Spring 4-19-2018

Identifying RmdA Protein Interactions in Streptomyces Using a Bacterial Two-Hybrid System

Rachel Nguyen
rachel.nguyen@otterbein.edu

Identifying RmdA Protein Interactions in *Streptomyces* Using a Bacterial Two-Hybrid System

Rachel T. Nguyen

Biochemistry and Molecular Biology Program

Otterbein University

Westerville, Ohio 43081

16 April 2018

Submitted in partial fulfillment of the requirements for graduation with Distinction

Advisory Committee:

Jennifer A. Bennett, Ph.D.	_____
Distinction Advisor	Advisor Signature
John Tansey, Ph.D.	_____
Second Reader	Second Reader Signature
David Robertson, Ph.D.	_____
Distinction Representative	Distinction Representative Signature

Acknowledgements

I would like to thank Dr. Jennifer Bennett for giving me the opportunity to learn from her both inside and outside the lab as a mentor; Dr. John Tansey for being my second reader and for supporting me these past three years at Otterbein University; Dr. David Robertson for serving as my Distinction advisor; Curtis Smith, Maker Space and Laboratory Operations Manager from the Point, for making a 3D model of the predicted structure of RmdA; Erin Ulrich for supporting me throughout my research by gathering last minute needed reagents. I would also like to thank The Bert and Jane Horn Endowed Student Research Fund for financing my research supplies in order to conduct the experiment; The Department of Biology and Earth Science Summer Research Stipend for awarding me with a stipend to continue my research over the summer; The Otterbein Student Research Fund for helping me take the first steps in this research project by supplying me with reagents and kits. I thank my fellow undergraduate researchers for keeping me sane while in lab. It has definitely been a pleasure working with you all. Specifically, I would like to thank Sean Kirk, the Bennett Lab Technician, for making much needed reagents and plates. Lastly, I would like to thank my mother, father, sister, and Angel for supporting me in all of my endeavors in life; you are all in my heart.

Abstract

Streptomyces is a genus of the phylum actinobacteria most commonly found as soil bacteria and used as a major source of antibiotics. RmdA and RmdB are phosphodiesterases that break down the ubiquitous second messenger cyclic-di-GMP which determines colony morphology and development of *Streptomyces*. The objective of this research is to identify whether RmdA will have interactions with itself using the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) System. Each gene was fused into one of two BACTH vectors that encode a different domain of a single protein (T18 and T25) and then cotransformed into the BACTH indicator strain. The transformants were plated on the indicator plates, LB-X-Gal and MacConkey-maltose, and incubated to qualitatively show their possible interactions. If the proteins interact, they will bring the separated T18 and T25 domains in close proximity to produce beta-galactosidase on LB-X-Gal or ferment maltose on MacConkey-maltose which will be seen as blue or red colonies respectively. The plasmids containing T18-RmdA and T25-RmdA were successfully created and cotransformed to determine whether RmdA interacts with itself. When testing RmdA fused to T18 with an open C-terminus (T18-RmdA) and RmdA fused to T25 with an open C-terminus (T25-RmdA), no interactions were detected. This could be due to the T18 or T25 fragments blocking or preventing the function of the N-terminus of RmdA which could be required for protein interactions. Further testing is being conducted to determine if the N-terminus is needed to interact.

Table of Contents

List of Figures

List of Tables

Introduction

Streptomyces is a genus of the phylum actinobacteria which is composed of Gram-positive bacteria. This genus is most commonly found as soil bacteria and with a high GC content of more than 70% (Hasani, et al., 2014). *Streptomyces* is a filamentous organism that reproduces by germinating a spore which grows into branched, vegetative hyphal filaments (primary hyphae). These filaments then anchor and send up aerial hyphae (secondary hyphae) where synchronous, evenly-spaced septation occurs within each aerial filament. Then the cells within the hyphae mature into chains of spores which will separate resulting in free spores that will repeat the lifecycle (Chater, 2006). It is important to note that the ungerminated spores undergo a process similar to mitosis of higher organisms (McGregor, 1954). Because of the complexity of the morphological differentiation within this model organism, it has been used as a representation of the bacterial life cycle.

Bis-(3′–5′)-cyclic-dimeric-guanosine monophosphate (c-di-GMP), an RNA dinucleotide second messenger, plays an important role in the development of *Streptomyces*. It was found to be involved in different cellular activities such as biofilm formation, motility, cell cycle progression, and virulence in a variety of bacteria (Ryan, et al., 2006). Its association with determining sessility or motility in bacteria is most noticeable. High concentrations of c-di-GMP resulted in an increased production of biofilms to adhere to the surface, while on the other hand, low concentrations of c-di-GMP promoted motility in the bacterium (Aswin, et al., 2010). Intracellular levels of c-di-GMP are regulated by either the GGDEF domain of diguanylate cyclases (DGCs) or by c-di-GMP specific EAL or HD-GYP domains of phosphodiesterases (PDEs) (Ryan, et al., 2006). DGCs are involved with c-di-GMP synthesis while PDEs are involved with their degradation (reviewed from Valentini & Filloux, 2016). The pathway begins

when a primary messenger binds to a receptor to activate DGCs to synthesize c-di-GMP from two molecules of guanosine triphosphate (GTP). At this point, PDEs could also cleave the molecule back into two GTPs. c-di-GMP would then bind to either a protein or a riboswitch to ultimately change gene expression (reviewed from Maxwell Dow, et al., 2006). Specifically for this research, PDEs of c-di-GMP include RmdA and RmdB (regulator of morphology and development A and B) which were originally named SCO0928 and SCO5495 based on their positioning on the *Streptomyces coelicolor* chromosomal map in Figure 1 (Hull et al., 2012).

RmdA and RmdB are GGDEF-EAL domain phosphodiesterases that break down the ubiquitous second messenger cyclic-di-GMP which determines colony morphology and development of *Streptomyces.* When looking at how these two proteins affect the development of *Streptomyces*, RmdA and RmdB were found to be responsible for regulating life cycle progression. When one of the two was inactivated by a transposon insertion, there was a subtle phenotype delay in the formation of aerial hyphae and therefore the rest of the life cycle including antibiotic production and sporulation. However, if both of the genes were inactivated, there was no production of aerial hyphae, resulting in a bald phenotype or an organism without any aerial filaments. This suggests that RmdA and RmdB have additive or even partially overlapping functions (Hull et al., 2012).

Regarding RmdA, nothing is known about its interaction with other proteins. This project will look at the interactions RmdA might have with itself and other proteins, such as the phosphodiesterase RmdB. This research is important because these interactions have not been previously determined. *Streptomyces coelicolor* is a model for the bacterial life cycle; therefore, any information gained about the organism can have many applications, including relevance to similar proteins in disease-causing *Streptomyces* species and other bacteria. The Bacterial

Adenylate Cyclase Two-Hybrid System kit (BACTH*)* is also a novel method for Dr. Bennett's research laboratory (Karimova et al., 1998). No one has used the BACTH assay in her lab to determine interactions between two proteins prior to this project. Using this method may reveal more answers the lab has been investigating in regards to how the phosphodiesterase RmdA cleaves c-di-GMP or whether it could have interacting partners. A mutant for *rmdA* has been made and characterized, but more information is needed and the BACTH assay would further answer questions about how this phosphodiesterase works in natural conditions. There is evidence of a phosphodiesterase that was able to dimerize with itself. In *E. coli*, the EAL domain of YahA, when isolated, was in fast thermodynamic monomer-dimer equilibrium. This domain was then only seen to be active in its dimer state (Sundriyal et al., 2014). This could possibly be seen in interactions of RmdA with itself. The RmdA and RmdB proteins have proven to be important in the development of *Streptomyces* due to their similar delayed mutant and drastic co-mutant changes in aerial hyphae development phenotype. Therefore, it would not be surprising if they were found to form heterodimers or interact with each other in some way during these processes.

The objective of this research is to determine whether or not RmdA will have interactions with itself and/or RmdB, using the BACTH System. Each gene was cloned into the plasmid vectors provided with the kit, then transformed into the BACTH strains provided. Once transformed, the transformants were plated on indicator plates and incubated. The current hypothesis is that RmdA will have interactions with itself through homodimerization as seen in other phosphodiesterases and possibly interact with RmdB because inactivation of either phosphodiesterase gene results in a similar phenotype. This study evaluated RmdA+RmdA interactions.

Materials & Methods

To address the objectives above, RmdA was tested to see if it interacted with itself using the BACTH System (Karimova et al., 1998).

