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C-di-GMP and Its Role in Regulation of Natural Products Production

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Abstract

Among clinical and industrial important microorganisms *Streptomyces* are the largest source of natural products. But only a fraction of all Biosynthetic Gene Clusters in their genome is active under laboratory conditions. During the last years research has clarified the role of cyclic dimeric 3`-5` guanosine monophosphate, c-di-GMP, as an ubiquitous secondary messenger. It is not only a regulator of morphological development but also of the production of natural products. This makes it an interesting starting point for the development of new natural products. The production and degradation of c-di-GMP builds up the first layer of regulation. A set of enzymes with redundant or at least overlapping function is responsible for these reactions. Subsequently several effectors such as BldD or σ^{WhiG} affect the expression of many target genes. Through countless interactions within this system - comprising feedback loops and bilateral regulation - a multilayered system makes it possible to have a finely regulated system that can react quickly to external and internal environments. In this system c-di-GMP helps to provide BldD its function as the so called "master regulator" in *Streptomyces*. Interfering in this system with genetic manipulations such as knockouts or overexpression of genes led to altering levels of c-di-GMP. Thus, it was possible to change the production levels of produced natural products and activate silent gene clusters.

Keywords: c-di-GMP, *Streptomyces*, Natural products, Antibiotics, Second messengers

Abbreviations: c-di-GMP: Cyclic dimeric 3`-5` Guanosine Monophosphate; CSR: Cluster Situated Regulator; BGC: Biosynthetic Gene Cluster; DGC: Diguanylate Cyclase; GAF: Mammalian cGMP-regulated PDEs, Anabaena adenylyl cyclases and *Escherichia coli* transcription activator FhlA; PAC: Photoactivated Adenylyl Cyclase; PAS: Per-Arnt-Sim; PDE: Phosphodiesterase.

Introduction

Natural products play a crucial role in the development of drugs. Over the last forty years one third of all approved drugs are natural products or derivatives from them. Additionally, another third uses at least a pharmacophore of a natural product. The therapeutic areas do not only cover the widely known fields of antibiotics and cytostatics, but also anticoagulants, anti-hypertensive or anti-diabetic drugs and many more [1]. Nevertheless, antibiotics are one of the most prominent fields for natural products. Increasing antibiotic resistance in the upcoming years is leading to an urgent need for the development of new antibiotics [2]. The World Health Organization stated the antibiotic resistance crisis to be a "global public health concern". Also, the Center for Disease Control and Prevention and the European Medicines Agency are substantially concerned about the course in the last decades [2-4]. *Streptomyces*, a genus of the family *Streptomycetaceae* and the class *Actinobacteria*, are the largest source of natural products among microorganisms. A large proportion of all antibiotics originally derive from them [5]. However, in the last years only a handful of new drugs in the antibiotic field were approved. So, there is an urgent need to search for new natural compounds beyond of the existing ones [6]. A high rate of rediscovered compounds is one problem of common techniques for screening for new

natural products with antibiotic effect [7]. Consequently, new techniques to discover completely new entities should be searched and used. *Streptomyces* have this potential because a large percentage of Biosynthetic Gene Clusters (BGC) remains inactive under laboratory conditions. Thus, undiscovered natural products are burrowed within their genome. Analysis using antiSMASH revealed that each sequenced *Streptomyces* genome has 36.5 BGCs on average [8], while producing only single natural product. Additionally, it is estimated that only 1 % of all *Actinobacteria* are cultivable and can be identified by using standard methods [9]. To use *Streptomyces* to their full potential we need techniques to activate more biosynthetic gene clusters. C-di-GMP is a secondary messenger involved in crucial pathways for natural products biosynthesis. Biotechnological interventions in this overarching regulation circuit are able to enhance yields and furthermore activate biosynthetic gene clusters as described below.

Streptomyces

Streptomyces are gram-positive, non-motile, soil-dwelling *Actinobacteria*, with a high G+C content genome. They are multicellular bacteria and undergo a complex life cycle [5,10,11]. The germination starts with a suitable trigger. From the unigenomic spore a germ tube emerges, which grows mainly at the tip. Through branching a tightly interwoven mycelium is formed and quasi-exponential growth is possible. Additionaly speta are formed in older parts of the mycelium [12-14]. After a few days the colonies begin to form aerial hyphae, which themselves mainly grow at their tips. The life cycle is summarized in Figure 1. A set of genes is involed in this process. According to the appearance of the respective knockout mutants the genes were called "*bld*" after their "bald" look on agar plates. The long, often curled aerial hyphae undergo multiple synchronus cell devisions to form spores. Responsible for this is another set of genes called "*whi*" after their knockout mutants, which are unable to produce pigmented spores and therefore look white [15-17]. These names bld and whi may be irritating because of the intricate effects of some enzymes. BldD does not only have an influence on the formation aerial hyphae but also plays a role in spore formation through interactions with WhiB and ssgR [18]. The names were given years ago just for phenotypic looks, but the underlying mechanisms were not elucidated yet.

