Radiation-sensitive Gene A (RadA) Targets DisA, DNA Integrity Scanning Protein A, to Negatively Affect Cyclic Di-AMP Synthesis Activity in Mycobacterium smegmatis*

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Background: Little is known about the control of cyclic di-AMP synthesis in bacteria.

Results: RadA targets DisA to inhibit its cyclic di-AMP synthesis activity that regulates mycobacterial growth.

Conclusion: We report a novel mechanism of control of c-di-AMP synthesis and its effects on bacterial growth in Mycobacterium smegmatis.

Significance: These findings enhance our understanding of c-di-AMP synthesis control and its roles in bacteria.

Cyclic di-AMP has been recognized as a ubiquitous second messenger involved in the regulation of bacterial signal transduction. However, little is known about the control of its synthesis and its physiological role in bacteria. In this study, we report a novel mechanism of control of c-di-AMP synthesis and its effects on bacterial growth in Mycobacterium smegmatis. We identified a DisA homolog in M. smegmatis, MsDisA, as an enzyme involved in c-di-AMP synthesis. Furthermore, MsRadA, a RadA homolog in M. smegmatis was found to act as an antagonist of the MsDisA protein. MsRadA can physically interact with MsDisA and inhibit the c-di-AMP synthesis activity of MsDisA. Overexpression of MsdisA in M. smegmatis led to cell expansion and bacterial aggregation as well as loss of motility. However, co-expression of MsradA and MsdisA rescued these abnormal phenotypes. Furthermore, we show that the interaction between RadA and DisA and its role in inhibiting c-di-AMP synthesis may be conserved in bacteria. Our findings enhance our understanding of the control of c-di-AMP synthesis and its physiological roles in bacteria.

In recent years, cyclic dinucleotides have been recognized as ubiquitous second messengers involved in regulation of multiple physiological processes including bacterial cell wall metabolism, biofilm formation, motility, virulence, and host immune response (1–7). However, most of our knowledge about the functions of these second messengers is derived from the study of cyclic di-GMP (c-di-GMP). Much less is clear about the regulation of c-di-AMP (c-di-AMP) synthesis and its physiological effects in bacteria. Cyclic di-AMP was observed for the first time in crystallization studies of DisA in Bacillus subtilis (8, 9). It is synthesized by the N-terminal diadenylate cyclase (DAC) domain of DisA via condensation of two ATP molecules (8). DisA functions as a DNA integrity scanning protein and it was first recognized as a checkpoint protein for responding to DNA damage and monitoring genomic integrity at the onset of sporulation in B. subtilis (9). DisA can form an octameric complex and its C-terminal HhH (DNA-binding helix-hairpin-helix) domain is responsible for the DNA-binding activity of the protein (8). Interestingly, branched DNA can strongly suppress the DAC activity of DisA (8). This is the only evidence found to date on the direct regulation of DAC activity of DisA protein in bacteria. Nevertheless, several lines of evidence have indicated that c-di-AMP could be involved in regulation of bacterial physiology and pathogenesis (1, 2, 10–12). For example, mutation of the phosphodiesterase gene in Staphylococcus aureus, which degrades c-di-AMP to 5’-pApA, causes an increase in cross-linked peptidoglycans and change in bacterial cell size (10, 11). It has also been reported that degradation of c-di-AMP greatly enhances the sensitivity of B. subtilis cells to β-lactam antibiotics (1). The c-di-AMP secreted by the pathogen Listeria monocytogenes was recently shown to stimulate an IFN-mediated host immune response (2). More recently, the first bacterial regulator, DarR, was characterized as a c-di-AMP receptor in Mycobacterium smegmatis, which could respond to the second messenger and regulate lipid metabolism and bacterial growth (12). However, although the importance of c-di-AMP signaling in bacteria is well established, evidence on direct regulation of the enzymes involved in c-di-AMP synthesis and its correlation with bacterial physiology are scant.

A disA ortholog exists in the genome of the human pathogen Mycobacterium tuberculosis as well as that of M. smegmatis, a fast-growing model mycobacterium (13, 14). Particularly, M. tuberculosis DisA has been shown to encode a DAC that can convert ATP or ADP to c-di-AMP (15). However, the effect of control of c-di-AMP synthesis on mycobacterial DisA protein remains to be clearly characterized. In this study, we show that...
RadA acts as an antagonist of DisA and can physically interact with DisA to inhibit its c-di-AMP synthesis activity in *M. smegmatis*. We report a novel mechanism of bacterial c-di-AMP synthesis control via the physical interaction between RadA and DisA, which affects bacterial growth and cell morphology. Furthermore, we show that the interaction between RadA and DisA, which affects bacterial growth and cell morphology.