Bacterial strains, media and growth conditions: The various *E. coli* strains that were used in this study are listed in Table 1. These strains were grown in either lysogeny broth (LB) or Super Optimal broth with Catabolite repression (SOC) media according to the protocol given by Agilent for chemically competent XL1-Blue and the general protocol for transforming calcium chloride competent cells for BTH101. If necessary, final concentrations of ampicillin (100µg/mL), kanamycin (50µg/mL), or streptomycin (100µg/mL) were added to the specific media. *E. coli* strains were grown at 37°C except the indicator strain BTH101 which was grown at 30°C because complementation was seen to be more efficient at this temperature (Euromedex). When testing the interactions between proteins, LB-X-Gal agar and MacConkey Maltose agar media were used. The LB-X-Gal medium contained final concentrations of X-Gal (40µg/mL), IPTG (0.5mM), and the concentrations of ampicillin and kanamycin listed above. Within the MacConkey media, final concentrations of maltose (1%), IPTG (0.5mM), and the concentrations of ampicillin and kanamycin listed above were added. It is important to note that plates containing X-Gal (40µg/mL) and IPTG (0.5mM) were overlaid with these two reagents directly before plating the bacteria. X-Gal was utilized as a colorimetric substrate when determining whether or not proteins interact. When using the BACTH system, if the proteins interact, they will aid in producing cyclic adenosine monophosphate (cAMP) which will turn on the *lacZ* gene in *E. coli* which codes for β-galactosidase. This enzyme will then cleave the β-galactoside within X-Gal into galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter is

then oxidized into 5,5′-dibromo-4,4′-dichloro-indigo which is blue in color (Burn, 2012). IPTG was used to help trigger the transcription of the lac operon. This occurs because the reagent is a molecular minic of allolactose which binds to the lac repressor thus allowing transcription of the lac operon to occur (van Hoek, 2007).

Cloning and plasmid preparation: The Qiagen Plasmid Mini-prep kit was used for the isolation of all plasmid DNA. For each *in vivo* ("in the cell") interaction (RmdA+RmdA), the genes that code for the desired proteins were amplified using the Platinum *Pfx* DNA Polymerase protocol (Invitrogen) for polymerase chain reactions (PCR). The Bio-Rad T100™ Thermal Cycler conditions used followed the Invitrogen protocol (denature 50µL reaction for five minutes at 94°C, then perform thirty cycles of denaturation at 94°C for fifteen seconds, then annealing at 55°C for thirty seconds, then extending at 68°C for two minutes due to the *rmdA* having 2,144 base pairs (bp); after completing thirty cycles, the reactions were kept at 68°C for five minutes and then left at 4°C to maintain the reaction) with one change: the annealing temperature was increased from 55°C to 57°C. MT1110 was used as the DNA template to amplify the desired genes (Table 2). Once these genes were cloned and purified, each one was fused into one of two BACTH vectors containing the DNA sequence corresponding to a single fragment of the catalytic domain for the adenylate cyclase gene from the bacterium, *Bordetella pertussis* (Figure 2A). The adenylate cyclase gene naturally possesses both fragments (T18 and T25). When these two domains are separated from each other, they are deemed inactive with no ability to make cyclic adenosine monophosphate (cAMP) (Figure 2B). One of the protein genes (*rmdA*) was fused with the BACTH vector pUT18C or pUT18 and the other gene (*rmdA*) was fused with the other vector pKT25 or pKNT25 (Figure 2C) to create pRN1, pRN2, pRN3, and pRN4 respectively (Table 3, Figure 4). It is important to note that the vectors were

dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) before fusion with the *rmdA* gene insertion. The primers needed for this amplification and fusion (Table 4) were designed carefully to form a product that ultimately created an inframe fusion to prevent the alteration of the reading frame in the plasmids.

Confirmation of cloned genes: After ligating each gene to its respective vector to form the plasmids, a restriction digest of each plasmid was done using their specific restriction enzymes (pRN1 and pRN3 were cut with *Pst*I and *Bam*HI, and pRN2 and pRN4 were cut with *Hin*DIII and *Bam*HI), and then gel electrophoresis was done to confirm the presence of the desired genes in the BACTH vectors. The presence of the desired DNA sequences were indicated by the size of the fragments shown on the gel. The expected band sizes for each vector were 3,442 base pairs (bp) for pKT25, 3,469 bp for pKNT25, 3,017 bp for UT18C, and 3,023 bp for pUT18. The expected band sizes for each DNA insertion of *rmdA* was 2,324 bp for pRN3, 2,215 bp for pRN4, 2,326 bp for pRN1, and 2,215 bp for pRN2. If the size of the fragments line up with the actual size of the desired gene, then the cloning and fusing of those genes into the BACTH vectors will be tentatively deemed successful and then confirmed by sequencing to assess whether the gene fusion is in-frame. Qualitatively, two bands were present in each lane of the gel and the presence of the cloned genes in the plasmids was confirmed. The expected sizes for the DNA insertion and vectors of pRN1 and pRN3 were found.

Qualitative Testing of Interactions and Formation of Controls: In order to qualitatively test the interactions between the two proteins, pRN1 and pRN3 were each individually transformed into XL1-Blue (Agilent) and plated on LB agar containing ampicillin (100µg/mL) or kanamycin (50µg/mL) respectively according to the antibiotic resistance gene on the plasmid (Figure 4). Then one plasmid containing the T25 fragment (pRN3) and one plasmid containing

the T18 fragment (pRN1) were picked from the LB ampicillin or LB kanamycin plates and cotransformed into BTH101. Four controls were also made by cotransforming pKT25-zip+pUT18C-zip (Euromedex) as a positive control, empty pKT25+empty pUT18 as a negative control, empty pKT25+pRN2 as a negative control, and empty pUT18+pRN3 as a negative control into BTH101. These four controls and the experimental cotransformation were plated on LB-X-Gal and MacConkey-maltose agar for qualitative analysis (Figure 4).

Bioinformatics Analyses: The SMART database was used to determine the predicted protein domains of RmdA. RaptorX was utilized to obtain the predicted model of RmdA in *S. coelicolor*. Once obtained, the pdb file was converted to an obj file using PyMOL. The obj file was then printed by the MakerBot Replicator (5th Generation) three dimensional printer to obtain a 3D model of the protein's predicted structure. Multiple sequence alignment data were acquired using the Clustal Omega Program. *Streptomyces* Genome Database was utilized to obtain the amino acid sequences for the multiple sequence alignment through Clustal Omega in order to determine how similar RmdA orthologs in *Streptomyces* were, and therefore how conserved the protein is within the genus. A protein BLAST was used to not only determine how conserved RmdA was in the genus but also outside the genus and the family (Altschul, 1997).

Results

Plasmids Constructed to Test Protein Interactions with RmdA: The plasmids pRN1 and pRN3 were constructed to determine whether RmdA interacted with itself as a homodimer. When pRN1 and pRN3 were digested with *Pst*I and *Bam*HI, the expected band sizes were found as seen in Figure 5A and 5C. The expected band sizes for pRN1 were 3,017bp for the vector pUT18C and 2,326bp for the DNA insertion of *rmdA* as seen in Figure 5A. The band sizes in

Figure 5C for pRN3 coincided with the expected band sizes of 3,442bp for the vector pKT25 and 2,324bp for the DNA insertion. However, for pRN2 and pRN4, all the expected band sizes were not found. In Figure 5B, the band size for the vector pUT18, 3,023bp, lined up with the band sizes shown in lanes 1 and 2, but the expected band size for the DNA insertion, 2,215bp, may not be present. Instead, the band appeared to be closer to about 2,000bp. For pRN4, Figure 5D only showed one band size that corresponded to the expected vector, pKNT25, band size of 3,442bp. The band for the DNA insertion (expected to be 2,215bp) was not seen in the gel.

Known Zipper Proteins Interacted in BACTH Experiment: When the desired protein genes are successfully fused into their specific BACTH vector fragments and they do show interactions with each other, they will bring the separated T18 and T25 domains in close proximity to produce cAMP. The cAMP will then bind to the promoter region and turn on the reporter gene (Figure 2D). When the reporter gene is turned on, it will produce β-galactosidase on LB-X-Gal or ferment maltose on MacConkey-maltose which will be seen as blue colonies or red colonies respectively.

Known interacting leucine zipper motifs from GCN4, a yeast transcriptional activator, were fused to the T18 and the T25 fragments to act as the positive control (O'Shea et al., 1991 and Euromedex). When pUT18C-zip and pKT25-zip were cotransformed into BTH101, they restored the adenylate cyclase gene to produce cAMP and therefore restored the phenotype on the indicator plates by producing blue or red colonies.

Tested Interactions Between pRN1+pRN3: When testing the interactions between pRN1 (T18-RmdA) and pRN3 (T25-RmdA), it was found that when RmdA was cloned to T18 (pRN1) and T25 (pRN3) with both having exposed C-termini, no interaction was detected and colonies did not turn blue or red (Figure 6). These results were strengthened by the positive control

colonies on the LB-X-Gal plates turning blue, and the colonies on the MacConkey-maltose plates turning red. All the negative controls did not change colors as expected.

