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How c-di-GMP controls progression through the *Streptomyces* life cycle

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Abstract

Members of the antibiotic-producing bacterial genus *Streptomyces* undergo a complex developmental life cycle that culminates in the production of spores. Central to control of this cell differentiation process is signaling through the second messenger c-di-GMP. So far, three proteins that are directly controlled by c-di-GMP in *Streptomyces* have been functionally and structurally characterized: the key developmental regulators BldD and σ^{WhiG}, and the glycogen-degrading enzyme GlgX. c-di-GMP signals through BldD and σ^{WhiG}, respectively, to control the two most dramatic transitions of the *Streptomyces* life cycle, the formation of the reproductive aerial hyphae, and their differentiation into spore chains. Later in development, c-di-GMP activates GlgX-mediated degradation of glycogen, releasing stored carbon for spore maturation.

c-di-GMP controls *Streptomyces* development

3′, 5′-cyclic diguanylic acid (c-di-GMP) is synthesized from two molecules of GTP by diguanylate cyclases (DGCs) characterized by GGDEF domains and degraded by c-di-GMP-specific phosphodiesterases (PDEs) carrying EAL or HD-GYP domains (Figure 1B). These domains are named after conserved residues found in their active sites [1]. Homologs of DGCs and PDEs are found in all major bacterial phyla [2], making c-di-GMP a nearly universal signaling molecule through which bacteria sense and respond to the environment. Despite its ubiquity, studies on c-di-GMP signaling have primarily been limited to Gram-negative bacteria, where it controls processes such as motility, biofilm formation and virulence [1,3-5].

Streptomyces, which belong to the phylum Actinobacteria (synonym Actinomycetota) are filamentous bacteria with a fascinating developmental life cycle involving progression from

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vegetative growth to the production of reproductive aerial hyphae, which differentiate into long chains of exospores (Figure 1A, D) [6-8]. In a key study, the endogenous c-di-GMP metabolism of *Streptomyces venezuelae* was overwhelmed by engineering the overexpression of either a DGC or a PDE, aiming to raise or deplete, respectively, c-di-GMP levels [9]. Both had dramatic consequences. Overexpressing the DGC blocked the initiation of development, giving a classic bald phenotype, while overexpressing the PDE caused precocious hypersporulation (Figure 1A). These results suggested that high c-di-GMP levels block differentiation, trapping *Streptomyces* in vegetative growth, whereas decreased levels of c-di-GMP accelerate entry into development, promoting sporulation [9]. Consistent with this conclusion, the loss of individual enzymes involved in c-di-GMP metabolism affects development [10-15]. *S. venezuelae* has 10 such enzymes (Figure 1B), and deletion studies show that loss of either of the DGCs, CdgB or CdgC, enhances sporulation whereas deletion of the PDEs RmdA or RmdB delays development (Figure 1C) [13]. The DGCs CdgA, CdgB and CdgC, and the PDE RmdB are the most highly conserved c-di-GMP metabolizing enzymes in the genus *Streptomyces* [13,14].

Consistent with c-di-GMP playing a central role in development, data showed that c-di-GMP levels are high in early vegetative growth and drop progressively to reach a minimum at around 14 h of growth in liquid sporulation medium, coinciding with the initiation of differentiation (Figure 1D) [16]. c-di-GMP levels then rise during spore formation, reaching a maximum when development is complete (Figure 1D). How these c-di-GMP levels are controlled across development is certain to be complex. As shown in Figure 1B, five of the 10 c-di-GMP metabolizing enzymes in *S. venezuelae* are composite GGDEF-EAL proteins carrying both the synthetic and degradative domains, and most of the enzymes carry multiple regulatory domains (e.g. GAF, PAS, PAC) that are likely to control enzymatic activity in response to unknown regulatory inputs [13,14,17]. In addition, four genes encoding DGCs – *cdgA*, *cdgB*, *cdgC* and *cdgE* – are direct targets of BldD regulation (see below), creating the possibility of negative regulatory feedback loops [9-11,13,17].

c-di-GMP binds BldD to control the onset of development

The striking phenotypic consequences of changing c-di-GMP levels in *Streptomyces* suggested that this nucleotide second messenger must interact directly with the regulatory network that controls the life cycle. The first direct target of c-di-GMP to be discovered in *Streptomyces* was BldD, identified as a c-di-GMP binding protein in affinity pull-down assays using a c-di-GMP capture compound [9,18]. BldD is the master repressor of *Streptomyces* development. It sits at the apex of the regulatory network, repressing a large set of sporulation genes, including many genes of the core transcriptional regulatory cascade itself, in addition to genes encoding proteins critical for sporulation septation and the segregation of chromosomes into spores [7,9,10,19]. Critically, the ability of BldD to bind DNA and repress its target genes requires complex formation with c-di-GMP, which acts as a to act as a “brake”, prolonging vegetative growth and blocking entry into development [9].