The genus *Streptomyces* was first proposed by Waksman und Henrici [19]. In the following years they began to isolate compounds such as streptothricin and actinomycin from *S. antibioticus* and *S. lavendulae*. Since the discovery of penicillin approximately 70,000 natural products from microbials have been identified of which 20,000 are from *Actinobacteria* [9,20]. The natural products have a large structural diversity as shown in Figure 2. The figure shows some of the most important natural products derived from *Streptomyces*.

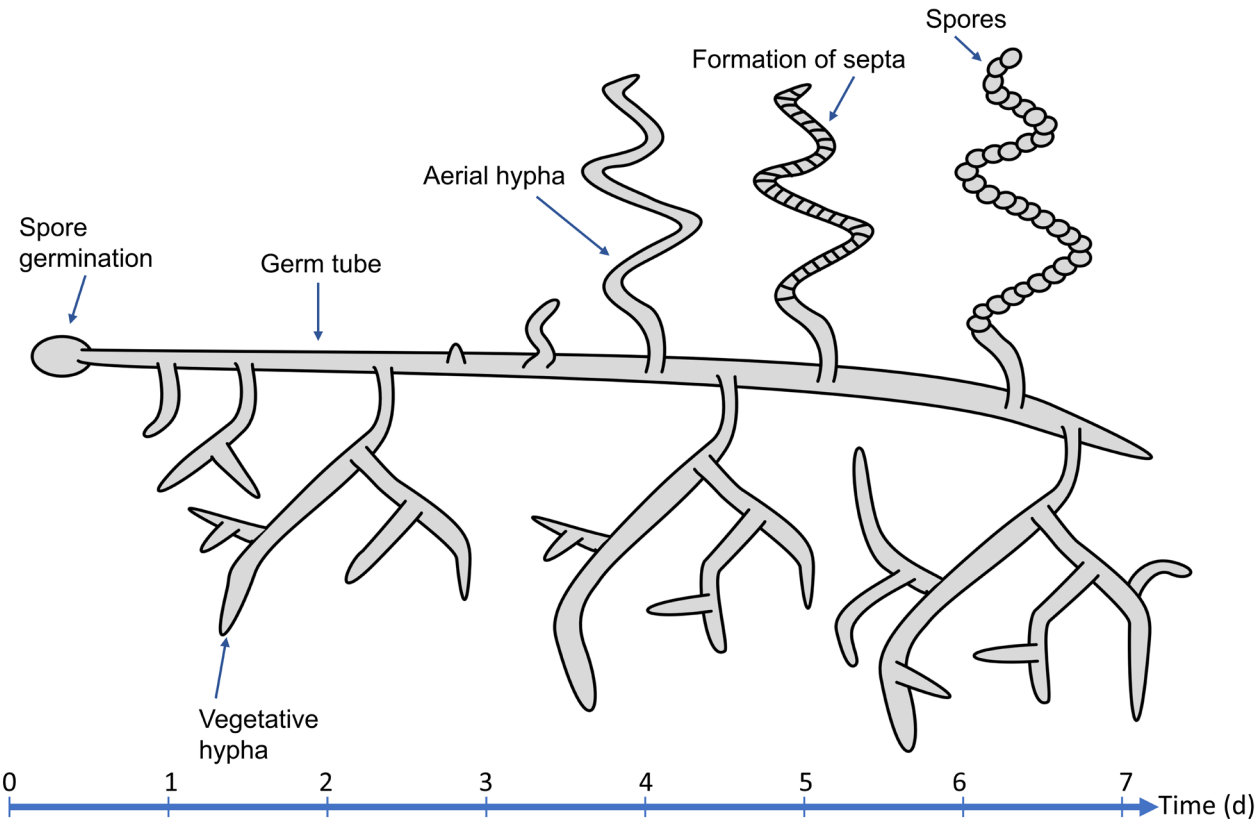

Figure 1: Lifecycle of Streptomyces. Development from a single spore into vegetative hyphae, aerial hyphae and formation of spores.