Bioinformatics Analyses: Using the SMART database, RmdA was predicted to have a PAS, a PAC, a GGDEF, and an EAL domain (Figure 7). The PAS domain is often involved with signaling proteins because it acts as a signal sensing domain. The PAS and PAC domains make up the PAS fold which binds ligands or cofactors to detect sensory input signals (Hefti, et al., 2004). RmdA also contains a GGDEF domain and an EAL domain within its protein structure. GGDEF domains are involved with DGCs which synthesize c-di-GMP; on the other hand, EAL domains are associated with PDEs which degrade c-di-GMP. These two domains that are typically active in separate proteins work together to regulate the levels of c-di-GMP within the cell (Simm, et al., 2004). Therefore, it is highly likely that when both domains are seen within a protein, one is enzymatically inactivated as seen in RmdA which is primarily a PDE with an active EAL domain (Hull, et al., 2012). The predicted protein structure of RmdA was determined using RaptorX and 3D printed using the MakerBot Replicator (5th Generation) three dimensional printer. In conjunction with the SMART database and PyMOL, all domains were found on the 3D structure (Figure 8).

RmdA was also sequence aligned with its orthologs (SCAB11501, KY5_7426, sle_07070, STRS4_02448, SVEN15_6684, SAV_7304, SGR_709, and SLI_1159) within *Streptomyces* using the Clustal Omega Database (Figure 9). The *Streptomyces* Genome Database was used to find RmdA's orthologs and all of their amino acid sequences for input into the Clustal Omega Database. In Table 5, the identity matrix between the orthologs and RmdA were high which suggests that RmdA is highly conserved within the *Streptomyces* genus.

To preface, the genus of *Streptomyces* belongs to the family *Streptomycetaceae*, and in order to also determine how conserved RmdA was within the genus and outside the genus and family, protein BLASTs were conducted (Ventura, et al., 2007). As seen in Table 6, RmdA was highly conserved within the genus due to the percentages of identity and similarity within the genus being in the upper nineties, with a high query coverage, and an error value of 0.0. When excluding *Streptomyces* (Table 7), although the amount of query coverage and percentages of identity and similarity were slightly lower, it could still be concluded that RmdA is conserved outside of the genus. One final protein BLAST was run excluding the family *Streptomycetaceae* with the results shown in Table 8. After examination, there was no error value and the query coverage was in the upper nineties. When evaluating the percentages of identity and similarity, the lowest values were 49% and 64% respectively. This indicated that this protein was still highly conserved outside of the family because according to Rost (1999), a protein sequence with an identity percentage of above 30% and a high query coverage was still considered a highly conserved sequence.

Discussion

Although under these conditions RmdA was not found to interact with itself, it is still believed that RmdA could form a homodimer as seen in other PDEs. YahA in *E. coli* was able to form a homodimer through its EAL domain. This homodimer state of the protein was needed in order for the PDE to be in its active state (Sundriyal, 2014). Not only has a PDE been seen to interact with itself as a homodimer through the EAL domain in YahA but also in the protein FimX found in *Pseudomonas aeruginosa* (Robert-Paganin, 2012). Therefore, although the pRN1+pRN3 cotransformants did not show interaction of RmdA with itself under the conditions

tested, it is possible that the N-terminus of RmdA is needed for an interaction to occur in this phosphodiesterase, because it could have been blocked by the T18 and/or T25 fragment. Future steps include creating the plasmids pRN2 and pRN4 to test the interactions with th efree N-terminus of RmdA. Once the plasmids pRN1, pRN2, pRN3, and pRN4 have successfully been created, they can be used in future experiments to test other RmdA interactions such as RmdA+RmdB to evaluate the second hypothesis of the study, and also by screening a library for other RmdA interacting partners using the BACTH assay.

It could also be interesting to see if RmdA interacted with other DGCs and PDEs within the *Streptomyces* genome, because Sarenko et al. (2017), found that within *E. coli* K-12, DGCs and PDEs not only worked as pairwise interactions, but were also able to form an interaction hub. The interaction hub was composed of three different DGCs and two PDEs which showed a very interconnected network of proteins. These five proteins went on to interact with other proteins to elicit a response from the c-di-GMP pathway (Sarenko, et al., 2017). Therefore, it would be fascinating to see if the same web of interacting DGCs and PDEs were to be found in *Streptomyces* as well.

Not only can qualitative analysis aid in identifying protein interactions, but quantitative techniques can also be utilized to confirm interactions between proteins by measuring levels of cAMP and β-galactosidase. Using ELISA assays, cAMP can be measured in boiled bacterial cultures, and β-galactosidase levels can also be measured by permeabilizing the cells using the substrate o-nitrophenol-β-galactoside (ONPG) to begin the reaction. ONPG will give the solution a yellow color which will help determine the level of β-galactosidase for each sample. The β-galactosidase level of non-interacting proteins is 150 units of β-galactosidase/mg of dry bacterial waste; if those hybrid proteins did interact, then the levels of β-galactosidase would be

between 700-7000 units/mg depending on the strength of the interaction. Immunoprecipitation assays, a complementary biochemical approach to find interacting proteins, could also be used to determine other RmdA protein interactions. In this approach, histidines (6X His) would be tagged onto the protein of interest, in this case RmdA, and combined with the proteome of *Streptomyces*. Antibodies to the histidine tag would then be introduced to cause RmdA to precipitate also pulling down any proteins that were interacting with it. The next steps would be to run a trypsin digest followed by HPLC and tandem mass spectroscopy to identify the interacting proteins. There is not much known about how the c-di-GMP pathway works within Gram positive bacteria like *Streptomyces*, so it will be interesting to uncover the protein interactions of RmdA and other cyclic di-GMP proteins.

Figures

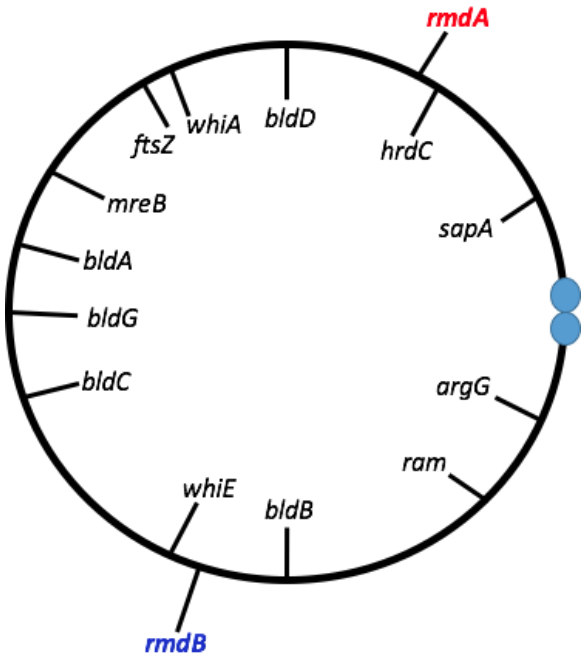


Figure 1: Chromosomal Map of *S. coelicolor* Specifying *rmdA* and *rmdB* Locations. For reference, known genes within *S. coelicolor* are positioned on the inside of the chromosomal map. *rmdA* (SCO0928) and *rmdB* (SCO5495) are positioned on the outer portion of the map (modified from Bennett, 2006).

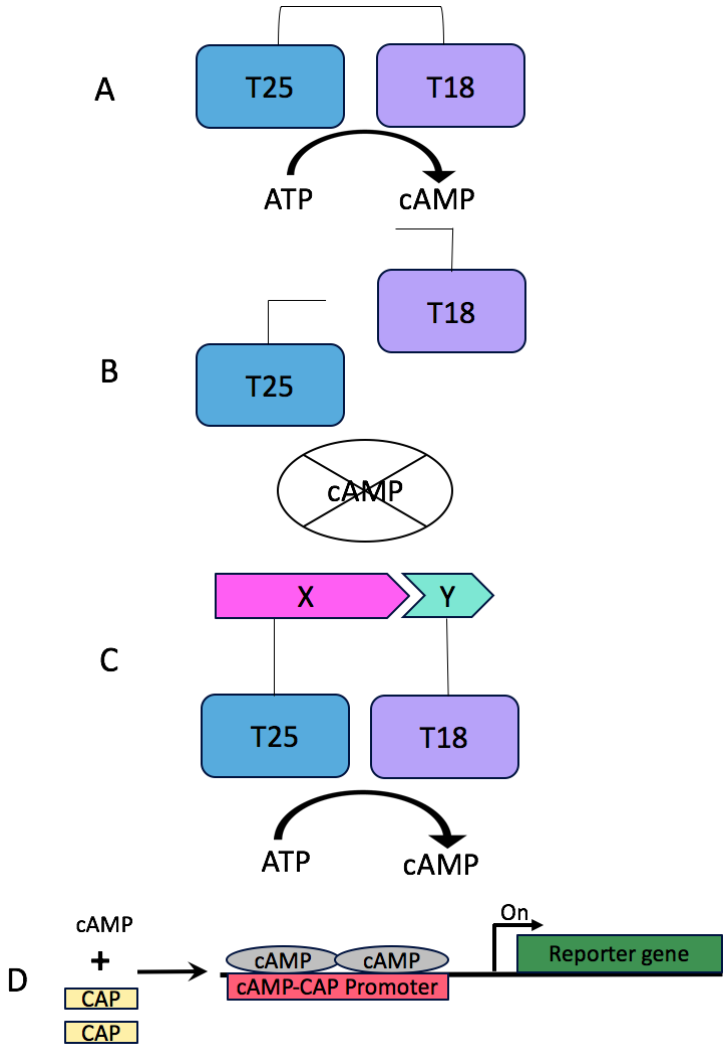


Figure 2: Importance of T18 and T25 fragments in Adenylate Cyclase. (A) The catalytic domain of adenylate cyclase contains two fragments (T18 and T25) whose interactions are necessary in order to produce cAMP. (B) If the fragments are physically separated, then the enzyme is not active and will not produce cAMP. (C) BACTH system takes advantage of this domain by fusing the interested genes into each of the fragments to test the interactions. If they interact then they will bring the fragments in close proximity to produce cAMP. (D) If cAMP is produced, then it can interact with the catabolite activator proteins which helps it bind to the cAMP-CAP promoter region to turn on the reporter gene which in our case is *lacZ*.

