Regulatory pathways in *Streptomyces* spp.

A closer look at the regulatory cascade started by the A-factor in Streptomyces griseus and the regulatory pathway in Streptomyces fradiae which leads to the production of tylosin.

Inez de Jong

Microbial Physiology

Andriy Kovalchuk

Eriko Takano

Abstract

Streptomyces species are the largest source of naturally produced antibiotics. Since resistance against the known antibiotics continues to grow there is always a need to find new antibiotics. The genes needed for antibiotic production are located in large gene clusters that take up a large area of the genome. The regulation of these biosynthesis clusters is often a complicated system. The synthesis of tylosin in *Streptomyces fradiae* for instance is controlled by a regulatory cascade with no less than five regulators. It starts with a γ-butyrolactone binding to its receptor TylP which in turn activates the transcription of three genes; *tylP, tylQ* and *tylS*. The interplay of these three genes, plus two more activated by TylS, leads to the activation of the tylosin biosynthesis pathway. The synthesis of streptomycin in *Streptomyces griseus* has a straightforward regulatory cascade leading to its synthesis. From the systems known today it is relatively rare in the amount of processes it activates ranging from numerous processes in morphological differentiation to many aspects of secondary metabolism. In both systems there is an important role for γ-butyrolactones, small diffusible molecules. Low concentrations of these molecules are enough to switch on their specific pathways. Another important role is for a rare TTA codon in some of the regulatory genes. Only one tRNA is able to translate this codon to leucine. Without this tRNA the pathways will not function and mutant strains sometimes display a very specific phenotype.

Table of Contents

1. Introduction 4

2. Regulatory pathway controlled by A-factor in *Streptomyces griseus* 6

2.1. The regulatory cascade starting with A-factor in *S. griseus* 7

2.2. Autoregulation of AdpA 7

2.3. The GBL system of *S. griseus* differs from other known GBL systems 7

3. The influence of the rare leucine codon TTA in *Streptomyces* spp. 9

3.1. The occurrence of the rare TTA codon in *Streptomyces* spp. 9

3.2. Influence of the rare TTA codon on the production of secondary metabolites and morphological differentiation in *S. griseus* 9

4. Regulatory pathway in *Streptomyces fradiae* 10

4.1. The master regulator TylP exerts its influence on multiple targets 10

4.2. Two SARPs are present in the tylosin cluster, only one influences the production of tylosin 11

4.3. The not yet fully understood function of TylQ 12

4.4. The presence of TylU, a SARP helper protein, optimizes the production of tylosin 12

4.5. The tylosin regulatory cascade in short 13

5. Discussion 14

5.1. GBLs are needed for the activation of the systems in both *S.griseus* and *S. fradiae* 14

5.2. The occurance of a TTA codon in the regulatory pathways of both *S.griseus* and *S. fradiae* 14

5.3. The A-factor cascade versus the TylP cascade, differing on the amount of processes activated and regulators requiered 15

5.4. The many unknowns in the tylosin regulatory pathway 15

5.5. Final remarks 15

References 16

1. Introduction

Streptomyces are soil-dwelling organisms from the order actinomycetes. In appearance *Streptomyces* spp. look like fungi and are able to form aerial mycelium and spores [1]. Their secondary metabolism yields a wide range of bio-active compounds amongst which numerous kinds of antibiotics. They are by far the main source of natural antibiotics in the world. The G+C rich genome of *Streptomyces* spp., over 70%, often contains one or more antibiotic biosynthesis clusters. Besides the antibiotics producing species there also are some pathogenic species like *Streptomyces scabies*.

In *Streptomyces* spp., and other actinomycetes, small diffusible signaling molecules are found which regulate antibiotic production and morphological differentiation [2]. These small molecules called γ-butyrolactones (GBLs) were first identified by Khokhlov and coworkers [3] in *Streptomyces griseus*. With this discovery, the GBL from *S. griseus*, named A-factor, became the example for GBLs later discovered in other actinomycetes. GBLs exert their influence by binding to their cytoplasmatic receptor proteins causing the latter to disassociate from their specific DNA targets which activates their transcription [2].

