*, which results in the production of biologically active A-factor.[45]

A-factor production appears to be limited by substrate availability, rather than transcriptional regulation of *afsA*,

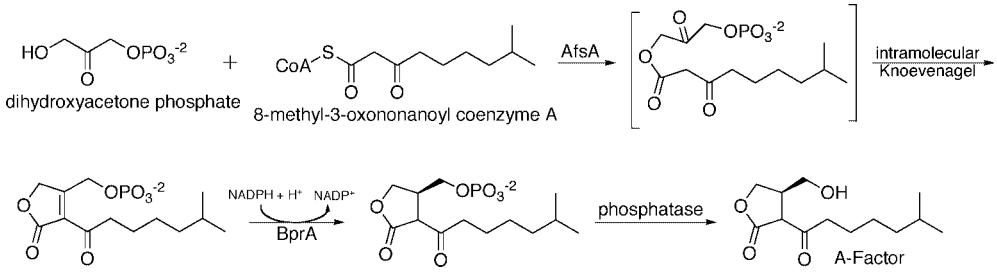


Figure 2. Proposed pathway for A-factor biosynthesis. AfsA catalyzes the condensation of DHAP and a β-keto acid derivative; this is followed by an aldol condensation and BprA-mediated reduction. Dephosphorylation yields A-factor.[45]

which is constitutively expressed. A-factor slowly accumulates during growth in liquid cultures, and reaches a maximum level of 25−30 ng/mL (about 100 nM) in the midexponential phase of growth.[46] The provision of AfsA substrates occurs throughout glycolysis and fatty acid biosynthesis, both of which are active during exponential growth. This has prompted the hypothesis that, by responding to the availability of primary metabolites, AfsA activity functions as a transmitter of the physiological state of the microbe, ultimately leading to the production of secondary metabolites and the onset of morphological differentiation.[16]

2.1.2. A-factor Receptor Protein

The A-factor receptor, ArpA, belongs to the TetR family of transcriptional repressors, and as such consists of a regulatory, ligand-binding domain and a helix-turn-helix domain that binds palindromic sequences within the target DNA.[47] Repression is relieved when A-factor binds. While considerable effort was made to crystallize ArpA, its proclivity for aggregation has so far foiled these attempts, and the *S. coelicolor* ortholog CprB has instead been crystallized (Figure 3).[48] CprB shares about 30% amino acid identity with ArpA, and although its inducing ligand remains undiscovered, it recognizes and binds to the same nucleotide sequence as ArpA.[18a] When the CprB crystal structure was determined at 2.4 Å resolution by Natsume et al.,[48a] it was discovered to function as a dimer. The DNA binding domain consists of three N-terminal helices, α1, α2, and α3; with α2, and α3 forming a helix-turn-helix motif. The amino acid sequence within α3 of CprB and AfsA is identical, consistent with the observation that they bind to the same nucleotide palindrome. The A-T rich, partially palindromic nucleotide sequences to which GBL receptors bind are sometimes referred to as autoregulatory elements, or AREs.

Helices α4−α8 of CprB are thought to form a ligand binding cavity that measures approximately 20 × 5 Å, large enough to accommodate a GBL. Because the functional receptor/repressor is a dimer (Figure 3), two GBLs would be bound. The inner surface of the binding pocket includes several residues that are conserved in ArpA (e.g., Q64, A94, W127, L157, G163, and L181). Mutational analysis shows that W127, which is universally conserved in the ligand-binding domains of the γ-butyrolactone receptors, is required for dissociation of the receptor from target DNA.[48a]

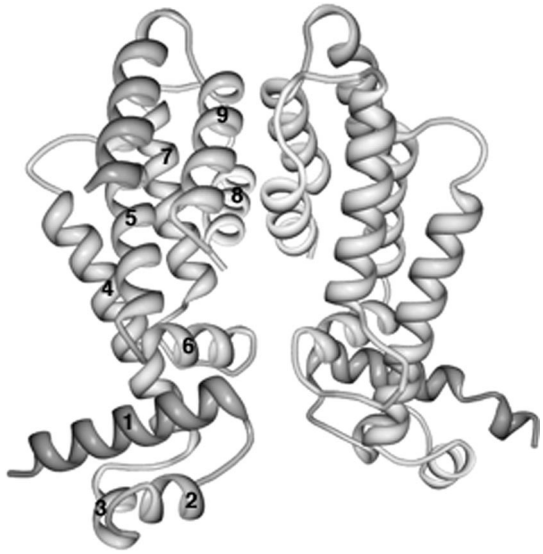


Figure 3. Crystal structure of dimerized CprB. α-Helices 1−3 bind DNA while helices α4−α8 form a pocket into which ligand can be bound. Neither the target DNA nor the regulatory ligand are known for CprB.[48a]

2.1.3. AdpA

To date, only one target for ArpA has been discovered: the *adpA* gene.[49] AdpA is a 405 amino acid protein, whose N-terminus features a ThiJ/PfpI/DJ-1-like dimerization/regulatory domain and an AraC/XylS-type DNA-binding domain at its C-terminal portion. AdpA is the one of the few members of the AraC/XylS family isolated that is sufficiently stable for extensive biochemical analysis.[50] AraC family proteins typically possess a single DNA binding domain that consists of two helix-turn-helix regions, bridged by an α-helix. Therefore, a single domain could be capable of binding a pair of adjacent major groove regions, so that when a functional dimer is produced, up to four major groove regions may be bound. A binding protein with such a large DNA contact area does not need to contact all bases. This built-in flexibility allows AraC-like regulators to bind different subsets of bases, so that genes from a wider regulon pool can be targeted. The alignment of the AdpA binding site[51] with mapped AraC sites[52] enabled the establishment of a consensus sequence for this large and important family of transcriptional activators.[51]