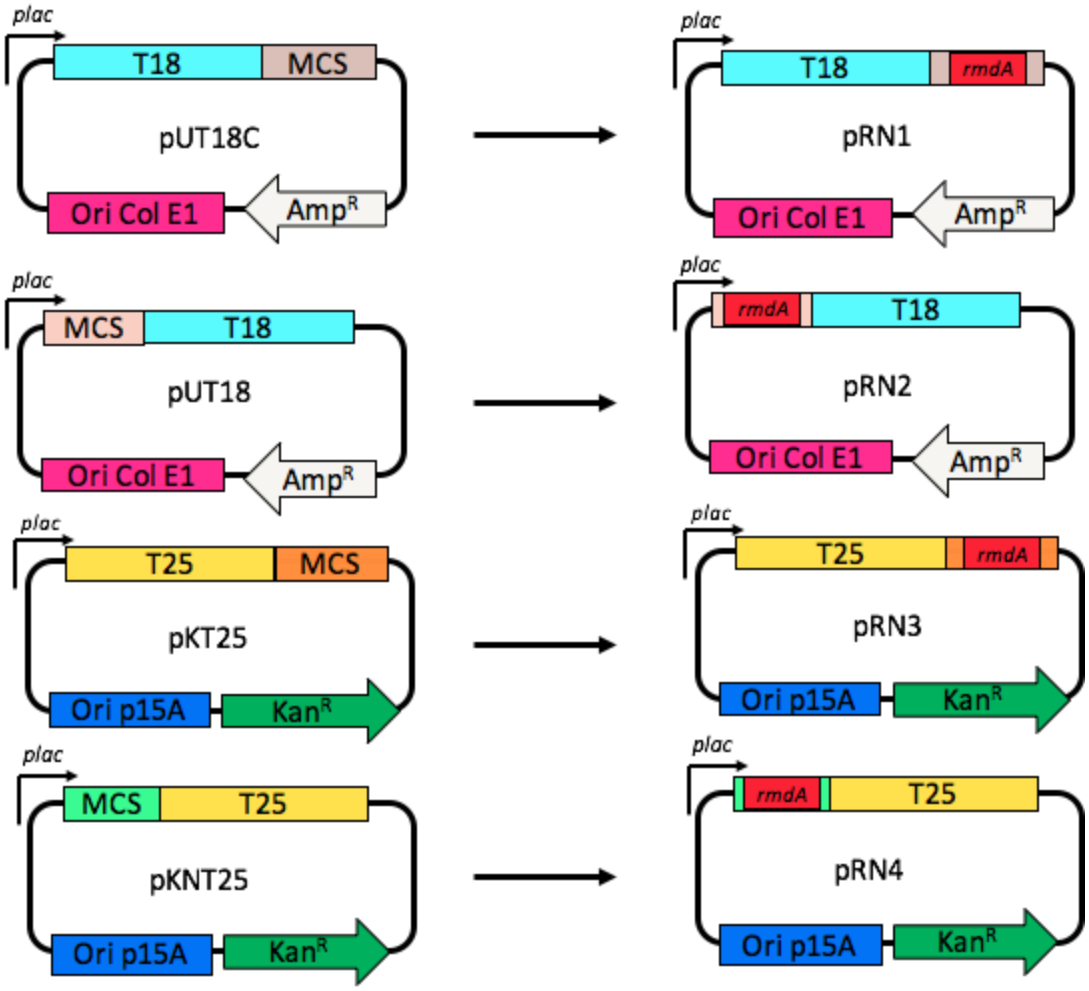


Figure 3: Ligation of *rmdA* into each BACTH plasmid. Each of the provided BACTH plasmids (pUT18C, pUT18, pKT25, and pKNT25) were digested with their restriction endonucleases (*Pst*I+*Bam*HI, *Hin*DIII+*Bam*HI, *Pst*I+*Bam*HI, and *Hin*DIII+*Bam*HI respectively). Using T4 DNA Ligase protocol (New England BioLabs), *rmdA* was ligated into the plasmids creating pRN1, pRN2, pRN3, and pRN4.

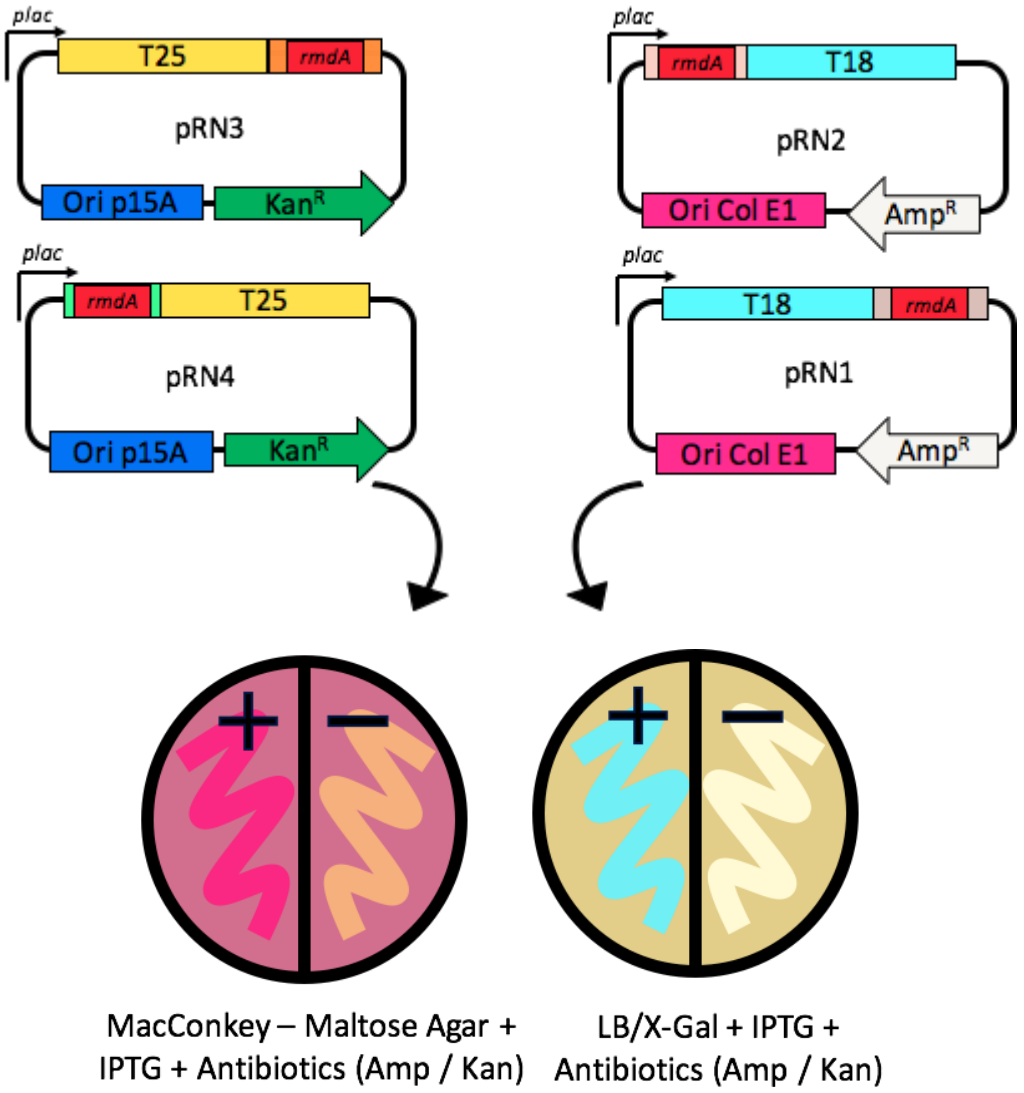


Figure 4: Qualitative Analysis Methods. Two plasmids each containing either the T25 fragment or the T18 fragment were cotransformed into BTH101 and plated on MacConkey-Maltose agar and LB/X-Gal agar both containing IPTG, ampicillin, and kanamycin to show a colorimetric qualitative analysis to determine interactions. On the MacConkey-Maltose plates, red colonies indicate an interaction, and on the LB/X-Gal plates, blue colonies signify an interaction.

