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# Cyclic Di-GMP Phosphodiesterases RmdA and RmdB Are Involved in Regulating Colony Morphology and Development in *Streptomyces coelicolor*

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Cyclic dimeric GMP (c-di-GMP) regulates numerous processes in Gram-negative bacteria, yet little is known about its role in Gram-positive bacteria. Here we characterize two c-di-GMP phosphodiesterases from the filamentous high-GC Gram-positive actinobacterium *Streptomyces coelicolor*, involved in controlling colony morphology and development. A transposon mutation in one of the two phosphodiesterase genes, SCO0928, hereby designated *rmdA* (*r*egulator of *m*orphology and *d*evelopment A), resulted in decreased levels of spore-specific gray pigment and a delay in spore formation. The RmdA protein contains GGDEF-EAL domains arranged in tandem and possesses c-di-GMP phosphodiesterase activity, as is evident from *in vitro* enzymatic assays using the purified protein. RmdA contains a PAS9 domain and is a hemoprotein. Inactivation of another GGDEF-EAL-encoding gene, SCO5495, designated *rmdB*, resulted in a phenotype identical to that of the *rmdA* mutant. Purified soluble fragment of RmdB devoid of transmembrane domains also possesses c-di-GMP phosphodiesterase activity. The *rmdA rmdB* double mutant has a bald phenotype and is impaired in aerial mycelium formation. This suggests that RmdA and RmdB functions are additive and at least partially overlapping. The *rmdA* and *rmdB* mutations likely result in increased local pools of intracellular c-di-GMP, because intracellular c-di-GMP levels in the single mutants did not differ significantly from those of the wild type, whereas in the double *rmdA rmdB* mutant, c-di-GMP levels were 3-fold higher than those in the wild type. This study highlights the importance of c-di-GMP-dependent signaling in actinomycete colony morphology and development and identifies two c-di-GMP phosphodiesterases controlling these processes.

Cyclic diguanosine monophosphate (c-di-GMP) is a ubiquitous second messenger in bacteria. Since its identification by Moshe Benziman and colleagues and characterization as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) (47), c-di-GMP has been shown to coordinate numerous cellular activities, including biofilm formation, motility, cell cycle progression, and virulence. c-di-GMP regulates these processes by affecting the synthesis and activities of flagella, pili, adhesins, exopolysaccharides, and extracellular DNA, as well as virulence factors. It plays important roles in the cellular transition from a motile to surface-attached lifestyle, from formation to dissolution of biofilms, and, in pathogens, from acute to chronic infections and from one host to another (reviewed in references 26, 45, 52, 55, and 65).

Intracellular c-di-GMP levels are inversely regulated by diguanylate cyclases (DGCs) involved in c-di-GMP synthesis and by c-di-GMP-specific phosphodiesterases (PDEs) involved in c-di-GMP degradation (46, 47). A conserved and widely spread GGDEF domain is responsible for the DGC activity. c-di-GMP formation is catalyzed by a homodimer formed from two GGDEF domains, each of which binds the substrate, GTP (11, 43, 51). The c-di-GMP-specific PDE activity is encoded by the EAL (11, 14, 53, 56) or HD-GYP (23, 34, 49) protein domains. A large number of c-di-GMP-metabolizing enzymes contain both GGDEF and EAL domains arranged in tandem (54). Most of these proteins possess only one enzymatic activity, either DGC or PDE. It is often possible to predict the enzymatic activity of the GGDEF-EAL proteins based on conservation of the amino acids essential for catal-

ysis. However, some enzymes are bifunctional, and their prevailing activity depends on the input signal (20, 32, 57).

Changes in c-di-GMP levels affect activities of diverse c-di-GMP receptors (effectors). Among protein-based c-di-GMP receptors are enzymes, transcription factors, and proteins controlling downstream output activities via protein-protein interactions. c-di-GMP also controls gene expression via two specific classes of riboswitches (reviewed in reference 38).

At present, our knowledge of c-di-GMP signaling pathways is limited almost exclusively to the Gram-negative bacteria (proteobacteria and spirochetes). The c-di-GMP signaling pathways are present in many Gram-positive bacteria (9), but functional studies have been done only in mycobacteria, where c-di-GMP signaling appears to play a relatively limited role and is involved in

long-term survival (32). Recent reports uncovered the involvement of c-di-GMP in the development of the Gram-positive actinomycete *Streptomyces coelicolor* (17, 59).

*S. coelicolor* is a nonmotile, nonpathogenic soil bacterium characterized by a complex cycle of morphological differentiation and therefore traditionally utilized as a model of bacterial development. The genus *Streptomyces* has significant pharmacological importance because more than two-thirds of the antibiotics currently in use are produced by its representatives. The *S. coelicolor* life cycle begins when a free spore germinates to produce long, branching vegetative filaments that grow into and on the substrate surface. These vegetative filaments rarely divide, yielding a network of multinucleated hyphae. Colony maturation leads to the development of aerial hyphae that are erected above the colony surface. These aerial hyphae undergo a sporulation-specific cell division process, resulting in an aerial mycelium comprised of uninucleoid prespores that metamorphose into gray-pigmented mature spores (13, 22).

Here we searched for developmental mutants of *S. coelicolor* following transposon mutagenesis. One mutant, resulting from transposon insertion in the gene designated *rmdA* (*r*egulator of *m*orphology and *d*evelopment A), produced a decreased amount of gray spore pigment relative to that produced by the wild-type strain, was delayed in sporulation, and had abnormal colony morphology. The *rmdA* gene was found to encode a GGDEF-EAL protein. Following genetic and biochemical analysis, we elucidated that RmdA functions as a c-di-GMP PDE. Bioinformatics analysis uncovered a potential second GGDEF-EAL PDE in *S. coelicolor*. We have shown here that this protein, designated RmdB, also acts as a c-di-GMP PDE and that the *rmdB* mutant has a phenotype similar to that of the *rmdA* mutant. The *rmdA rmdB* double mutant is completely blocked in aerial mycelium formation, which suggests that RmdA and RmdB are critical, partially redundant, signaling enzymes regulating c-di-GMP-dependent development in *S. coelicolor*.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** Strains used in this work are listed in Table 1. The prototrophic SCP1⁻ SCP2⁻ strain MT1110 of *S. coelicolor* A3(2) was used as the wild type (6, 30). Liquid cultures were grown in baffled flasks containing YEME medium with 10.3% sucrose at 30°C (30). The agar media R2YE and mannitol soya flour (MS) were prepared as described previously (30). For *S. coelicolor*, neomycin, apramycin, and kanamycin were used at final concentrations of 10 μg/ml, 25 μg/ml, and 160 μg/ml, respectively.

*Escherichia coli* SURE was utilized for the preparation of cosmid DNA. The *dam dcm* mutant strains ET12567 and ER²-1 were used to circumvent the methyl-specific restriction system of *S. coelicolor* for propagation of cosmid or plasmid DNA (35). *E. coli* strains were grown at 37°C in Luria-Bertani medium containing, where necessary, ampicillin (100 μg/ml), apramycin (100 μg/ml), and/or kanamycin (50 μg/ml).