**Control of c-di-AMP Synthesis in Mycobacteria**

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Media, and Growth Conditions—*Escherichia coli* BL21(DE3) cells and the plasmid pET28a (purchased from Novagen) were used for expressing mycobacterial proteins. The pBT and pTRG plasmids and *E. coli* XR host strains were purchased from Stratagene for bacterial one-hybrid assays. All bacterial strains and plasmids used in this study are listed in the Table 1. *M. smegmatis* mc²155 genes, including *MsdisA*, *Msrada*, and their mutants, were amplified by PCR from mycobacterial genomic DNA using appropriate primers (Table 2). The corresponding recombinant strains were constructed (Table 1). *E. coli* BL21(DE3) cells transformed with recombinant plasmids were grown up to 1.0 at 37 °C in LB cultures containing ampicillin (100 μg/ml) or kanamycin (30 μg/ml). Protein expression was induced at 18 °C for 10 h by the addition of 0.5 mM D-1-thiogalactopyranoside. Cells were harvested, resuspended, and sonicated. The cell extract was further clarified by centrifugation and His₆-tagged or GST-fused proteins were purified from the supernatant by using nitrilotriacetic acid (NTA)-affinity column or GST-affinity purification column, respectively.

**Protein Expression and Purification—*M. smegmatis* mc²155**

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**TABLE 1**

Strains and plasmids used in this study

| Plasmid or strain | Relevant genotype or features | Source or Ref. |
|-------------------|-------------------------------|---------------|
| *M. smegmatis* mc²155 | | |
| Msm/WT | | |
| Msm/pMV261 | mc¹55with pMV261 | This study |
| Msm/pMV261-disA | mc¹55with pMV261-disA | This study |
| Msm/pMV261-D84A | mc¹55with pMV261:disA D84A | This study |
| Msm/pMV261-H137A | mc¹55with pMV261:disA H137A | This study |
| Msm/pMV261-radA-disA | mc¹55with pMV261:radA-disA | This study |
| Msm/pMV261-MTdisA | mc¹55with pMV261:MTdisA | This study |
| Msm/pMV261-BSdisA | mc¹55with pMV261:BSdisA | This study |
| Msm/pMV261-BTdisA | mc¹55with pMV261:BTdisA | This study |
| Msm/pMV261-SCdisA | mc¹55with pMV261:SCdisA | This study |
| Msm/pMY261-pTRG-MTdisA | mc¹55with pMV261:MTRG-MTdisA | This study |
| Msm/pMY261-BSradA-BSdisA | mc¹55with pMV261:BSradA-BSdisA | This study |
| Msm/pMY261-BTradA-BTdisA | mc¹55with pMV261:BTradA-BTdisA | This study |
| Msm/pMY261-pTRG-SradA-SCdisA | mc¹55with pMV261:pTRG-SradA-SCdisA | This study |
| Msm/pMind-bbP | mc¹55with pMV261::bbP | This study |
| Msm/pMind-bbP-D171A | mc¹55with pMV261::bbP-D171A | This study |
| Msm/pMind-bbP-radA | mc¹55with pMV261::bbP-radA | This study |
| Msm/pMind-disA | mc¹55with pMV261::disA | This study |
| pET28a(+) | Kan’, T7 lac promoter, N-terminal His₆ | Novagen |
| pET28a-disA | disA in EcoRl-Xbal sites of pET28a | This study |
| pET28a-disA-D84A | disA D84A in EcoRl-Xbal sites of pET28a | This study |
| pET28a-disA-H137A | disA H137A in EcoRl-Xbal sites of pET28a | This study |
| pET28a-BSdisA | BDisA in EcoRl-Xbal sites of pET28a | This study |
| pGEX-4T-1 | Amp’, T7 tac promoter, N-terminal GST-tag | Stratagene |
| pGEX-RadA | RadA in EcoRl-Xbal sites of pGEX-4T-1 | This study |
| pGEX-RadA-K106M | K106 M in EcoRl-Xbal sites of pGEX-4T-1 | This study |
| pGEX-Rada D176A | D176A in EcoRl-Xbal sites of pGEX-4T-1 | This study |
| pBT | ch1², p15A replicon, lac-UV5 promoter | Stratagene |
| pBT-MSradA | Msrada in EcoRl-Xbal sites of pBT | This study |
| pBT-MTradA | MTrada in EcoRl-Xbal sites of pBT | This study |
| pBT-BSradA | Bsrada in EcoRl-Xbal sites of pBT | This study |
| pBT-BTradA | BTrada in EcoRl-Xbal sites of pBT | This study |
| pBT-SradA | SradA in EcoRl-Xbal sites of pBT | This study |
| pTRG | tet’, ColE1 replicon, lpp/lac-UV5 promoter | Stratagene |
| pTRG-MSdisA | MDisA in EcoRl-Xbal sites of pTRG | This study |
| pTRG-MTdisA | MTrA in EcoRl-Xbal sites of pTRG | This study |
| pTRG-BSdisA | BDisA in EcoRl-Xbal sites of pTRG | This study |
| pTRG-BTradA | BTrada in EcoRl-Xbal sites of pTRG | This study |
| pTRG-SradA | SradA in EcoRl-Xbal sites of pTRG | This study |
| pTRG-MS655 | MS655 in EcoRl-Xbal sites of pTRG | This study |
| pMV261 | Kan’, pAL5000 replicon, bap60 promoter operon | This study |
| pMind | Kan’, pAL5000 replicon, tetRO operon | This study |

