# EshA Accentuates ppGpp Accumulation and Is Conditionally Required for Antibiotic Production in *Streptomyces coelicolor* A3(2)

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Disruption of *eshA*, which encodes a 52-kDa protein that is produced late during the growth of *Streptomyces coelicolor* A3(2), resulted in elimination of actinorhodin production. In contrast, disruption of *eshB*, a close homologue of *eshA*, had no effect on antibiotic production. The *eshA* disruptant accumulated lower levels of ppGpp than the wild-type strain accumulated. The loss of actinorhodin production in the *eshA* disruptant was restored by expression of a truncated *relA* gene, which increased the ppGpp level to the level in the wild-type strain, indicating that the reduced ppGpp accumulation in the *eshA* mutant was solely responsible for the loss of antibiotic production. Antibiotic production was also restored in the *eshA* mutant by introducing mutations into *rpoB* (encoding the RNA polymerase β subunit) that bypassed the requirement for ppGpp, which is consistent with a role for EshA in modulating ppGpp levels. EshA contains a cyclic nucleotide-binding domain that is essential for its role in triggering actinorhodin production. EshA may provide new insights and opportunities to unravel the molecular signaling events that occur during physiological differentiation in streptomycetes.

One of the most intriguing challenges in biology today is elucidation of the mechanisms by which cells detect and respond to extracellular nutritional conditions. Among the prokaryotes, *Bacillus subtilis* and *Streptomyces* spp. provide tractable experimental systems for studying such mechanisms because they exhibit a wide range of adaptations to extreme nutrient limitation, including the production and secretion of antibiotics and enzymes and the formation of aerial mycelium (*Streptomyces* spp.) and endospores (*Bacillus* spp.) (12). Nutritional status and sporulation have been successfully linked in *B. subtilis* (45), in which CodY detects and responds to nutrient limitation by monitoring the level of the intracellular GTP pool as an overall indicator of cellular physiology. Recent work by Inaoka and Ochi (20) supported this proposal. The stringent response, a general and ubiquitous response to nutrient limitation in prokaryotes, plays a central role in responding to nutrient stress, mediating its effect through the nucleotide guanosine 5′-diphosphate 3′-diphosphate (ppGpp) (5). By analyzing mutants with impaired abilities to elicit the stringent response, we have shown that ppGpp plays a role in triggering the onset of antibiotic production in both *B. subtilis* (21) and *Streptomyces* spp. (6, 7, 15, 29, 37, 39, 41, 52, 55), whereas morphological differentiation is triggered by reduced levels of GTP. Streptomycetes are gram-positive, filamentous soil bacteria that have a complex process of morphological differentiation and the ability to produce a wide variety of secondary metabolites (referred to as physiological differentiation) that include antibiotics and other useful medicinal compounds. Morphological differentiation and physiological differentiation

in streptomycetes often coincide and occur in response to environmental signals that include nutrient limitation. *Streptomyces coelicolor* A3(2), the streptomycete that has been genetically characterized most, is particularly appropriate for studying the regulation of morphological and physiological differentiation. This strain produces at least four antibiotics, two of which, the blue-pigmented polyketide actinorhodin and the red-pigmented prodiginine (formerly called prodigiosin) complex, are usually produced in the stationary phase (9, 10). There has been much progress in elucidating not only the organization of antibiotic biosynthetic gene clusters in many *Streptomyces* species but also the roles of pathway-specific regulatory genes that are required for the activation of their cognate biosynthetic genes (3, 19). In *S. coelicolor* A3(2), ActII-ORF4 (a positively acting regulatory protein) is crucially important for the expression of all of the biosynthetic genes that encode the enzymes of the actinorhodin pathway (1, 14).

Kwak et al. (31) and we (28) independently discovered a novel 52-kDa protein that is required for initiating several developmental processes in streptomycetes. Disruption of the corresponding gene, *eshA*, eliminated antibiotic production in *S. coelicolor* A3(2) and in *Streptomyces griseus* (28, 47). At the same time, Kwak et al. (31) reported that EshA is required for extension of sporogenic hyphal branches (thus the origin of the gene designation *eshA*) in *S. griseus*. We found later that disruption of *eshA* causes a defect in aerial mycelium formation in *S. griseus* that appears to result from an aberrant accumulation of deoxynucleoside triphosphates that accompanies the arrest of DNA synthesis in the late growth phase (47). In contrast, disruption of *eshA* in *S. coelicolor* did not result in aberrant accumulation of deoxynucleoside triphosphates and did not affect the formation of aerial hyphae, implying that there are qualitative differences between the EshA proteins of the two species (47). Abundant EshA accumulates during sporulation

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *Streptomyces* strains | | |
|   *S. coelicolor* A3(2) strain 1147 | SCP1$^+$ SCP2$^+$ (prototrophic wild type) | Laboratory stock |
|   *S. coelicolor* A3(2) strain KO-350 | *eshA::tsr* (*eshA* null mutant) | 28 |
|   *S. coelicolor* A3(2) strain KO-351 | *eshA::tsr rpoB* | This study |
|   *S. coelicolor* A3(2) strain KO-541 | *eshB* (*eshB* null mutant) | This study |
|   *S. coelicolor* A3(2) strain KO-542 | *eshA::tsr eshB* | This study |
|   *S. griseus* IFO13189 | Prototrophic streptomycin-producing wild type | Laboratory stock |
|   *S. griseus* KO-390 | *eshA* disruptant derived from 13189 | 47 |
| Plasmids | | |
|  *E. coli* replicons | | |
|   pBluescript SK(+) | Amp$^r$; cloning vector | Toyobo (Osaka, Japan) |
|   pGEM-T | Amp$^r$; cloning vector for PCR product | Promega |
|   pSCP52 | Amp$^r$; pBluescript SK(+) with 7.1-kb PstI fragment containing *S. coelicolor eshA* gene | 28 |
|   pBL52SG | pBluescript SK(+) with 3.4-kb BamHI fragment containing *S. griseus eshA* gene | This study |
|   pNS80 | pBluescript SK(+) with *eshA* gene of *S. coelicolor* under control of the *eshA* promoter of *S. griseus* | This study |
|  *Streptomyces* replicons | | |
|   pV1 | Tsr$^r$ Hyg$^r$ Amp$^r$; pIJ941 carrying pBluescript SK(+) sequence | 27 |
|   pV52SC | pV1 with 2.2-kb PstI fragment containing *S. coelicolor eshA* gene | 28 |
|   pIJ8600 | Apr$^r$ Tsr$^r$; *tipAp attP* | 53 |
|   pIJ6084 | Apr$^r$ Tsr$^r$; *tipAp::relA attP* | 52 |
|   pNS66 | pV1 with 1.7-kb PstI fragment containing *S. coelicolor eshA* lacking cyclic nucleotide-binding domain sequence | This study |
|   pNS82 | pV1 with 3.4-kb fragment from pNS80 | This study |
|   pTYM18 | Km$^r$; *attP* | 44 |
|   pTYM52SC | pTYM18 with 2.2-kb PstI fragment containing *S. coelicolor eshA* gene | This study |