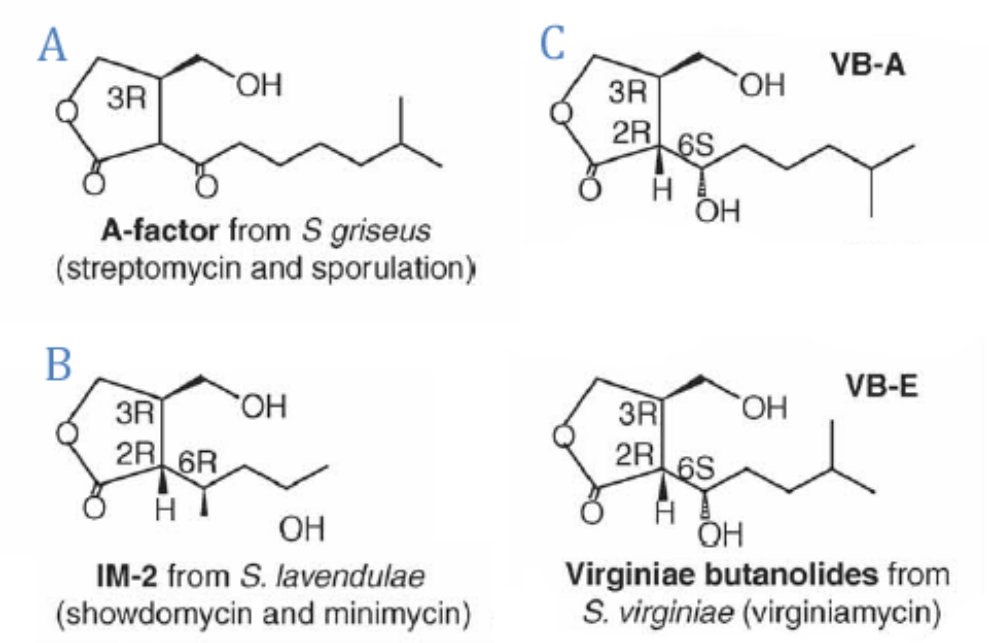

Fig.1: Chemical structures of three γ-butyrolactones

Four examples of structures of γ-butyrolactones. A, the A-factor from *S. griseus*. B, an example of GBL from *Streptomyces lavendulae*. C, two examples of Virginiae butanolides of *Streptomyces virginiae*. Adapted from [2]

The structures of only a few GBLs have been discovered until now, see fig.1 for some examples. Nanomolar concentrations of the GBLs are enough for intracellular recognition [2]. These low concentrations make it extremely difficult to extract them from the medium and characterize them. There are many putative GBL receptor protein found in many different actinomycetes but finding their cognate GBL is a difficult task [4],[2].

An often found regulatory gene involved with the regulation of antibiotic production is SARP, *Streptomyces* antibiotic regulatory protein [5],[6]. They form a family of pathway specific activators in for example *Streptomyces coelicolor* and *Streptomyces fradiae,* where they are incorporated in the regulatory biosynthesis pathway of actinorhodin and tylosin respectively [7],[4]. Most SARPs found are the last step needed to switch on an antibiotic biosynthesis cluster where they function as the transcriptional activator towards the biosynthesis of that specific antibiotic.

Antibiotic biosynthesis in *Streptomycetes* is often regulated via (complicated) pathways [5]. In this paper two of these pathways will be described. As first example the regulatory cascade in *Streptomyces griseus* starting with the A-factor will be discussed, the example of a regulatory pathway starting with a GBL. This is a relatively uncomplicated system with some small but important steps required to keep the pathway functional. As a second example the regulatory pathway in *Streptomyces fradiae* leading to the production of tylosin will be discussed. This second example is rather more complicated then the first and contains some unique features not found in any other known pathway. To conclude the two different pathways will be compared with each other to show that though there are quite a few similarities, the differences are numerous as well.

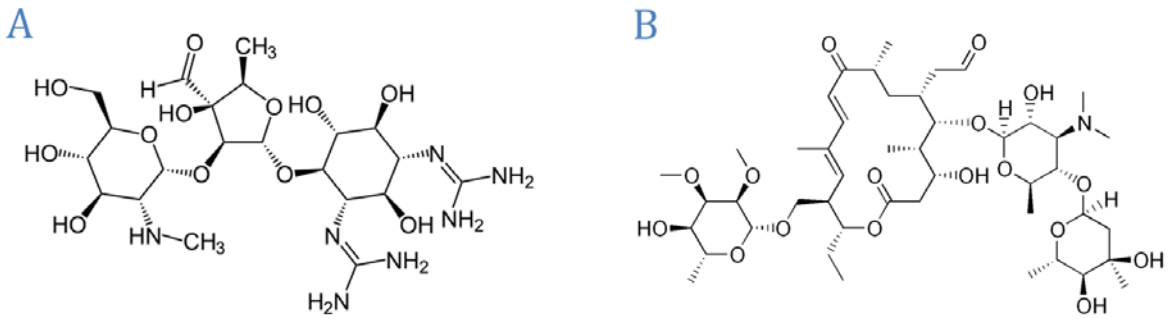

Fig.2: Chemical structures of streptomycin and tylosin

A: Chemical structure of Streptomycin, secondary metabolite of *Streptomyces griseus*