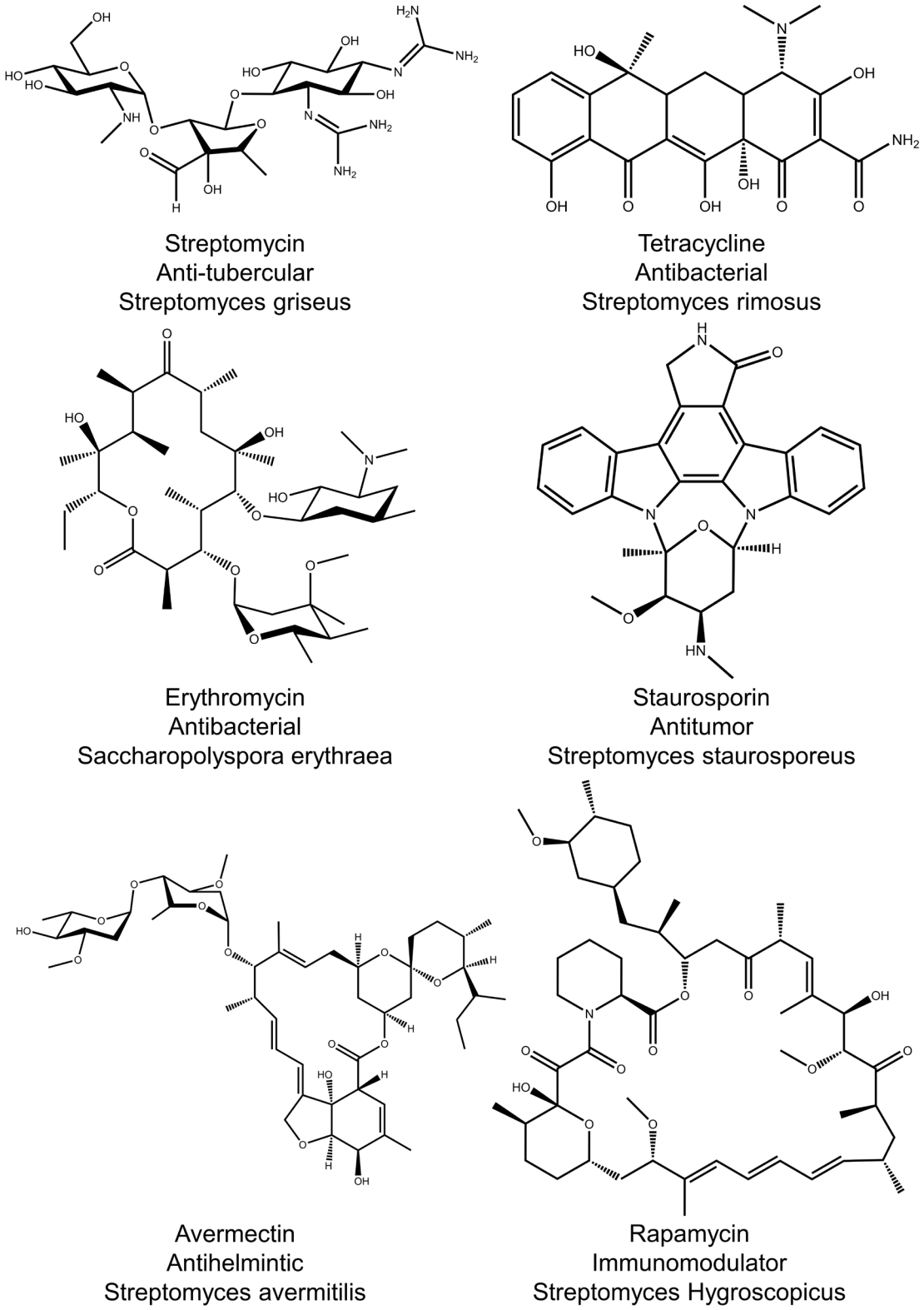

Figure 2: Structural diversity of natural products produced by the named strains. The description shows the name of the compound, the medical use, and the originating strain. Streptomycin is one of the first compounds isolated from *Streptomyces*. Ivermectin is a semi-synthetic derivative from Avermectin. The others show different structure types occurring as natural products of *Streptomyces*.

The synthesis of natural products in *Streptomyces* is organized in biosynthetic gene clusters. These clusters are 20 kb to over 100 kb in size and contain most of the essential genes for biosynthesis [21]. Recent genome sequencing efforts coupled with advances in bioinformatics indicate that the majority of biosynthetic gene clusters are not expressed under normal laboratory conditions. Termed 'silent' or 'cryptic', these gene clusters represent a treasure trove for discovery of novel small molecules, their regulatory circuits and their biosynthetic pathways [22,23]. A lot of different techniques have been deployed to generate compounds from these silent gene clusters. For example, co-cultivation with other microorganisms to mimic interactions between species or applying chemical elicitors with metabolome analysis revealed new compounds [24-27]. One problem with these approaches is that they are governed to an analytical scale.

That is why we take a look at a global technique to activate new biosynthetic gene clusters or increase amounts of produced natural products through intervention in a higher-level, pleiotropic regulator. For this it is crucially important to not only be able to detect the gene clusters in the genomic sequence, but also to know regulatory cascades of their activation. In most of the BGCs so-called cluster sited regulators (CSRs) are located directly within the biosynthetic genes. They are necessary for transcription or activity of the other genes. But some gene clusters do not have any CSRs and the number of so-called "CSR-free" BGCs is growing through advances in sequencing.

It is important to note that a regulator within a BGC can have an effect on more than one BGC, meaning that it has a more global function. Additionally, there are global regulators governing production of natural products and also morphological development. The current state of research will be described in the following chapters.