induced by phosphate starvation and nutritional downshift (31) and also when cells reach the middle to late growth phase (28, 47). In *S. griseus* at least, EshA exists as multimers (~20-mers) with a diameter of 27 nm (47). Homologues of EshA in two other bacterial species are also induced in a stress- or growth-phase-dependent manner; SrpI of the cyanobacterium *Synechococcus* sp. is induced under sulfur deprivation conditions (34, 35), while MMPI is induced during infection by *Mycobacterium leprae* (57, 61). These observations led Triccas et al. (57) and Kwak et al. (31) to propose that EshA, SrpI, and MMPI constitute a new family of bacterial stress response proteins. The EshA proteins of both *S. coelicolor* and *S. griseus* have a central domain that exhibits considerable amino acid sequence identity (30% to 32%) to eukaryotic-type cyclic nucleotide (cNMP)-binding domains (25, 56). Although the biochemical function of EshA is not known, it was conceivable that the protein exerts its influence via this putative nucleotide-binding domain. In this study, we used both genetic and biochemical approaches to demonstrate that EshA has a role in secondary metabolism in *S. coelicolor*. We found that EshA is a cyclic AMP (cAMP)-binding protein and that it is required for sustaining levels of ppGpp during the late growth phase that are sufficient for activation of actinorhodin biosynthesis.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains used in this study are listed in Table 1. *S. coelicolor* A3(2) strain 1147, a prototrophic wild-type strain, was used as the parental strain. *Escherichia coli* DH5α was used for plasmid construction. *E. coli* GM2163 (*dam dcm*) was used for preparation of nonmethylated DNA for transformation of *S. coelicolor*. *S. coelicolor* strains were

grown at 30°C on GYM agar (37) or R5$^-$ agar (17) without sucrose (R5MS agar). Strains of *S. griseus* were grown on TSB agar (47). Spontaneous rifampin-resistant (*rif* [*rpoB*]) mutants were obtained from colonies that grew within 7 days after spores were spread on R5MS agar containing rifampin (100 μg/ml). The mutants were used for studies after single-colony isolation. Mutations in the *rpoB* gene were detected by DNA sequencing as described previously (62).

**Plasmid construction.** Plasmids used or constructed in this study are listed in Table 1, and the oligonucleotide primers used are listed in Table 2. General DNA manipulations, such as plasmid isolation and transformation of *E. coli*, were performed as described by Sambrook et al. (48). Plasmid pNS66, which contained an *S. coelicolor eshA* gene that lacked the putative cyclic nucleotide-binding domain, was constructed as follows. Two DNA fragments containing sequences upstream (820 bp) and downstream (920 bp) of the cyclic nucleotide-binding domain were amplified using primers ΔCNBD-F1 and ΔCNBD-R1 and primers ΔCNBD-F2 and ΔCNBD-R2, respectively. The PCR products were digested with PstI and ApaI and cloned into the PstI site of *E. coli-Streptomyces* shuttle vector pV1 by three-fragment ligation, resulting in pNS66. Plasmid pNS82, which contained *S. coelicolor eshA* fused to the *S. griseus eshA* promoter, was constructed as follows. The *S. griseus eshA* promoter (0.6 kb) and terminator (1.4 kb) regions were amplified from pBL52SG using primers M13 forward and f1-R and primers f2-F and M13 reverse, respectively. The *S. coelicolor eshA* open reading frame (1.3-kb) was amplified from pSCP52 using primers f3-F and f3-R. The three PCR-generated fragments were cloned into the BamHI site of pBluescript SK(+) to obtain plasmid pNS80. Finally, the 3.4-kb BamHI fragment from pNS80 was inserted into the BamHI site of pV1, resulting in pNS82.

**Site-directed mutagenesis of *eshA*.** Site-directed mutagenesis of *eshA* was performed with a Quickchange II site-directed mutagenesis kit (Stratagene) used according to the manufacturer's instructions. pTYM52SC was used as a template for all the mutagenesis reactions. The primers used in the reactions are shown in Table 2. After DNA sequence confirmation, each mutant *eshA* allele was introduced into the Δ*eshA* strain KO-350 for phenotypic analysis.

**Construction of *eshB* mutant.** A 1,130-bp *eshB* gene fragment was amplified from genomic DNA using primers eshB-F1 and eshB-R1 and cloned into TA cloning vector pCR2.1 (Invitrogen), resulting in pXU21. This plasmid was digested at the unique SalI site in the *eshB* coding region, treated with the Klenow enzyme, and religated to introduce a frameshift mutation into *eshB*. A 1.7-kb