The crystal structure of BldD bound to c-di-GMP together with biochemical studies revealed a unique molecular mechanism of c-di-GMP signaling (Figure 2). BldD has two domains, an N-terminal DNA-binding domain (DBD) and a C-terminal domain (CTD) that was of

unknown function [9,20,21]. Biochemical experiments demonstrated that the CTD is a c-di-GMP-binding domain, and that binding c-di-GMP causes the CTD to dimerize [9]. Remarkably, the crystal structure showed that the subunits of the CTD dimer are separated by ~10 Å, with no protein-protein contacts. Instead, a tetrameric cage of c-di-GMP bridges between the two subunits (Figure 2) [9]. Consequently, high levels of c-di-GMP drive dimerization of BldD (Figures 1D and 2), leading to repression of the BldD regulon of sporulation genes during the vegetative growth stage, thereby acting as a checkpoint to control the initiation of development. As c-di-GMP levels begin to drop at the start of the transition to sporulation, the BldD dimer dissociates into monomers, causing it to dissociate from DNA, thus allowing the transcriptional cascade leading to sporulation to become activated (Figure 1D) [9,22]. c-di-GMP can assume different oligomeric states to execute different functions [1,3,5], but to date the tetrameric form of c-di-GMP seen in BldD is unique [22].

c-di-GMP is monomeric in solution at physiological concentrations [23], and the pathway that leads to the fully assembled $BldD_2$-$(c\text{-}di\text{-}GMP)_4$ complex is not completely understood. It is clear, however, that BldD binds c-di-GMP through an ordered sequential mechanism [22]. The structure of a BldD assembly intermediate bound to one c-di-GMP dimer was captured, showing that BldD still forms a dimer in this intermediate. Guided by these structures, it was possible to prevent the binding of the second c-di-GMP dimer by mutating Asp116 to Ala, trapping the half-loaded assembly intermediate for functional assessment. Both *in vitro* and *in vivo*, the BldD(D116A) retained some weak DNA-binding activity [22]. However, the *bldD D116A* mutant has a null phenotype, showing that the ability to regulate entry into development requires assembly of the full $BldD_2$-$(c\text{-}di\text{-}GMP)_4$ complex [22].

BldD is present throughout most filamentous Actinobacteria, and its $RxDx_8Rx_2D$ c-di-GMP-binding signature motif (Figure 2) is conserved in all homologs [9]. Further, all these bacteria have DGCs, suggesting that BldD-(c-di-GMP) is likely to control key developmental processes in filamentous species throughout the phylum. Consistent with this suggestion, BldD-(c-di-GMP) has been shown to control sporangium formation in *Actinoplanes missouriensis*, an intriguing filamentous actinobacterial species that produces flagellated, motile spores [24,25].

In addition to their fascinating developmental life cycles, *Streptomyces* and their filamentous actinobacterial relatives are also of importance because they are the most abundant source of antibiotics and other natural products used in medicine [26]. Although BldD does not directly control antibiotic biosynthesis in the model species *S. venezuelae*, in other filamentous Actinobacteria it has been shown to directly control the expression of the biosynthetic gene clusters (BGCs) for several clinically important antibiotics. These include erythromycin in *Saccharopolyspora erythraea* [27], avermectin in *Streptomyces avermitilis* [28], and daptomycin in *Streptomyces roseosporus* [28], showing that the BldD-(c-di-GMP)-mediated regulation of developmental processes in Actinobacteria extends to their medically and commercially important specialized metabolism.

c-di-GMP binds RsiG-σ^{WhiG} to control the differentiation of aerial hyphae into spores

While $BldD_2$-$(c\text{-}di\text{-}GMP)_4$ regulates the onset of development, a 2020 study revealed that c-di-GMP also directly intervenes later in the *Streptomyces* life cycle by regulating the activity of the sporulation-specific sigma factor σ^{WhiG} [29]. This alternative sigma factor is highly conserved in the genus *Streptomyces*, where it is required for the differentiation of aerial hyphae into spores [29,30]. The specific role of σ^{WhiG} in *Streptomyces* was revealed using ChIP-seq (chromatin immunoprecipitation followed by sequencing) combined with global transcriptional profiling, which showed that the main function of σ^{WhiG} is to activate transcription of just two genes, *whiH* and *whiI.* WhiH and WhiI are both transcription factors, each responsible for regulating a large set of late-stage sporulation genes [31,32], explaining how σ^{WhiG} controls the differentiation of aerial hyphae into spores.