Originally the strain *S. coelicolor* was used as a model organism and was also the first fully sequenced genome of all *Streptomyces* [28]. It was and is the best overall studied strain. Until now almost 20 different secondary metabolites of this strain were analyzed. Among others are the polyketide actinorhodin and the red tripyrrole prodiginines [29,30]. Recently, *S. venezuelae*, producer of chloramphenicol and the polyketide jadomycin, is emerging as a new model strain. Because it also sporulates in liquid culture, it is considerably more suitable for the evaluation of developmental processes [31,32]. This is especially true, because surface grown colonies, which are necessary for *S. coelicolor* to undergo the full development circle, are compromised by developmental asynchrony and heterogeneity making statements about regulons difficult. Because some *Streptomyces* strains are extremely difficult to handle, it is easier to express the biosynthetic gene cluster of interest in a heterologous host. Well studied strains like *S. coelicolor*, *S. lividans*, *S. albus* and *S. avermiltilis* have been used and also specifically modified for this purpose by deleting their BGCs to minimize interactions [33,34]. Other strains from the so called "rare Actinobacteria" (for example *Saccharopolyspora erythraea*) are used because of the interesting natural products they are producing.

In particular the investigations of the direct effects of c-di-GMP were performed with the two model organisms *S. coelicolor* and *S. venezuelae* [35-38]. Recently also a different, *S. ghanaensis*, known for the production of moenomycin, an antibiotic which was used for decades as a feedstuff without rising resistance levels [39], was also investigated [11,40,41].

The second messenger C-di-GMP

Cyclic dimeric 3´-5´ guanosine monophosphate (c-di-GMP) was first identified in 1987 as an allosteric activator of a bacterial cellulose synthase in the alphaproteobacterium *Gluconacetobacter xylinus*. It took quite a while until the first signs did show the impact of c-di-GMP. Ten more years were needed for the characterization of the main domains of the first response regulators in *Caulobacter crescentus* and *Bordetella pertussis* [42,43]. These domains are conserved throughout different species. Later it was shown, that the amino acid sequence GGDEF, in the so-called GGDEF-domain, has diguanylate cyclase (DGC) activity, building up c-di-GMP out of two molecules of GTP [43]. In contrast to this the EAL amino acid sequence, in the EAL-domain and also the HD-GYP domain, are catalyzing the degradation of c-di-GMP in pGpG and subsequently into GMP and therefore has the function of a phosphodiesterase (PDE) [5,44–46]. The c-di-GMP turnover is summarized in Figure 3.

It is important to note that some of the enzymes carry both necessary domains within their sequence, but in most cases only one of them is active. The other one is degenerate in the surrounding of the active site and does not carry out its original function. In some rare cases it was shown that both domains are still active and the protein can carry out both reactions. Their activity is mediated by stimuli that alter the activity of either one of the domains [48].

In the following years its role was enlarged to a ubiquitous secondary messenger in bacteria.

C-di-GMP is responsible for different kinds of changes in morphology like transition from sessility to motility in *Salmonella enterica*, *Pseudomonas aeruginosa* and *Escherichia coli* [49,50]. Associated with sessility is also the biofilm formation [51]. Another function of c-di-GMP is to modulate pathogeny in among others *Yersinia pestis* and *Vibrio cholerae* [52–54].

The downstream effectors are very diverse reaching from PilZ domains [55,56] to riboswitches [57]. In *Streptomyces* two special complexes carry out the main function, which are described below.