The temporal regulation of AdpA production is not completely controlled by A-factor. The *bldA* gene encodes the only tRNA that recognizes the leucine codon UUA, which is seldomly used in the high G + C streptomycetes.[53] Because *bldA* is developmentally regulated, genes containing this codon are thus regulated at the translational level.[54] It was therefore of great interest when it was revealed that *adpA* in *S. griseus* has a TTA codon. Interestingly, while its ortholog in *S. coelicolor* (*bldH/adpA*$_c$) is not known to respond to GBL signaling, it too is *bldA* dependent.[55]

The identity of AdpA as the ArpA target was discerned by its capacity to bind the promoter of the pathway specific regulator of streptomycin production, StrR, which was known to be A-factor dependent.[49] Since that time, the list of AdpA targets has continued to grow. In addition to those targets discussed in section 2.1.1, recent analysis of the extracellular proteome of *S. griseus* indicates that AdpA may directly control the secretion of 11 proteins and indirectly influence the secretion of 27 others. Many of the proteins are hydrolytic (e.g., proteases, esterases), so it has been suggested that A-factor mediates the catabolism of the substrate hyphae during morphogenesis[40,56] as suggested in Figure 1.

More recently, a global approach to discerning the effects of A-factor was explored by DNA microarray analysis of transcriptomes obtained from an *afsA*-deficient mutant. The phenotype of this mutant can be rescued by the exogenous addition of A-factor in the midexponential phase of growth. Thus, the transcriptome of A-factor treated hyphae can be compared with that of those left untreated. Microarray results, verified by competitive electrophoretic mobility shift assays, suggest that AdpA binds to 37 DNA sites to activate (directly and indirectly) 72 genes. Perhaps not surprisingly, many of these A-factor-inducible genes encode proteins of unknown function.[57] The A-factor regulon may also indirectly involve the expression of seven small RNAs.[58] These results clearly suggest that the A-factor regulatory cascade of *S. griseus* may be more complex than previously thought.

2.2. γ-Butyrolactones in Other *Streptomyces* spp

The presence of GBL signaling in other *Streptomyces* spp. can be unequivocally demonstrated only by isolating the GBL, resolving its structure, and observing its capacity to alter secondary metabolism and/or morphological differentiation when added exogenously. However, despite the assumption that a diverse range of GBLs is produced by a variety of streptomycetes, GBLs have been purified from just a few species (Table 1). This is largely due to the difficulty in obtaining sufficient material for purification; GBLs are typically recovered by organic extraction of large (e.g., >400 L) volumes of spent culture medium.[59] Thus, a more feasible approach is to search for homologues of the *arpA* receptor gene. These genes are often discovered during library screens for genes that influence antibiotic biosynthesis or through the use of degenerate PCR primers designed to amplify these conserved genes.[60] However, several recent reports describe potentially powerful new receptor-based methodologies. For instance, an *E. coli* based cell-free system has been developed in which the structural gene for green fluorescence protein is fused downstream of the DNA binding site for the *S. coelicolor* GBL receptor ScbR. Transcription is governed by a T7 promoter and purified His-tagged ScbR; addition of a GBL capable of binding ScbR results in fluorescence.[61] A slightly different approach has been taken by Hsiao and coworkers,[62] who use ScbR as a sensor in combination with its target DNA, which is engineered to control the expression of a kanamycin resistance gene. Proof of concept was demonstrated by the characterization of two previously identified *S. coelicolor* GBL molecules (SCB2 and SCB3), about which little was known. Finally, Joo et al.[63] employ the ScbR receptor protein as an affinity capture matrix to enrich GBLs from *S. coelicolor*. These workers then apply diagnostic peak lists generated from tandem mass spectrometric fragmentation analysis of known GBLs to detect the presence of the enriched butyrolactones. These new approaches should facilitate the discovery of additional GBLs.

Although ArpA homologues can be used to explore potential GBL production, the presence of an *arpA*-like gene by no means can be considered evidence for GBL signaling. Among the ArpA homologues that have been described, only those that are acidic (pH ~5) bind autoregulator when tested; basic (p*I* ~10) proteins do not.[64] For example, *S. virginiae* possesses two receptor homologues that govern the expression of genes involved in the biosynthesis of the streptogramin complex virginiamycin. BarA, like ArpA, binds its cognate GBL as well as DNA, but BarB lacks GBL binding activity.[65] BarA regulation of virginiamycin production is hierarchical, as it governs the transcription of *vmsR*, a member of the family of *Streptomyces* antibiotic regulatory proteins (SARPs).[66] SARPs possess an OmpR-like DNA binding domain, and may represent the most common targets of GBL regulation.[67] Accumulated evidence for a number of species suggests that, in most cases, the GBL receptor directly regulates a SARP (or SARPs), rather than derepressing an *adpA*-like signal amplification gene. In the case of virginiamycin, VmsR regulates the transcription of a second SARP-encoding gene, *vmsS*, which in turn is needed for transcription of an atypical response regulator, *vmsT*.[66]

As more GBL receptors are identified, it appears that complex GBL regulation of antibiotic production and/or morphological differentiation may be the rule and the more straightforward A-factor pathway the exception.[68] For instance, in the case of tylosin production by *S. fradiae*, an unidentified inducer binds the receptor TylP. This effector molecule is deduced to be a GBL based on the amino acid sequence of TylP and the DNA sequences to which TylP binds. Indeed, TylP activity initiates a hierarchical cascade of events that results in production of the antibiotic. In addition to binding its own promoter, it represses transcription of the genes *tylS* and *tylQ*; TylS is a SARP-like protein that activates the expression of two genes, *tylU* and *tylR*, and which encodes the pathway specific activator for tylosin biosynthesis. Both TylU and TylS are needed for full expression of TylR. Conversely, TylQ negatively regulates *tylR*.[69] Thus, the GBL-responsive repressor TylP directly governs both an activator and a repressor of tylosin production. Presumably, the complexity of this network reflects the need to integrate multiple extra- and intracellular signals. There is certainly no a priori reason to suspect that GBLs that regulate morphogenesis do so in a more direct fashion.