TABLE 2. Primers used in this study

| Primer | Sequence[a] | Relevant enzyme |
|--------|-------------|-----------------|
| ΔCNBD-F1 | GCTCCTCCGGTGTCAC**CTGCAG**TCCCTTCG | PstI |
| ΔCNBD-R1 | GCAACCGTCGGTG**GGGCCC**GGTAGGC | ApaI |
| ΔCNBD-F2 | CG**GGGCCC**AGCGGCAGACCAAGCAC | ApaI |
| ΔCNBD-R2 | GGGGCGGGCACGTC**CTGCAG**GGTTGTGGGC | PstI |
| f1-R | CGAGTCAACAG**CCATGG**GGC | NcoI |
| f2-F | TGCAGATCGCC**AGATCT**CCCAGGTGAGG | BglII |
| f3-F | CTCC**CCATGG**CCGTCGACAG | NcoI |
| f3-R | GTGGGCGGTGCG**AGATCT**CAGCG | BglII |
| eshB-F1 | GAGCAACTCCTATTACTTGTTCG | |
| eshB-R1 | TACTCCCAGATGGCGTCA | |
| Act-F | ACCGATGCGGGATGTGTAATTCCG | |
| Act-R | GTGCGCGATATTGCTTTCGAGCAC | |
| relA/NdeI-F | CGCCTACAC**CATATG**CGCACCATGC | NdeI |
| relA/Bam-R | CG**GGATCC**TCGGTCTACTTCTGTCAGTCGAG CAGC | BamHI |
| eshA-Fw | CTCGTCAACAACCGCGAGTTC | |
| eshA-Rv3 | CGGAGTGGGCGATGTGTTG | |
| eshA-G127V-F | GTCCTCGTCGCACGCGTCGGCCCCGCAGAC | |
| eshA-G127V-R | GTCTGCGGGGCCGACGCGTGCGACGAGGAC | |
| eshA-G161M-F | GGTGCTCGCGGACATGAGCCACTTCGGC GAGC | |
| eshA-G161M-R | GCTCGCCGAAGTGGCTCATGTCCGCGAG CACC | |
| eshA-G165N-F | GACGGCAGCCACTTCAACGAGCAGGCGCTG TCGGAC | |
| eshA-G165N-R | GTCCGACAGCGCCTGCTCGTTGAAGTGGCT GCCGTC | |
| eshA-F196S-F | CTCACGCGCGCCGACTCCACCGCGGTCCAG | |
| eshA-F196S-R | CTGGACCGCGGTGGAGTCGGCGCGCGTGAG | |

[a] Boldface type indicates restriction enzyme sites.

BglII fragment from pIJ963 containing a hygromycin resistance cassette was introduced into this plasmid to generate pXU25. pXU25 was passed through *E. coli* GM2163 and introduced into *S. coelicolor* 1147 protoplasts. Single-crossover transformants were selected on R2YE medium containing hygromycin. After two rounds of nonselective growth on GYM medium, spores were plated to obtain single colonies and screened for hygromycin sensitivity. The expected frameshift mutation in *eshB* was confirmed by PCR and nucleotide sequencing. The *eshB* mutation constructed was introduced into strain KO-350 (*eshA*::*tsr*) by conjugation to generate the *eshA eshB* double mutant KO-542.

**RNA isolation and RT-PCR analysis.** RNA was isolated from cells grown on R5MS agar covered with cellophane sheets (42). DNase I-treated RNA (0.5 μg) was used as a template for reverse transcription (RT) at 65°C with the Thermo-Script RT-PCR system (Invitrogen) and a specific reverse primer, primer Act-R (62). The resulting cDNA was used for PCR amplification with primers Act-F and Act-R under the following conditions: 3 min at 96°C, followed by 25 cycles of 98°C for 10 s and 65°C for 30 s. DNase-treated RNA samples that had not been subjected to reverse transcription were used for PCR amplification as negative controls.

**Western blot analysis.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (43).

**Biacore assay.** The instrument used for a Biacore assay was a BIACORE 3000 (Biacore AB, Uppsala, Sweden), which is used for measurement of surface plasmon resonance in real time. CM5 sensor chips were first washed with HBS-EP buffer (10 mM HEPES [pH 7.4], 0.15 M NaCl, 3.0 mM EDTA, 0.005% surfactant P20; Biacore AB) for 7 min and then activated by mixing 70 μl of activating reagent with a mixture of 0.2 M 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide and 0.05 M *N*-hydroxysuccinimide prior to immobilization. 8-(6-Aminohexyl)aminoadenosine 3′,5′-cyclic monophosphate (a derivative of cAMP) was diluted to obtain a concentration of 1 mM with 10 mM borate buffer (pH 8.5), and the resulting solution (70 μl) was injected at a flow rate of 10 μl/min. Excess reactive groups were deactivated with 1 M ethanolamine. The blanks were running buffer and either the CM5 sensor surface or the surface treated with 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide–*N*-hydroxysuccinimide and ethanolamine without any added ligand. All buffers were filtered and degassed before use. Analysis of the sensorgrams was performed with the BIA-evaluation software (version 4.1), which allows a range of quantitative kinetic analyses and automatically calculates the binding parameters, taking into account negative control and experimental readings.

**Expression and purification of the *S. coelicolor* RelA protein.** Plasmid pPRO-relA, used for overexpression of the *S. coelicolor* RelA protein in *E. coli*, was constructed as follows. The *relA* gene was amplified from genomic DNA isolated from *S. coelicolor* using primers relA/NdeI-F and relA/Bam-R. The NdeI- and BamHI-digested PCR fragment was ligated into pPROEX-1. The recombinant RelA protein with an N-terminal His tag was purified on nickel-nitrilotriacetic acid matrices (QIAGEN). The RelA protein was further purified by SDS-poly-acrylamide gel electrophoresis using a 10% polyacrylamide gel and was extracted with phosphate-buffered saline containing 0.1% SDS. The purified RelA protein obtained (1.8 mg) was used to raise rabbit antibodies.

**Measurement of the intracellular nucleotide pools.** For plate culture assays, ca. $2 \times 10^7$ spores were spread on R5MS agar covered with a cellophane sheet. The methods used for extraction of intracellular nucleotides from mycelia which were grown on a cellophane sheet or in liquid medium have been described previously (36, 37). Nucleotide pool sizes were analyzed using a high-performance liquid chromatography system (Hitachi D-7000 HPLC series with an L-7100 pump and an L-7400 UV detector) with an ion-exchange column (Partisil-10 SAX; 4.6 mm by 25 cm; GL Science) as described previously (37). Elution was performed at a flow rate of 1 ml/min by using a gradient consisting of a low-ionic-strength buffer (7 mM $KH_2PO_4$, adjusted to pH 4.0 with $H_3PO_4$) and a high-ionic-strength buffer (0.5 M $KH_2PO_4$ and 0.5 M $Na_2SO_4$, adjusted to pH 5.4 with KOH). UV detection was performed at 260 nm. The following gradient conditions were used: the percentage of the high-ionic-strength buffer was increased during the initial 20 min from 0 to 100% and then was kept at 100% for the next 20 min, reduced from 100 to 0% in 3 min, and kept at 0% for 10 min to return to the initial conditions. The intracellular concentrations of nucleotides in cells grown on cellophane were expressed in pmol per mg (dry weight) of cells. A wet weight of 1 g was equivalent to a dry weight of 134 mg.