B: Chemical structure of Tylosin, secondary metabolite of *Streptomyces fradiae*

2. Regulatory pathway controlled by A-factor in *Streptomyces griseus*

S. griseus is like many other *Streptomyces* spp. a producer of antibiotics. The production of streptomycin lies at the end of a relatively short and straightforward regulatory cascade [1]. However what is remarkable about the system is the multitude of pathways activated by the first regulators. It is a very tiny snowball on top of the hill which gathers a lot of extra snow rolling down hill. As is shown in fig.3 a whole range of processes of secondary metabolism and morphological differentiation are organized in this system. The regulatory cascade starting with the A-factor will be discussed here and some points as to why this system is not completely as straightforward as it looks.

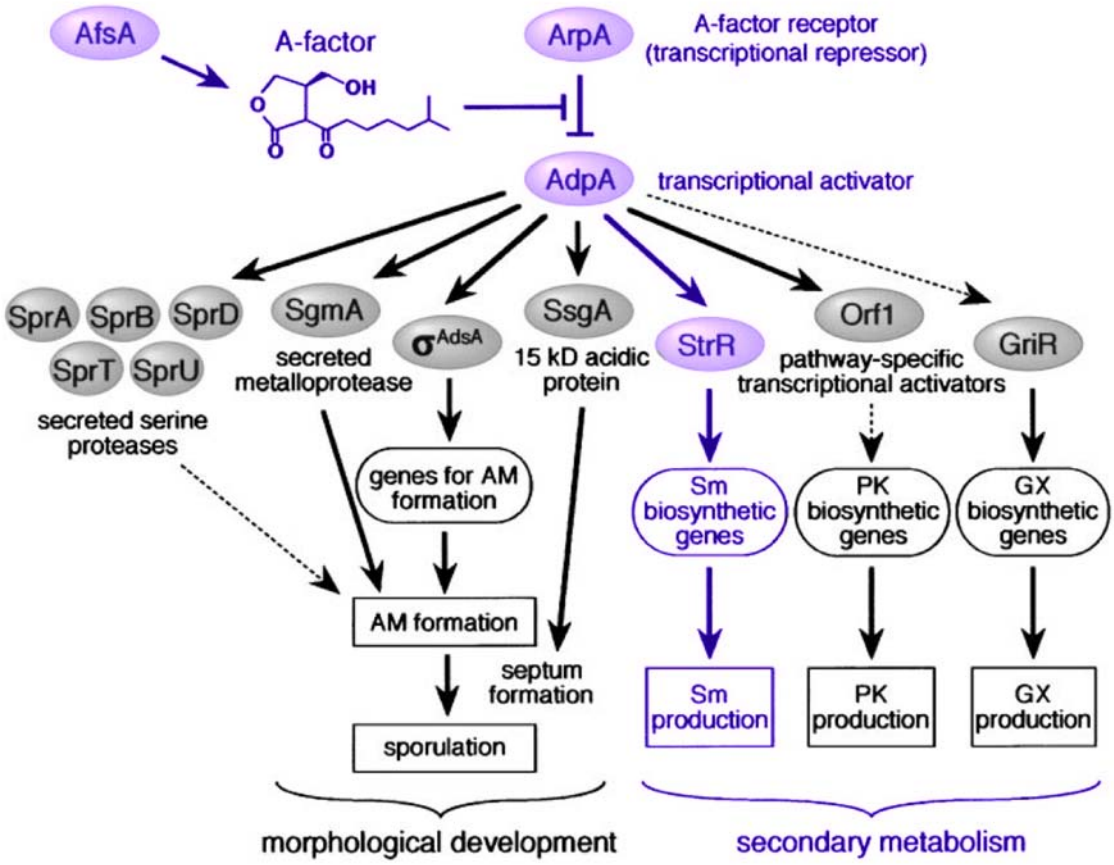

Fig.3: The A-factor regulatory cascade

The A-factor regulatory cascade with all its effects from morphological development to secondary metabolism, at the top starting with the A-factor and its receptor ArpA. By binding of the A-factor to its receptor the transcription of AdpA is activated. AdpA in turn activates the transcription of StrR leading towards the activation of streptomycin synthesis. AdpA is also the activator of all the other processes of morphological development and secondary metabolism shown. Adapted from [8]