During vegetative growth, σ^{WhiG} is present in *Streptomyces* cells, yet the expression of its target genes does not increase until later in development. This suggested σ^{WhiG} is post-translationally regulated.. It was initially unclear how σ^{WhiG} activity is regulated until a bacterial two-hybrid genomic library screen was used to identify its cognate antisigma factor, RsiG [29]. Unlike many antisigma factors, which are often encoded adjacent to their cognate sigma partners [33], *rsiG* lies distant from *whiG* in *Streptomyces* genomes (in *S. venezuelae* the two genes are found ~1.5 Mbp apart). The structure of the RsiG-σ^{WhiG} complex revealed that binding to RsiG results in compaction of σ^{WhiG}, which prevents its interaction with RNA polymerase. Crucially, the structure also revealed a c-di-GMP dimer at the RsiG-σ^{WhiG} interface (Figure 3), even though no c-di-GMP was added prior to crystallization, meaning it copurified with the complex following overexpression in *E. coli*. This represents the first, and to date only example of a sigma-antisigma pair whose association requires c-di-GMP. Most of the direct contacts to the c-di-GMP dimer are mediated by the antisigma factor RsiG, and RsiG alone is able to bind c-di-GMP (with a K_d of 6.5 μM). This binding affinity increases to a K_d of 0.4 μM with the addition of σ^{WhiG}, most likely because σ^{WhiG} provides additional direct contacts to c-di-GMP.

The manner in which RsiG interacts with the c-di-GMP dimer is unprecedented: it binds the second messenger via two copies of a novel $Ex_3Sx_2Rx_3Qx_3D$ motif, one on each of two helices that form an antiparallel coiled coil (Figure 3) [29,34]. Typically, in other effector proteins that bind c-di-GMP dimers, the four guanine bases make stacking interactions to adopt a fully intercalated conformation [35-39]. In contrast, when bound to RsiG, the two molecules of c-di-GMP are partially intercalated, with only two of the guanine bases stacked, and the other two rotated out to stack against conserved glutamine residues and interact with conserved aspartic acid residues found in the repeated signature motif (Figure 3). The c-di-GMP dimer functions to bind the sigma-antisigma pair together through direct contacts and by promoting the folding of a loop in RsiG that forms part of the interface with σ^{WhiG}, thereby stabilizing the complex. A mutant RsiG protein that cannot bind c-di-GMP (having alanines substituting for arginine and aspartic acid residues in both signature motifs) was unable to impede σ^{WhiG} activity, both *in vitro* and *in vivo*, confirming that c-di-GMP is required for stable complex formation [29]. Consequently, formation of the RsiG-(c-di-

GMP)$_2$-σ^{WhiG} complex is favored during vegetative growth when c-di-GMP levels are high, resulting in sequestration of σ^{WhiG} from RNAP. When c-di-GMP levels drop at the onset of reproductive growth, the complex disassociates, releasing σ^{WhiG} to direct RNAP to transcribe the genes encoding the late-stage sporulation regulators WhiH and WhiI (Figure 1D). The K_d values of BldD and RsiG-σ^{WhiG} for c-di-GMP provide a potentially simple explanation for why BldD dissociates from DNA before σ^{WhiG} is released from RsiG. BldD binds c-di-GMP with a K_d of 2.5 μM [9], whereas the K_d for [RsiG + σ^{WhiG}] is 0.4 μM [29]. Therefore, as c-di-GMP levels decline, BldD would become inactive as a repressor before σ^{WhiG} is released from its antisigma (Figure 1D).

Distribution and evolution of the antisigma factor RsiG

RsiG is unique among both antisigma factors and c-di-GMP receptors, sharing no homology with characterized examples of either. A search of representative bacterial genomes found that distribution of this novel c-di-GMP-binding antisigma factor is restricted to the phylum Actinobacteria [40]. The majority of the actinobacterial homologs identified resemble *S. venezuelae* RsiG with twin c-di-GMP-binding motifs. However, five of the representative actinobacterial genomes were found to encode RsiG homologs that have only one c-di-GMP-binding motif. The structure of one of these, from *Rubrobacter radiotolerans,* revealed that the single-motif RsiG homologs dimerize to form an antiparallel coiled coil such that they bind c-di-GMP and cognate σ^{WhiG} partners in a similar manner to the monomeric twin-motif form of RsiG. Based on the distribution of the two forms of RsiG within the Actinobacteria, it seems likely that the single-motif protein is the ancestral form of RsiG and that an intragenic duplication event gave rise to the twin-motif form of the protein inherited by many Actinobacteria [40].