S. rochei is one of the few microbes (in addition to *S. griseus*) in which the putative GBL signaling cascade regulates morphogenesis as well as antibiotic production. This microbe produces two unrelated polyketide antibiotics, lankamycin and lankacidin, which are encoded on a large, linear plasmid pSLA-2. This plasmid also bears all the components of an A-factor-like signal transduction system including an *afsA* homologue (*srrX*), six genes bearing homology to *arpA* (*srrA*, *srrB*, *srrC*, *orf92*, *orf99*, *orf126*), two transcriptional activators including one with similarity to *adpA* and the other to *strR*, and three SARP genes (*srrY*, *srrW*, and *srrZ*).[70] As the presence of so many genes suggests, the signaling network appears to be more complex than the *S. griseus* A-factor system. *srrX* is needed for production of both antibiotics, and like ArpA, SrrB negatively regulates lankacidin and lankamycin synthesis. By contrast, the other ArpA homologues, SrrA and SrrC, positively influence morphological differentiation in an unspecified manner. Deletion of *ssrX* has no effect on morphological differentiation, and a *srrA*, *srrX* double mutant appears wild-type with respect to both secondary metabolism and development. However, an *srrC*, *srrX* double mutant sporulates well but fails to produce antibiotics. Insight into antibiotic regulation is gained by analogy to A-factor, as SrrA functions as both putative GBL receptor and SARP repressor, binding directly to sequences upstream of *srrY*; this repression is relieved by the addition of the GBL-containing solvent fraction.[70b] To date, there have been no reports describing the means by which these proteins influence morphogenesis. Interestingly, SrrC bears more similarity to the methylenomycin furan receptor MmfR (discussed in section 3) than any of the known γ-butyrolactone receptor proteins.[70a]

The GBL receptor system of the phytopathogen *S. acidiscabies* provides another recent example in which both secondary metabolism and morphological differentiation are regulated.[60b] This microbe has a gene cluster that includes two GBL receptor-like genes, *sabR* and *sabS*, as well as an *afsA* homologue, *sabA*. Deletion of *sabA* or *sabS* results in overexpression of yellow pigment as well as a conditional block in aerial hyphae production, which phenocopies *S. coelicolor bldA* mutants. Interestingly, a TTA codon is present in the 5′ end of *sabS* gene, representing the first GBL receptor homologue that is *bldA* dependent. Like the receptors involved in tylosin and lankamycin/lankacidin, no GBL has yet been identified. In all these cases, while it is tempting to assume the accumulation of a GBL triggers signal transduc-

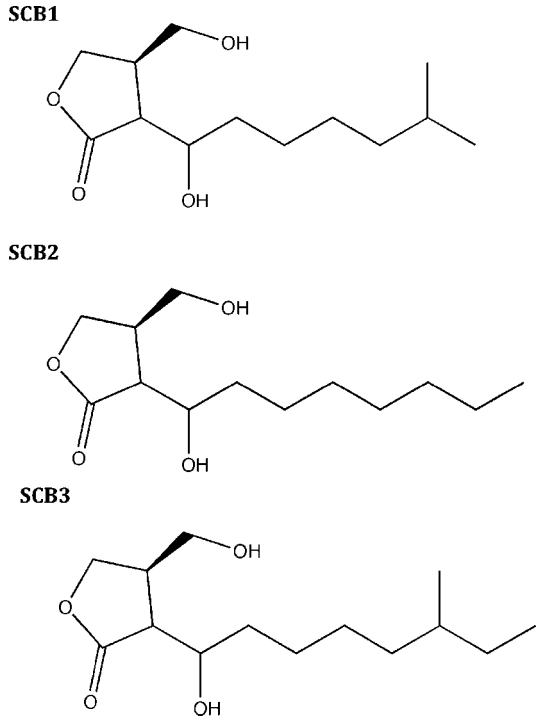


Figure 4. The γ-butyrolactones of *S. coelicolor*. Unlike A-factor in *S. griseus*, SCB1−3 regulate only secondary metabolism in *S. coelicolor*.[118]

tion, it is prudent to remember that not all GBL receptor homologues bind to a GBL.

Three GBLs have been isolated from *S. coelicolor*, of which SCB1 is the most abundant and best understood (Figure 4). SCB1 influences the biosynthesis of actinorhodin and undecylprodigiosin,[59] as well as a less-well characterized molecule produced by the *cpk* polyketide synthase gene cluster.[71] *scbA*, an *afsA* homologue, is induced late in the transition phase; this distinguishes SCB1 production from that of A-factor, which must accumulate throughout exponential phase to reach a threshold concentration.[46] SCBs exert their effects in a narrow concentration range of 0.25−0.5 μM. The *S. coelicolor arpA* homologue, *scbR*, is adjacent and divergent to *scbA*. It regulates the biosynthesis of the pigmented antibiotics actinorhodin and undecylprodigiosin via an unknown pathway; to date only three targets have been identified: its own promoter, that of *scbA*, and the SARP gene *cpkO*, which controls the expression of the cryptic polyketide biosynthetic cluster *cpk*, located next to *scbR*.[71,72]

In contrast to the SCB system, two additional *S. coelicolor* ArpA-like receptors, CprA and CprB are involved in morphological differentiation as well as pigmented antibiotic production. These two proteins share 90.7% amino acid identity to one another and about 35% identity to ArpA.[73] The ligand for these receptors remains unknown, but the effects of *cprB* deletion are similar to those of *arpA* in that antibiotic (actinorhodin and undecylprodigiosin) production is precocious and elevated and differentiation is also accelerated by about a day. Thus, like ArpA, CprB is a repressor. Conversely, *cprA* deletion generates the opposite phenotype with delayed and reduced antibiotics and aerial hyphae formation occurring about a day later than wild-type. Apart from the observation that *ramC* expression is indirectly dependent on CprA,[74] little is known about how CprA and CprB exert their effects. As discussed in section 4, RamC is thought to be the enzyme that post-translatinally modifies the lantibiotic-like morphogenetic peptide SapB.[28]

3. Methylenomycin Furans

Methylenomycin A is a cyclopentanone antibiotic first identified in *Streptomyces violaceoruber* SANK 95570[75] and later found to be produced by *S. coelicolor* A3(2).[76] The antibiotic accumulates at the end of active growth and its synthesis can be induced by acid shock.[77] In both microbes, the gene cluster encoding methylenomycin synthesis is found on plasmids, the circular plasmid pSV1 and the linear plasmid SCP1, respectively. The plasmids share about 99% identity at the DNA level within the methylenomycin biosynthetic operons, but there is very little similarity outside the biosynthetic gene cluster,[3a,78] indicating that the plasmids acquired the gene clusters independently via horizontal gene transfer.