**Bioinformatics analysis.** The *S. coelicolor* genome database was searched for annotated gene and protein sequences. Protein sequences were analyzed by the SMART (33) and Pfam (21) domain databases. BLAST software was employed to identify homologous proteins in other bacteria. Multiple sequence alignments were constructed using ClustalW on the Biology Workbench 3.2 software platform (http://workbench.sdsc.edu/).

**Colony morphology and sporulation analysis.** Ten colonies of confirmed *S. coelicolor* mutants grown on MS agar plates for 5 days at 30°C were observed using a low-power photomicroscope (Meiji). Representative colonies were photographed with a color digital camera (model

### TABLE 1 Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristic(s) | Reference or source |
|---|---|---|
| **Strains** | | |
| *S. coelicolor* | | |
| MT1110 | Prototrophic, SCP1⁻, SCP2⁻ | 30 |
| TH1 | SCO0928::Tn*5* | This work |
| TH100 | SCO5495::Tn*5062* | This work |
| TH101 | SCO5495::Tn*5062* | This work |
| TH105 | SCO0929::Tn*5062* | This work |
| TH106 | SCO5495::Tn*5062* SCO0928::Tn*5* | This work |
| | | |
| *E. coli* | | |
| SURE | Cloning strain | Stratagene |
| ER²-1 | *dam dcm* mutant to avoid *Streptomyces* methyl restriction | 5 |
| ET12567 | *dam dcm* mutant strain with mating helper plasmid pUZ8002 | 30 |
| DH5α | Host for overexpression of MBP fusions | NEB |
| MG1655 | K12 strain used in motility assays | ATCC |
| MG1655 *yhjH* | MG1655 *yhjH*::Kanʳ | 19 |
| | | |
| **Plasmids** | | |
| pJA77 | Mini-Tn*5* delivery vector, Neoʳ | 4 |
| pMAL-c5x | MBP overexpression vector | NEB |
| pMAL-*rmdA* | MBP::(PAS9-GGDEF-EAL) from RmdA | This work |
| pMAL-*rmdB* | MBP::(GGDEF-EAL) from RmdB | This work |
| pMAL-*sco1397* | MBP::SCO1397 | This work |
| | | |
| **Cosmids** | | |
| SCM10 | SCM10.2.B08 (03071421MU); contains SCO0928 | 7 |
| SCM10 | SCM10.1.G03 (03021117RA); contains SCO0929 | 7 |
| SC8D9 | 8D9.1.F04 (8BF-1 022 2007-04-18); contains SCO5495 | 7 |
| SC1A8A | 1A8A.2.B09; contains SCO1397 | 7 |

CFW01312C) from the Scion Corporation. *S. coelicolor* spore chains were observed under phase-contrast microscopy at total magnification ×1,000. Aerial mycelium was prepared on glass cover slides and mounted in 50% glycerol (36). Images were captured with an Olympus BX 40 light microscope equipped with an Olympus color digital camera (model DP72) and cellSens software. Micrographs were processed in the software packages ImageJ and Adobe Photoshop.

**DNA preparation and manipulation.** Chromosomal DNA from *S. coelicolor* was isolated using the Wizard genomic DNA purification kit (Promega). Plasmid and cosmid DNA were prepared with the QIAprep miniprep kit (Qiagen). PCR products were prepared for DNA sequencing using the QIAquick PCR purification kit (Qiagen). The DyeEX 2.0 spin kit (Qiagen) was used for the cleanup of all DNA sequencing reactions, and the Applied Biosystems ABI Prism 310 genetic analyzer was used to analyze sequencing reactions.

**Transposon mutagenesis and insertion site analysis.** The pJA77 minitransposon system is based on the Tn*5* transposon containing the *aphI* gene (neomycin resistance [Neoʳ]) (4). pJA77 was isolated from *E. coli* ER²-1 and transformed into wild-type *S. coelicolor* MT1110 (30) by polyethylene glycol-mediated transformation (41). Following selection of Neoʳ colonies, we screened for Apramycin sensitivity (Apraˢ) to ensure transposition as opposed to plasmid integration via a single crossover. Transformants were also plated on minimal medium, and auxotrophic mutants were eliminated. To establish linkage between the transposon insertion and the mutant phenotype, chromosomal DNA from the insertional mutants was isolated and transformed into MT1110 protoplasts. The Neoʳ transformants were screened visually and by phase-contrast microscopy (4).

To determine the transposon insertion locations, we used inverse PCR and DNA sequencing. Chromosomal DNA from the transposon insertion mutant was digested with Sau3AI to generate short DNA fragments that were ligated into small circular molecules. This DNA served as the template for an inverse PCR (invPCR) reaction utilizing the following transposon-specific primers: Tn5InvF2 (5′-AGGAGACTCGGGCGCCTTCG

TCTACCAG) and Tn5InvR3 (5′-CATCATGGCAGAGGCGGAGACGC CGTTC). The PCR products were sequenced. The nested primer Tn5InvSeq4 (5′-GTAGGGGTTTCGGACATTCTGTG) was used to sequence the unknown chromosomal DNA surrounding the transposon insertion, utilizing the known transposon sequence as a priming site. The Basic Local Alignment Search Tool (BLAST) was used to determine the site of transposon insertions. The *Streptomyces* genome database (http://www.sanger.ac.uk/Projects/S_coelicolor/) was used for gene and protein identification (6).

Confirmation of the transposon insertion site in SCO0928 (*rmdA*) was accomplished by PCR amplification of the transposon (Tn)-containing fragment using the following primers: SCO0928F (5′-AGGCGGCCCGT AACGGTGCTTGAG), which anneals downstream of the predicted transposon insertion site, and Tn5Inv3R (5′-CATCATGGCAGAGGCGGAG ACGCCGTTC), which is transposon specific. The PCR product was sequenced. Insertions of the Tn*5062* transposon in SCO0928, SCO0929, and SCO5495 were verified in a similar manner using the following primer pairs: SCO0928 forward (5′-TTCTCCAGGCGTTCGAAGAAG AAG) and SCO0928/SCO0929 reverse 5′-GATGAAACCAAGCCAACCA GGAAG); SCO0929 forward (5′-ACCATGTCGTACCCCTTGAAGAAC) and SCO0928/SCO0929 reverse; SCO5495 forward (5′-CGCCCTGCTC TGGTACATCCAGAC) and SCO5495 reverse (5′-TTGCCGTCGTCCTT GAAGAAGATG).

**Mutant construction.** Cosmid DNA mutagenized by *in vitro* transposon mutagenesis with a Tn*5* derivative, Tn*5062*, was obtained from Paul Dyson (7). ET12567 containing the helper plasmid pUZ8002 was used as a donor in the *oriT*-mediated conjugal transfer of cosmid DNA from *E. coli* to *S. coelicolor* (42). To select for exconjugates and to eliminate *E. coli*, the plates were overlaid with 25 μg/ml apramycin and 20 μg/ml nalidixic acid, respectively. Apramycin-resistant (Apra^r) exconjugates were streaked on MS medium containing kanamycin to screen for kanamycin sensitivity (Kan^s). Since *S. coelicolor* cosmids contain both Kan^r and Amp^r cassettes on the Supercos-1 backbone (44), screening for Apra^r Kan^s colonies allowed the selection of mutant *S. coelicolor* colonies that had undergone gene disruption via double-crossover recombination.