**37°C in the 7H9 Middlebrook liquid medium or on 7H10 agar plates.**
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TABLE 2
Primer usage

| Name        | Sequence 5’–3’                                | Usage          |
|-------------|------------------------------------------------|----------------|
| BsdA-f      | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| BsdA-r      | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| BsrA-f      | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| BsrA-r      | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| BSradA-f    | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| MSdisA-f    | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| MSdisA-r    | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| MstradA-f   | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| MstradA-r   | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| PtradA-f    | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |
| PtradA-r    | AGAGGAAATTTCGATGCTGAAAAGAGAAAGAAAAGG          | Clone          |

**Thin Layer Chromatography (TLC) Assays for c-di-AMP Synthesis Activity**—The activity of MsDisA or B. subtilis DisA (BsuDisA) was measured by monitoring the formation of c-di-AMP in the presence of [α-32P]ATP and proteins. Reaction mixtures (50 μl) containing 100 mM NaCl, 20 mM Tris–HCl (pH 7.5), and 10 mM MgCl2, 0.1 mM ATP, 5 μCi of [α-32P]ATP, and MsDisA or BsuDisA were incubated at 30 °C for 0–45 min. Small aliquots (1.0 μl) were then placed onto TLC PEI-cellulose F plates (MERCK, Germany) that had been developed in 1.5 M KH2PO4 buffer for 30 min. Formation of c-di-AMP was analyzed using a Typhoon Scanner (GE Healthcare).

**Co-immunoprecipitation Assays**—The in vivo association between MsDisA and MsRadA were assayed by co-immunoprecipitation as described in a previous report (17) with some modifications. Exponentially growing cells of M. smegmatis were harvested, resuspended, and lysed with lysis buffer (0.5% Nonidet P-40, 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl). Co-immunoprecipitations were performed by incubating M. smegmatis cell extract with MsRadA antiserum in 100 μl of buffer for 3 h at 4 °C. A total of 20 μl of protein A-Sepharose slurry was then added and incubation was continued for

lyzed overnight and stored at −80 °C. Protein concentration was determined by spectrophotometric absorbance at 260 nm according to Gill and von Hippel (16).

**Co-immunoprecipitation Assays**—The in vivo association between MsDisA and MsRadA were assayed by co-immunoprecipitation as described in a previous report (17) with some modifications. Exponentially growing cells of M. smegmatis were harvested, resuspended, and lysed with lysis buffer (0.5% Nonidet P-40, 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl). Co-immunoprecipitations were performed by incubating M. smegmatis cell extract with MsRadA antiserum in 100 μl of buffer for 3 h at 4 °C. A total of 20 μl of protein A-Sepharose slurry was then added and incubation was continued for
another hour. Immune complexes were collected, and the beads were washed with buffer. Finally, the beads were re-suspended in SDS-PAGE sample buffer. After boiling, the samples were analyzed by Western blotting using an MsDisA antiserum. For assessing the quality of the experiment, we have used ParB, a unrelated protein, as a control to keep basically equal amounts of total protein for each cell sample.