2.1. The regulatory cascade starting with A-factor in *S. griseus*

It all starts with the A-factor (2-isocapryloyl-3Rhydroxymethyl-γ-butyrolactone). The A-factor is a GBL which has been discovered in the 1960s by Khokhlov [3]. It has become the example of GBLs in *Streptomyces* spp. AfsA is the enzyme responsible for the production of the A-factor. When the A-factor has reached a certain concentration it binds to ArpA (A-factor receptor protein) and causes it to disassociate from the *adpA* promoter. With this the transcription of *adpA* is activated and it results in AdpA (A-factor dependent protein) being formed. AdpA acts as a positive transcriptional regulator and is responsible for the activation of multiple pathways for secondary metabolism and morphological differentiation. AdpA forms dimers and binds to one or more binding sites downstream of the transcriptional start points of the target genes. RNA polymerase is then recruited to the specific promoter regions where it facilitates the isomerization of the RNA polymerase-DNA complex into an open complex ready for transcriptional initiation.[8] AdpA was first recognized as a protein responsive/dependant on A-factor by Vujaklija and coworkers in 1993, though it was not yet given the current name [9]. The expression of *strR,* pathway-specific activator for the streptomycin biosynthesis cluster, is also regulated by binding of AdpA. Near the promoter region of *strR* two AdpA binding sides are found which by binding of AdpA activate the transcription [10]. The transcription of all the streptomycin biosynthesis genes is dependent on StrR. Not all the genes in the streptomycin biosynthetic cluster have a recognized binding site. However no genes are transcribed from this cluster in a *strR* knock-out strain. Since no other DNA-binding proteins are found in the streptomycin biosynthetic cluster StrR is alleged to be the only transcription activator for all the genes in this cluster.[10]

2.2. Autoregulation of AdpA

This cascade described above looks pretty simple, perhaps too simple when there might be up to 152 genes belonging to 74 transcriptional units that have been found by Hara and co-workers to be probably induced by the A-factor [11]. There is certainly more to this simple system which is strictly secured by internal regulation. When ArpA is bound to AdpA the transcription of the latter is repressed. However the transcription of *adpA* is not only repressed by ArpA but also by its own product AdpA by means of a negative feedback loop. [12]. In fig.4 is shown how low intracellular levels of AdpA exert no influence on its own transcription and only one regulator is bound to its own promoter. When the concentration of AdpA rises, two more AdpA molecules bind to the *adpA* promoter and together they suppress the transcription of *adpA.*

2.3. The GBL system of *S. griseus* differs from other known GBL systems

There are four other systems known in *Streptomyces* spp. that contain a GBL system with its cognate receptor as there is in *S. griseus*. In three of those systems the GBL has no influence on aerial mycelium formation [13]. However a recent discovery in *Streptomyces clavuligerus* shows that in an *adpA* knock-out strain no spore formation is observed and only sparse aerial mycelium are formed [14]. So *S. griseus* is not unique in that it influences both secondary metabolism and morphological

differentiation. When first research was done on *adpA* mutants the finding that no aerial mycelium and spores could be formed anymore came quite as a surprise [15]. So not only is the A-factor important for the production of streptomycin, it also influenced the morphological changes of the organism. Another remarkable difference between the A-factor regulatory cascade and the other four known systems is that the regulation of the production of the A-factor itself is regulated directly or indirectly by AdpA[13], hence placing it under similar control as the synthesis of for instance streptomycin. In the other known systems the A-factor receptor negatively regulates itself and directly influences the production of its cognate GBL. Experiments by Kato and coworkers have shown that deletion of *arpA* does not influence the production of the A-factor whereas the deletion of *adpA* gives rise to an increased amount of A-factor formed [13]. Probably AdpA acts as a kind of repressor on the formation of A-factor, However how precisely the production of A-factor is regulated by AdpA is not discovered yet.