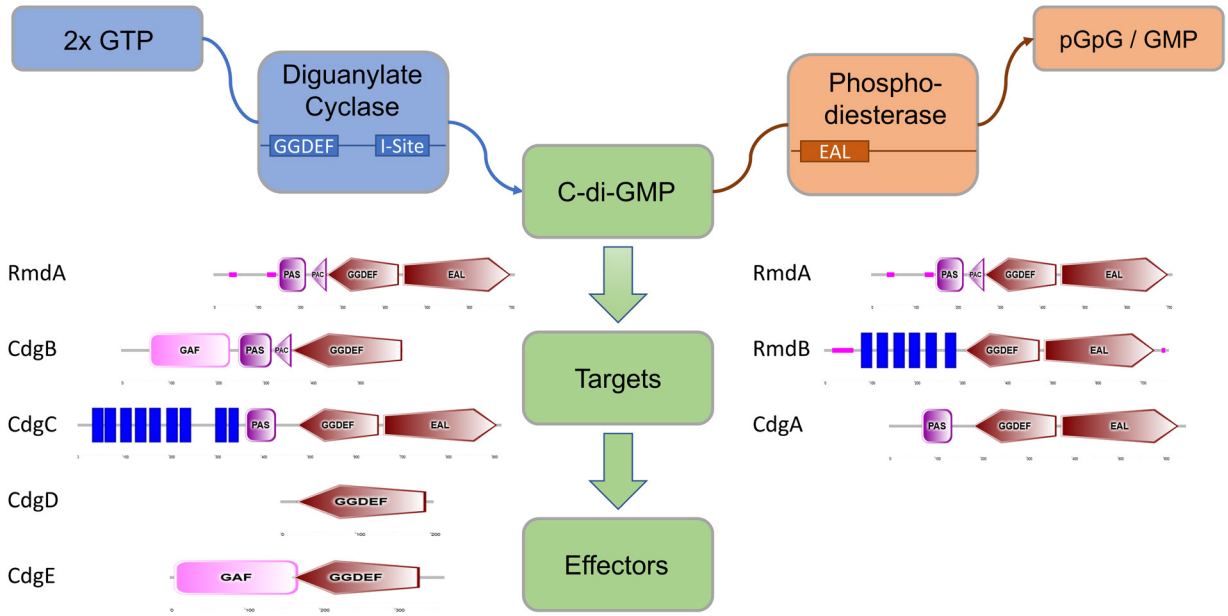

Figure 3: Overview of c-di-GMP turnover. C-di-GMP is produced by DGCs out of two molecules of GTP and degraded by PDEs into pGpG or GMP. In its active form it forms complexes with target proteins, which in turn regulate the expression of the effectors. The schemes of the enzymes listed are from *S. ghanaensis* [47].

About ten years ago first research about the involvement of c-di-GMP in *Streptomyces* was published [10]. At first the research in *Streptomyces* focused on the impact of c-di-GMP on morphological development. During the past years, additionally the effects on the production of secondary metabolites were analyzed more detailed [11,36,40,41,58]. Each *Streptomyces* strain has a certain set of multiple enzymes, varying from five to twelve enzymes in total in all sequenced *Streptomyces* [5,38,59]. Some of these enzymes like CdgA, CdgB and RmdB are existing in almost every strain, while others are present in only a few [5].

C-di-GMP is able to integrate a wide range of different internal and external signals through numerous additional domains. Three of these sensory and regulatory domains play the main role in *Streptomyces*: PAS (Per-Arnt-Sim)/ PAC (Photoactivated adenylyl cyclase) and GAF (mammalian cGMP-regulated PDEs, Anabaena adenylyl cyclases and *Escherichia coli* transcription activator FhlA) [40]. PAS is a common signal sensing domain, with various ligands, such as heme, FMN or FAD, mainly present in archea and prokaryotes. They can occur both intracellularly and extracellularly. In *S. ghanaensis* an ortholog of CdgC was shown to possess a heme, potentially sensing signals from O_2, Co or NO [40]. Maybe analogous to a PAS domain from *E. coli*, which showed oxygen dependent activity in the past [60]. GAF domains are also small molecule binding domains. They are also found in mammalian phosphodiesterases with cGMP as one of the known ligands [61,62]. In *Streptomyces* and in a lot of bacteria these domains have not been analyzed in detail, meaning that a lot of uncertainty exists about potential ligands and their influence on the activity of the enzymes [5]. For better understanding how changes in intracellular c-di-GMP levels impact the production of secondary metabolites we need to take a look at the effectors of c-di-GMP first.

C-di-GMP effectors and targets

C-di-GMP is the active secondary messenger. In its main functions it is building complexes with enzymes, which subsequently bind their target DNA sequences. These targets play roles in different pathways and build a multilayered system of regulation to be able to adapt to different internal and external effects [63]. BldD was proven to create a complex out of four molecules of c-di-GMP and two molecules of BldD bind to their signature DNA-sequence (5`-TNAC[N]$_5$GTNA-3´) via their DNA binding domain. The conserved amino acid sequence motif RXD-X_8-RXXD binds c-di-GMP [35,36]. When enough c-di-GMP is present the complex is formed and binds the DNA. First it was shown that the binding represses the transcription of the regulon. Just recently in 2019 also a mode of c-di-GMP dependent activation of genes was published [64]. Studies on the regulation and the interaction revealed that in *S. coelicolor* 261 Genes were changed over twofold in expression through the knockout of *bldD*. Some of the genes are involved in life cycle development or secondary metabolites production, while other proteins still have

unknown functions [10]. The resulting effects on secondary metabolites vary from complete stop to enhancement of production between different strains and the underlying causes remain to be elucidated [10,41,64].

One important target of BldD is BldA. BldA is the only tRNA in the genome of *Streptomyces* that can translate the UUA-Codon [65,66]. Because of the high GC-content of approximately 72%, this codon is used in only 2% of all genes, which still amounts to 145 genes. None of these genes is crucial for survival, so a *bldA* null mutant is still viable. Also, most of these genes are not conserved throughout *Streptomyces*. Some of them are in species specific BGCs for example. This makes it hard to predict general effects for BldA. The amount of BldA can be a limiting factor for the translation of a protein and proceeding the production of a natural product. Almost 50% of *Streptomyces* with partially dysfunctional *bldA* gene produced a new compound after restoring its function. This is not only due to direct presence of a UUA codon in a BGC but also indirect effects through regulators containing the codon [67,68].