**Surface Plasmon Resonance Analysis**—Interaction between MsDisA and MsRadA in vitro was analyzed on a BLACore 3000 instrument (GE Healthcare) as described previously (18, 19). Briefly, the purified GST-tagged MsRadA fusion protein, to be used as the ligand, was diluted in HBS buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 50 mM EDTA, 0.005% BLACore surfactant P20). As a negative control, the GST protein was substituted for the RadA protein. His<sub>6</sub>-tagged MsDisA proteins were immobilized onto NTA chips. An overlay plot was then produced to depict the interaction between the two proteins. Each analysis was performed in triplicate.

**Bacterial Aggregation and Mobility Assays**—To observe cell aggregation, *M. smegmatis* cells were transferred to 5 ml of fresh 7H9 liquid medium containing 10 µg/ml of Congo red with an initial turbidity of 0.05 at 600 nm. Cells were cultured at 37 °C, 160 rpm/min for 2 days. Bacterial mobility assays were performed following a previously described procedure (20). Briefly, *M. smegmatis* cells were grown up to A<sub>600</sub> = 1.0 and then spotted onto BM-2 plates (62 mM potassium phosphate, 2 mM MgSO<sub>4</sub>, 10 mM FeSO<sub>4</sub>, 0.1% casamino acid, 0.4% glucose, and 0.5% Bacto-agar). Mobility was measured after 48 h. Three plates were tested for each culture.

**Bacterial Two-hybrid Assay**—Bacterial two-hybrid analysis was carried out according to the procedures described in a previous report (19). pBT and pTRG vectors containing the disA and radA genes were generated. pBT/pTRG plasmids were co-transformed into the reporter strain and spotted onto the screening medium plate containing 5 mM 3-amino-1,2,4-triazole (Stratagene), 8 g/ml of streptomycin, 15 g/ml of tetracycline, 34 g/ml of chloramphenicol, and 50 g/ml of kanamycin. The plates were then incubated at 30 °C for 3–4 days. Strains co-transformed with pBT-LGF2 and pTRG-Gal11P (Stratagene) were used as positive controls (CK<sup>+</sup>) for expected growth on the screening medium. Strains co-transformed with the empty vector pBT and pTRG were used as negative controls (CK<sup>−</sup>).

**Scanning Electron Microscopy (SE) Assay and Morphological Observation**—SE experiments were performed according to previously published procedures (21). Mycobacterial cells were harvested at later log phase and treated with 2.5% glutaraldehyde solution at 4 °C for 2 h. Fixed cells were washed, then dehydrated by sequential treatments in 30, 50, 75, 85, 95, and 100% ethanol for 15 min each. Samples were dried up, sputter-coated with gold, and observed using a scanning electron microscope (S570; Hitachi, Tokyo, Japan). Colony morphologies of the *M. smegmatis* strains were investigated by spot-inoculating 3 µl of overnight cultures on 7H10 medium and subsequent incubation at 37 °C for 2–3 days.

**Quantitative Real-time PCR**—Quantitative real-time PCR assays were performed as described previously (21, 22). Gene-specific primers (Table 2) were used, and first-strand cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Each PCR (20 µl) contained 10 µl of 2X SYBR Green Master Mix Reagent (Applied Biosystems), 1.0 µM of cDNA samples, and 200 nM gene-specific primers. The reactions were performed in a Bio-Rad IQ5 RT-PCR machine and the thermocycling condition consisted of an initial step of 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. All quantitative RT-PCR experiments were performed in triplicates. Amplification specificity was assessed using melting curve analysis. Expression levels of all genes were normalized to the levels of *parB* gene transcripts.

**RESULTS**

*M. smegmatis* DisA Homolog Is a c-di-AMP Synthesis Enzyme—We searched the genome of *M. smegmatis* and found a *B. subtilis* DisA homolog, MsDisA. MsDisA has high amino acid sequence similarity with *B. subtilis* DisA and contains three clear domains (Fig. 1A). In particular, the N-terminal DAC domain of MsDisA contains two conserved amino acid residues essential for c-di-AMP synthesis activity (8), of which Asp-84 is putatively involved in Mg<sup>2+</sup>-binding and His-137 is necessary for ATP binding. This indicates that MsDisA could encode a DAC enzyme. Using time course experiments, we further determined the c-di-AMP synthesis activity of MsDisA together with the previously reported *B. subtilis* DisA protein.
As shown in Fig. 1B, when 2 μM BsuDisA or MsDisA was mixed together with 5 μCi of [α-32P]ATP, the amount of c-di-AMP synthesized progressively increased as the reaction time was extended, indicating MsDisA possesses a similar function as BsuDisA and is capable of utilizing ATP to produce c-di-AMP. As expected, MsDisA-D84A and MsDisA-H137A were deficient in catalyzing c-di-AMP synthesis activity because no c-di-AMP production was observed under similar reaction conditions (Fig. 1C). Taken together, these results indicate that MsDisA encodes a c-di-AMP synthesis enzyme.