To construct the *rmdA rmdB* double mutant, the *rmdB*::Tn*5062* cosmid was conjugated from *E. coli* ET12567/pUZ8002 into the *rmdA* mutant, and Kan^s Apra^r Neo^r colonies were identified and analyzed further. For complementation, a wild-type version of one of the genes (*rmdA* or *rmdB*) was introduced into the double mutant strain by interspecies conjugation, and single-crossover integrants of the cosmid were selected.

**Protein overexpression and enzymatic assays.** The DNA fragments encoding the PAS9-GGDEF-EAL module of RmdA, the GGDEF-EAL module of RmdB, and full-length SCO1397 were PCR amplified using, respectively, the following primer pairs: SCO0928-F (5′-AATCATATGCATCAT CATCATCATCACTTCGAGGGCGCCGCCATAG) and SCO0928-R (5′-A TTGAATTCTCAGCCCGTCGCGTCC); SCO5495-F (5′-AATCATATG CATCATCATCATCATCACCAGTTGCGCGACCCGCTC) and SCO5495-R (5′-ATTGAATTCTCACTCGTCGCTCGCCG); and SCO1397-F (5′-AA TCATATGCATCATCATCATCATCACATGACCGGCGGGGTCGC) and SCO1397-R (5′-ATTGAATTCTCAGAGAGCCGTACGACGCC). The amplified PCR fragments were digested with NdeI and EcoRI and cloned into the same restriction sites in pMal-c5x (NEB).

Maltose-binding protein (MBP) fusions were overexpressed in *E. coli* DH5α and affinity purified on amylose resin according to the specifications of the manufacturer (NEB). Briefly, the overnight cultures expressing MBP fusions were grown to an $A_{600}$ of 0.6 in LB supplemented with 100 μg/ml ampicillin at 30°C. The cultures were transferred to room temperature, and 0.5 mM (final concentration) isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added. Following a 15-h incubation, cells were harvested by centrifugation and resuspended in amylose column binding buffer (50 mM Tris-HCl, pH 8.0, 350 mM NaCl, 10 mM MgCl₂, 0.5 mM EDTA, 10% glycerol). Cells were disrupted using a French pressure cell, and cell debris was removed by centrifugation at 35,000 × *g* for 45 min at 4°C. Two milliliters (bed volume) of amylose resin (NEB) pre-

equilibrated with the binding buffer was added to the soluble cell extract derived from a 1.5-liter culture and agitated for 1 h at 4°C. The mix was loaded onto a column, and the resin was washed with 200 ml of column binding buffer. Fractions were eluted with 12 ml of binding buffer containing 10 mM maltose. The protein was either used immediately or stored at −80°C in 20% (vol/vol) glycerol (final concentration). Protein concentrations were measured using a Bradford protein assay kit (Bio-Rad) with bovine serum albumin as the protein standard. Proteins were analyzed using SDS-PAGE.

The DGC assays *in vitro* were performed by measuring the rate of GTP conversion to c-di-GMP as described by Ryjenkov et al. (51), and c-di-GMP PDE activity assays were performed by measuring the rate of c-di-GMP hydrolysis as described by Schmidt et al. (53). The PDE assays were performed at room temperature in PDE assay buffer (50 mM Tris-HCl [pH 8.5], 50 mM NaCl, 0.5 mM EDTA) containing various concentrations of $Mg^{2+}$ and/or $Mn^{2+}$. The initial c-di-GMP concentration was 200 μM. The reactions were started by adding 1 μmol purified enzyme. Aliquots were withdrawn at different time points, $CaCl_2$ was added to 10 mM to stop the reaction, and samples were boiled for 5 min. Postreaction processing and nucleotide separation and quantification were accomplished by high-performance liquid chromatography (HPLC) as described earlier (51).

**Protein reconstitution with hemin and UV-vis spectroscopy.** Reconstitution of the MBP-RmdA (PAS9-GGDEF-EAL) protein fusion with hemin and flavins *in vitro* was performed essentially as described earlier (24, 39). A reduced deoxy [Fe(II)] form of the hemoprotein was obtained by reduction with dithionite followed by removal of the reductant using gel filtration on a Sephadex G-25 column. The oxidized [Fe(III); met] form was obtained after protein incubation with an equimolar amount of potassium ferricyanide and subsequent ferricyanide removal by gel filtration. The reduced deoxy forms with CO [Fe(II)-CO] and oxygen [Fe(II)-O₂] were obtained by gentle bubbling (for 10 min) through protein solution of CO or air, respectively. Electronic absorption UV-visible (UV-vis) spectra were recorded using a Shimatzu UV-1601PC spectrophotometer.

**Intracellular c-di-GMP extraction and measurements.** Intracellular nucleotides were extracted with 40% methanol– 40% acetonitrile in 0.1 N formic acid, following the protocol described by Bobrov et al. (8). *S. coelicolor* was grown for 70 h at 30°C on MS agar overlaid with cellophane disks, roughly corresponding to the time of sporulation in the wild type. Cells were scraped from the cellophane with a sterile scalpel blade, and the wet weights were determined and matched, followed by extraction. c-di-GMP was quantified by liquid chromatography-tandem mass spectrometry carried out at the Mass Spectrometry Core at Michigan State University. The levels of extracted c-di-GMP were determined based on c-di-GMP standards. Average data from three independent measurements are presented.

## RESULTS

**Identification of the *rmdA* gene as a regulator of morphology and development in *S. coelicolor*.** To identify *S. coelicolor* sporulation mutants, we conducted *in vivo* Tn*5* mutagenesis of the wild-type strain, MT1110, using the protocol described by us earlier (4). Colony morphology of one of the mutants, TH1 (Fig. 1B), was aberrant compared to that of the wild-type strain. While the wild type is characterized by a central gray region of aerial mycelium surrounded by a ring of less-pigmented mycelium (Fig. 1A), TH1 had a white aerial mycelium with little gray pigment. Because gray pigment is present exclusively in spores, low pigmentation of TH1 indicated a potential sporulation defect.