AdpA, also known as BldH, is an important regulator of core promoters within BGCs and furthermore one of the most versatile transcription factors in *Streptomyces* and contains a conserved TTA codon [23,69,70]. This global regulation is particularly important in CSR-free BGCs [23]. The activity of AdpA is also regulated by RNase III, AbsB (Antibiotic biosynthesis gene B), by cleavage of its mRNA and through direct interaction with BldD. The knockout of *absB* in *S. ghanaensis* more than doubled the production of moenomycin whereas the knockout of *adpA* completely abolished the production [70].

The second important direct target of c-di-GMP is the complex between σ^{WhiG} and RsiG, which is only formed in the presence of c-di-GMP. It was first analyzed in *S.venezuelae* but is conserved throughout all *Streptomyces*. The σ^{WhiG}/RsiG complex binds two molecules of c-di-GMP with two conserved $E(X)_3S(X)_2R(X)_3Q(X)_3D$ domains. At lower intracellular levels of c-di-GMP σ^{WhiG} is released of the complex and can subsequently bind an RNA polymerase. The consensus sequence for DNA binding of the RNA polymerase and σ^{WhiG} is very similar, but not identical to the phylogenetically related sigma factors for flagellum biosynthesis [71,72]. The transcription of *whiG* itself is regulated inhibitory directly through BldD and enhancing through WblA. For the latter it is unclear whether the interaction is direct or indirect [10,73]. The two main targets of σ^{WhiG} are WhiH and WhiI. These two on the other hand, affect more than 100 genes involved in late sporulation [72]. The WhiH protein has similarities to GntR family transcription factors, a family with other members known to be involved in developmental regulation, including DevA and DasR [74-76]. Until now only a few connections between σ^{WhiG} and the production of secondary metabolites have been made. In *S. chattanoogensis L10* σ^{WhiG} binds to CSR within the natamycin BGC. Additionally, indirect effects on pathway specific positive regulators were shown [77]. How this indirect regulation is enabled remains unclear. In contrast to that in the same strain a second BGC was downregulated and in *S. coelicolor* the overexpression of σ^{WhiG} leads to inhibition of the production of actinorhodine [77,78]. In the future, it will be interesting to find out through which regulatory elements σ^{WhiG} controls the production of secondary metabolites.

One possibility would be, that it is achieved through the WhiB-like (Wbl) regulators, a family of transcription factors. They were shown to have an influence in both sporulation and secondary metabolites production. The knockout of the most abundant member, *wblA*, led to a more than 10-fold increase in the production of tiancimycins in *S.* sp. CB03234 [79]. The function of WblA is a transcription repressor, nevertheless also activation and dual repressor activation have been reported [80]. In *S. ansochromogenes 7100* and *S. somaliensis SCSIO ZH66* the inactivation of *wblA* led to activation of cryptic BGCs. In both studies, a BGC was inactivated in addition to the one being activated for unknown reason [73,81]. The knockout also almost abolished the expression of several other *Whi* and *Wbl* genes. Which of these effects are direct and which are indirect still needs to be investigated [73]. WblA is negatively regulated through BldD [41] but contrary results have been observed for the interaction between AdpA and WblA. In the model strain *S. coelicolor* and in *S. ghanaensis* AdpA negatively regulates *wblA* transcription [41,82] on the other hand in *S.* griseus and *S. chattanoogensis L10*, AdpA shows a positive regulation on WblA [83,84]. In both cases direct interactions with the promotor region have been shown. Also, the overexpression of *wblA* led to increased production of natamycin in *S. chattanoogensis L10*, contrary to the results mentioned before [84].

Furthermore, there is a complex of BldM, regulated by BldD, and WhiI, which is regulated by WhiG [10,18]. The fact alone that BldM used to be called WhiK shows that it is under heavy regulation and is interwoven in multiple pathways [85]. *bldM* is under direct control of BldD and additionally the sigma factor directing *bldM* transcription, BldN, is also under control of BldD [10,18]. BldM has two different DNA binding motifs, one is a homo-dimer, the other one is a hetero-dimer with BldM and WhiI. WhiI is one of the main targets of WhiG and is connected to the BldD pathway again. The targets of the complexes simultaneously occur during the development of aerial hyphae and in sporulation [18]. Although the influence of BldM on morphological development is clear, it remains unclear whether there is an additional link to antibiotic production. Because the knockout of BldM is leading to a "bald" morphology there is a connection to genes responsible for the formation of aerial hyphae [18,85]. Further research is needed to evaluate whether this connects to natural product regulation pathways. The pathways described above are shown in Figure 4.