## RESULTS

**Disruption of *eshA* but not disruption of *eshB* eliminates actinorhodin production.** Although we previously reported that *eshA* disruption suppressed antibiotic production in *S. coelicolor* A3(2) (28), we found recently that the effect was more evident when cells were grown on R5MS agar instead of R2YE agar (Fig. 1A); production of the blue-pigmented anti-
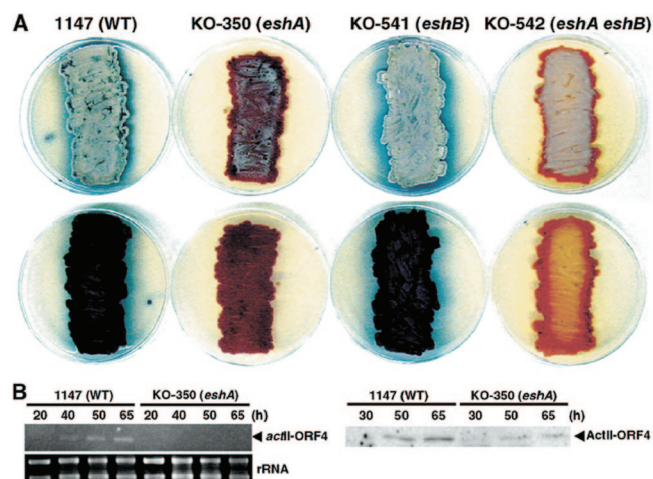


FIG. 1. (A) Effects of disruption of *eshA*, *eshB*, and *eshA* plus *eshB* on antibiotic production. Spores were inoculated onto R5MS agar plates, and this was followed by 4 days of incubation. The reverse sides of the plates (lower plates) are shown to better illustrate the amounts of antibiotic produced. The blue and red compounds are actinorhodin and undecylprodigiosin, respectively. (B) RT-PCR analysis (left panel) and Western blotting (right panel) for the *act*II-ORF4 transcript and ActII-ORF4 protein. rRNA indicates the position of 5 μg of total RNA. RNA and crude cell extracts were prepared from wild-type and mutant cells grown on R5MS agar for the times indicated. WT, wild type.
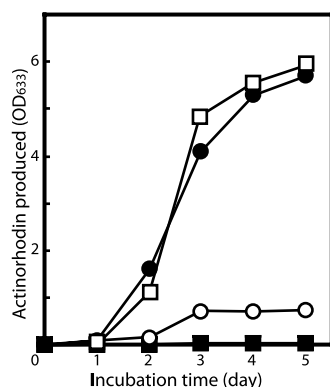
FIG. 2. Time course of actinorhodin production in R5MS liquid medium. The total actinorhodin produced within and outside cells was measured after treatment of the culture broth with 1 M NaOH. Symbols: ●, wild-type strain 1147; ○, *eshA* disruptant KO-350; □, *eshB* mutant KO-541; ■, *eshA eshB* double mutant KO-542. $OD_{633}$, optical density at 633 nm.

biotic actinorhodin was eliminated when *eshA* was disrupted (throughout 10 days of incubation), which also resulted in reduced formation of aerial mycelium. In contrast, production of the red-pigmented prodiginine antibiotic complex was not affected. Inspection of the genome sequence of *S. coelicolor* revealed a gene encoding EshB, a close homologue of EshA with 64% amino acid sequence identity. A frameshift mutation was introduced into *eshB* to assess its role in morphological and physiological differentiation. On R5MS medium, the *eshB* mutant produced both antibiotics at levels comparable to the levels produced by the wild-type strain (Fig. 1A). The *eshA eshB* double mutant KO-542 did not produce actinorhodin, but it appeared to produce the red-pigmented antibiotic at wild-type levels (lack of pigmentation in the middle of the KO-542 streak reflected lower levels of mycelial growth). Thus, *eshA*, but not *eshB*, is required for actinorhodin production on R5MS agar. Similar results were obtained when strains were grown in R5MS liquid medium (Fig. 2). Actinorhodin production is regulated positively by a pathway-specific regulatory protein, ActII-ORF4 (1, 14). Analysis of *act*II-ORF4 expression by RT-PCR and Western blotting revealed a dramatic decrease in the level of the *act*II-ORF4 transcript (Fig. 1B, left panel) and a marked decrease in the level of the ActII-ORF4 protein (Fig. 1B, right panel) in the *eshA* mutant compared to the wild-type strain, presumably accounting for the loss of actinorhodin production.

**eshA disruptant accumulates lower levels of ppGpp.** ppGpp plays an essential role in initiating the onset of antibiotic production in several *Streptomyces* spp., including *S. coelicolor* A3(2) (24, 38, 52). We therefore postulated that the *eshA* disruptant KO-350 might have an impaired ability to accumulate intracellular ppGpp. To test this hypothesis, the parental strain (strain 1147) and a disruptant strain (strain KO-350) were grown on R5MS agar, and changes in the levels of the intracellular nucleotide pool were monitored (Fig. 3A). While the growth of the two strains was virtually identical, the *eshA* disruptant KO-350 accumulated lower levels of ppGpp than parental strain 1147 accumulated through the early, middle, and late phases of growth. The GTP pool size decreased
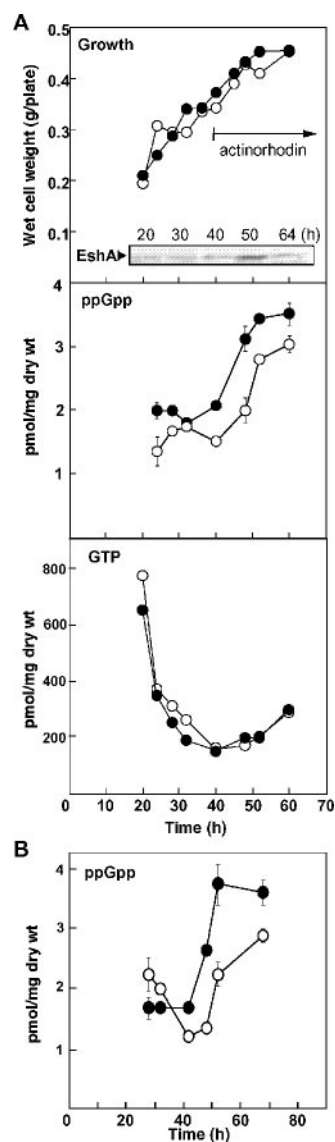


FIG. 3. Changes in the intracellular concentrations of ppGpp and GTP and levels of the EshA protein during growth on agar medium. (A) Strains of *S. coelicolor* A3(2) were grown on R5MS agar covered with a cellophane sheet. Cells were harvested at different times to determine biomass, nucleotide concentrations, and the amount of EshA produced. Nucleotides were separated by high-performance liquid chromatography, whereas EshA protein levels were determined by Western analysis. The error bars indicate the standard deviations of the means of triplicate samples. Symbols: ●, wild-type strain 1147; ○, *eshA* disruptant KO-350. (B) Strains of *S. griseus* were grown on TSB agar as described above for panel A. ppGpp levels were determined by high-performance liquid chromatography. Symbols: ●, wild-type strain 13189; ○, *eshA* disruptant KO-390.

sharply in both strains as growth progressed, presumably reflecting curtailment of purine nucleotide synthesis due to the reduced availability of substrates from the medium.