MsRadA Physically Interacts with MsDisA—To further study the regulation of MsDisA activity, a genomic location analysis was first performed. As shown in Fig. 2A, MsdisA is situated close to the MsradA gene, suggesting that they might be located within the same operon. This idea was confirmed by a series of reverse transcription PCR assays and the two genes were found to be co-transcribed in M. smegmatis (Fig. 2, B and C). In addition, a search of the STRING database revealed that MsDisA physically interacts with MsRadA. We further conducted co-immunoprecipitation experiments to investigate whether interaction between these two genes occur in M. smegmatis. Protein A beads that were conjugated with an antibody raised against MsDisA were used for the assays. MsDisA clearly associated with MsRadA as indicated by a specific and expected size of hybridization signal (Fig. 3A) detected by anti-MsRadA antibody in M. smegmatis cell extracts (lane 2). No signal could be detected when the pre-immune sera (lane 3), which does not contain anti-MsDisA antibody, was mixed with protein A beads. In addition, no significant difference was observed for

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**FIGURE 2. Assays for the radA-disA co-transcription by reverse transcription-PCR.** A, the operon structure of MsradA-MsdisA and their relative genomic location. B, the operon structure of radA-disA and its genomic location. Primers were designed for assays and indicated by black arrows. C, reverse transcription PCR assays for radA-disA co-transcription. Total DNA were used as positive controls and mRNA (DNA-free) were used as negative controls. The PCR procedure was as follows: reactions were degenerated at 95 °C for 30 s, annealed at 60 °C for 30 s, extended at 72 °C for 30 s and under 35 cycles.

**FIGURE 3. Assays for the physical interaction between MsRadA and MsDisA.** A, co-immunoprecipitation assay for examining in vivo interactions between MsRadA and MsDisA in M. smegmatis. Co-immunoprecipitation assays were carried out using Protein A conjugated with anti-MsDisA antiserum. Preimmune sera (lane 3) does not contain anti-MsDisA antiserum and was used as a negative control. The amount of total loading protein in each sample was detected using an anti-MsParB antibody. B, surface plasmon resonance assays for the interaction between MsDisA and MsRadA. His6-tagged MsDisA was immobilized on an NTA chip and different concentrations of GST-MsRadA were passed over the chip on a BIAcore 3000. 1000 nM GST alone was used as negative control. Overlay plots depicting the interactions were produced. C, surface plasmon resonance assays for interactions between MsDisA and two MsRadA mutant proteins, K106M and D176A, in which the Mg2⁺-binding site and the ATP-binding site were mutated, respectively. Heat-denatured MsRadA protein and GST protein were used as negative controls.
the amount of total protein in each sample when an unrelated anti-MsParB antibody was used (Fig. 3A, lower panel). These results indicate that MsRadA physically interacts with MsDisA in vivo in *M. smegmatis*.

Furthermore, we performed a surface plasmon resonance assay to confirm the interaction between MsRadA and MsDisA in vitro. As shown in Fig. 3B, a His$_6$-tagged MsDisA protein was immobilized on an NTA chip. MsRadA proteins were flowing on the surface of the chip and the affinity values were evaluated by BIAcore 3000 evaluation software. When an increasing amount of GST-tagged MsRadA protein (125–1000 nM) was passed over the chip, a response of about 300 response units was observed. However, no significant response was observed when 1000 nM GST protein was used to replace recombinant GST-tagged MsRadA protein. In addition, no response was observed when 500 nM heat-denatured MsRadA protein was passed over the chip. Interestingly, significant responses were observed for two mutant MsRadA proteins, namely K106M and D176A, in which the Mg$^{2+}$-binding site and the ATP-binding site were mutated (Fig. 3C). Thus, native MsRadA as well as its two mutant variants physically associate with MsDisA in vitro.