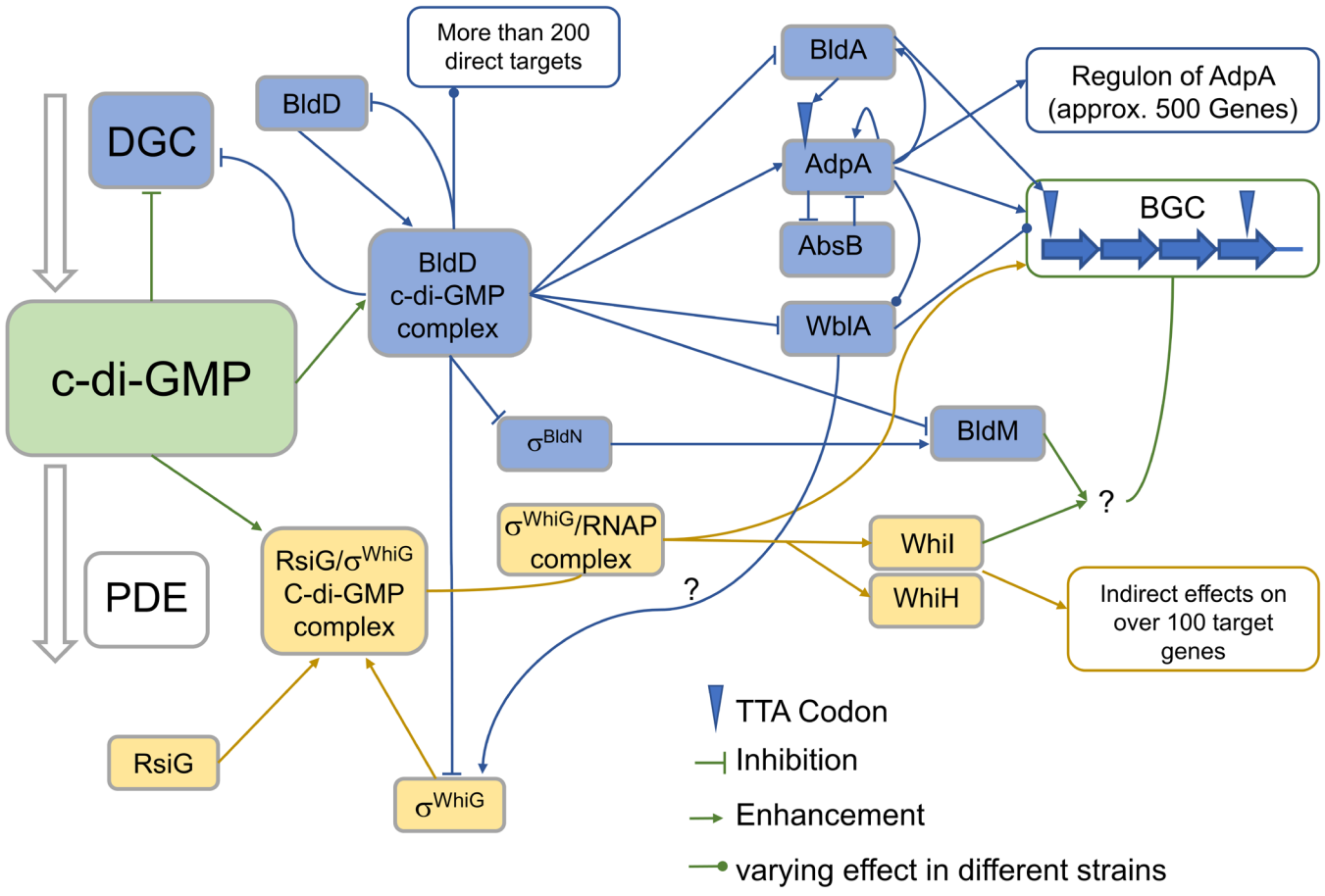

Figure 4: Overview of the most important known interactions of c-di-GMP and their downstream effectors. The effect is only shown if it is a conserved interaction. For example, BldA has more individual interactions in certain strains if they contain other TTA-codons. The effects of the BldM-WhiI complex are not yet analyzed in regards to natural products synthesis. AdpA and WblA show different effects on each other in different strains. WblA also showed activating and inhibiting effects on BGCs. It has to be analyzed whether the effect of WblA and σ^{WhiG} is conserved in *Streptomyces*.

Influence of changes in synthesis and degradation on effectors of c-di-GMP

In recent years it was shown that not only changes on the effector levels are a possibility to influence the production of natural products. In several strains, as discussed above, knockout and overexpression studies were conducted on DGCs and PDEs [38]. However, most of them focused on the morphologic influence of c-di-GMP and not the production of secondary metabolites [5,15,86]. Only recently, first works on this topic have been published [11,38,41].