Early analysis of *S. coelicolor* methylenomycin production suggested that a diffusible signal was involved in its biosynthesis. It was observed that methylenomycin deficient mutants display different cosynthesis capabilities when two such strains are grown in close proximity. Strains that rescue other nonproducers are classified as secretors, while convertors are those mutants that regain the capacity to produce the antibiotic when grown near secretors.[79] Recent studies unequivocally show that secretor strains retain the ability to synthesize small signaling molecules akin to GBLs, termed methylenomycin furans (MMFs), but lack the methylenomycin biosynthetic genes, while the opposite is true of converters.[80] Similar to GBLs, the MMFs are extractable in organic solvent as well as being heat, protease, and acid resistant. However, the MMFs are not inactivated by alkaline treatment, a key observation in their structural elucidation as this suggested that the MMF family of signaling molecules were not lactones, distinguishing them from the GBLs.[80a] Indeed, this new class of signaling molecule is structurally distinct in that they all share a common 2-alkyl-4-hydroxymethylfuran-3-carboxylic acid (AHFCA) core structure but differ in the identity of the C2 alkyl group.[80a,81] Five distinct MMFs have been isolated from *S. coelicolor* (Figure 5); however, their production depends only on a single enzyme, MmfL. Two other biosynthetic genes, *mmfH* and *mmfP* carry out later steps in MMF biosynthesis that may be also be catalyzed by the paralogues *actVA* and SCO3558, respectively, albeit at a lower efficiency.[80a]

The discovery of the MMFs demonstrates the power of genome mining, and its role in methylenomycin production was inferred by its genomic context. Specifically, the MMF biosynthetic three-gene operon, *mmfLHP*, is adjacent to genes known to be required for methylenomycin production. MmfL shows sequence similarity to AfsA, the butenolide synthase required for A-factor biosynthesis in *S. griseus*.[45,80a] The analogy to the GBL system is further extended by the

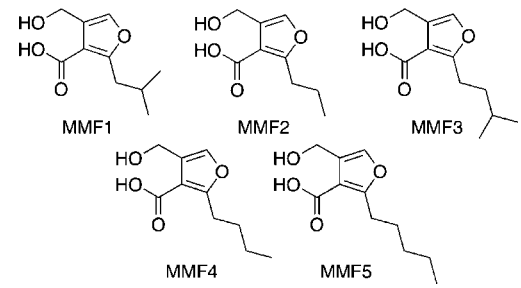


Figure 5. MMF structure. Like A-factor synthesis, MMF biosynthesis begins with the condensation of DHAP and a coenzyme β-keto acid derivative. This is followed by ring closure and dephosphorylation to yield one of five MMFs.[80a,81]

presence of two regulatory genes (*mmyR*, *mmfR*) whose products are similar to ArpA-like receptor/repressor proteins.[80b] MmyR and MmfR are proposed to form a hetero-oligomeric repressor through which the diffusible signals exert their effect.

Given the similarities between the MMF and GBL systems, it is not surprising that both A-factor and the MMFs are derived from the same starting material, a coenzyme A β-ketothioester intermediate in fatty acid biosythesis and dihydroxyacetone phosphate (DHAP; see Figure 1). The origin of the ketones was established by analyzing the incorporation of radiolabeled precursors resulting in varying alkyl substituents at C-2. The involvement of fatty acid intermediates was further confirmed by the heterologous expression of the MMF biosynthetic pathway in *E. coli*, which results in molecules with linear alkyl side chains. This reflects the use of the straight chain starter acetyl-CoA by *E. coli* to synthesize fatty acids. By contrast, *Streptomyces* spp. begin fatty acid synthesis with both linear and branched chain units, so this microbe can generate a variety of alkyl side chain species.[80a] On the basis of the similarity of the starting materials and the homology of the implicated biosynthetic machinery, it is conceivable that the pathways initially proceed via common steps. Following an initial aldol reaction of the CoA β-ketothioester and DHAP, chemoselective ring closure results in either a furan or butenolide.[81] Although synthetic studies pointed to a ketal intermediate that undergoes dehydrative aromatization, the reactive α-hydroxyl group does not attack the electrophilic carbonyl, thus, resulting in furan ring formation without detectable butenolide product. Recent chemical synthesis of MMF supports this proposed mechanism.[81]

MMF-like compounds are not confined to *S. coelicolor*. A similar three-gene cluster was identified when the genome of *Streptomyces avermitilis* was sequenced and annotated.[3b] In addition, related rhamnolylated AHFCAs have been isolated from several *Strepomyces* isolates.[82] The biological function of these compounds has yet to be determined, raising the exciting possibility that a new class of signaling molecules awaits investigation.