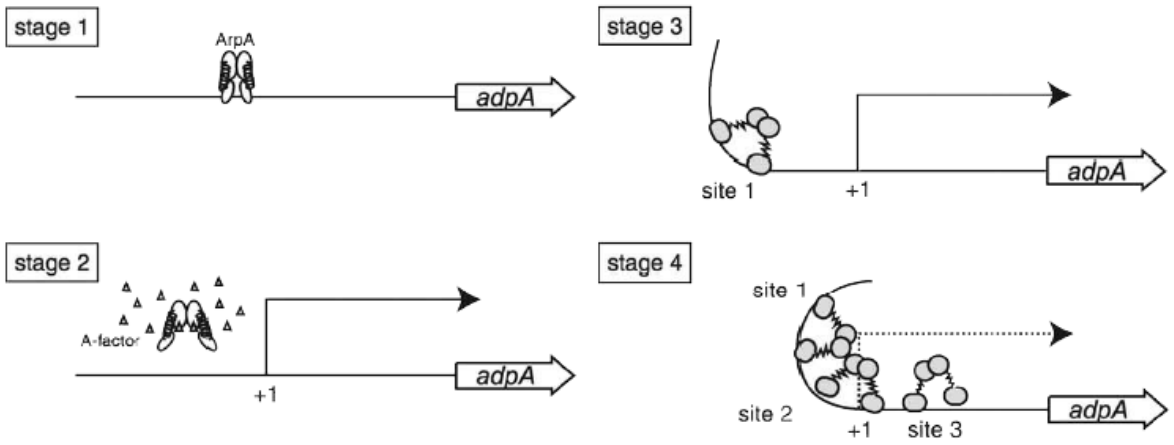

Fig.4: Self-regulation of *adpA*

Stage 1: A-factor concentration is low during early growth which makes it possible for ArpA to bind and repress the expression of *adpA*. Stage 2: When the A-factor reaches a critical concentration it binds to ArpA causing it to disassociate from *adpA*, making transcription of *adpA* possible. Stage 3: When intracellular concentration of AdpA is still low it binds one time to the promoter of *adpA*, still allowing for its transcription. Stage 4: When the concentration of AdpA becomes high enough it binds to two other binding sites in its own promoter region and represses the further transcription of *adpA.* Adapted from [12]

3. The influence of the rare leucine codon TTA in *Streptomyces* spp.

3.1. The occurrence of the rare TTA codon in *Streptomyces* spp.

The above described mechanism of the production of streptomycin is not the whole story about the regulatory pathway to produce this antibiotic. A single gene *bldA*, coding for a tRNA specific for the leucine codon UUA, has a great impact on a large part of the secondary metabolism and morphological development shown in fig.3

bldA encodes for the only tRNA in the genome which is specific for the leucine codon UUA. The codon recognized by this tRNA, TTA, is a very rare codon considering that Streptomyces spp. generally have a G+C content over 70%. The *bldA* gene was originally discovered in *Streptomyces coelicolor* where research was done in a non-sporulating, non-antibiotic producing strain. The TTA codon was discovered to be present in pathway-specific regulatory genes in each of the three biosynthetic clusters in *S. coelicolor* for antibiotic production. The lack of antibiotic biosynthesis in the *bldA* mutant is caused by the lack of a tRNA to translate the TTA codon to build in a leucine. An example that proofs this is the change of the TTA codon to TTG in the cluster activator gene *actII-ORF4* which resulted in the restored function of this gene [16]. The change to another codon for leucine made it possible for the *bldA* mutant to resume the production of actinorhodin.

The transcription of the UUA codon is not necessarily needed for normal vegetative growth. The different phenotypes in *bldA* mutant strains compared to wild-type strains during stationary phase are the result of a TTA codon in regulatory genes such as *adpA* and a*ctII-orf4*.[17] With a non functioning central regulator the necessary proteins for aerial mycelium formation and sporulation cannot be produced anymore.

3.2. Influence of the rare TTA codon on the production of secondary metabolites and morphological differentiation in *S. griseus*

The influence of *bldA* in *S. griseus* starts nearly right at the top of the hill. *adpA* contains a TTA codon [15] and hence falls under the regulation of *bldA*. And because of that all the following pathways are influenced by this single codon even if most of them do not contain a TTA codon themselves.

AdpA is its own synthesis repressor [12] so in the absence of *bldA, adpA* will be transcribed but will not be translated resulting in building up of the *adpA* mRNA. The translation of this mRNA starts with a rapid burst once *bldA* expression starts. The switch on of *bldA* appears to happen in an opposite way then for most tRNAs and starts with stationary growth phase [18]. With this activation all those other pathways shown in fig.3 are enabled. *strA*, the activator of the streptomycin biosynthesis, contains a TTA codon as well, so streptomycin production falls under double control of *bldA*. Without this rare tRNA the regulatory cascade starting with ArpA does not function anymore and prevents secondary metabolism and morphological differentiation in *S. griseus*. One molecule has a big impact on this important system.

4. Regulatory pathway in *Streptomyces fradiae*

The tylosin biosynthetic cluster comprises ~1% of the genome of *S. fradiae* (85kb). In the 43 open reading frames that lie in the biosynthetic cluster is a block of five polyketide synthase genes for the synthesis of the polyketide lactone part of tylosin. Also three genes for its own resistance are found there. This all is pretty normal for an antibiotic biosynthetic cluster but then there comes the most interesting part about this cluster. The tylosin biosynthetic cluster contains a surprising amount of six regulatory genes. Out of that the question arises what the main pathway-specific activator is of the whole cluster and what the influence of the other regulatory genes is. Since until now no exception is found to the pattern that there is one activator per cluster, which is specific to that pathway alone [7]. Here the research that is done on the different regulatory genes of this cluster will be discussed and it soon becomes clear that this regulation is a bit more complicated than that of the A-factor cascade in *S. griseus*.