Like disruption of *eshA* in *S. coelicolor* A3(2), disruption of *eshA* in *S. griseus* resulted in the loss of antibiotic (streptomycin) production (47). Thus, it was possible that an *S. griseus* *eshA* disruptant would also exhibit an impaired ability to accumulate ppGpp. Growth of wild-type *S. griseus* and growth of

a congenic *eshA* mutant on TSB agar (a medium that supports streptomycin production) demonstrated that the *eshA* disruptant, like its *S. coelicolor* A3(2) counterpart, did indeed have a reduced ability to accumulate ppGpp during the middle and late growth phases (40 to 70 h) (Fig. 3B). Thus, *eshA* disruptants of both *S. coelicolor* and *S. griseus* accumulate lower levels of ppGpp, which in turn might result in loss of antibiotic production. To assess whether accumulation of reduced levels of ppGpp might reflect lower levels of the ppGpp synthetase RelA, we also determined the level of RelA in *S. coelicolor* 1147 (wild type) and KO-350 (*eshA* mutant) by Western blotting of proteins derived from a P100 precipitate (ribosomal) fraction (RelA exists predominantly in a ribosome-associated form [5]). RelA was detected throughout growth; while the level of RelA decreased in the late growth phase (i.e., 40 h), there were no significant differences between 1147 and the *eshA* disruptant (data not shown).

**Induction of ppGpp synthesis in the *eshA* mutant restores actinorhodin production.** To assess whether reduced levels of ppGpp were primarily responsible for the *eshA* phenotype, we used the method of Sun et al. (52) to induce elevated levels of ppGpp during growth. pIJ6084 contains the truncated *S. coelicolor relA* gene (and thus produces a ribosome-independent RelA) under control of the thiostrepton-inducible *tipA* promoter. pIJ6084 and the pIJ8600 vector control were introduced into *eshA* disruptant KO-350, and the strains were grown on R5MS agar lacking or containing 0.05 μg/ml of thiostrepton. Production of actinorhodin was completely restored in the presence of pIJ6084 but not in the presence of pIJ8600, but this occurred only when cells were grown in the presence of thiostrepton (Fig. 4A). Lower or higher concentrations of thiostrepton were less effective for restoring actinorhodin production (data not shown). We next determined ppGpp levels in mycelium harboring pIJ6084 or pIJ8600 that had been grown in the presence of thiostrepton. Cells harboring pIJ6084 had a higher level of ppGpp than cells containing pIJ8600 in both early-growth-phase (20 h) and late-growth-phase (50 h) cultures (Fig. 4B); the levels observed in induced cultures containing pIJ6084 were comparable to the levels seen in wild-type strain 1147 (Fig. 3A). Thus, although relatively low, the reduced level of ppGpp in the *eshA* mutant appeared to be solely responsible for the observed defect in antibiotic production. Further evidence that supports this conclusion was derived from introduction of particular *rpoB* mutations into the *eshA* disruptant. Recent X-ray crystallography of the RNA polymerase-ppGpp complex demonstrated unambiguously that there is binding of ppGpp adjacent to the RNA polymerase active center (2). We reported previously that the loss of actinorhodin production observed in *relA* or *relC* mutants (which are deficient in ppGpp synthesis) could be suppressed by introducing particular mutations into the *rpoB* gene that encodes the β subunit of RNA polymerase, which is believed to be a primary target of ppGpp in eliciting changes in gene expression (40, 62). Therefore, we assumed that introduction of certain *rpoB* mutations into KO-350 should restore actinorhodin production to levels that exceed the level in the wild-type strain. To assess this assumption, we isolated 25 rifampin-resistant (*rif*) mutants of KO-350, which developed spontaneously on the plates containing rifampin (see Materials and Methods), since *rpoB* mutations have been found frequently among *rif* mutants
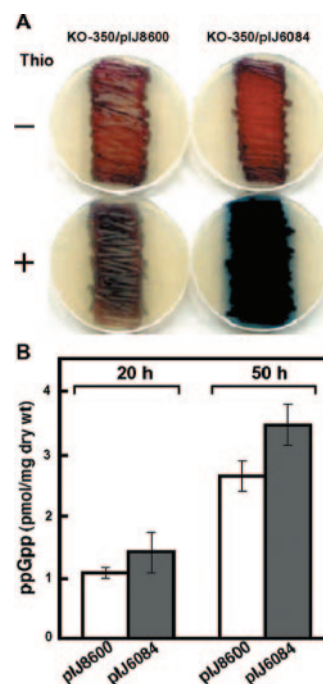


FIG. 4. Effect of inducing the truncated *relA* gene on antibiotic production (A) and intracellular ppGpp pool size (B). Plasmid pIJ6084 harbors a truncated (ribosome-independent) *relA* gene under control of the *tipA* promoter, while pIJ8600 is the vector control. Cells were grown on R5MS agar plates to the early growth phase (20 h) or late growth phase (50 h) with or without thiostrepton (0.05 μg/ml) (Thio), as described in the legend to Fig. 1. The ppGpp pool size was determined as described in the legend to Fig. 3. The error bars indicate the standard deviations based on data from six independent experiments.

(16, 22, 23, 32, 49, 51, 59). We found that the *rif* mutants examined all had a point mutation or a deletion mutation in the *rpoB* gene. Of the 25 isolates, 12 exhibited complete restoration of actinorhodin production (or produced even higher levels than the parental strain produced), and this was accompanied by restoration of *act*II-ORF4 transcription, as determined by RT-PCR. The effective *rpoB* mutations were 416F→L, 427D→N, 440R→C, 475P→L, deletion 410V, deletion 414K–416F, and deletion 426M, whereas other mutations (427D→G, 427D→A, 433S→T, and 485S→F) did not restore actinorhodin production. One of the effective mutations, the mutation in KO-351 (427D→ N), is shown in Fig. 5A and B.