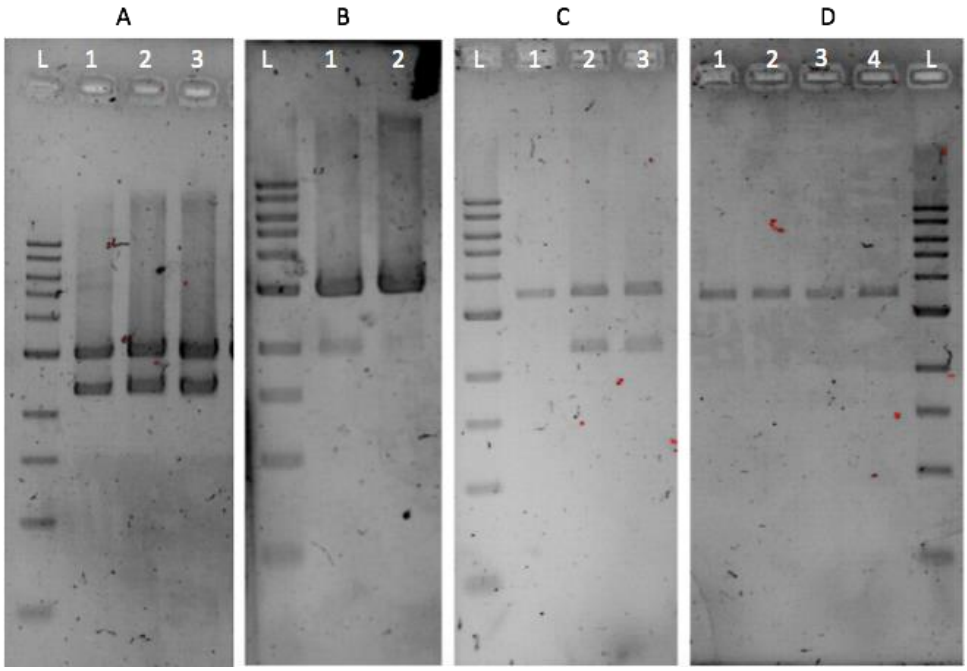


Figure 5: Restriction Digest Confirmation of pRN1, pRN2, pRN3, and pRN4. pRN1 and pRN3 were cut with *Pst*I and *Bam*HI, and pRN2 and pRN4 were cut with *Hin*DIII and *Bam*HI. They were then visualized using gel electrophoresis with the one kilobase ladder in lane L (New England BioLabs). Each lane in the gels represent a different plasmid candidate for each of the four plasmids (A) The gel for pRN1 showed the fragments for the vector and the DNA insertion. All lanes (1-3) agree with the predicted sizes of 3,017bp for the vector and 2,326bp for the *rmdA* gene insertion. (B) The gel for pRN2 showed that the fragments for the vector and the DNA insertion in lanes 1-2 do not agree with the predicted sizes of 3,023bp for the vector and 2,215bp for the gene insertion. (C) The gel for pRN3 showed the fragments for the vector and the DNA insertion in lanes 2-3 agree with the predicted sizes of 3,442bp for the vector and 2,324bp for the gene insertion. However, lane 1 only showed one band that corresponds with the predicted vector size. (D) The gel for pRN4 showed that the band sizes in all lanes (1-4) did agree with the predicted size of 3,469bp for the vector, but not for the *rmdA* gene insertion band size of 2,215bp.

Figure 6: Macroscopic phenotype of pRN2+pRN3 interaction. Row A - pRN1+pRN3; Row B - pUT18C-zip+pKT25-zip; Row C - pKT25+pUT18; Row D - pKT25+pRN1; Row E - pUT18+pRN3. No interactions were seen in Row A as confirmed by having the same phenotype as the negative controls in Rows C-E.

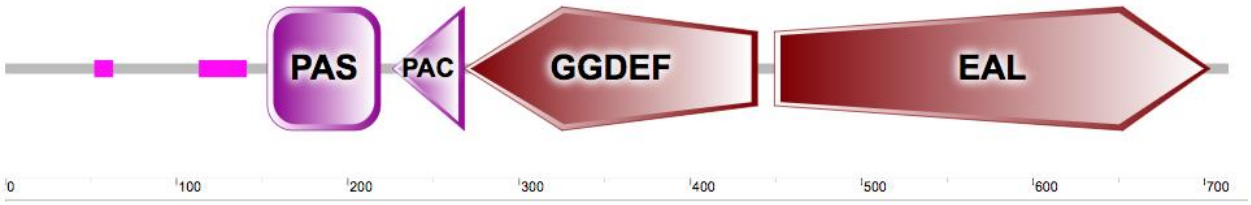


Figure 7: Domains within *S. coelicolor* RmdA. Using the SMART database, *S. coelicolor* RmdA was predicted to have a PAS domain, a PAC domain, a GGDEF domain, and an EAL domain.

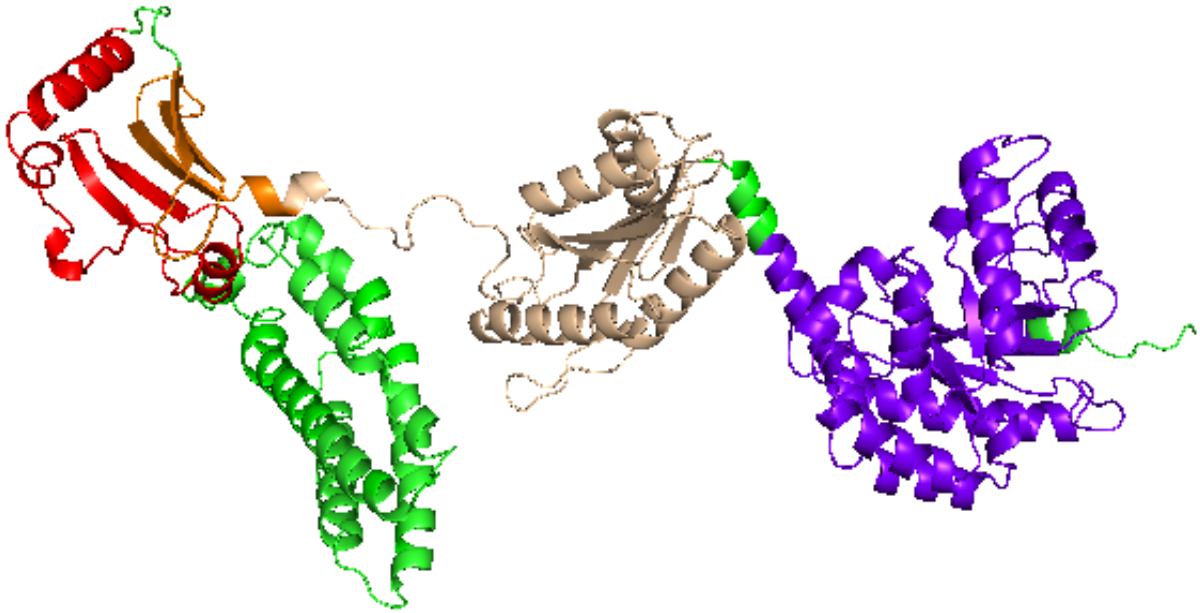
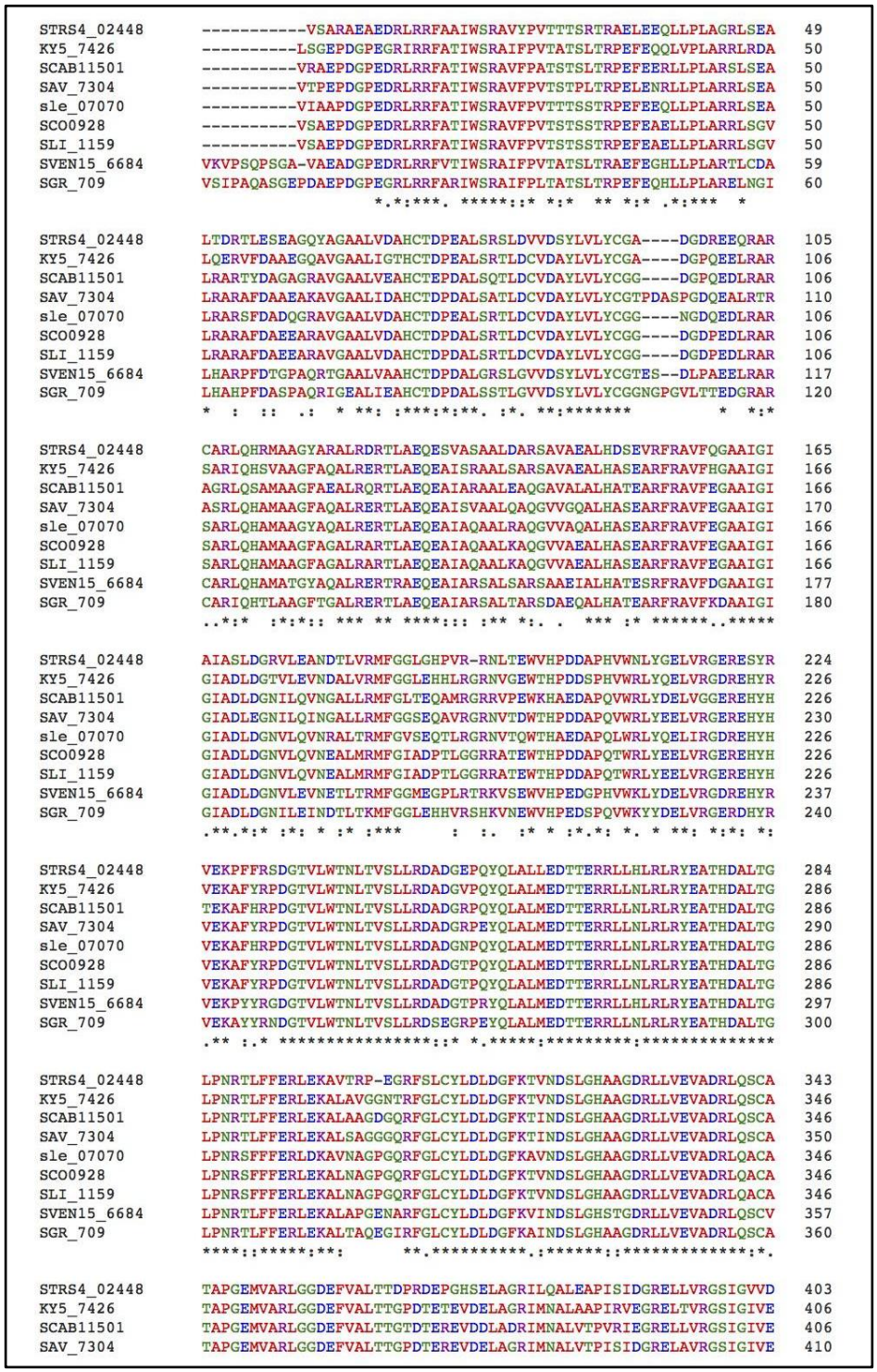