4.1. The master regulator TylP exerts its influence on multiple targets

When the *tyl* genes were first sequenced, sequences of GBL receptors were already known which made it possible to put a label on TylP as a likely GBL receptor [7]. With that discovery TylP is placed on top of the regulatory cascade towards tylosin synthesis. GBL receptors bind to AutoRegulatoryElements (ARE) in the promoter region of target genes, thereby repressing the transcription of these genes. Upon binding of the GBL to their conjugate receptor the latter will release its binding to the ARE sequence and leave the genes open for transcription. Bignell and coworkers found 'ARE'-like sequences in the promoter regions of *tylP, tylQ* and *tylS*. TylP is the only protein to bind to this sequence, changes in this sequence result in the loss of binding of TylP to the promoter regions [19]. Stratigopoulos and coworkers reported in 2002 that the over-expression of *tyl*P leads to the switch off of the three genes, *tylP, tylQ* and *tylS* [20]. Which makes TylP not only a repressor of other genes but it also is its own repression in an auto regulatory way.

A search was started to find the GBL that would influence its cognate receptor TylP. Extracts of the cultures at different stages of development shows that the GBL for this pathway is already present during mid-exponential growth phase when the production of tylosin has not started yet [19]. This leads to the conclusion that the synthesis of the GBL is not regulated via the TylP regulatory cascade. Upon exposure of TylP, bound to the PARE (TylP AutoRegulatoryElements) sequence, to four known GBLs no binding is affected [19]. The GBL from *S. fradiae* appears to be a completely new compound and has not yet been identified. It is not even sure whether it is a true GBL, though the signs point in that direction, so the search continues. For examples of known GBLs see fig.1.

Possible genes involved in the putative GBL biosynthesis are *orf18** and *orf16**, respectively coding for an acyl-CoA oxidase and a cytochorome P450. Both contain a TTA codon [21] which can have a great influence on their translation as is shown before. Knock-out strains of the orfs failed to lift the binding of TylP to the PARE sequences and resulted in a lower tylosin production [19]. So tylosin synthesis still can take place even without the putative GBL.

In the *tylP* knock-out strain normal transcription of other *tyl* genes takes place apart from *tylQ*. Tylosin is produced earlier then in the wild-type strains and also the level of production is increased. Surprisingly on solid medium the morphological differentiation is initiated earlier and the aerial mycelium formed are less abundant and more highly fragmented then in wild-type strains [20]. So not only does TylP repress multiple regulatory genes in the tylosin biosynthetic cluster it also has a stimulatory effect on aerial mycelium growth.

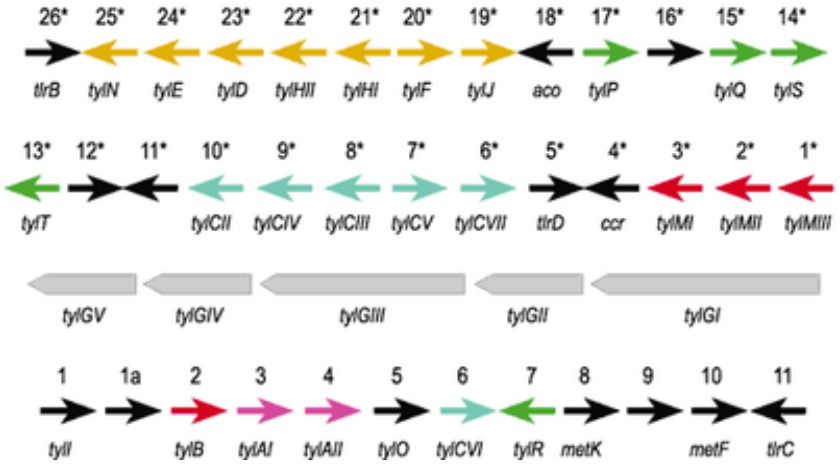

Fig.5: The tylosin biosynthetic gene cluster

The tylosin biosynthetic gene cluster of *S. fradiae*. Containing 43 genes (~85kb) occupying 1% of the genome (Not drawn to scale)

4.2. Two SARPs are present in the tylosin cluster, only one influences the production of tylosin

Two SARPs were found in the tylosin biosynthetic regulatory cluster in *S. fradiae*, *tylS* and *tylT*. Upon disrupting the expression of *tylT* no effects are observed on sporulation and growth. However a 20% reduction in tylosin production is observed when grown on tylosin production medium [22]. Apparent from this is that TylT is not essential for the function of the tylosin regulatory pathway. However upon disruption of the expression of *tylS* no tylosin is produced anymore but no effect is noticeable on morphological differentiation [22]. Of the two SARPs present in the tylosin biosynthetic cluster only one is essential for the production of tylosin. When in the *tylS*-KO *tylR* was over-expressed tylosin production was restored be it in minimal amounts. So *tylR*, the direct activator of tylosin synthesis, is positively regulated by TylS. This is a rare finding for SARPs which were only known to be the last regulators in regulatory cascades [22].