4. SapB and Other Lanthionine-Containing Peptides

The small hydrophobic peptide SapB was first reported in 1989[83] as one of five so-called spore-associated proteins, identified by their attachment to the surface of *S. coelicolor* spores. With a molecular weight of only 2027 Da, SapB was easily gel purified, and by 1991, its role as an extracellular morphogenetic peptide was shown in two ways.[13] First, it was demonstrated that the diffusion of SapB from wild-type and *whi* colonies of *S. coelicolor* was, at least in part, responsible for accelerating differentiation of other, less robust wild-type strains and restoring aerial hyphae formation to Bld mutants plated in close proximity. However, the most compelling evidence that the peptide has an important role in aerial hyphae formation is the ability of purified peptide to restore to Bld mutants the capacity to erect aerial structures. As a hydrophobic, amphiphilic molecule that was shown to self-assemble,[25] it was hypothesized that SapB functions by reducing the surface tension at the colony−air interface, thereby releasing nascent aerial filaments from the confines of the aqueous milieu of the vegetative mycelium. Evidence for this was at least twofold; first, SapB is highly surface active, reducing the surface tension of water (78 mJ/m^2) to 30 mJ/m^2 at 50 μg/mL,[25] roughly the amount that would be delivered in a 20 μL drop containing 1 μg of peptide.[13] The second line of evidence was obtained by examining the ultrastructure of SapB-rescued Bld mutants. Here, undifferentiated vegetative hyphae stand erect; in these mutants, SapB cannot trigger complete morphogenesis that culminates in sporulation.[25] Further evidence awaited structural resolution, which was not to follow for some years.

In fact, structural and genetic elucidation of SapB was quite elusive due to its extreme structural stability; indeed, the peptide is resistant to Edman degradation, is heat and protease resistant,[13] and tandem mass spectrometry fails to yield fragments that could be easily defined chemically. However, in 2004, it was noted that the gene *ramC* (the *S. coelicolor amfT* ortholog) was predicted to encode an enzyme whose C-terminal half bears similarity to lantibiotic modification enzymes.[28] Lantibiotics are ribosomally synthesized peptide antibiotics that undergo specific post-translational modifications that include dehydration of serine and threonine residues followed by intramolecular cyclization with cysteine residues to yield stable lanthionine or methyllanthionine bridges, respectively. In most cases, the number of dehydrated residues outnumbers available cysteines, so unreacted didehydroalanines (Dha) and/or didehydrobutyrines (Dhb) often remain.[84]

The *ram* gene cluster (Figure 6) had been reported a decade earlier when it was discovered in a screen for *S. coelicolor* genes that when expressed in low copy could accelerate differentiation in the closely related species *Streptomyces lividans*.[85] The initial annotation of the *ram*

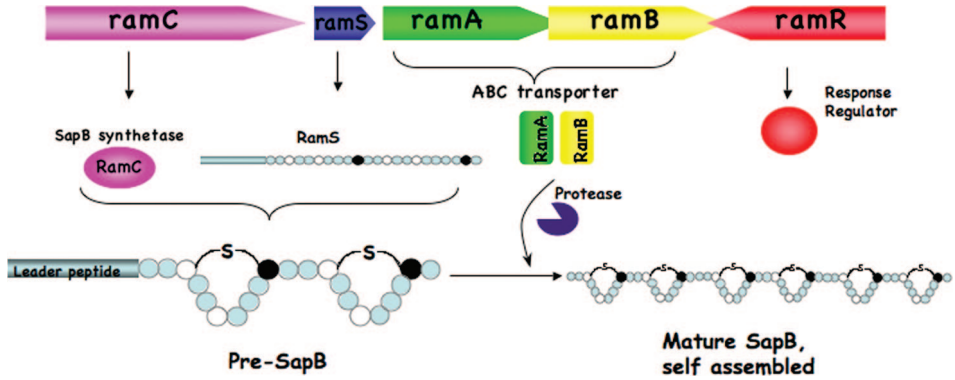


Figure 6. SapB bioynthesis. The SapB prepeptide is encoded by *ramS*, which is part of a five-gene cluster. RamC is thought to encode the enzyme that dehydrates four serine residues (shown in RamS as white circles), and catalyzes the formation of lanthionine bridges between the two cysteine residues (black circles). An unidentified protease cleaves the leader sequence and the peptide is exported.[28]

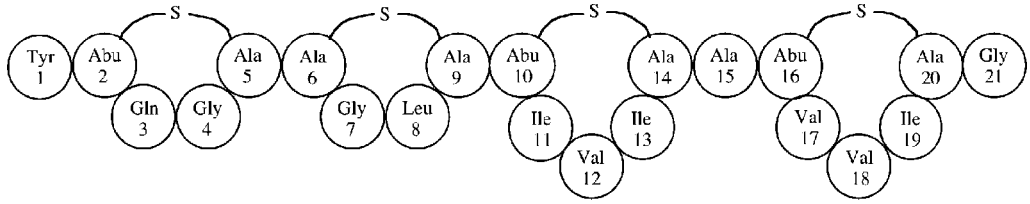


Figure 7. SapT. Unlike SapB produced by *S. coelicolor*, which has two lanthionine bridges, the *S. tendae* peptide SapT has only one lanthionine linkage, but three methyllanthionine bridges form between dehydrated threonine residues (dideoxybutyrate) and cysteines. Nonetheless, when self-assembled, both peptides present similar amphipathic structures and are biologically active when applied to either species.[95]

genes (for rapid aerial mycelium) focused on *ramR*, a response regulator, and *ramAB*, which encode components of an ABC transporter. Homology with the *amf* locus of *S. griseus*[86] was noted; amino acid identity ranges from 38% between *ramR* and *amfR* to 51% between *ramA* and *amfA*.[85] Annotation and analysis of the other two genes in the cluster, *ramC*, thought to encode a serine-threonine kinase and *ramS*, a gene capable of coding for a 42 amino acid peptide, were reported in a series of subsequent papers published by other groups[74,87] some of whom had independently discovered the *ram* genes in screens designed to identify genes involved in the transition from vegetative to aerial growth.[87a,b] However, because the predicted structure of the *ramS* translational product is twice the size of mature SapB and lacks agreement with what at the time was the only reported amino acid composition,[13] the possibility that RamS could be pre-SapB was dismissed.[30]