To investigate the nature of this defect, we closely monitored the sporulation process in TH1. In the wild type, mature spore chains are comprised of individual spores that are evenly sized, shaped, and spaced. By day three, the wild type produces long chains with distinct compartments containing spores separated by
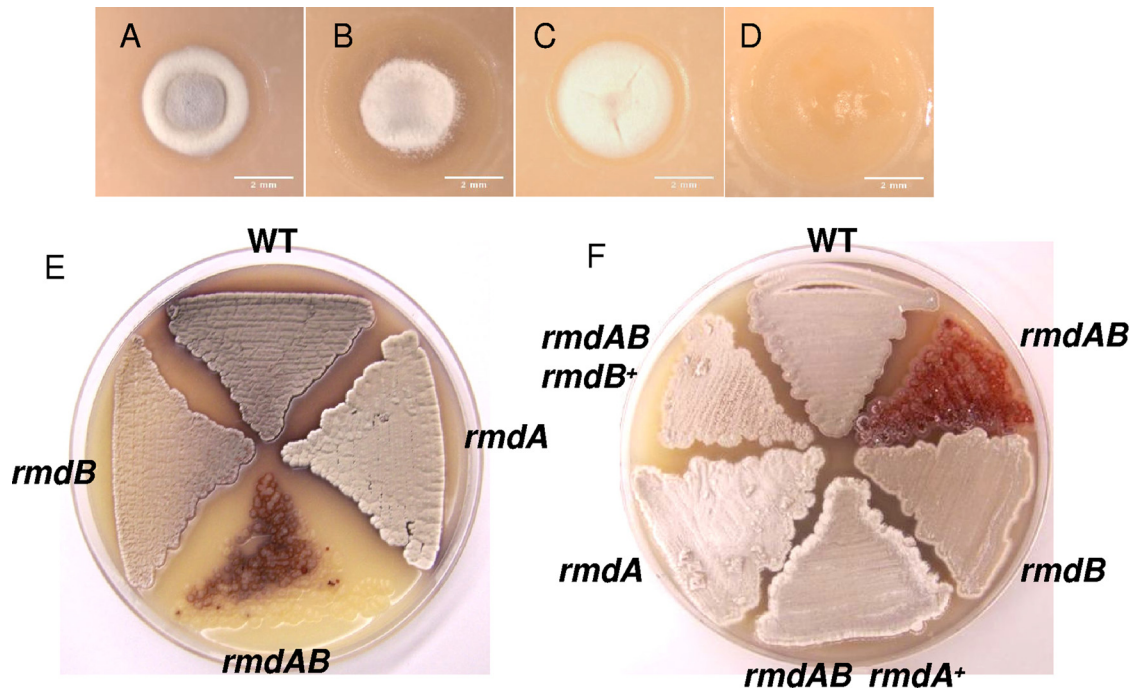
**FIG 1** Effects of inactivation of *rmdA* and *rmdB* on *S. coelicolor* colony morphology and aerial hyphae. (Top) Colony morphology. (A) wild type; (B) TH1 (*rmdA*::Tn*5*); (C) TH101 (*rmdB*::Tn*5062*); (D) TH106 (*rmdA*::Tn*5* *rmdB*::Tn*5062*). Single colonies that were well separated from neighboring colonies were selected for observation using stereomicroscopy. Images were taken on MS media after inocula of dilute spores were grown for 5 days at 30°C, yielding a few colonies per plate. (Bottom) Aerial hyphae. (E) Reduced gray spore pigment production in *rmdA* and *rmdB* single mutants and a complete block in aerial hypha formation in the *rmdA rmdB* double mutant. WT, wild type. (F) Complementation of the *rmdA rmdB* double mutant with the *rmdA* or *rmdB* gene. Strains were grown at 30°C for 5 days on MS medium.

fully developed septa, which can be observed by phase-contrast microscopy. However, only very rare, short spore chains were visible in TH1 by day three (Fig. 2). On day five, most aerial filaments in TH1 had initiated sporulation, but many spore chains contained incompletely separated spores. However, by day seven, the majority of these spore chains were fully separated (Fig. 2). These results show that the mutation in TH1 resulted in a considerable delay for the onset of sporulation in comparison to the wild-type strain.

We determined that the Tn*5* insertion in TH1 occurred in codon 581 of SCO0928, hereby designated *rmdA* (Fig. 3A). To test whether the sporulation defect in TH1 resulted from the Tn*5* insertion, we performed linkage analysis. The chromosomal DNA from the TH1 mutant was transformed into the wild type, and transformants expressing the Tn5-encoded Neo^r marker were selected and assayed further. All seven analyzed transformants displayed a phenotype that was indistinguishable from that of the TH1 mutant (data not shown). To further verify that inactivation of *rmdA* is responsible for the sporulation defect in TH1, we constructed a new *rmdA* mutant, TH100, using a cosmid that contains a Tn*5062* insertion in codon 120 of *rmdA* (7). In accord with our expectations, the phenotype of the newly generated *rmdA*::Tn*5062* mutant, TH100, displayed a sporulation phenotype indistinguishable from that of TH1 (data not shown).

*rmdA* is located downstream of SCO0929 in a putative two-gene operon. SCO0929 encodes a conserved protein of 270 amino acids containing a domain of unknown function, DUF574 (33). To assess a possible role of SCO0929, we constructed a mutant with a Tn*5062* transposon mutation in SCO0929. We found that

inactivation of SCO929 did not affect sporulation (data not shown), which suggests that *rmdA* is solely responsible for the developmental delay in TH1 and TH100.

**RmdA (SCO0928) functions as a c-di-GMP PDE.** The *rmdA* gene encodes a 714-amino-acid (aa) protein with the following domain architecture: X-PAS9-GGDEF-EAL (Fig. 4; (21). The N-terminal domain X of approximately 160 aa is present in signaling proteins from numerous species in the genus *Streptomyces* and other actinomycetes; however, it shows no significant similarity with known protein domains. PAS9 belongs to a large superfamily of PAS domains that often bind small molecules and are involved in signal sensing and/or in protein-protein interactions (58). Sequence analysis of the GGDEF and EAL domains of RmdA revealed that both domains contain residues essential for DGC and PDE activities, respectively, and neither domain contains identifiable deleterious mutations. Since both the GGDEF and EAL domains are potentially enzymatically active, RmdA may possess either one activity or another or may potentially be bifunctional.

To test for enzymatic activity of RmdA, we expressed *rmdA* (as an MBP fusion to the PAS9-GGDEF-EAL domain fragment) in two *E. coli* test strains, MG1655 and MG1655 *yhjH* (19) (Table 1). MG1655 is a highly motile strain. The inactivation of *yhjH*, which encodes a dominant c-di-GMP PDE in *E. coli*, impairs motility in the semisolid agar (19, 50). If RmdA had DGC activity, it would be expected to increase intracellular c-di-GMP levels in strain MG1655 and decrease the diameter of the swimming zone in the semisolid agar. However, no such decrease was detected (not shown). If RmdA had PDE activity, it would be expected to restore
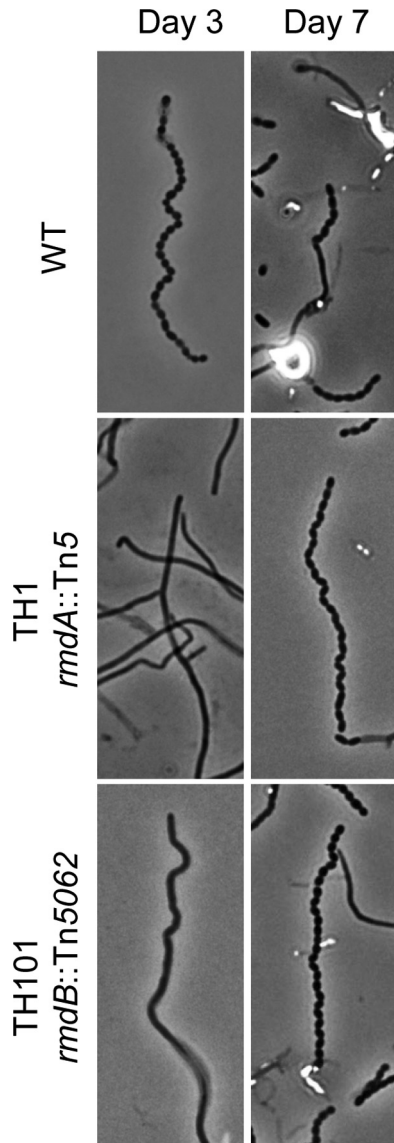
FIG 2 The *rmdA* and *rmdB* mutations delay *S. coelicolor* sporulation. Shown are representative phase-contrast micrographs (×1,000) of *S. coelicolor* aerial hyphae viewed from impression slides after 3 and 7 days of growth at 30°C on MS agar. The wild-type (WT) strain is characterized by aerial filaments that have undergone division to produce evenly spaced, consistently sized spores separated by complete septa at day 3. The spores begin to disperse by day 7. The *rmdA* and *rmdB* mutants exhibit a delay in sporulation, producing primarily undifferentiated aerial hyphae on day three and many long spore chains by day seven.