4.3. The not yet fully understood function of TylQ

TylQ is a transcriptional repressor with some similarities to GBL receptor proteins, like TylP, but no conclusive evidence has been found yet that TylQ is a GBL receptor protein. Together with *tylS, tylQ* is expressed before the other 3 regulatory genes in the tylosin biosynthetic cluster. On the contrary to the constitutively expressed *tylS* the expression of *tylQ* is switched off before the tylosin biosynthesis regulatory pathway is activated [23]. *tylQ* is presumably repressed by TylP but a TylP knock-out is not sufficient to inhibit the repression on TylQ, suggesting that there is something else influencing the expression of TylQ [20]. The possibility of TylQ being a GBL receptor protein would fit into the picture here since then there would be an outside influence fitting with the now unexplained behavior of *tylQ* expression.

In *tylQ* over-expression strains the expression of *tylR*, the transcriptional activator needed for all the aspects of tylosin production, is repressed. Due to this repression no tylosin is produced anymore. Conclusive with that is that the *tylQ* knock-out strain produce tylosin earlier now that the repressor is absent. What is in conclusive is that the production of tylosin in this case starts even before TylP is formed [23] suggesting again that, until now, the regulation mechanism of tylosin production is not fully understood.

4.4. The presence of TylU, a SARP helper protein, optimizes the production of tylosin

A surprise influence in the production of tylosin is TylU. In *tylU* knock-out strains morphological differentiation is not influenced but the amounts of tylosin produced drop dramatically by 80% [24]. TylU is a positive regulator which function is required for optimal production of tylosin but is not necessarily needed for tylosin to be produced. The production of tylosin is strongly down-regulated when TylU is absent. However when *tylU* is up-regulated the tylosin surprisingly is not over produced [24]. Apparently the TylU produced by wild-type saturates the effect mechanism and cannot be increased further.

Over-expression of TylS in a *tylU* knock-out strain does not bring the tylosin yield back to the wild-type production level. When TylR is over-expressed in a *tylU* knock-out strain the tylosin production is strongly increased to 60% above wild-type production level [24]. It appears that TylR is really the last step in the regulatory cascade before the production of tylosin is switched on. To fully activate TylR production both TylS and TylU are necessary.

Upon over-expression of TylU in a *tylS* knock-out, no tylosin production takes place [24]. Normally TylU would only be produced when TylS is present but its function alone, when expressed in the *tylS* knock-out, is not enough to activate *tylR*. To summarize TylS positively regulates TylU and they're both needed for optimal activation of *tylR*. TylS can activate TylR by itself but TylU cannot activate TylR at all. TylU appears to be a helper that is optimizing the activation of TylR but is not essential.

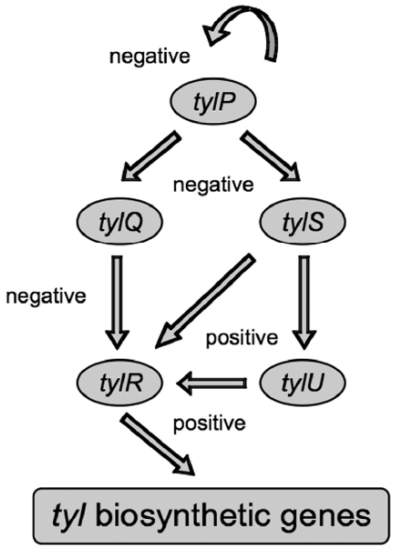

Fig.6: The tylosin regulatory cascade

The regulatory cascade of the tylosin cluster beginning with *tylP* and ending with *tylR*, the latter being the general activator for the *tyl* biosynthetic genes. See text for detailed information.

4.5. The tylosin regulatory cascade in short

To summarize, TylP is a repressor of *tylP, tylQ* and *tylS*, the repression is lifted when a putative GBL binds to TylP. With TylS being produced the regulatory cascade proceeds with TylS positively regulating the production of TylU and together they activate the production of TylR. TylR is the final regulator to switch on the synthesis of the tylosin biosynthetic cluster. During the tylosin production *tylQ* has to be switched off otherwise no tylosin will be produced. How the expression of tylQ is down-regulated at the stationary phase is not clear at the moment.