Members of the genus *Rubrobacter* are not filamentous and do not sporulate. This means that in this genus, RsiG$_2$-(c-di-GMP)$_2$-σ^{WhiG} must regulate a different biological process from the homologs first characterized in *Streptomyces*. Indeed, it was shown that in *Rubrobacter*, RsiG$_2$-(c-di-GMP)$_2$-σ^{WhiG} regulates the biosynthesis of type IV pili [40], surface appendages that mediate diverse functions in bacteria including motility, biofilm formation, surface sensing, and DNA uptake [41]. Additionally, in *Rubrobacter* multiple DGCs and PDEs were predicted to be under the control of RsiG$_2$-(c-di-GMP)$_2$-σ^{WhiG}, suggesting the presence of regulatory feedback loops evocative of those observed for *Streptomyces* BldD-(c-di-GMP). This example thus illustrates how homologous transcriptional regulators can undergo major structural and functional shifts over the course of their evolution.

c-di-GMP binds GlgX to stimulate enzymatic degradation of glycogen stores during spore maturation

In addition to BldD- and σ^{WhiG} -mediated regulation of gene expression, recent studies revealed that c-di-GMP controls the timing of glycogen degradation by acting as an allosteric activator of the glycogen debranching enzyme GlgX [16]. Like BldD, GlgX was identified as a c-di-GMP binding protein by affinity pull-down assay using a c-di-GMP capture compound. Glycogen is a highly branched homopolysaccharide consisting of α-1,4-

linked glucose subunits in the linear oligosaccharide chains, and α-1,6-linked glucose at branching points [42]. Many bacterial species accumulate glycogen for carbon and energy storage, especially under conditions of carbon excess while limited for another nutrient (e.g. nitrogen) [43,44].

The storage and degradation of glycogen in time and space is linked to developmental functions and regulation in *Streptomyces* [45]. In *Streptomyces coelicolor*, deposition of the biopolymer occurs in two discrete 'tissues' of the developing colony. Phase I deposition takes place at the interface of the vegetative and aerial hyphae, likely serving as an energy source for aerial growth. Phase II storage occurs in immature spore chains within aerial hyphae. Notably, *S. coelicolor* possesses two isoforms of glycogen branching enzyme, GlgBI, associated with Phase I deposition, and GlgBII, associated with Phase II deposition [46.47]. In *S. venezuelae*, uncontrolled degradation of glycogen caused by overexpressing the glycogen debranching enzyme GlgX results in delayed sporulation and a reduced number of viable spores [16]. Interestingly, the glycogen content of spores decreases as they mature. Spores harvested from *S. venezuelae* plates three days after inoculation contain about 160 μg glycogen per mg total protein, whereas those harvested after 14 days after inoculation contain only 5 μg glycogen per mg total protein (Katrin Wrede and Natalia Tschowri, unpublished), suggesting that glycogen degradation is part of the spore maturation process, and that carbon released from the biopolymer supports spore formation and vitality.

Together with the glycogen phosphorylase GlpP, GlgX constitutes the core of glycogen catabolism. While GlpP catalyzes the depolymerization of α-(1→4)-glucosidic linkages, GlgX removes α-(1→6)-linked glucose residues in glycogen [42]. Two mechanisms account for the correct timing of GlgX-mediated glycogen degradation during spore maturation. First, *glgX* expression is developmentally regulated so that the enzyme accumulates during sporulation. Second, activation of GlgX requires binding of c-di-GMP [16]. A GlgX-c-di-GMP structure showed that the enzyme utilizes a novel $ExRx_6R$ signature motif to bind the second messenger (Figure 4). In complex with c-di-GMP, GlgX forms an antiparallel, head-to-tail dimer in which monomeric c-di-GMP binds at each end of the protein dimer to stabilize an active conformation (Figures 1D and 4). Dimerization of GlgX is stimulated by c-di-GMP binding but is not strictly dependent on it, and activation of the enzyme occurs through c-di-GMP-induced long-range conformational changes resulting in structural rearrangements at the active site of the enzyme. Importantly, GlgX has a relatively moderate affinity for c-di-GMP (~ 8 μM) explaining why enzyme activity depends on relatively high levels of the second messenger, which reach their peak during sporulation [16] (Figure 1D). Thus, the accumulation of GlgX and of c-di-GMP in the late stages of development ensures the controlled release of stored carbon for spore maturation. Notably, glycogen debranching enzymes are widespread in eukaryotes, archaea and bacteria [42], but the $ExRx_6R$ c-di-GMP binding motif is only conserved in glycogen debranching enzymes from streptomycetes and a few other Actinobacteria. This suggests that the link between c-di-GMP signaling and GlgX-mediated glycogen degradation evolved as a specific feature of *Streptomyces* development.