swimming of MG1655 *yhjH* in semisolid agar, and this is what was observed (Fig. 5A).

To verify the predicted PDE function of RmdA, we overexpressed and purified the MBP-RmdA fusion using affinity chromatography. We tested enzymatic activity of the protein *in vitro* (53). RmdA proved to possess c-di-GMP-specific PDE activity; however, the relative activity in standard, $Mg^{2+}$-containing buffers was surprisingly low. Addition of $Mn^{2+}$, which is known to improve catalytic activity of c-di-GMP PDEs (3), enhanced the PDE activity of MBP-RmdA by approximately 2-fold (Fig. 5B).

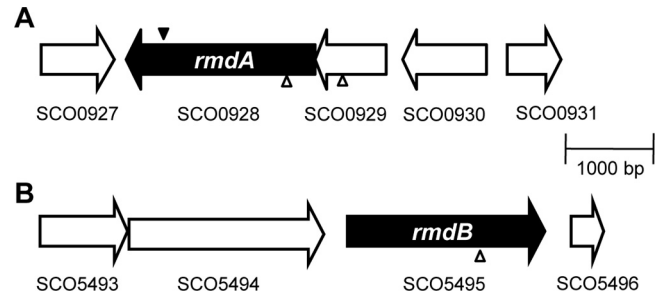Based on the PDE activity of RmdA, we expected that the *rmdA*



FIG 3 Transposon insertion sites. Genetic maps showing the *rmdA* (SCO0928) and *rmdB* (SCO5495) regions. SCO0929 and *rmdA* form a potential operon. The solid wedge represents the location of the Tn*5* minitransposon insertion through which TH1 (*rmdA*::Tn*5*) was originally isolated by *in vivo* mutagenesis. The hollow wedges represent the location of the *in vitro* Tn*5062* transposon insertions in *rmdA*, SCO0929, and *rmdB* (7).

mutation would increase intracellular levels of c-di-GMP. To test this prediction, we extracted total nucleotides from the wild-type and mutant TH1 grown on MS agar overlaid with cellulose membranes for 70 h at 30°C and analyzed c-di-GMP content via liquid chromatography-tandem mass spectrometry (LC-MS/MS). We found that c-di-GMP levels did not differ significantly between the *rmdA* mutant and the wild type (Fig. 6), suggesting that only a local pool of c-di-GMP may have been affected. Because the *rmdA* mutation resulted in no gross alteration in c-di-GMP levels, we conclude that RmdA is specifically involved in regulating *S. coelicolor* development.

**Bioinformatics analysis of putative *S. coelicolor* c-di-GMP PDEs.** To investigate whether additional c-di-GMP PDEs are involved in controlling c-di-GMP-dependent development in *S. coelicolor*, we performed a bioinformatics analysis of the EAL and HD-GYP domain proteins encoded in the *S. coelicolor* genome. In addition to RmdA, four other proteins, SCO1397, SCO2817, SCO5495, and SCO5511, contain EAL domains (21) (Fig. 4), and one, SCO5218, contains an HD-GYP domain.

The sequence of the HD-GYP domain in SCO5218 appears to be consistent with it being enzymatically active. However, our ability to predict activities of this class of proteins based on sequence analysis remains limited (34, 49). In this study, we did not pursue experimental investigation of SCO5218.

Sequence analysis of the EAL domains in SCO1397 and SCO5511 strongly suggested that they are incapable of hydrolyzing c-di-GMP because they miss residues involved in c-di-GMP binding or coordination of essential divalent metals (Fig. 4B) (3, 53). For example, SCO5511 lacks the signature "EAL" motif, while SCO1397 lacks the N-terminal residues of the EAL domain (Fig. 4B). Of the remaining two proteins, SCO2817, designated CdgA, was recently shown to function as a DGC (17). Therefore, among the *S. coelicolor* EAL domain proteins, only RmdA and SCO5495 may be enzymatically active (Fig. 4B). Below, we show that SCO5495 is a c-di-GMP PDE involved in colony morphology and developmental control, and therefore we designated it RmdB. We used SCO1397, predicted to be enzymatically incompetent in the initial stages of experimental analysis, as a negative control.

RmdA and RmdB appear to be highly conserved among streptomycetes. Orthologs of both proteins are encoded in the genomes of *Streptomyces avermitilis, S. venezuelae, S. hygroscopicus, S. griseus, S. flavogriseus, S. bingchenggensis, S. violaceusniger*, and *S.*
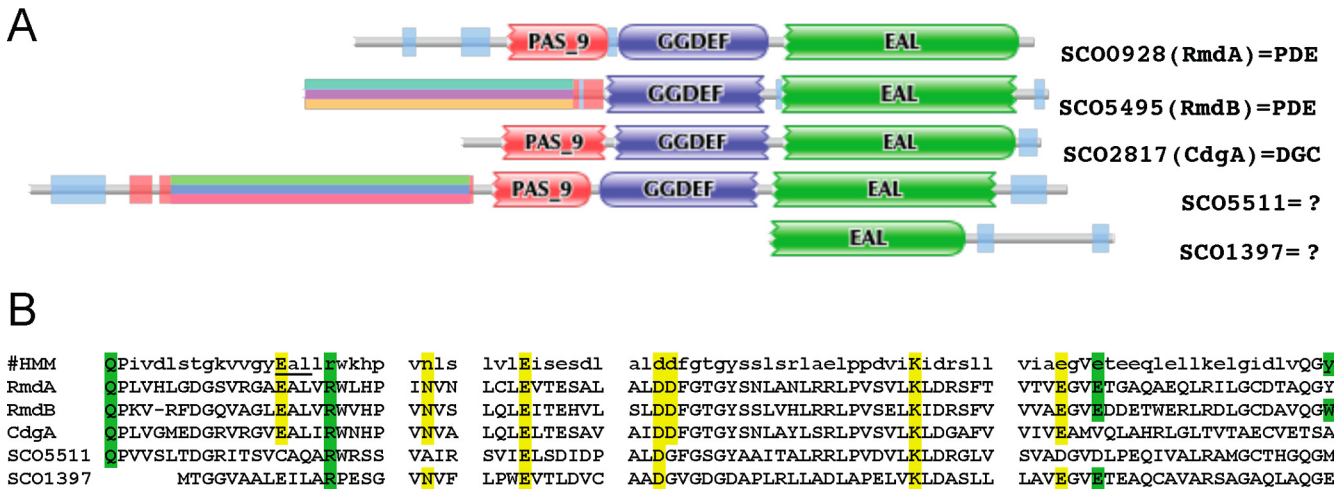
**FIG 4** Analysis of *S. coelicolor* EAL domain proteins. (A) Protein domain architectures derived from the Pfam database (21). (B) Sequence alignment of the most conserved regions of the EAL domains. #HMM, hidden Markov model-derived consensus sequence of the EAL domain. Residues involved in c-di-GMP binding inferred from the X-ray structure of an EAL domain PDE are shown on a green background; residues involved in coordinating catalytic metals are shown on a yellow background (3).