Figure 8: Predicted RmdA Structure in *S. coelicolor*. Using RaptorX, the predicted structure of RmdA was found. Then the PAS (red), PAC (orange), EAL (purple) and GGDEF (tan) domains were identified from utilizing PyMOL and SMART database.


STRS4_02448 ----------VSARAEAEDRLRRFAAIWSRAVYPVTTTSRTRAELEEQLLPLAGRLSEA 49
KY5_7426 ----------LSGEPDGPEGRIRRFATIWSRAIFPVTATSLTRPEFEQQLVPLARRLRDA 50
SCAB11501 ----------VRAEPDGPEDRLRRFATIWSRAVFPATSTSLTRPEFEERLLPLARSLSEA 50
SAV_7304 ----------VTPEPDGPEDRLRRFATIWSRAVFPVTSTPLTRPELENRLLPLARRLSEA 50
sle_07070 ----------VIAAPDGPEDRLRRFATIWSRAVFPVTTTSSTRPEFEEQLLPLARRLSEA 50
SCO0928 ----------VSAEPDGPEDRLRRFATIWSRAVFPVTSTSSTRPEFEAELLPLARRLSGV 50
SLI_1159 ----------VSAEPDGPEDRLRRFATIWSRAVFPVTSTSSTRPEFEAELLPLARRLSGV 50
SVEN15_6684 VKVPSQPSGA-VAEADGPEDRLRRFVTIWSRAIFPVTATSLTRAEFEGHLLPLARTLCDA 59
SGR_709 VSIPAQASGEPDAEPDGPEGRLRRFARIWSRAIFPLTATSLTRPEFEQHLLPLARELNGI 60
LTDRTLESEAGQYAGAALVDAHCTDPEALSRSLDVVDSYLVLYCGA----DGDREEQRAR 105
LQERVFDAAEGQAVGAALIGTHCTDPEALSRTLDCVDAYLVLYCGA----DGPQEELRAR 106
LRARTYDAGAGRAVGAALVEAHCTEPDALSQTLDCVDAYLVLYCGG----DGPQEDLRAR 106
LRARAFDAAEAKAVGAALIDAHCTDPDALSATLDCVDAYLVLYCGTPDASPGDQEALRTR 110
LRARSFDADQGRAVGAALVDAHCTDPEALSRTLDCVDAYLVLYCGG----NGDQEDLRAR 106
LRARAFDAEEARAVGAALVDAHCTDPDALSRTLDCVDAYLVLYCGG----DGDPEDLRAR 106
LRARAFDAEEARAVGAALVDAHCTDPDALSRTLDCVDAYLVLYCGG----DGDPEDLRAR 106
LHARPFDTGPAQRTGAALVAAHCTDPDALGRSLGVVDSYLVLYCGTES--DLPAEELRAR 117
LHAHPFDASPAQRIGEALIEAHCTDPDALSSTLGVVDSYLVLYCGGNGPGVLTTEDGRAR 120
CARLQHRMAAGYARALRDRTLAEQESVASAALDARSAVAEALHDSEVRFRAVFQGAAIGI 165
SARIQHSVAAGFAQALRERTLAEQEAISRAALSARSAVAEALHASEARFRAVFHGAAIGI 166
AGRLQSAMAAGFAEALRQRTLAEQEAIARAALEAQGAVALALHATEARFRAVFEGAAIGI 166
ASRLQHAMAAGFAQALRERTLAEQEAISVAALQAQGVVGQALHASEARFRAVFEGAAIGI 170
SARLQHAMAAGYAQALRERTLAEQEAIAQAALRAQGVVAQALHASEARFRAVFEGAAIGI 166
SARLQHAMAAGFAGALRARTLAEQEAIAQAALKAQGVVAEALHASEARFRAVFEGAAIGI 166
SARLQHAMAAGFAGALRARTLAEQEAIAQAALKAQGVVAEALHASEARFRAVFEGAAIGI 166
CARLQHAMATGYAQALRERTRAEQEAIARSALSARSAAEIALHATESRFRAVFDGAAIGI 177
CARIQHTLAAGFTGALRERTLAEQEAIARSALTARSDAEQALHATEARFRAVFKDAAIGI 180
AIASLDGRVLEANDTLVRMFGGLGHPVR-RNLTEWVHPDDAPHVWNLYGELVRGERESYR 224
GIADLDGTVLEVNDALVRMFGGLEHHLRGRNVGEWTHPDDSPHVWRLYQELVRGDREHYR 226
GIADLDGNILQVNGALLRMFGLTEQAMRGRRVPEWKHAEDAPQVWRLYDELVGGEREHYH 226
GIADLEGNILQINGALLRMFGGSEQAVRGRNVTDWTHPDDAPQVWRLYEELVRGEREHYH 230
GIADLDGNVLQVNRALTRMFGVSEQTLRGRNVTQWTHAEDAPQLWRLYQELIRGDREHYH 226
GIADLDGNVLQVNEALMRMFGIADPTLGGRRATEWTHPDDAPQTWRLYEELVRGEREHYH 226
GIADLDGNVLQVNEALMRMFGIADPTLGGRRATEWTHPDDAPQTWRLYEELVRGEREHYH 226
GIADLDGNVLEVNETLTRMFGGMEGPLRTRKVSEWVHPEDGPHVWKLYDELVRGDREHYR 237
GIADLDGNILEINDTLTKMFGGLEHHVRSHKVNEWVHPEDSPQVWKYYDELVRGERDHYR 240
VEKPFFRSDGTVLWTNLTVSLLRDADGEPQYQLALLEDTTERRLLHLRLRYEATHDALTG 284
VEKAFYRPDGTVLWTNLTVSLLRDADGVPQYQLALMEDTTERRLLNLRLRYEATHDALTG 286
TEKAFHRPDGTVLWTNLTVSLLRDADGRPQYQLALMEDTTERRLLNLRLRYEATHDALTG 286
VEKAFYRPDGTVLWTNLTVSLLRDADGRPEYQLALMEDTTERRLLNLRLRYEATHDALTG 290
VEKAFHRPDGTVLWTNLTVSLLRDADGNPQYQLALMEDTTERRLLNLRLRYEATHDALTG 286
VEKAFYRPDGTVLWTNLTVSLLRDADGTPQYQLALMEDTTERRLLNLRLRYEATHDALTG 286
VEKAFYRPDGTVLWTNLTVSLLRDADGTPQYQLALMEDTTERRLLNLRLRYEATHDALTG 286
VEKPYYRGDGTVLWTNLTVSLLRDADGTPRYQLALMEDTTERRLLHLRLRYEATHDALTG 297
VEKAYYRNDGTVLWTNLTVSLLRDSEGRPEYQLALMEDTTERRLLNLRLRYEATHDALTG 300
LPNRTLFFERLEKAVTRP-EGRFSLCYLDLDGFKTVNDSLGHAAGDRLLVEVADRLQSCA 343
LPNRTLFFERLEKALAVGGNTRFGLCYLDLDGFKTVNDSLGHAAGDRLLVEVADRLQSCA 346
LPNRTLFFERLEKALAAGDGQRFGLCYLDLDGFKTINDSLGHAAGDRLLVEVADRLQSCA 346
LPNRTLFFERLEKALSAGGGQRFGLCYLDLDGFKTINDSLGHAAGDRLLVEVADRLQSCA 350
LPNRSFFFERLDKAVNAGPGQRFGLCYLDLDGFKAVNDSLGHAAGDRLLVEVADRLQACA 346
LPNRSFFFERLEKALNAGPGQRFGLCYLDLDGFKTVNDSLGHAAGDRLLVEVADRLQACA 346
LPNRSFFFERLEKALNAGPGQRFGLCYLDLDGFKTVNDSLGHAAGDRLLVEVADRLQACA 346
LPNRTLFFERLEKALAPGENARFGLCYLDLDGFKVINDSLGHSTGDRLLVEVADRLQSCV 357
LPNRTLFFERLEKALTAQEGIRFGLCYLDLDGFKAINDSLGHAAGDRLLVEVADRLQSCA 360
TAPGEMVARLGGDEFVALTTDPRDEPGHSELAGRILQALEAPISIDGRELLVRGSIGVVD 403
TAPGEMVARLGGDEFVALTTGPDTETEVDELAGRIMNALAAPIRVEGRELTVRGSIGIVE 406
TAPGEMVARLGGDEFVALTTGTDTEREVDDLADRIMNALVTPVRIEGRELLVRGSIGIVE 406
TAPGEMVARLGGDEFVALTTGPDTEREVDELAGRIMNALVTPISIDGRELAVRGSIGIVE 410