Conclusions

Research from the last decade on c-di-GMP signaling in *Streptomyces* has revolutionized our view of the regulatory mechanisms that control progression through the life cycle of these biotechnologically important bacteria. The rise and fall of c-di-GMP levels, in concert with the K_ds of the three effectors BldD, RsiG-σ^{WhiG} and GlgX, determine the timing and order of events during differentiation. Across development, the measured c-di-GMP levels range from 10-75 pmol c-di-GMP per mg total protein (Figure 1D) [16]. If the protein concentration of the *S. venezuelae* cytoplasm were ~100 μg μl^{-1}, this would equate to c-di-GMP concentrations of 1-7.5 μM, and the K_d values of the three known *Streptomyces* c-di-GMP binding proteins sit in this approximate range (Figure 1D). To allow c-di-GMP dynamics to be monitored at the single-cell level – especially important in a differentiating, multicellular organism like *Streptomyces* – it will be important in the future to use c-di-GMP biosensors to visualize signaling heterogeneity by live cell microscopy [48,49].

The combination of physiological and structural analyses of c-di-GMP signaling in *Streptomyces* has proven to be extremely powerful for the discovery of unprecedented and unpredictable mechanisms through which a nucleotide second messenger can affect protein function, as exemplified by the $BldD_2$-$(c\text{-}di\text{-}GMP)_4$ and RsiG-$(c\text{-}di\text{-}GMP)_2$-$\sigma^{WhiG}$ complexes. BldD, RsiG-σ^{WhiG}, and GlgX all bind c-di-GMP via distinct and novel motifs, and none of the three proteins share homology with any previously identified c-di-GMP binding proteins or with each other. These examples suggest that the true diversity of c-di-GMP effectors is likely vastly underappreciated. As c-di-GMP is a nearly universal second messenger in bacteria, further characterization of the molecular mechanisms that underpin c-di-GMP signaling in diverse phyla will be an important focus of future research efforts aimed at understanding how bacteria coordinate responses to dynamic environmental conditions.

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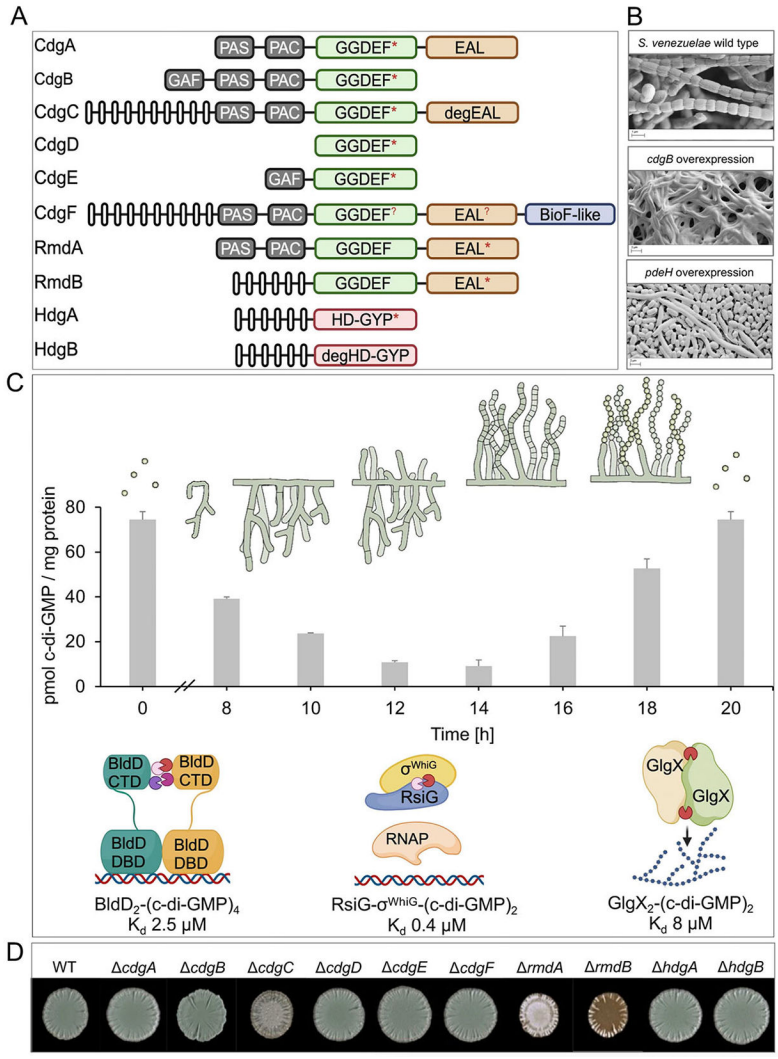

Figure 1. c-di-GMP levels control *Streptomyces* development.