**FIG 5** c-di-GMP PDE activity of RmdA and RmdB. (A) Increase in swimming zones in semisolid agar of *E. coli* strain MG1655 *yhjH* with MBP-RmdA and MBP-RmdB. Assays were run as described earlier (19). (B) Kinetics of c-di-GMP hydrolysis by MBP-RmdA and MBP-RmdB. The PDE assays shown here were performed in the PDE assay buffer containing 5 mM $Mg^{2+}$ and 5 mM $Mn^{2+}$ (MBP-RmdA) or 5 mM $Mg^{2+}$ (MBP-RmdB) (53).

*scabiei.* They are also present in other filamentous actinomycetes. Furthermore, the potential *rmdA-SCO0929* operon is preserved in streptomycetes and representatives of the suborder *Frankineae*, e.g., *Geodermatophilus obscurus* and *Nakamurella multipartita*. The high degree of conservation suggests that the regulatory mechanism(s) through which RmdA and RmdB are involved in colony morphology and development is likely conserved in filamentous actinomycetes.

**RmdA is a hemoprotein.** We noticed that in addition to RmdA, two *S. coelicolor* GGDEF and/or EAL domain proteins, CdgA and SCO5511, contain PAS9 domains (Fig. 4A), which suggests that they may bind a common ligand and respond to the same primary signal. To investigate the nature of the putative ligand bound to the PAS9 domain of RmdA, we reconstituted the
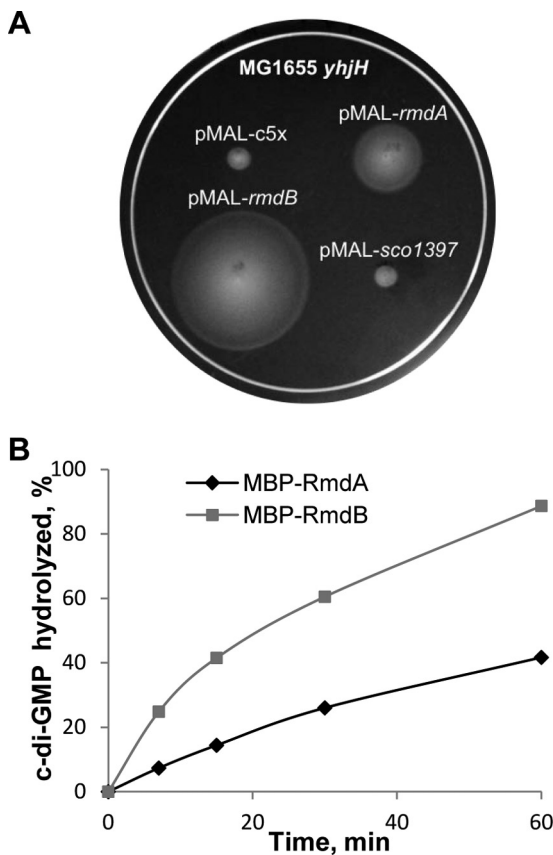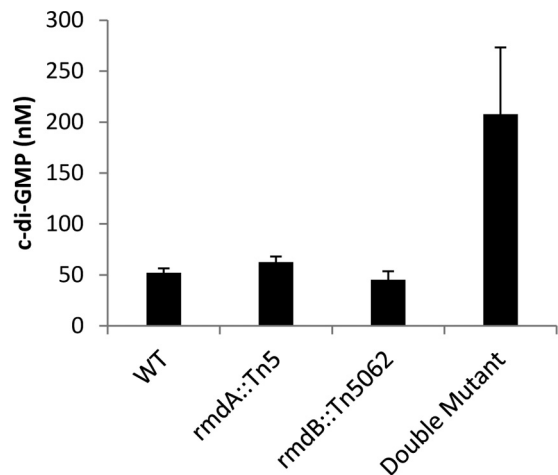


**FIG 6** Intracellular c-di-GMP levels in the *S. coelicolor* PDE mutants. The level of c-di-GMP for the *rmdA rmdB* double mutant was approximately three times that of the wild type and single mutant strains. c-di-GMP was extracted as described previously (8) from cells grown on cellulose membranes on MS agar for 70 h at 30°C and quantified by LC-MS/MS.
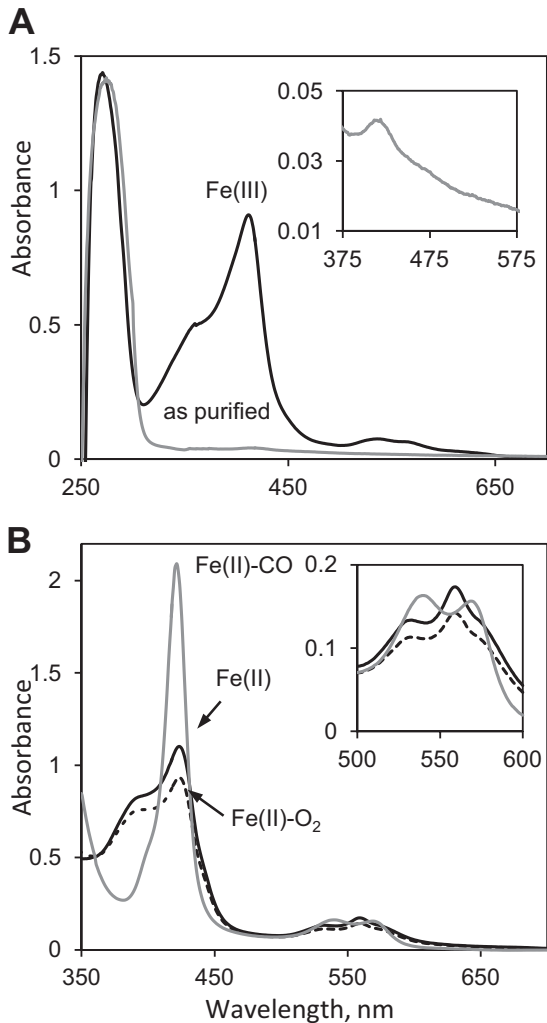
FIG 7 Characterization of RmdA as a heme-binding protein. (A) Electronic absorbance spectra of the MBP-RmdA protein. Gray trace, as purified from *E. coli*; black trace, after *in vitro* reconstitution with hemin. Inset, expanded version of the spectrum showing a Soret band indicative of traces of heme present in the protein purified from *E. coli*. (B) Reconstituted with hemin MBP-RmdA protein. Black solid trace, Fe(II), deoxy form; dotted trace, Fe(II), oxy form; gray trace, Fe(II), carbonmonoxy form.