The discovery that led to the structural resolution of SapB was the finding that while the N-terminus of RamC possesses a serine-threonine kinase domain, its C-terminus is similar to the cyclase domains of the enzymes MrsM and CinM,[28] involved in the production of the lantibiotics mersacidin and cinnamycin, respectively. Prior to this observation, it was reported that a synthetic C-terminal fragment of AmfS, the *S. griseus* RamS ortholog, triggers aerial hyphae formation in Bld mutants,[24] in a manner similar to SapB, albeit at a much higher concentration. Together, this led to the hypothesis that RamS might be the SapB prepeptide. Confirmation of this was based on chemical modification of purified peptide that enabled Edman degradation[88] and its alignment with the C-terminal half of RamS.[28] Although SapB is far too insoluble to permit NMR analysis, a combination of biochemical approaches, including tandem MS, resulted in the proposed structure and resulting biosynthetic pathway shown in Figure 6.[28]

Interestingly, the annotation of RamC as a serine threonine kinase has unusual relevance in this case. Chatterjee et al.[89] show that the lacticidin 481 synthetase, LctM, employs a two-step mechanism for the dehydration of serine and threonine residues in the biosynthesis of its lantibiotic peptide. As the hydroxyl is a poor leaving group, phosphoserine/phosphothreonine are better substrates for the second step, formation of double bond between the α and β carbons. In fact, recent analysis of the lantibiotic synthetase VenL from *S. venezuelae* reveals that, unlike LctM and all other previously studied lantibiotic synthetases, VenL (and by homology RamC) use two separate domains to catalyze the dehydration step: a phosphoserine/phosphothreonine lyase domain and a Ser/Thr kinase domain. Together, with a cyclase domain, these introduce the lanthionine and methyllanthionine bridges found in mature lantibiotics.[90] Curiously, RamC is so far unique in that it lacks a prototypical C-terminal cyclase-like domain with a characteristic zinc binding site.[91]

The presence of SapB-like molecules in other *Streptomyces* spp. is suggested by Western blot analysis and bioinformatics. Specifically, anti-SapB reacts with spore proteins from *S. lividans*, *Streptomyces parvulus*, and *S. viridochromogenes*;[13] genome annotation reveals the presence of *ramS* homologues in *Streptomyces scabies* (which has two such genes), *S. avermitilis*, *Streptomyces griseoflavus*, *Streptomyces albus*, *Streptomyces roseosporus* as well as *amfS* in *S. griseus*. Amino acid identity ranges from 73% (*S. griseus* and *S. roseosporus*) to 100% (*S. greseofulvus* Tu4000 and *S. avermitilis*). In all cases, the serine and cysteine residues involved in dehydration and lanthionine bridging are conserved.

The lack of homologues of SapB in bacteria outside the streptomycetes presumably reflects the specific requirement of the filamentous microbes as they erect aerial hyphae. The capacity of SapB to self-assemble[25] has led to the model whereby SapB and other hydrophobic proteins called chaplins[92] form a polymeric film around the aerial hyphae.[28,93] Not only would this reduce the surface tension at the colony at the surface during initial aerial hyphae formation, but it has also been suggested that the continued presence of this film might form a channel through which nutrients and other metabolites can diffuse from the substrate to the growing tips of the aerial filaments.[94]

Despite the lack of homologues, there is at least one other, structurally distinct, but functionally homologous peptide. SapT (MW 2032), isolated from *S. tendae* which shares the canonical lantibiotic features, but has four smaller heterocyclic loops formed by three methyl-lanthionine bridges and one lanthionine bridge[95] (Figure 7). Unlike SapB, SapT demonstrates limited antibiotic activity, inhibiting *Bacillus cereus* at high concentrations (0.5 μg peptide spotted directly on a lawn of cells; due to limited solubility, standard minimal inhibitory concentration assays are not possible). SapT, like SapB, rescues aerial hyphae formation in Bld mutants. This is also true of the fungal hydrophobin SC3,[25] which bears no structural similarity to the streptomycete peptides, but is also capable of self-assembly at an air−water interface.[96] Surprisingly, all three proteins also restore complete differentiation, that is, sporulation, to *ram* null mutants.[95] Although these molecules are structurally diverse, there appears to be some specificity as other natural and synthetic surfactants fail to elicit morphogenesis.[97]

The supermolecular structure produced upon self-assembly and the amphipathic nature of these peptides may account for this specificity. Models of both SapT[95] and SapB[28] suggest that the macrocyclic rings prevent the hydrophobic side chains from burrowing within the cores of the peptides. This gives the molecules a distinctly amphiphilic character, where the polar backbone atoms would be capable of forming

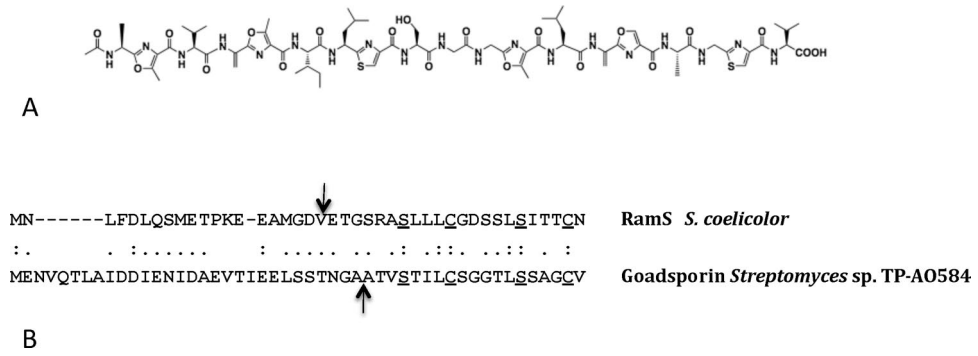


Figure 8. Goadsporin structure and alignment with RamS. (A) Goadsporin is a thiopeptide with two thiazole and three oxazole rings;[99] however, when aligned with RamS (B), there is striking conservation of the serine and cysteine residues that are modified in both molecules. Arrows mark sites of leader cleavage.

extensive hydrogen bonds with surrounding water molecules, while the hydrophobic side chains could project out of the water layer. This alignment might favor the observed self-assembly of SapB[25] through the formation of large, stable hydrophobic interfaces between SapB monomers. Indeed, such a model has been proposed for the multimerization of the fungal surfactant HFBII[98] and is consistent with the capacity of the peptides to trigger the emergence of upwardly growing hyphae.