(A) Scanning electron micrographs showing that overexpression of a c-di-GMP-synthesizing enzyme (CdgB) prevents the initiation of development, trapping *Streptomyces* in vegetative growth (the classic 'Bald' phenotype), whereas overexpression of a c-di-GMP-degrading enzyme (PdeH) causes precocious hypersporulation. Wild-type *S. venezuelae* is shown for comparison. Cells were grown on maltose-yeast extract-malt extract (MYM) agar for four days at 30 °C prior to imaging. (B) Domain organization of the 10 c-di-GMP-metabolizing enzymes in *S. venezuelae*. GGDEF domains are shown in green, EAL domains in brown, HD-GYP domains in pink and the BioF-like domain of CdgF in purple. Predicted transmembrane helices are shown as open bars and N-terminal GAF, PAS and PAC signaling domains are shown in grey. WT, wild type; deg, degenerate; *, enzymatically active domain; ?, enzymatic activity unknown. (C) Colony phenotypes of mutants for each of the 10 genes encoding c-di-GMP-metabolizing enzymes in *S. venezuelae*. (D) Representation of the *Streptomyces* life cycle showing the three main developmental stages: vegetative growth, aerial growth, and sporulation. c-di-GMP levels (grey bars) vary across the life cycle [16]. During the vegetative phase, high c-di-GMP levels mediate the oligomerization

of the developmental master repressor BldD into an active dimer, blocking the expression of a large regulon of sporulation genes. High c-di-GMP levels also directly mediate the sequestration of the sporulation-specific sigma factor σ^{WhiG} by the antisigma factor RsiG. As c-di-GMP levels begin to drop at the start of the transition to sporulation, the BldD dimer dissociates, resulting in the loss of DNA binding, and thereby allowing the transcriptional cascade leading to sporulation to become activated [9,22]. Subsequently, as c-di-GMP levels drop further, σ^{WhiG} is released from the antisigma factor RsiG, allowing the activation of late sporulation gene expression [29]. As sporulation proceeds, c-di-GMP levels begin to rise, and the binding of c-di-GMP to GlgX leads to a long-range conformational change in its active site, activating the enzyme and causing the degradation of glycogen in maturing spores [16]. The K_ds of BldD, RsiG-σ^{WhiG} and GlgX for c-di-GMP shown are consistent with this order of events. Note that GlgX is abundant only during sporulation. CTD, C-terminal domain; DBD, DNA-binding domain; RNAP, RNA polymerase.