MBP-RmdA (PAS9-GGGDEF-EAL) fusion with common PAS domain ligands, FAD, FMN, and hemin (27). MBP-RmdA did not bind FAD or FMN *in vitro* (not shown), but it did bind hemin with a nearly equimolar stoichiometry (Fig. 7A). Further, upon close inspection of the spectra of the MBP-RmdA protein purified from the *E. coli* overexpression strain, we noticed that it already contained traces of heme (Fig. 7A, inset).

To investigate the specificity of heme binding, we reduced the protein-hemin complex with dithionite under anoxic conditions. We observed a shift in the Soret band (from 412 nm to 424 nm) and sharpening of the α- and β-peaks in the 500- to 600-nm region of the spectrum. These changes are characteristic of the formation of the reduced, Fe(II), deoxy form with hexacoordinated heme iron, where two axial ligands are provided by amino acids of the protein (Fig. 7B). Addition of CO, a gaseous ligand that has high affinity to the majority of hemoproteins, resulted in further sharpening of the Soret band (421.5 nm) and noticeable

changes in the α- and β-peaks of heme, suggestive of changes in the heme binding pocket that most likely result from CO replacing a distal (sixth) amino acid ligand of heme (Fig. 7B). Addition of oxygen to the deoxy Fe(II) form also resulted in spectral changes, albeit smaller in scope than those that originated with the addition of CO (Fig. 7B). These results establish that RmdA binds heme specifically and is capable of responding to gaseous ligands, which in turn suggest that conformational changes in the heme-binding pocket might affect the activity of RmdA.

We tested whether PDE activity of the holo-MBP-RmdA protein is affected by CO or oxygen, but we observed no changes using several independently purified protein preparations, nor did we observe changes in PDE activity when the oxidized, Fe(III), met form of the protein was compared to the reduced, Fe(II), deoxy form or the apoprotein was compared to the holoprotein. At present, we don't understand why activity of RmdA appears to be decoupled from the sensory input. Several possibilities exist (see Discussion) and will be explored in the future.

To gain insight into the possibility that CdgA or SCO5511 binds heme, we compared the sequences of their PAS9 domains with that of RmdA. We found these sequences to be highly divergent and lacking conserved residues (e.g., His) that often ligate heme iron. The highest similarity (>30% identity) to the PAS9 domain of RmdA was displayed by the RmdA homologs from *Streptomyces* species, as well as PAS9 proteins from cyanobacteria and proteobacteria. Therefore, we could not predict whether CdgA or SCO5511 binds heme. It appears that PAS domains have evolved various ways to coordinate the heme ligand. For example, the heme-PAS domains from oxygen-sensing c-di-GMP PDE, *E. coli* DosP (16, 62), and histidine kinase *Bradyrhizobium* FixL (25, 29) have low, <16% identity to PAS9 of RmdA.

**RmdB (SCO5495) functions as a c-di-GMP PDE.** To investigate the involvement of SCO5495 in *S. coelicolor* development, we constructed a Tn*5062* mutant (Fig. 3B), designated TH101. We observed that colonies of TH101 produced significantly less gray spore pigment than the wild type (Fig. 1C and E). Microscopic analysis revealed that after 3 days of growth, TH101 produced few sporulated aerial filaments compared to the wild type (Fig. 2). After 5 days, the number of spore chains increased, yet most of them displayed a delay in the completion of septation. By day seven, the quantity of spore chains was comparable to that observed for the wild type at 3 days (Fig. 2). These phenotypes were identical to the phenotypes of the *rmdA* mutants, TH1 and TH100, thus suggesting that RmdB also modulates development in *S. coelicolor*. The similarity between the *rmdA* and *rmdB* phenotypes suggested that RmdB functions as a c-di-GMP PDE.

The 746-aa RmdB protein contains an approximately 300-aa-long N-terminal domain comprising seven transmembrane motifs followed by the GGDEF-EAL domain tandem (Fig. 4A). Neither the GGDEF nor EAL domains of SCO5495 contain readily identifiable residues inconsistent with DGC or PDE activities, respectively. To test for RmdB activity, we cloned its cytoplasmic GGDEF-EAL module downstream of the MBP tag. The effects of this fusion on motility in the semisolid agar were assayed with the *E. coli* test strains, MG1655 and MG1655 *yhjH*, and the effect of MBP-RmdB was consistent with it acting as a PDE (Fig. 5A). The MBP-SCO1397 fusion, used as a negative control, showed no PDE activity (Fig. 5A), consistent with the sequence analysis presented above.

We overexpressed and purified the cytoplasmic fragment of

RmdB and found that it has PDE activity *in vitro*. The relative activity of the RmdB fragment was higher than that of RmdA (Fig. 5B), which is consistent with the motility-based assay (Fig. 5A). It is noteworthy that activities of the truncated fusion proteins measured here are only approximations of the *in vivo* activities of full-length proteins, which are further influenced by their respective sensory domains. The relative contributions of RmdA and RmdB to regulation of development likely depend on environmental conditions.

We measured the effect of the *rmdB* mutation on intracellular c-di-GMP levels and found no significant differences between TH101 and the wild type (Fig. 6). The lack of perturbations in intracellular c-di-GMP levels is an indication that RmdB is specifically involved in controlling *S. coelicolor* development.

**RmdA and RmdB additively regulate colony morphology, sporulation, and aerial mycelium development.** To investigate the relationship between RmdA and RmdB, we constructed the *rmdA rmdB* double mutant by inactivating the *rmdB* gene in strain TH1. The colony morphology of the double mutant, TH106, was strikingly different from those of the wild type and individual *rmd* mutants in that the double mutant had abnormally large colonies with the bald (*bld*) phenotype (Fig. 1D), i.e., it was completely blocked in the development of aerial hyphae (Fig. 1E). The introduction into the *rmdA rmdB* mutant of a cosmid containing the wild-type *rmdA* or *rmdB* gene complemented the defect in aerial mycelium formation (Fig. 1F).

We investigated the effect of the double mutant on intracellular c-di-GMP levels and found that these levels were increased by approximately 3-fold compared to those for the wild type (Fig. 6). Taken together, these data demonstrate that two PDEs, RmdA and RmdB, have at least partially overlapping functions in controlling colony morphology, sporulation, and aerial mycelium development in *S. coelicolor*.