**MsRadA Inhibits c-di-AMP Synthesis Activity of MsDisA—**Physical interaction between MsRadA and MsDisA suggests a functional link between these two proteins. We further investigated the effect of MsRadA on the c-di-AMP synthesis activity of MsDisA. As shown in Fig. 4A, when increasing amounts of MsRadA protein (0.25–2 μM) were co-incubated with 2 μM MsDisA and 5 μCi of [α-32P]ATP, the amount of c-di-AMP produced steadily decreased (lanes 8–11), suggesting that MsRadA inhibited the c-di-AMP synthesis activity of MsDisA. Interestingly, MsRadA was found to weakly degrade ATP, concomitant with an increase in its protein concentration. This suggested that the inhibitory effect of MsRadA on c-di-AMP synthesis might have been caused by its ATPase activity. To exclude this possibility, we examined the effects of the mutant MsRadA-D176A protein, which lacks ATPase activity but retains physical interaction with MsDisA (Fig. 3C), on the c-di-AMP synthesis activity of MsDisA. Strikingly, MsRadA-D176A also significantly inhibited the c-di-AMP synthesis activity of MsDisA (lanes 13–16). In comparison, GST protein did not exhibit the inhibitory activity (Fig. 4A, lanes 3–6) when the same amount of protein was added into the reactions. In addition, both wild type and mutant MsRadA proteins lacked the ability to hydrolyze c-di-AMP when similar amounts of protein were incubated with radioactively labeled c-di-AMP molecules (Fig. 4B). Based on these data, we conclude that MsRadA physically interacts with MsDisA and inhibits its c-di-AMP synthesis activity.

**Overexpression of MsDisA, but Not Co-expression of MsDisA-MsRadA, Induces Cell Aggregation and Inhibits Bacterial Motility—**We investigated the effects of MsDisA and its interaction with MsRadA on mycobacterial growth and physiological characteristics. As shown in Fig. 5A, when *M. smegmatis* strains were spotted on the surface of solid agar medium, a typical bacterial lawn was observed for the wild type strains containing the pMV261 empty plasmid (Msm/pMV261) and the mutant *MsdisA*-D84A-overexpressing strains (Msm/pMV261-D84A). However, only a thin and small bacterial lawn was observed for the *MsdisA* overexpressing strain, indicating it grew at a slower rate than the wild type strains. Strikingly, co-expression of *MsdisA* and *MsradA* could completely rescue the slow growth and small bacterial lawn phenotype of the *MsdisA*-overexpression strain. In addition, we confirmed the difference in growth rates of these mycobacterial strains by determining their growth curves in liquid 7H9 medium (Fig. 5B). Increase in expression levels of *MsdisA* and *MsradA* in *MsdisA* and *MsdisA-MsradA* co-expression strains were verified by RT-PCR assays (Fig. 5C) and Western blotting assays (Fig. 5D).

Interestingly, recombinant *M. smegmatis* strains overexpressing *MsdisA*, but not the mutant D84A or wild type strains, were found to have formed cell aggregates when stained by Congo red. However, when co-expressing *MsradA* together with *MsdisA* in *M. smegmatis*, no cell aggregates were observed and the growth of co-expression strains was very similar to that of the wild type (Fig. 5A).

The change in cell aggregation phenotype suggested that *MsdisA* overexpression had an effect on the motility of *M. smegmatis*. To test this hypothesis, we compared the growth of wild type, *MsdisA*-overexpression and *MsdisA-MsradA* co-expression strains. As shown in Fig. 5E, the wild type strains dispersed well on the surface of BM-2 plates (left panel), indicating that it has good motility, which is consistent with previous reports (23). In contrast, no obvious dispersion was observed for *MsdisA* overexpression strains, indicating that overexpression of *MsdisA* inhibited the motility of *M. smegmatis* (Fig. 5E, middle panel). This result is consistent with the above observation of cell aggregations. Again, co-expression of *MsdisA* and *MsradA* could rescue the phenotype of the *MsdisA*-overexpression strains (Fig. 5E, right panel).

In summary, overexpression of *MsdisA* induced cell aggregations, and inhibited bacterial growth and motility. However, co-expression of *MsradA* and *MsdisA* could successfully rescue these growth phenotypes to levels comparable with those of the wild type.

**Overexpression of MsDisA, but Not Co-expression of MsDisA-MsRadA, Induces Cell Expansion—**We further used SE assays to characterize the effects of the interaction between MsRadA and MsDisA on bacterial cell morphology. *MsdisA* or *MsdisA-MsradA* genes were cloned into the TetR-controlled expression plasmid pMind (24). Then, we compared the morphological differences of the recombinant strains harboring these plasmids. As shown in Fig. 6A, upon 25 ng/ml of tetracycline induction, *MsdisA* overexpressing *M. smegmatis* strains showed obvious cell expansion (right panel) compared with those without tetracycline induction (Fig. 6A, left panel). We evaluated the total amount of 350 *MsdisA* overexpressing *M. smegmatis* cells and found that ~27% cells were expanded. No significant morphological change was observed for wild type strains under the same induction condition (Fig. 6B). Strikingly, when the *MsradA* gene was co-expressed with *MsdisA*, the cell morphology of recombinant *M. smegmatis* strains became normal and similar to those of the wild type strains (Fig. 6C), indicating that *MsradA* could rescue the morphological changes induced by *MsdisA* overexpression.