**EshA exerts its influence via a nucleotide-binding domain.** The EshA proteins of *S. coelicolor* and *S. griseus* exhibit 76% amino acid identity and are characterized by the presence of a putative cyclic nucleotide-binding domain (28, 47) that is absent from the otherwise homologous proteins MMPI and SrpI (35, 61). To investigate if the EshA domain is essential for restoration of antibiotic production, the putative cNMP-binding domain of *S. coelicolor* A3(2) was deleted (Fig. 6A), yielding pNS66. pNS66 and pV52SC (harboring the entire *eshA* gene) were introduced separately into the *eshA* disruptant KO-350, and the resulting strains were grown on R5MS agar. While pV52SC restored actinorhodin production to wild-type levels, pNS66 did not, despite the production of wild-type levels of the deleted EshA derivative (delEshA) (35 kDa compared to the 52-kDa wild-type protein) (Fig. 6B, C). Thus, the putative
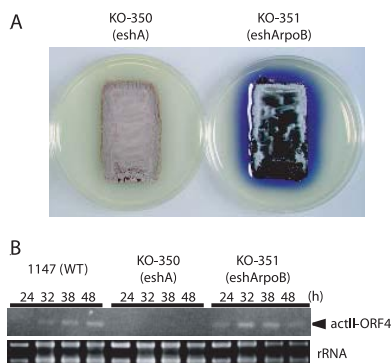
FIG. 5. Effect on actinorhodin production of introducing an *rpoB* mutation into the *S. coelicolor eshA* mutant (A) and RT-PCR analysis of the *act*II-ORF4 transcript (B). Strains were grown on R5MS agar as described in the legend to Fig. 1. rRNA indicates the position of 5 μg of total RNA. RNA was isolated from cells grown for different times. WT, wild type.



FIG. 7. Effect of the *eshA*[F196S] mutation on actinorhodin produc-tion. Δ*eshA* cells complemented by either the wild-type or F196S mu-tant *eshA* allele were grown for 5 days on the R5MS agar plate. Wild-type and Δ*eshA* strains harboring the empty vector were included as controls. WT, wild type.

cNMP-binding domain is essential for EshA functioning. As observed for the native EshA protein, the majority of the domain-deleted EshA was recovered in the P100 precipitate fraction when it was examined by Western analysis (data not shown), probably reflecting the formation of multimers, as shown previously for *S. griseus* EshA (47). Neither EshA nor the deleted derivative delEshA was detected in the S100 su-pernatant or membrane fraction.

To demonstrate further the functional importance of the cNMP-binding domain of EshA, we used site-directed mu-tagenesis to replace several highly conserved amino acids (28). We created four mutant alleles, G127V, G161M, G165N, and F196S. One of the mutants, the F196S mutant, failed to com-plement the Δ*eshA* mutation, as assessed by actinorhodin pro-
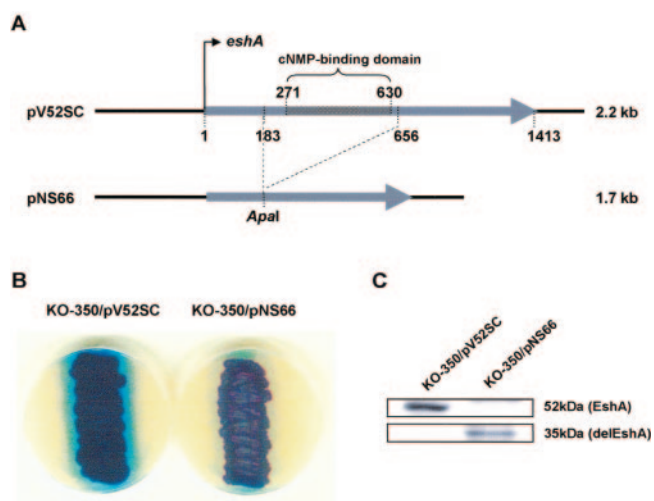


FIG. 6. Effect of deleting the cyclic nucleotide-binding domain of EshA on actinorhodin production in *S. coelicolor* A3(2). (A) pV52SC contains the entire *eshA* gene, while pNS66 lacks the nucleotide-bind-ing domain. (B) Growth of KO-350 harboring pV52SC or pNS66 on R5MS agar after incubation for 3 days. (C) Western analysis of EshA and a deleted derivative (delEshA). Cells were grown to the late growth phase (50 h) on R5MS agar plates. Western blotting was performed using the whole-cell extract.
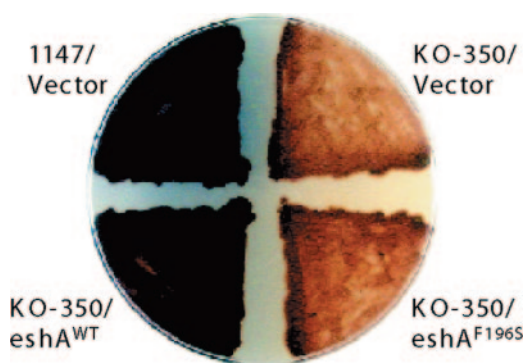
duction (Fig. 7), demonstrating that the cNMP-binding do-main is essential for EshA functioning. The other three mutants produced actinorhodin at the same level as the *eshA* wild-type allele (data not shown).

**Kinetic study of the interaction between EshA and cAMP.** To study the kinetics of binding of cAMP to EshA, surface plasmon resonance was used. The method of Wilchek and Selinger (60) was employed with a cAMP derivative and EshA monomer (purified by gel filtration) (see Materials and Meth-ods and reference 47 for details). The cAMP derivative bound to EshA (Fig. 8A) with an association rate constant of 6.9 $M^{-1}$ $s^{-1}$ and a dissociation rate constant of 0.0002 $s^{-1}$, as estimated by fitting the binding curves to the Langmuir binding model. The dissociation equilibrium constant was 28.9 μM.

The association of EshA with the cAMP derivative was elim-inated by external addition of 400 to 800 μM cAMP (Fig. 8B), whereas this association was not affected by any other nucle-oside triphosphate, diphosphate, or monophosphate, including cyclic GMP and ppGpp (each added at a concentration of 1 mM). AMP had a slight effect when it was added at a concen-tration greater than 500 μM. Thus, EshA appears to bind specifically to cAMP.