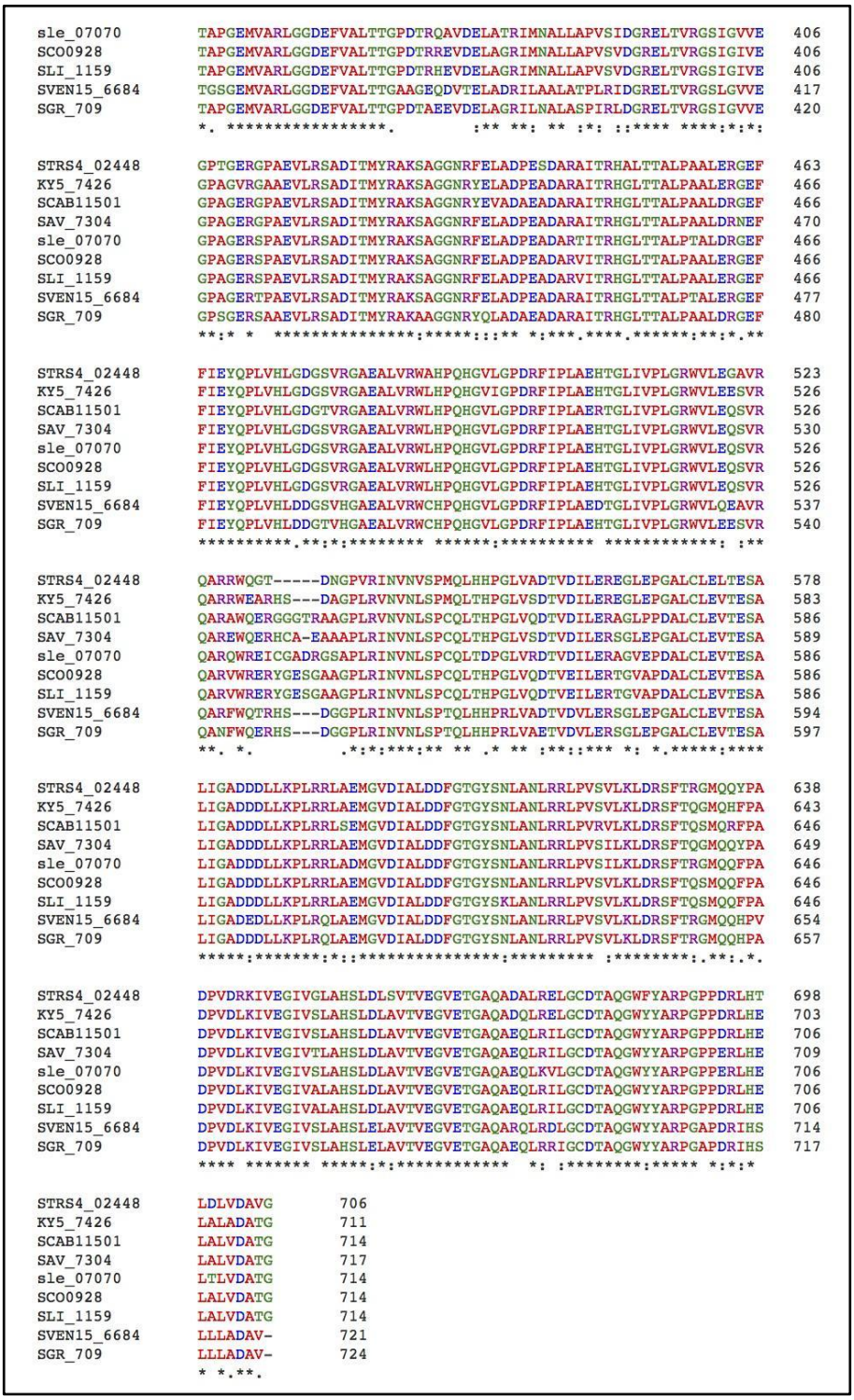


Figure 9: Multiple Sequence Alignment of RmdA and Orthologs within *Streptomyces*. RmdA of *S. coelicolor* was aligned with its orthologs, as determined by the *Streptomyces* Genome Database, STRS4_02448, KY5_7426, SCAB11501, SAV7304, sle_07070, SLI_1159, SVEN15_6684, and SGR_709 to deduce whether the protein is conserved within the genus, *Streptomyces* (Table 5).

Tables

Table 1: *E. coli* Strains Used

Strain	Genotype	Reference
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [*F´*, proAB, lacI*^{q}*ZΔM15,* Tn*10* (Tet^{r})]	Agilent
BTH101	F´*, cya-99, araD139, galE15, galK16, rpsL1 (Str*^{r}*), hsdR2, mcrA1, mcrB1*	Euromedex

Table 2: *S. coelicolor* Strain Used

Strain	Genotype	Reference
MT1110	Prototrophic, SCP1^{-}, SCP2^{-}	Kieser, et al., 2000

Table 3: Plasmids Used

Plasmid	Description	Reference
pUT18C	Codes for the T18 fragment of CyaA that will fuse at the T18 C-terminal end to the desired protein.	Euromedex
pUT18	Codes for the T18 fragment of CyaA that will fuse at the T18 N-terminal end to the desired protein.	Euromedex
pKT25	Codes for the T25 fragment of CyaA that will fuse at the T25 C-terminal end to the desired protein.	Euromedex
pKNT25	Codes for the T25 fragment of CyaA that will fuse at the T25 N-terminal end to the desired protein.	Euromedex
pUT18C-zip	Derivative of the pUT18C plasmid with the leucine zipper of GCN4 fused inframe at the T18 C-terminal. The gene is inserted between the *Kpn*I and *Eco*RI site in the plasmid.	Euromedex
pKT25-zip	Derivative of the pKT25 plasmid with the leucine zipper of GCN4 fused inframe at the T25 C-terminal. The gene is inserted between the *Kpn*I and *Eco*RI site in the plasmid.	Euromedex
pRN1	*S. coelicolor rmdA* inserted into pUT18C using *Pst*I and *Bam*HI	This study
pRN2	*S. coelicolor rmdA* inserted into pUT18 using *Hin*DIII and *Bam*HI	This study
pRN3	*S. coelicolor rmdA* inserted into pKT25 using *Pst*I and *Bam*HI	This study
pRN4	*S. coelicolor rmdA* inserted into pKNT25 using *Hin*DIII and *Bam*HI	This study

Table 4: Primers Used to Create In-Frame Fusions

Primer	Sequence	Application
pUT18C-RmdA-F	AAA CTG CAG AGG AAG AGG ACC CGT ACG CGT TC	Cloning *S. coelicolor* RmdA into pUT18C
pUT18C-RmdA-R	AAA GGA TCC GAA GCG GCC CGT AAC GGT GCT TGA	Cloning *S. coelicolor* RmdA into pUT18C
RmdA-pUT18-F	AAA AAA AAG CTT GAG GAA GAG GAC CCG TAC GCG TTC	Cloning *S. coelicolor* RmdA into pUT18
RmdA-pUT18-R	AAA GGA TCC CCC GTC GCG TCC ACC AGG GCC AG	Cloning *S. coelicolor* RmdA into pUT18
KT25-RmdA-F	AAA CTG CAG GAG GAA GAG GAC CCG TAC GCG TTC	Cloning *S. coelicolor* RmdA into pKT25
KT25-RmdA-R	AAA GGA TCC GAA GCG GCC CGT AAC GGT GCT TGA	Cloning *S. coelicolor* RmdA into pKT25
RmdA-KNT25-F	AAA AAA AAG CTT GAG GAA GAG GAC CCG TAC GCG TTC	Cloning *S. coelicolor* RmdA into pKNT25
RmdA-KNT25-R	AAA GGA TCC CCC GTC GCG TCC ACC AGG GCC AG	Cloning *S. coelicolor* RmdA into pKNT25