Furthermore, overexpressing the *ybbP* gene would be expected to produce a similar cell expansion phenotype if the morphological
change induced by overexpressing disA is caused by increased c-di-AMP synthesis in the mycobacterial strains. As expected, the ybbP-overexpressing strains exhibited cell expansion phenotypes upon 25 ng/ml of tetracycline induction (Fig. 6D). However, no cell expansion was observed upon overexpression of the ybbP-D171A mutant gene, in which the Mg$^{2+}$-binding site was mutated and c-di-AMP synthesis activity was lost (8), indicating that c-di-AMP synthesis activity is essential for the cell expansion phenotype induced by ybbP in M. smegmatis.

This observation was confirmed by further HPLC-MS assays for the intracellular c-di-AMP concentrations of several recombinant expressing strains. As shown in Fig. 6F, the disA-overexpressing strain had a 13-fold higher c-di-AMP concentration (10.4 μM) compared with the wild type strain (0.8 μM) or the mutant disA-overexpressing strain (0.8 μM). However, when co-expressing disA and radA in M. smegmatis, the recombinant strain only obtained a lower c-di-AMP level (0.4 μM) compared with the wild type strain.

Therefore, overexpression of MsdisA, but not co-expression of MsdisA-MsradA, induces cell expansion. This phenotype was found to be dependent on c-di-AMP synthesis activity of MsDisA.
Interaction between DisA and RadA and Their Regulations on Cell Morphology Are Conserved in Bacteria—We further investigated the conserved interaction between DisA and RadA in several bacterial species and their effects on mycobacterial cell morphology. Pairs of radA and disA genes were also found to exist in the genomes of many bacterial species including Mycobacterium tuberculosis, Bacillus thuringiensis, and Streptomyces coelicolor and B. subtilis in addition to M. smegmatis in the NCBI database (Fig. 7A). We used bacterial two-hybrid experiments to examine if RadA and DisA also interacted in these representative bacterial species. Co-transformants containing radA/disA grew well on the screening medium, indicating interaction between RadA and DisA (Fig. 7B). No growth was observed for their self-activation controls. Therefore, similar to the case in M. smegmatis, a conserved interaction between DisA and RadA exists in other bacterial species.

When these disA genes were expressed through a strong promoter hsp60 within the plasmid pMV261 in M. smegmatis, similar cell expansion phenotypes were observed for all tested disA genes (Fig. 7C, left panels), indicating that they could play a similar role as MsdisA. Furthermore, when disA genes were co-expressed with their respective radA genes, the cell morphology of recombinant M. smegmatis strains was found to be similar to that of the wild type strains (Fig. 7C, right panels), indicating that these bacterial radA genes could rescue the morphological phenotypes induced by disA overexpression. These results strongly suggest that the interactions between DisA and RadA are conserved across multiple bacterial species.

DISCUSSION

In the present study, we report a potentially conserved interaction between RadA and DisA that could negatively affect the promotion of c-di-AMP synthesis by DisA in M. smegmatis. Overproduction of disA alone led to cell expansion, aggregation, and loss of motility, whereas co-expression of radA-disA rescued these phenotypes. Thus, we found a novel mechanism of control of bacterial c-di-AMP synthesis via RadA-DisA interaction in M. smegmatis.
In recent years, accumulating evidence has shown that cyclic dinucleotides widely function as second messengers that control multiple microbial phenotypes and physiological characteristics (4, 5, 25–28). Compared with the clearly characterized functions for c-di-GMP in many bacteria (4, 5, 28), much less is known about the control of c-di-AMP synthesis and its effects on bacterial growth. An important aspect of the current study is the discovery of a link between RadA and control of c-di-AMP synthesis. The radA/sms gene was initially identified as a radiation-sensitive gene in Escherichia coli and its main function was considered to play a synergistic role in DNA damage repair and recombination (29, 30). Mutation of radA has been reported to confer profound sensitivity to various DNA-damaging agents in E. coli (30). In addition, the RadA protein has been shown to be required for efficient recombination of donor DNA during transformation (31). In the present study, we report a new and additional function of RadA. We present data showing that RadA physically and functionally interacts with DisA and affects the c-di-AMP synthesis activity of DisA in Mycobacterium smegmatis.