## DISCUSSION

*S. coelicolor* has been a model organism for studying cell division and development in bacteria because mutants in the complex cycle of morphological development can be readily identified both visually and microscopically. Here we relied on decreased gray pigmentation of Tn mutants as an indication of a potential defect in sporulation to identify the *rmdA* gene. We showed that *rmdA* encodes a c-di-GMP PDE. Another c-di-GMP PDE identified here, RmdB, is also involved in developmental control and morphological differentiation of *S. coelicolor*. According to our bioinformatics analysis, RmdA and RmdB are the only EAL domain-containing c-di-GMP PDEs present in *S. coelicolor*. The membrane-localized SCO5218 containing an HD-GYP domain may potentially function as yet another c-di-GMP PDE in this bacterium. Given the high degree of conservation among RmdA and RmdB homologs, it is likely that c-di-GMP signaling pathways modulate development in various filamentous actinomycetes. This notion emphasizes the need to understand c-di-GMP signaling in this group of bacteria, which includes many species that produce pharmacologically important antibiotics.

Numerous questions regarding the role of c-di-GMP in *S. coelicolor* remain. One set of questions concerns environmental and intracellular signals that affect expression and activity of the enzymes involved in c-di-GMP signaling. In *Streptomyces*, development is initiated by nutrient starvation and the production of secondary metabolites like antibiotics (22). How these signals affect c-di-GMP synthesis and degradation remains to be investigated.

In this study, we noticed that RmdA is a hemoprotein and therefore is expected to sense gaseous ligands or redox changes. Interestingly, in several proteobacterial species, including *Gluconacetobacter xylinus*, *E. coli*, and *Bordetella pertussis* (12, 62, 63), the oxygen concentration affects c-di-GMP synthesis or hydrolysis via heme-containing enzymes. However, studies of the role for oxygen in *Streptomyces* development have been mainly confined to antibiotic production in industrial cultures, not mechanistic studies (2, 37, 64). While RmdA is capable of sensing gaseous ligands, its PDE activity is surprisingly nonresponsive to perturbations. The apparent disconnect between the sensory input and output activity of RmdA may be due to various reasons. It is possible that proper experimental conditions were not found or that the MBP-RmdA fusion was impaired due to the MBP tag or the lack of the N-terminal domain X. It is also possible that RmdA gains responsiveness only in a complex with its cognate DGC. Several c-di-GMP PDEs have now been documented to function in signaling complexes (48, 61). Yet another scenario is that RmdA is a bifunctional GGDEF-EAL protein with a constitutive PDE activity. An inactivation of the EAL domain (either by proteolysis or by interactions with an RmdA partner) may be required to unmask the DGC activity of the GGDEF domain, and the DGC activity may be designed to be regulated via a heme-PAS9 domain. Examples of such unusual regulation in the bifunctional GGDEF-EAL proteins are beginning to emerge (57, 60).

One potential interaction partner of RmdA could be SCO0929, whose gene is located in an apparent operon with *rmdA* (Fig. 3), and the operon is conserved among filamentous actinomycetes. While in this study we did not observe visible developmental defects of the SCO0929 mutant, additional experimental conditions may be required to uncover its role. Signal sensing and processing in SCO0929-RmdA and the PAS9 domain proteins CdgA and SCO5511 also deserve further investigation.

A second set of questions concerns organization and specificity of c-di-GMP signaling pathways in *S. coelicolor*. While the issue of whether unique modules comprising DGCs and PDEs signal via dedicated c-di-GMP receptors (effector proteins) to unique targets or whether all DGCs and PDEs contribute to a total intracellular pool remains somewhat controversial, more and more evidence points toward specific cascades as a prevalent regulatory modality. A recent study where each DGC of the tiny bacterium *Bdellovibrio* was shown to have a specific phenotype that did not overlap with phenotypes controlled by other DGCs (28) provided yet another convincing argument in support of target-specific c-di-GMP signaling modules. We found that either the *rmdA* or *rmdB* mutation could cause a pronounced developmental delay, yet neither mutation resulted in significant perturbations in the total intracellular c-di-GMP levels (Fig. 6). This is consistent with the interpretation that a local, as opposed to a total, c-di-GMP pool is important for developmental regulation in *S. coelicolor*. It further shows a measure of specificity with which the RmdA and RmdB PDEs jointly guard c-di-GMP-dependent targets involved in development.

Recent experiments by the Buttner group showed that overexpression of *S. coelicolor* DGCs encoded by *cdgA* or *cdgB* (SCO4281) block aerial mycelium formation (17, 59), i.e., produce the same bald phenotype as does the *rmdA rmdB* double mutant. It is likely that RmdA and RmdB counterbalance the effects of CdgA and

CdgB. Whether CdgA, CdgB, RmdA, and RmdB are organized in specific signaling modules and, if so, how these modules operate remain to be investigated. It is peculiar that expression of *cdgA* and *cdgB* is regulated by BldD, the transcription factor affecting multiple developmentally regulated genes. BldD binding sites are also present upstream of SCO5511 (60), yet another predicted DGC (unpublished data), but they are not present upstream of *rmdA* or *rmdB*.

The third set of questions concerns molecular targets of c-di-GMP that result in developmental and colony morphology phenotypes and mechanisms through which c-di-GMP exerts its effects. In order to erect upward into the air and to form aerial hyphae, *S. coelicolor* filaments must break the surface tension created by the aqueous environment of the vegetative mycelium. Two overlapping mechanisms are involved in overcoming surface tension in *S. coelicolor*, i.e., chaplin proteins and the SapB protein (10, 15, 18, 31). We hypothesize that these may constitute c-di-GMP targets. However, at present little is known about regulatory pathways affecting synthesis or activities of these proteins.

Another important question that cannot yet be answered concerns identities of the *S. coelicolor* c-di-GMP receptors/effectors that mediate the effects of c-di-GMP on ultimate targets. The readily recognizable c-di-GMP receptors are represented by PilZ domains (1, 50), I sites in the enzymatically inactive GGDEF domains (11), or enzymatically inactive EAL domains (40). c-di-GMP receptors that are not based on PilZ, EAL, or GGDEF domains are difficult to recognize because their c-di-GMP-binding patterns are not readily identifiable (38). The PilZ domains are not encoded in the *Streptomyces* genomes. Among the GGDEF domain proteins, only CdgB and SCO5511 contain I sites (60), and both function as a DGC (60; unpublished data). SCO1397 is the only enzymatically inactive EAL domain protein that is not a DGC. While we cannot completely exclude the possibility that it binds c-di-GMP, this is unlikely. Overexpressed SCO1397 did not improve motility of MG1655 *yhjH* in the semisolid agar (Fig. 5A), in contrast to the case with other c-di-GMP receptors that serve as c-di-GMP sinks and decrease intracellular c-di-GMP levels (29). It therefore appears that identification of c-di-GMP receptors in streptomycetes will present a formidable challenge.

Finally, we would like to note that while the importance of the c-di-GMP signaling pathways in lifestyle changes in the proteobacteria has been well appreciated and the mechanisms of regulation are being elucidated (27, 39, 46), the studies of c-di-GMP signaling pathways in Gram-positive bacteria are still in their infancy. Our study, along with those of Buttner and colleagues (17, 59), reveals an apparently conserved function of c-di-GMP signaling in modulation of development in actinomycetes. This extends the realm of processes controlled by c-di-GMP and suggests that future studies in the Gram-positive bacteria may result in new, yet-unanticipated processes controlled by this second messenger, new kinds of c-di-GMP receptors, and new regulatory mechanisms.

## ACKNOWLEDGMENTS

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