The idea was that by changing levels of c-di-GMP, it could be possible to alter the activity of the respective complexes with BldD and σ^{WhiG}/RsiG and subsequently the expression of genes relevant for natural products biosynthesis. A knockout of a DGC and accordingly the overexpression of a PDE is supposed to lower the levels of c-di-GMP and consecutively leads to the breakdown of the complexes. In the case of BldD the disintegration of the complex is preventing the DNA binding. In the case of σ^{WhiG}/RsiG the breakdown releases σ^{WhiG}, which is then able to recruit an RNA polymerase on its respective target genes. The exact opposite is assumed for a knockout of a PDE and overexpression of a DGC [11,37]. This is a simplified view, not taking into account that there are potential feedback loops or enzymes with overlapping function impairing the impact on the level of c-di-GMP [15,40,87]. Liu et al. listed an overview on the effects in *S. venezuelae* and *S. coelicolor* with contrary effects on the production of actinorhodin in different *S. coelicolor* DGC mutants [38]. The formation of the complexes of c-di-GMP with BldD and σ^{WhiG}/RsiG is supposed to prevent the transition into aerial hyphae and sporulation respectively. Until now it remains still unclear whether the new findings of BldD enhancing the transcription are playing an important role in this regard [64]. But it would support the fact, that the overexpression of different DGC enzymes leads to different results in terms of natural products production. Another unclarity arises from studies from *S. ghanaensis*. In this strain,

opposing to the findings in *S. coelicolor*, the enzymatic activity of CdgA was a PDE and not a DGC, although they share high amino acid identity [11]. This means it is not possible to predict the activity of these enzymes just from their overall sequence similarity. On the other hand, it is possible to see degenerate domains, when crucial residues are missing [11]. Further evaluations in *S. ghanaensis* revealed that the knockout of rmdB led to the activation of cryptic BGCs. Two new natural products are produced. The same procedure in *S. albus* led also to the production of new compounds [41].

Table 1 summarizes knockouts, overexpression experiments and enzyme activities of *S. coelicolor*, *S. venezuelae* and *S. ghanaensis* and their impact on natural products synthesis [5,10,11,15,38,40,41,59,86].

Conclusion and Outlook

Cyclic-di-GMP has demonstrated to be an interesting starting point for the generation of new natural products. It was shown that through changes in the effector proteins as well as through changes in the turnover proteins it was possible to activate cryptic gene clusters.

C-di-GMP metabolizing enzymes are the upstream targets in this complex and interwoven pathway. The underlying cause

Table 1: Overview of c-di-GMP turnover enzymes in *S. ghanaensis*, *S. coelicolor* and *S. venezuelae*. Target of BldD means, that a BldD binding site is located in the promoter region of the respective gene. Arrows show the effect of overexpression and knockout on the production of secondary metabolites. "-" means that it was not yet analyzed. * = no *in vitro* validation. CdgB does not contain an EAL domain, so it is not able to have PDE activity.

Strain			Production of SMs	
Protein	Target of BldD	Enzym Activity	Overexpression	Knockout
S. ghanaensis				
CdgA		PDE	↓	↑
CdgB	x	DGC*	↑	↓
CdgC	x	DGC	↑	↓
CdgD		-	↓	↑
CdgE		DGC	→	→
RmdA		Bifunctional	→	↑
RmdB	x	PDE	↓	↑
S. venezuelae				
CdgA	x	DGC*	-	-
CdgB	x	DGC*	-	↓
CdgC	x	DGC	-	↓
CdgD		DGC*	-	-
CdgE	x	DGC*	-	-
RmdA		PDE [residual DGC activity]	-	→
RmdB		PDE	-	→
S. coelicolor				
CdgA	x	DGC*	↓	-
CdgB	x	DGC	↓	→
CdgC	/	DGC*	↑	-
CdgD	/	DGC	↓	-
CdgE		DGC*	-	-
RmdA		PDE	-	-
RmdB		PDE	-	-

why *Streptomyces* have multiple enzymes with redundant function is not yet clarified. A possible explanation would be, that they are expressed in a time-dependent manner and have different tasks throughout the development of *Streptomyces* enzymes [5,40]. Alternatively, the global pool of c-di-GMP is not the central point, but local pools and modules of enzymes play their role in the regulatory pathways [5,15]. This is in contradiction to the fact, that overexpression of other enzymes diminishes the effect of a knockout of redundant ones. But it is safe to say that redundant enzymes have different localizations, through for example transmembrane domains. This also makes it possible that external stimuli are integrated into the cells through extracellular sensing domains [88]. Additionally, intercellular proteins with their own domains are able to integrate other signals. But little is known about the natural ligands and their effect on the activity of most of these domains.

Further analyses are required, if the activation of cryptic BGCs - as shown above - is a general principle or if it was a hit by chance. In case, it can be confirmed, it will be a profound tool for the generation of new natural products of *Streptomyces* in general. This is especially true, because it is a conserved pathway and specifically rmdB occurs in more than 90 % of all *Streptomyces*.

Furthermore, it will be interesting to see which modulators are involved to connect c-di-GMP turnover enzymes and the activation of BGCs. Hundreds of enzymes are in the regulon of c-di-GMP effectors, but which are the decisive ones? This implies also an advantage the turnover enzymes as a target point: Not only single effectors are influenced, but the whole regulon. There is to note that this also can have adverse impact, because it is not precisely targeted.

Declarations of Interest

None.

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