Therefore, our results suggest that, in addition to its roles in DNA recombination and repair, RadA could play more extensive roles in bacterial growth through its effect on the synthesis of the c-di-AMP second messenger. The functional interaction between RadA and DisA is also consistent with their genomic location. MsdisA is situated close to the MsradA gene in the M. smegmatis genome. We carried out reverse transcriptional PCR assays and confirmed that these two genes are indeed co-transcribed in M. smegmatis. Interestingly, among available genome sequences in the NCBI database, the disA gene is always next to the radA gene in more than 45 eubacterial genera or 85 species. In addition, we confirmed the interaction between RadA and DisA in at least five different bacterial species (Fig. 7). Taken together, these findings strongly suggest that the physical and functional interaction between DisA and RadA is likely to be extensively conserved in bacteria.

A few recent studies have suggested that the precise control of intracellular c-di-AMP levels is essential for bacterial cell growth. For example, at least one of the three B. subtilis DAC is required for growth, with depletion followed by cell lysis occurring in mutants (1), whereas high-level accumulation of c-di-AMP is also detrimental for cell growth (32). In both S. aureus and B. subtilis, c-di-AMP has been found to be required for normal cell wall peptidoglycan homeostasis (1, 11, 32). In the present study, we found that the control of c-di-AMP synthesis could be linked to bacterial morphology and motility in M. smegmatis. Overproduction of disA induced cell expansion and bacterial aggregation as well as loss of motility. MsRadA acted as an effective antagonist of MsDisA and co-expression of radA-disA could rescue the disA overexpression phenotypes. Thus, the current findings further extend our knowledge of the physiological roles of c-di-AMP in bacteria. However, although the possibility is very small, these morphology phenotypes might be partially derived from inadequate DNA repair because RadA has been sequestered by DisA. This remains to be done in future work. In a recent study, we reported the identification of a c-di-AMP receptor, DarR, in bacteria and confirmed that DarR can respond to c-di-AMP signaling to negatively regulate fatty acid synthesis in M. smegmatis (12).
fatty acids is essential for bacterial growth, overexpression of DarR is detrimental to the growth of M. smegmatis. In the current study, we found that overproduction of disA inhibited M. smegmatis growth. This finding is consistent with the previously observed negative regulation of M. smegmatis growth by DarR. Overexpression of disA may enhance c-di-AMP synthesis and, therefore, promote the negative regulation of synthesis of essential fatty acids by DarR in M. smegmatis. Thus, taken together with previous findings, our results suggest that c-di-AMP can participate in the control of multiple physiological processes including peptidoglycan homeostasis, lipid metabolism, cell morphology, cell aggregation, and cell motility.

In mycobacteria, the Ser/Thr protein kinase PknA/B and its substrate Wag31 (a homolog of DivIVA in B. subtilis) are involved in the regulation of bacterial cell morphology (33). Overexpression of pknA or pknB causes defects in bacterial shape (33). Likewise, alteration of the Wag31 expression level also results in loss of mycobacterial rod shape and impairment of cell growth (33-35). In the present study, we found that overexpression of DisA significantly induced cell expansion, suggesting that c-di-AMP signaling may regulate bacterial morphology. However, compared with the wild type strains, no obvious change was observed in the expression levels of pknB, pknA, and Wag31 (data not shown) in disA overexpression strains. Thus, c-di-AMP may regulate mycobacterial cell morphology through a novel but unknown signaling pathway that is distinct from the pknA-pknB-wag31 pathway. Interestingly, when disA was overexpressed in a darR-deleted strain, no...
changes in cell morphology or bacterial motility were observed if compared with the WT/pMV261-disA strain (data not shown). This suggests that control of c-di-AMP signaling by disA could be unrelated to the function of DarR, the only c-di-AMP receptor characterized so far. Future studies should further characterize the signaling pathway underlying the effects of disA overexpression or disA-rada co-expression on mycobacterial growth.

In summary, we have successfully characterized the physical and functional interaction between RadA and a c-di-AMP synthesis enzyme, DisA, in M. smegmatis. We identified a novel mechanism for the control of bacterial c-di-AMP synthesis and bacterial growth via RadA-DisA interaction. These findings expand our knowledge of the physiological function of the c-di-AMP second messenger and provide important clues to novel mechanisms of control of bacterial morphology and growth.

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