5. Discussion

In this paper two regulatory pathways have been described, showing two different kinds of complexity that are found in regulatory pathways in *Streptomyces* spp. Every regulatory system has its unique features added to some similar features that are found in more species. Looking at all the systems known so far gives an astonishing view on all the different ways in which Streptomyces spp. produces its secondary metabolites and initiates morphological differentiation. The two examples described here show a different kind of regulation each with its own complexity. But besides the differences in the systems there are some similarities as well.

5.1. GBLs are needed for the activation of the systems in both *S.griseus* and *S. fradiae*

The A-factor was the first GBL system to be discovered over 40 years ago. It has become the model system for regulatory pathways in which binding of a GBL to its conjugate receptor activates the said pathway. But this system has so many small differences between the different organisms with these kinds of regulators found in for example *Streptomyces virginiae and Streptomyces lavendulae*. Most often the GBL are auto-regulatory and promote the transcription of their own gene(s). However in the example of the A-factor it does not auto regulate its own transcription. The synthesis of the A-factor is regulated by AdpA in a way similar to the regulation leading to the synthesis of streptomycin.

The regulatory pathway in *S.* fradiae is also activated by a GBL. The influence of the GBL in *S. fradiae* is unique in the interplay between the different regulators observed in its regulatory pathway. To start with it is a possible GBL that binds to TylP, no conclusive evidence has been found yet to say it is an actual GBL. TylP does show a high similarity to other previously known GBL receptor proteins, but so does TylQ. It might be that this system is under the control of two different GBLs which increases the difficulty to characterize them further. The impact of the putative GBL on this regulatory system is quite large since when the GBL-receptor complex is formed three genes of this system are de-repressed.

5.2. The occurance of a TTA codon in the regulatory pathways of both *S.griseus* and *S. fradiae*

Another common feature in both *S. griseus* and *S. fradiae* is the occurrence of the TTA codon in the genes coding for regulatory proteins. This rare codon adds another level of regulation to both systems where the TTA codon appears in regulators that are necessary to keep the pathways activated, *adpA* in *S. griseus* and *tylS* in *S. fradiae*[21] . Upon deletion of the only gene *bldA* coding for the leucine tRNA the formation of secondary metabolites and morphological differentiation is impaired. This single tRNA has a very important role in the pathways containing regulatory genes with the TTA leucine codon.

5.3. The A-factor cascade versus the TylP cascade, differing on the amount of processes activated and regulators requiered

The example of the regulatory cascade in *S. griseus,* starting with the A-factor leading to the activation of the Streptomycin biosynthetic pathway, shows its complexity in the amount of processes under its controls. Besides the production of streptomycin, which has been used as an example here, the A-factor cascade influences numerous regulatory pathways leading to many aspects of morphological differentiation and secondary metabolism. As becomes clear from fig.3 the complexity of the A-factor regulatory cascade lies in the snow ball effect. With only a few regulators at the top it ends with a huge impact.

The other example of the regulatory system in *S. fradiae* shows a whole different level of complexity. It is rather more the inverse of the first example at first glance. The regulatory system as shown in fig.6 shows the complexity that lies in the amount of regulators that have a role in this system. All these regulators work together to activate one biosynthetic gene cluster to produce tylosin. Because of the interplay between all those regulators the functioning of the whole system is far from being understood.

5.4. The many unknowns in the tylosin regulatory pathway

A lot of what is known today is based on hypothesis' which are not able to explain for many unexpected findings. For example there is the disappearance of TylQ from the cytoplasm before tylosin biosynthesis can start. *tylQ* is under the same regulation of *tylS* by repression of TylP, yet TylS is present at all time whereas TylQ appears to be repressed by something else then TylP alone. After all what would explain for the down regulation of TylQ when actually the transcription should be activated by the disassociation of TylP from its promoter region. This is just one example of an unidentified regulation in this pathway, which in turn makes fig.6 rather incomplete.

5.5. Final remarks

To conclude this summary, it is obvious from what is stated above that besides the many similarities in the different systems there are numerous differences to be found as well. A lot of work is still needed to elucidate the specificities of the systems leading to morphological differentiation and secondary metabolism. The A-factor cascade might not need that much research anymore since it has been studied intensively, but the tylosin regulatory cascade is an example of a pathway that does need a lot of work still. Understanding the regulatory pathways will hopefully lead to a better understanding to produce useful antibiotics from these organisms, after all, the search for more useful compounds always continues.

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