**EshA from *S. coelicolor* does not function in *S. griseus*.** Pre-viously, we failed to detect phenotypic expression of *S. coeli-color eshA* in *S. griseus* and speculated that this might reflect inactivity of the *S. coelicolor eshA* promoter in *S. griseus* (47). To address this issue further, we constructed a plasmid in which the coding sequence of *S. coelicolor eshA* was located downstream of the *S. griseus eshA* promoter. The resulting plasmid, pNS82, was introduced into the *S. griseus eshA* dis-ruptant KO-390. As expected, the pNS82 transformant of KO-390 produced abundant amounts of EshA, as determined by Western analysis (Fig. 9C). Despite the marked accumulation of EshA, neither aerial mycelium formation (Fig. 9A) nor streptomycin production (Fig. 9B) was restored in the *S. griseus eshA* disruptant (47). In contrast, introduction of pV52SG (harboring the *S. griseus eshA* gene) into KO-390 resulted in the formation of abundant aerial mycelium (Fig. 9A) and ex-tensive production of streptomycin (47; data not shown). Thus, despite the high level of amino acid sequence identity to its homologue and assumed orthologue, EshA from *S. coelicolor*
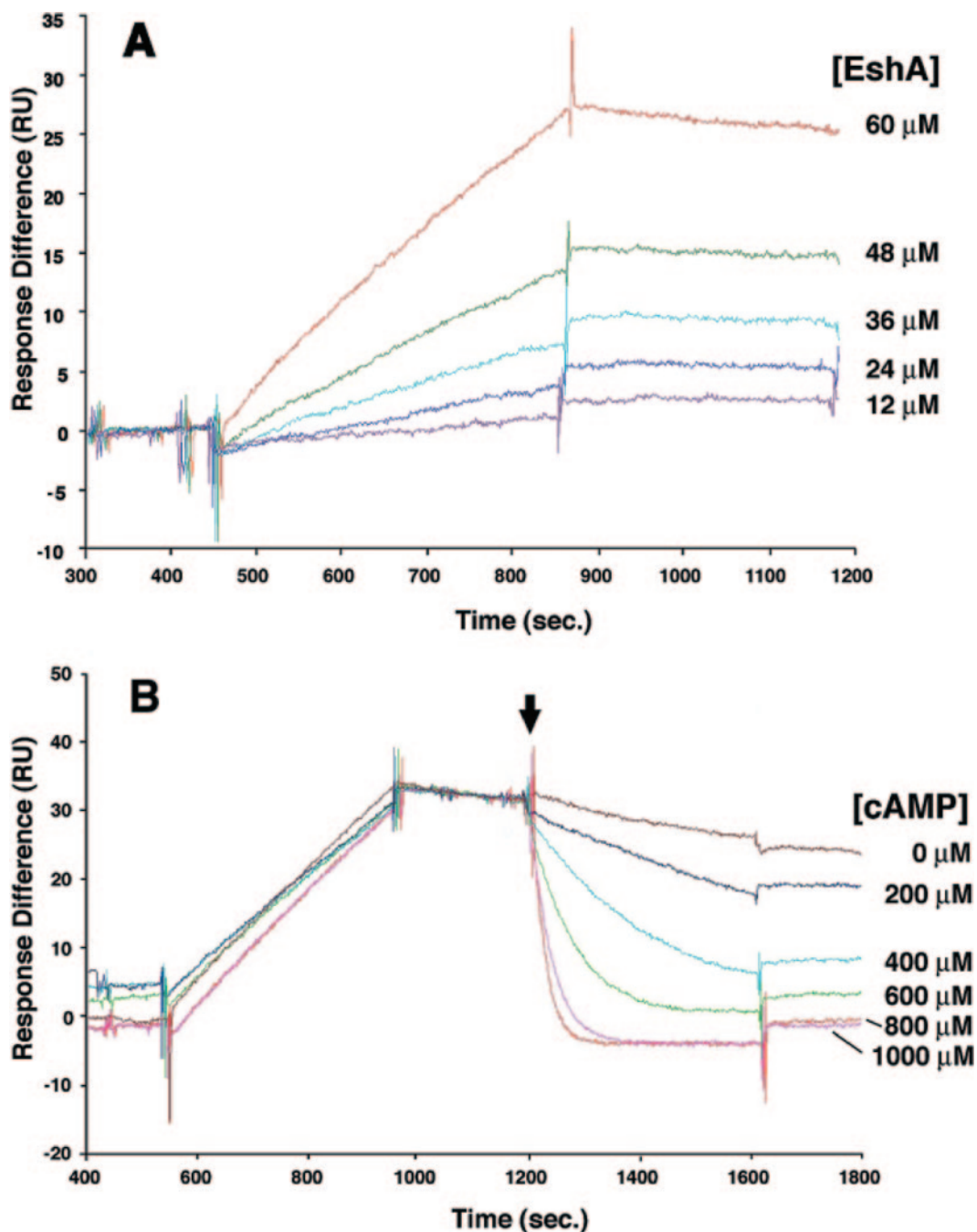
FIG. 8. Biacore analysis of the interaction between EshA and an immobilized cAMP derivative (A) and inhibition of EshA binding by externally added cAMP (B). The data were fitted globally to a 1:1 Lagmuir interaction model. The arrow indicates the time of external addition of cAMP at the concentrations indicated on the right. RU, resonance unit.

is not able to function in *S. griseus*, at least in the context of morphological and physiological differentiation.

**Temperature-induced deletion of *eshA* in *S. coelicolor* results in loss of actinorhodin production.** Chromosome instability is a characteristic of many *Streptomyces* species, in which deletions of up to 2 Mb have been observed (11), and it is believed to account for at least some instances of loss of antibiotic production. The apparent absence of essential genes in the less conserved terminal regions of the linear chromosomes of