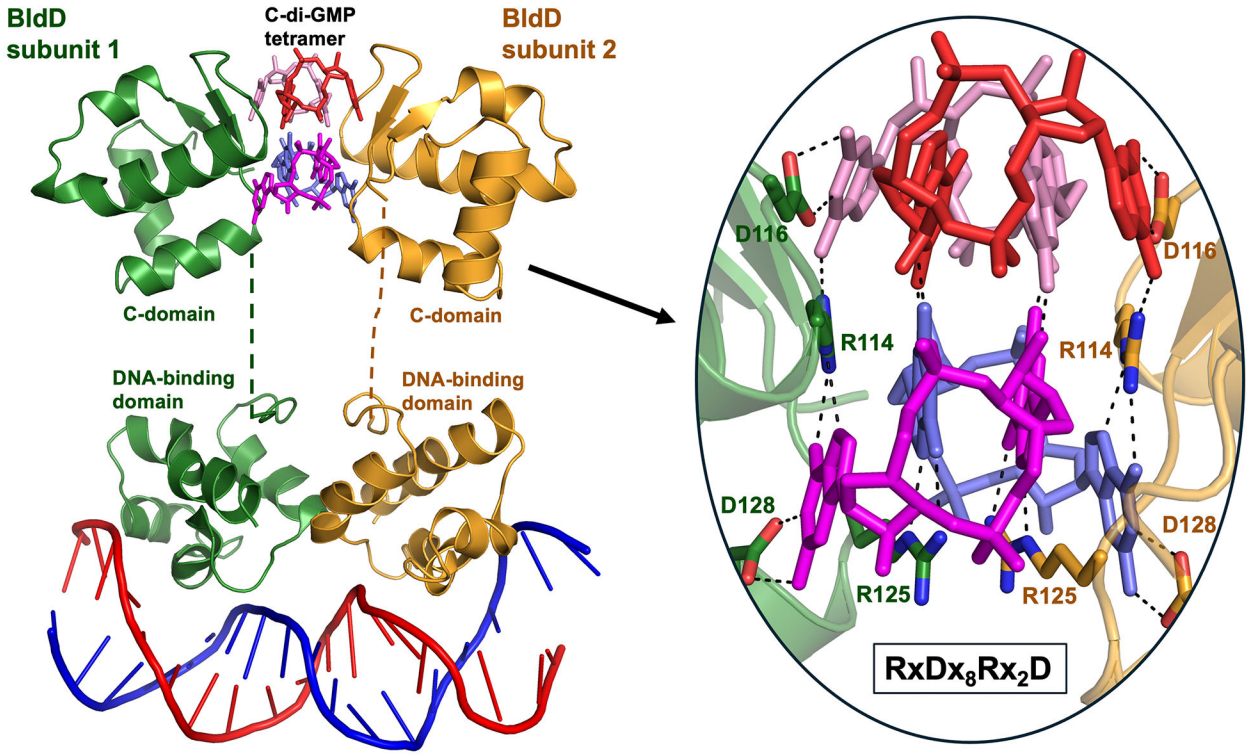

Figure 2. Dimerization of the developmental master repressor BldD is mediated by the formation of tetrameric c-di-GMP bound between the two CTDs.

Model of the $BldD_2$-$(c\text{-}di\text{-}GMP)_4$-DNA complex, based on the ~2 Å resolution structure of the CTD_2-$(c\text{-}di\text{-}GMP)_4$ complex combined with a ~4.5 Å resolution structure of the DBD-DNA region [9]. One BldD subunit is colored wheat and the other green. For clarity, each of the four c-di-GMP molecules is colored differently. The c-di-GMP molecules are shown as sticks and the DNA as a cartoon. The linker region between the CTD and the DBD (shown as dashed lines in wheat or green) is disordered in both subunits, indicating their conformational flexibility. The inset shows the mechanism of selective binding of the c-di-GMP tetramer by the $RxDx_8Rx_2D$ BldD signature motif. Dashes indicate polar interactions.

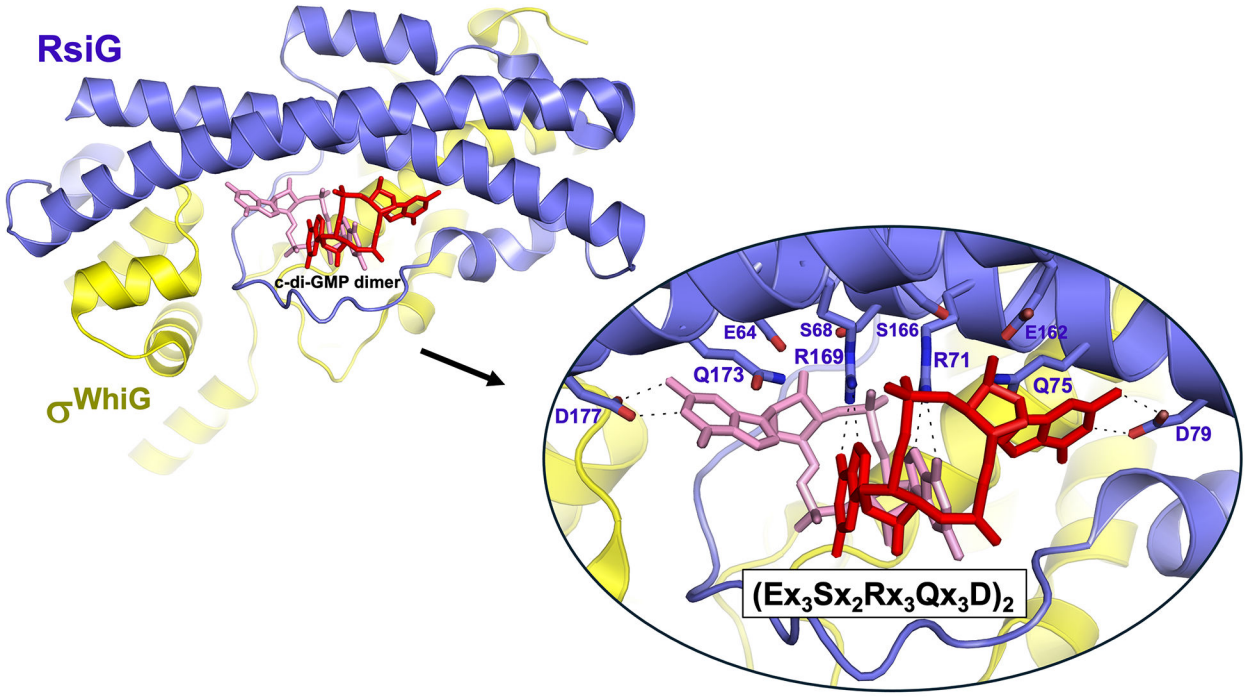

Figure 3. c-di-GMP arms RsiG as an antisigma, allowing it to bind and sequester the sporulation-specific σ factor, σWhiG.