5. Goadsporin

The post-translationally modified peptide goadsporin, produced by *Streptomyces* sp. TP-A0584, further extends the structural and functional diversity of small molecules that influence streptomycete morphogenesis (Figure 8A). Onaka and co-workers[99] discovered goadsporin in a screen designed to identify compounds that could stimulate actinorhodin production by *S. lividans* TK23, which does not express its actinorhodin biosynthetic genes under normal laboratory conditions. In paper disc assays, 10 μg of purified goadsporin accelerated sporulation in 36 out of 42 streptomycete isolates and promoted pigment production in 20 of these strains. At concentrations greater than 1 μg/mL, goadsporin also inhibited growth in 32 of the isolates, such that morphogenetic effects were seen just beyond a zone of growth inhibition suggesting that the peptide is active at a specific and narrow range of concentrations. Goadsporin displays antibiotic activity only against other actinomycetes.[100] Unlike other morphogenetic peptides, goadsporin is not secreted, nor does it have any effect on the producing strain when applied exogenously.[99]

Strikingly, the peptide is a linear oligopeptide that possesses four oxazole and two thiazole residues.[99] In this regard, goadsporin (MW 1161.66) is more similar to the microcin group of antimicrobial peptides produced by Gram-negative bacteria.[101] However unlike the microcins, in which thiazole, oxazole, oxazole-thiazole, and thiozole-oxazole structures derive from Gly-Cys, Gly-Ser, Gly-Ser-Cys, and Gly-Cys-Ser motifs, respectively, goadsporin biosynthesis cyclizes Cys, Ser, and Thr residues neighboring Gly, Ser, Ala, or Leu. In addition to dehydratation and cyclization, the N-terminus of goadsporin is acetylated. Given these differences between goadsporin and the microcins, it is perhaps not surprising that only one (*godE*) of the five genes thought to catalyze these modifications is similar to a microcin modification gene (*mcbC*).[102]

Goadsporin bears no similarity with any other peptide when the full-length sequence is queried. However, manual alignment with RamS (SapB prepropeptide) raises a curious evolutionary question (Figure 8B). Intriguingly, although there is only about 18% amino acid identity between the two peptides, the serine residues that become dehydrated to didehydroalanines but are not cyclized in either SapB or goadsporin align, as do the two modified cysteine residues. In SapB, these cysteines contribute to lanthionine bridge formation, whereas in goadsporin, they become thiazoles. Such similarity may suggest a common evolutionary history involving divergence at the level of post-translational modification. In fact, the G + C content of the goadsporin gene cluster (65.7%) is lower than the average streptomycete (70−72%) and the cluster is bounded by transposase-encoding genes, suggesting acquisition by horizontal gene transfer.[102]

6. Factor C

Factor C is a much larger extracellular protein (MW 31 038) that induces morphogenesis. The protein was first isolated from the spent medium of what was believed to be *S. griseus* 45H, but has recently been identified as *Streptomyces albidoflavus* 45H.[103] As little as 0.5 ng/mL of broth is sufficient to trigger sporulation in strains capable of differentiating in liquid, such as *S. griseus* 52-1. In addition, the direct addition of factor C to colonies of *S. griseus bld* mutants restores their ability to sporulate.[104] Curiously, when factor C is heterologously expressed in A-factor deficient *S. griseus bld* mutants, A-factor production is restored along with biosynthesis of proteins in the A-factor regulon.[105] More recent proteomic analysis reveals that, in the absence of factor C expression, such mutants overproduce a number of nutrient-scavenging and stress-related proteins. Factor C dependent A-factor production restores biosynthesis of these proteins to wild-type levels..[106]

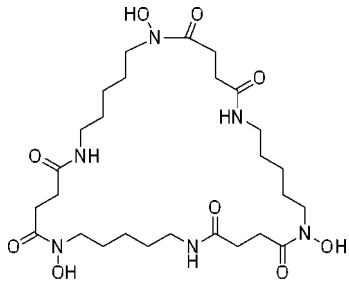
Although it is much larger than goadsporin and the SapB-like peptides, factor C also undergoes cleavage of a 38 amino acid residue N-terminal leader sequence.[107] The leader is clearly required for export as it bears a TAT secretion signal.[105] Its large size makes factor C an unusual signaling compound. Indeed, it does not appear to be a diffusible signal as its N-terminal 60−90 residues may span the membrane such that the N-terminus is cytoplasmic and the C-terminus is extracellular.[108] When factor C is added exogenously to medium, it rapidly disappears, possibly because it becomes inserted in the membrane or it is cleaved into smaller (active?) peptides.[109]

7. Siderophores

The acquisition of iron can be especially problematic for aerobic microorganisms like the streptomyctes, as iron generally occurs as insoluble precipitates in oxic environments. The use of high-affinity, iron-scavenging siderophores is a common strategy used by microbes to capture and take up iron from the environment.[110] Somewhat surprisingly, the siderophore desferrioxamine E has been shown to modulate streptomycete development as well[111] (Figure 9). This molecule belongs to a family of structurally related trishydroxamate siderophores that form strong hexadentate complexes with ferric iron.[112] Unlike most well-characterized siderophores, the desferrioxamines are not assembled by nonribosomal peptide synthetase (NRPS) multienzyme complexes.[110] Desferrioxamine E is the major desferrioxamine siderophore produced by *S. coelicolor* M145,[112] which also produces desferrioxamine B and ceolichelin, a NRPS-dependent siderophore.[113]