streptomycetes may well predispose these regions to deletion and rearrangement, events that may be induced by a variety of physiological stresses. *eshA* is located close to one of the ends of the *S. coelicolor* A3(2) chromosome (130 kb from the end; located on cosmid 1A4 [46]). To examine whether *eshA* was prone to such a deletion event, we grew *S. coelicolor* at 42°C (the highest temperature that allowed growth) for 7 days on GYM agar. A total of 150 randomly selected colonies were assayed for the presence of *eshA* by PCR using primers
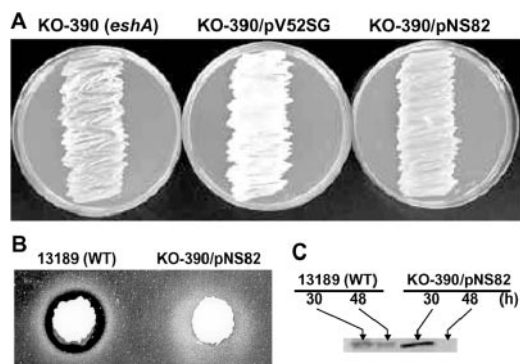
FIG. 9. Expression of *S. coelicolor* A3(2) *eshA* in the *S. griseus eshA* disruptant KO-390. pV52SG harbors *S. griseus eshA*, while pNS82 harbors *S. coelicolor eshA* expressed from the promoter of *S. griseus eshA*. (A) Strains were grown on TSB agar for 2 days. The white appearance of the growth in the middle plate reflects the development of aerial mycelium. (B) Streptomycin production by *S. griseus* wild-type strain 13189 and the *eshA* disruptant KO-390 harboring pNS82. Agar plugs (diameter, 8 mm) were cut from each of the TSB agar plates after 48 h of incubation and placed on the assay plate, and this was followed by 12 h of incubation. (C) Western analysis of EshA. The strains were grown on TSB agar plates for 30 h or 48 h. A Western analysis was performed as described in the legend to Fig. 6 using whole-cell extracts. WT, wild type.

eshA-Fw and eshA-Rv3, and 18 of these isolates (12%) lacked *eshA*. Of the smaller-colony types found in these 150 colonies, 55% had lost *eshA*. All of the deletion mutants, like the *eshA* disruptant KO-350, were totally or severely impaired for actinorhodin production on R5MS agar. Moreover, actinorhodin production was restored upon introduction of pV52SC harboring *eshA* (data not shown), suggesting that the loss of antibiotic production in the temperature-induced deletion mutants was attributable solely to deletion of *eshA* and not to deletion of flanking genes.

## DISCUSSION

We demonstrated that EshA plays a crucial role in establishing an intracellular level of ppGpp sufficient to trigger actinorhodin production in *S. coelicolor* A3(2) at least under some nutrient conditions and that the cyclic nucleotide-binding domain of EshA is essential for this activity. Thus, as demonstrated in this study (Fig. 1) and in previous work (28, 47), EshA functions as a positive regulator for antibiotic production in both *S. coelicolor* and *S. griseus*. Strikingly, relatively small differences in ppGpp pool sizes resulted in dramatic changes in antibiotic productivity (Fig. 4). This suggests that there is a threshold level of ppGpp that is required for triggering secondary metabolism and that ppGpp concentrations in the cell can be finely tuned by factors such as EshA. Thus, at least some of the physiological instability that is sometimes associated with antibiotic production in streptomycetes could be attributable to factors that influence EshA activity. Despite a high level of amino acid sequence identity, EshA of *S. coelicolor* was unable to complement an EshA mutant of *S. griseus*. However, there are other functional differences between the two proteins. While EshA plays a role in sustaining DNA synthesis

during the late growth phase in *S. griseus*, no such function is evident for its homologue in *S. coelicolor* (47).

Disruption of *eshA* resulted in the elimination of antibiotic production in both *S. coelicolor* and *S. griseus*, but only in some culture conditions. This conditional phenotype is reminiscent of that observed for ppGpp itself, which is required for triggering antibiotic production under nitrogen limitation conditions but not under phosphate limitation conditions (6, 41). Interestingly, a *relA* null mutant of *S. coelicolor* also does not produce actinorhodin on R5MS agar (unpublished data), mimicking the phenotype of the *eshA* mutant, which is consistent with our interpretation that EshA has a role in modulating ppGpp levels. Although not required for antibiotic production under any of the growth conditions used in these experiments, we cannot rule out the possibility that EshB plays a role in triggering antibiotic production under other conditions. A recent proteomic analysis of *S. coelicolor* demonstrated that the level of SCO5249 (= EshB) was dramatically reduced in a *bldA* mutant (30).

*eshA* is located close to one end of the linear *S. coelicolor* A3(2) chromosome, in a region of the genome predicted to be more susceptible to deletion and rearrangement. Indeed, when organisms were subjected to elevated growth temperatures, no fewer than 12% of the resulting colonies had lost *eshA* and did not produce actinorhodin, suggesting that loss of *eshA* (if it is located near the end of the chromosome of other streptomycetes) may account for the genetic instability of antibiotic production observed in many different streptomycetes.

EshA appears to be a member of a protein superfamily (31, 57) whose activities are modulated by binding cyclic nucleotides (33, 50), and the cyclic nucleotide-binding domain of EshA is clearly required for activity (Fig. 6 and 7). Moreover, we successfully demonstrated binding of cAMP to EshA with high specificity (Fig. 8). In prokaryotes, only Crp and DnaA are known to bind cAMP (4, 8, 18). *dnaA* of *E. coli* is essential for the initiation of DNA replication at the chromosomal origin, *oriC* (58), and interaction of DnaA with *oriC* is stimulated by binding of cAMP (13). Recent studies with *Streptomyces* spp. (26, 54) have demonstrated that extracellular levels of cAMP are high during the late growth phase, prompting speculation that cAMP is involved, directly or indirectly, in the onset of secondary metabolism. Moreover, Kang et al. (26) reported that addition of a low level of cAMP enhanced the formation of aerial mycelium by the *S. griseus* mutant HO2 (although accumulation of very high levels of cAMP, mediated through a cloned copy of the adenylate cyclase gene *cyaA*, eliminated both formation of aerial mycelium and production of streptomycin during the late growth phase, accompanied by severely reduced accumulation of ppGpp). The stimulatory effect on the formation of aerial mycelium by a low level of exogenous cAMP may be accounted for, at least partially, by activation of EshA via cAMP binding to the cAMP-binding domain.

*eshA* (or its homologue) genes are widely distributed and occur in streptomycetes (28), including *S. griseus* and *Strepotmyces avermitilis*, the actinobacterium *Frankia* sp. strain EAN1pec, and the cyanobacterium *Nostoc punctiforme* PCC73102, as determined by searching the nucleotide sequence database, although the *eshA* gene was not found in *E. coli*, *Bacillus subtilis*, and *Thermus thermophilus*. Therefore, EshA may offer new opportunities for uncovering and analyzing the unknown regulatory events that occur

at the onset of the stationary phase in these physiologically and morphologically complex organisms.

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