Ribbon diagram of the overall structure of the *S. venezuelae* RsiG-(c-di-GMP)$_2$-σ^{WhiG} complex [29]. The antisigma RsiG is colored blue and σ^{WhiG} is colored yellow. The two partially intercalated c-di-GMP molecules are shown as pink or red sticks. The inset shows the selective binding mechanism of the c-di-GMPs by residues of the two copies of $Ex_3Sx_2Rx_3Qx_3D$ RsiG signature motif, one present on each of the two helices of the RsiG antiparallel coiled coil, selectively bind the c-di-GMP nucleotides. Dashes indicate polar interactions.

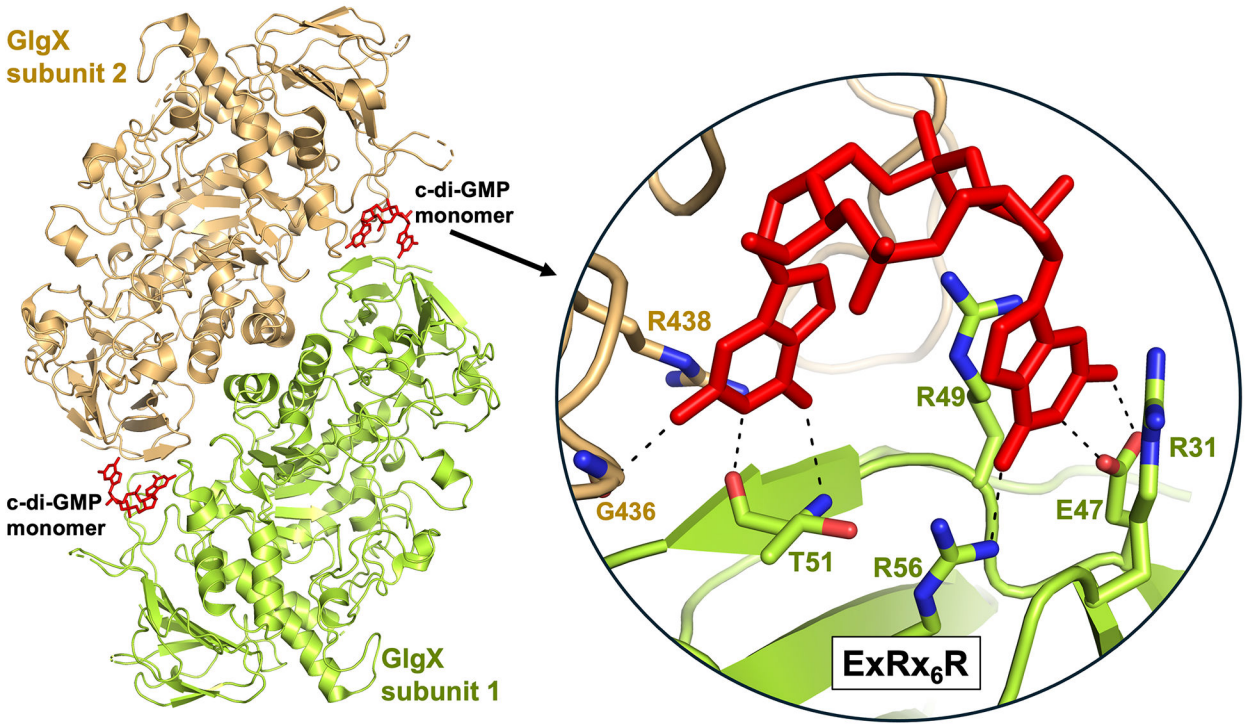

Figure 4. c-di-GMP controls the enzymatic activity of the glycogen debranching enzyme GlgX.

Ribbon diagram of the dimeric S. *venezuelae* $GlgX_2$-c-di-GMP_2 complex [16], with one GlgX subunit colored wheat and the other green. Shown as red sticks are the c-di-GMP molecules bound at the subunit interfaces at each end of the antiparallel GlgX dimer. The inset shows how the residues of the GlgX $ExRx_6R$ signature motif selectively bind c-di-GMP. Dashes indicate polar interactions.