Morphogenetic activity of this siderophore was discovered when it was noted that *S. griseus* could stimulate growth and development of *Streptomyces tanashiensis*. Desferrioxamine E purified from *S. griseus* culture supernatant was responsible for the observed effect, although other siderophores (e.g., ferrichrome and nocobactin) and free ferric ion had no effect. *S. coelicolor* also produces this siderophore, so it too can extracellularly influence *S. tanashiensis* growth and development. It follows that a desferrioxamine E-deficient *S. coelicolor* strain cannot rescue *S. tanashiensis* differentiation. Tellingly, the mutant is impaired in both growth and morphological differentiation, but appears wild-type upon the addition of purified desferrioxamine E. This raises the possibility that *S. tanashiensis* may be defective in production of siderophores but can use those produced by other microbes.[111] Indeed, others have reported the ability of *S. coelicolor* to use other, exogenous siderophores to acquire iron.[113] Given the central role of iron in primary metabolism, it is perhaps not surprising that iron-deficient strains show morphological defects. Interspecies exchange of siderophores is well-known in other microbial systems[114] so it may not be an unusual means by which microbes influence growth and development of others in the same microbial community.

There is some evidence, however, that the means by which siderophores mediate streptomycete morphogenesis may be more complex than simply the need for iron as a key cofactor. Recent work shows that two metals, zinc and iron, influence antibiotic production[115] and sporulation[116] in *S. coelicolor*. The role of zinc on antibiotic biosynthesis is mediated through a novel pleiotropic regulator AbsC, which represses expression of the gene cluster encoding coelibactin biosynthetic genes. Coelibactin is an uncharacterized NRPS-dependent peptide thought to have siderophore activity. When the *absC* gene is deleted, both actinorhodin and undecylprodigiosin production cease, but only if zinc levels are low. This is due to elevated expression of the coelibactin gene cluster, which possesses predicted binding motifs for the zinc-responsive regulator Zur. Mutant analysis confirms that the gene cluster is under dual regulation by Zur and AbsC.[115] *S. coelicolor zur* mutants are defective in development, but this phenotype is suppressed when the mutant also is deficient in coelibactin biosynthesis.[115] Thus, repression of the coelibactin gene cluster by both AbsC and Zur appears to be required for intracellular zinc homeostasis in *S. coelicolor*.[115,116]



desferrioxamine E

Figure 9. Desferrioxamine E. Several streptomycete siderophores have been implicated in modulating morphological differentiation of their production strains as well as other *Streptomyces* spp.[111−113]

8. Evidence of Other Extracellular Morphogenetic Molecules

While the list of extracellular molecules that influence streptomycete development continues to grow, there is most probably an untapped reservoir of such compounds in nature. Perhaps the best evidence for this was reported by Ueda and colleagues,[117] who describe the ability of 32 out of 33 wild streptomycete isolates to stimulate antibiotic production in 11 laboratory *Streptomyces* strains, and morphological differentation in 19 strains. Significantly, in no case could the observed complementation be induced by the application of a γ-butyrylactone, including A-factor. Indeed, the γ-butyrylactones are typically species specific,[118] so the study of interspecific streptomycete communication may open the door to a suite of structurally diverse signaling molecules.

Recent reports of the effect of antibiotics on nontarget microbes[119] make it tempting to speculate that subinhibitory concentrations of antibiotics may serve as morphogenetic factors. The notion that well-characterized antibiotics might prove to be a source of morphogenetic molecules is further suggested by the capacity of subinhibitory concentrations of antibiotics from diverse structural classes to modulate the activity of nearly 5% of bacterial promoters in promoter-*lux* libraries, including those that govern quorum sensing genes in a number of bacteria.[120] Indeed, the pamamycins, a group of macrolide antibiotics produced by *Streptomyces alboniger* demonstrate this capability. At low concentrations, these antibiotics induce precocious aerial hyphae formation, but at higher concentrations, growth of nonproducing streptomycetes and other Gram-positive bacteria is inhibited.[121] Like siderophore-induced development, pamamycin signaling is interspecific, rescuing or stimulating aerial hyphae formation in 20 different *Streptomyces* spp.[122] Such data together with the discovery that over 450 wild soil-dwelling bacterial isolates carry the resistance genes for at least seven antibiotics[123] seem to suggest that antibiotics are produced in abundance in nature and may have other functions apart (or in addition to) growth inhibition.

9. Future Directions

Since the initial discovery of A-factor,[2] the exchange of extracellular signaling molecules has become recognized as an essential regulatory mechanism both within and between many microbial species, including the streptomycetes. The next decade will surely reveal an even greater assortment of compounds that serve as morphogenetic signals. For instance, the streptomycetes have recruited the ubiquitous compound, *N*-acetylglucosamine, as a signaling molecule.[124] The GntR-

like regulator, DasR, transmits the signal to antibiotic pathway specific activators as well as to *N*-acetylglucosamine related catabolic genes,[125] providing a tangible link between the onset of secondary metabolism and development. At the same time, the technical capacity to identify extracellular products and dissect their function has greatly improved. A recent example can be found in a report by Yang et al.[126] who used new methodologies to probe interspecies interactions. Here, the authors use thin-layer agar natural product MALDI-TOF imaging to visualize the suppression of aerial hyphae formation in *S. coelicolor* when plated close to a colony of *Bacillus subtilis* that secretes the lipopeptide surfactin. These striking images show that SapB is produced only outside the zone of surfactin diffusion.[126]

A central question that remains unanswered is the identity of predicted sporulation signals in *S. coelicolor*.[14,127] So far only an unidentified oligopeptide imported by the BldK permease has been implicated as signal in this cascade.[128] It has been suggested that some of these molecules may be proteases that in *S. griseus* are part of the A-factor regulon (Figure 1) and the BldK-depended peptide might result from proteolytic cleavage of a precursor protein.[40] Clearly, the extracellular biology of the streptomycetes is complex as recently reviewed by Chater et al.[129] However, the level of integration of multiple physiological and environmental factors involved in eliciting morphological and physiological differentiation in the streptomycetes, as evidenced by the structural and functional diversity of the morphogenetic factors that have been characterized to date, will continue to make this group of microbes valuable model organisms.

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