Table 5: Identity Matrix of SCO0928 and Orthologs

	STRS4_02448	KY5_7426	SCAB11501	SAV_7304	sle_07070	SCO0928	SLI_1159	SVEN15_6684	SGR_709
STRS4_02448	100.00	81.30	77.34	79.60	79.18	79.32	79.18	78.58	76.03
KY5_7426	81.30	100.00	84.67	86.36	84.11	85.94	85.79	81.52	81.83
SCAB11501	77.34	84.67	100.00	88.36	85.99	87.68	87.39	78.70	79.58
SAV_7304	79.60	86.36	88.36	100.00	87.66	88.92	88.64	78.90	80.25
sle_07070	79.18	84.11	85.99	87.66	100.00	90.06	89.92	78.42	78.03
SCO0928*	79.32	85.94	87.68	88.92	90.06	100.00	99.72	78.56	79.15
SLI_1159	79.18	85.79	87.39	88.64	89.92	99.72	100.00	78.42	79.01
SVEN15_6684	78.58	81.52	78.70	78.90	78.42	78.56	78.42	100.00	83.77
SGR_709	76.03	81.83	79.58	80.25	78.03	79.15	79.01	83.77	100.00

*****Comparing SCO0928 to its orthologs in *Streptomyces*

Table 6: Protein BLAST of RmdA

Organism	E-value	Query Coverage	Percent Identity	Percent Similarity
S. lividans	0.0	99%	99%	99%
S. canus	0.0	99%	98%	99%
S. coelicoflavus	0.0	99%	98%	98%
S. diastaticus	0.0	99%	97%	98%
S. violaceorubidus	0.0	99%	97%	98%
S. parvulus	0.0	99%	97%	98%
S. pactum	0.0	99%	95%	97%
S. olivaceus	0.0	99%	95%	97%
S. ambofaciens	0.0	99%	95%	97%
S. bicolor	0.0	99%	91%	94%

Table 7: Protein BLAST of RmdA Excluding *Streptomyces*

Organism	E-value	Query Coverage	Percent Identity	Percent Similarity
Kitasatospora aureofaciens	0.0	100%	98%	98%
Actinospica acidiphila	0.0	99%	91%	93%
Kitasatospora albolonga	0.0	99%	79%	86%
Microtetraspora glauca	0.0	99%	77%	83%
Streptoalloteichus hindustan	0.0	98%	52%	66%
Streptoalloteichus hindustanus	0.0	94%	53%	67%
Streptacidiphillus oryzae	0.0	98%	52%	66%
Saccharopolyspora erythrae	0.0	97%	51%	65%
Lechevalieria xinjiangensis	0.0	96%	51%	65%
Actinopolyspora mortivallis	0.0	97%	50%	65%
Actinoalloteichus spitiensis	0.0	98%	50%	64%
Actinopolyspora mzabensis	0.0	97%	49%	65%
Saccharopolyspora flava	0.0	96%	51%	64%

Table 8: Protein BLAST of RmdA Excluding *Streptomycetaceae*

Organism	E-value	Query Coverage	Percent Identity	Percent Similarity
Actinospica acidiphila	0.0	100%	91%	93%
Streptoalloteichus hindustan	0.0	98%	52%	66%
Streptoalloteichus hindustanus	0.0	94%	53%	67%
Saccharopolyspora erythrae	0.0	97%	51%	65%
Lechevalieria xinjiangensis	0.0	97%	51%	65%
Actinopolyspora mortivallis	0.0	97%	50%	65%
Actinoalloteichus spitiensis	0.0	98%	50%	64%
Actinopolyspora myzabensis	0.0	97%	49%	65%
Saccharopolyspora flava	0.0	96%	51%	64%
Actinopolyspora erythraea	0.0	97%	49%	64%

References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zheng, Z., Miller, W., and Lipman, D. J. (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Becker, N. A., Peters, J. P., Lionberger, T. A., & Maher, L. J. (2012). Mechanism of promoter repression by Lac repressor–DNA loops. Nucleic Acids Research, 41(1), 156-166. doi:10.1093/nar/gks1011

Bennett, J. (2006). Molecular Genetic Analysis of Division and Development in Streptomyces coelicolor (Doctoral dissertation, Duquesne University). Retrieved from https://dsc.duq.edu/etd/301.

Burn, S. F. (2012). Detection of β-Galactosidase Activity: X-gal Staining. Kidney Development Methods in Molecular Biology, 886, 241-250. doi:10.1007/978-1-61779-851-1_21

Chater, K. F. (2006). Streptomyces inside-out: A new perspective on the bacteria that provide us with antibiotics. Philosophical Transactions of the Royal Society B: Biological Sciences, 361(1469), 761-768. doi:10.1098/rstb.2005.1758

Dow, J. M., Fouhy, Y., Lucey, J. F., & Ryan, R. P. (2006). The HD-GYP Domain and Cyclic Di-GMP Signaling. MPMI, 19(12), 1378-1384. doi:10.1094/ MPMI -19-1378

Hasani, A., Kariminik, A., Issazadeh, K. (2014). Streptomycetes: Characteristics and Their Antimicrobial Activities. International Journal of Advanced Biological and Biomedical Research, 2(1), 63-75.

Hefti, M. H., Françoijs, K., Vries, S. C., Dixon, R., & Vervoort, J. (2004). The PAS fold. European Journal of Biochemistry, 271(6), 1198-1208. doi:10.1111/j.1432-1033.2004.04023.x

Hull, T. D., Ryu, M., Sullivan, M. J., Johnson, R. C., Klena, N. T., Geiger, R. M., . . . Bennett, J. A. (2012). Cyclic Di-GMP Phosphodiesterases RmdA and RmdB Are Involved in Regulating Colony Morphology and Development in Streptomyces coelicolor. Journal of Bacteriology, 194(17), 4642-4651. doi:10.1128/jb.00157-12

Karimova, G., Pidoux, J., Ullmann, A., & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proceedings of the National Academy of Sciences, 95(10), 5752-5756. doi:10.1073/pnas.95.10.5752

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. 2000. Practical Streptomyces genetics. The John Innes Foundation, Colney, United Kingdom

Mcgregor, J. F. (1954). Nuclear Division and the Life Cycle in a Streptomyces sp. Journal of General Microbiology, 11(1), 52-56. doi:10.1099/00221287-11-1-52

Oshea, E., Klemm, J., Kim, P., & Alber, T. (1991). X-Ray Structure Of The Gcn4 Leucine Zipper, A Two-Stranded, Parallel Coiled Coil. Science, 254(5031), 539-544. doi:10.2210/pdb2zta/pdb

Robert-Paganin, J., Nonin-Lecomte, S., & Rety, S. (2012). Crystal structure of the FimX EAL domain in complex with reaction product pGpG. PLOS ONE. doi:10.2210/pdb4afy/pdb

Rost, B. (1999). Twilight zone of protein sequence alignments. Protein Engineering, Design and Selection, 12(2), 85-94. doi:10.1093/protein/12.2.85

Ryan, R. P. (2013). Cyclic di-GMP signalling and the regulation of bacterial virulence. Microbiology, 159(Pt_7), 1286-1297. doi:10.1099/mic.0.068189-0

Sarenko O, Klauck G, Wilke FM, Pfiffer V, Richter AM, Herbst S, Kaever V, Hengge R. 2017.

More than enzymes that make or break Cyclic Di-GMP—local signaling in the interactome of GGDEF/EAL domain proteins of Escherichia coli. mBio 8:e01639-17. https://doi.org/10.1128/mBio.01639-17.

Seshasayee, A. S., Fraser, G. M., & Luscombe, N. M. (2010). Comparative genomics of cyclic-di-GMP signalling in bacteria: Post-translational regulation and catalytic activity. Nucleic Acids Research, 38(18), 5970-5981. doi:10.1093/nar/gkq382

Simm, R., Morr, M., Kader, A., Nimtz, M., & Römling, U. (2004). GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Molecular Microbiology, 53(4), 1123-1134. doi:10.1111/j.1365-2958.2004.04206.x

Sundriyal, A., Massa, C., Samoray, D., Zehender, F., Sharpe, T., Jenal, U., & Schirmer, T. (2014). Inherent Regulation of EAL Domain-catalyzed Hydrolysis of Second Messenger Cyclic di-GMP. Journal of Biological Chemistry, 289(10), 6978-6990. doi:10.1074/jbc.m113.516195

Valentini, M., & Filloux, A. (2016). Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from Pseudomonas aeruginosa and Other Bacteria. The Journal of Biological Chemistry, 291(24), 12547–12555. http://doi.org/10.1074/jbc.R115.711507

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F., & Sinderen, D. V. (2007). Genomics of Actinobacteria: Tracing the Evolutionary History of an Ancient Phylum. Microbiology and Molecular Biology Reviews, 71(3), 495-548. doi:10.1128/mmbr.00005-07