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Differential Stringent Responses of *Streptomyces coelicolor* M600 to Starvation of Specific Nutrients

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Abstract This study focused on the involvement of the unusual nucleotide (p)ppGpp, a stringent factor, during the morphological and physiological differentiation of *Streptomyces coelicolor*. Two genes, *relA* and *rshA*, were disrupted to demonstrate the roles of the stringent factor in the differentiation. The intracellular concentration of (p)ppGpp in the wild-type (M600) and disrupted mutants was measured in relation to the intentional starvation of a specific nutrient, such as carbon, nitrogen, and phosphate or the *in situ* depletion of nutrients in a batch culture. As a result, it was found that the morphological characteristic of the *ΔrelA* mutant was a *bld* phenotype forming condensed mycelia, whereas the *ΔrshA* mutant grew fast-forming spores and straightforward mycelia. In both mutants, the production of actinorhodin (Act) was completely abolished, yet the undecylprodigiosin (Red) production was increased. Intracellular (p)ppGpp was detected in the *ΔrelA* mutant in the case of limited phosphate, yet not with limited carbon or nitrogen sources. In contrast, (p)ppGpp was produced in the *ΔrshA* mutant under limited carbon and nitrogen conditions. Therefore, (p)ppGpp in *S. coelicolor* was found to be selectively regulated by either the RelA or RshA protein, which was differentially expressed in response to the specific nutrient limitation. These results were also supported by the *in situ* ppGpp production during a batch culture. Furthermore, it is suggested that RelA and RshA are bifunctional proteins that possess the ability to both synthesize and hydrolyze (p)ppGpp.

Key words: (p)ppGpp, *relA*, *rshA*, actinorhodin, clavulanic acid, cephamycin C

Streptomycetes are Gram-positive bacteria that exhibit unique morphological differentiation, forming filamentous mycelia and exospores. The morphological differentiation in the microorganism normally coincides with the production of antibiotics (physiological differentiation) initiated under nutrient starvation in the culture medium [4, 13, 14, 26, 28]. Although microorganisms adapt to nutrient limitations in different ways, it is thought that the appearance of (p)ppGpp is the first response when microorganisms encounter a nutrient-limited environment, making (p)ppGpp a key factor in regulating the metabolism of microorganisms facing stringent culture conditions [3].

The metabolism of (p)ppGpp in *E. coli* is regulated by two proteins; (p)ppGpp synthetases (RelA protein) and ppGpp synthetase/hydrolases (SpoT protein) [22, 23]. It has already been established that the RelA protein in *E. coli* binds to ribosomes, senses the amount of uncharged tRNAs, and then synthesizes (p)ppGpp under conditions of limited amino acid, yet does not degrade (p)ppGpp [9]. The SpoT protein (ppGpp synthetase/hydrolase) in *E. coli* is a cytosolic bifunctional enzyme that synthesizes (p)ppGpp in a ribosome-independent mode under limited carbon or phosphate conditions, yet also degrades (p)ppGpp. However, the synthetic activity of the SpoT protein is obscured by its more abundant degrading activity [7, 25]. Other proteins, named Rel and RshA (because of their RelA/SpoT homology), have also been identified from the Actinobacteria and *Bacillus/Clostridium* groups, and found to be bifunctional enzymes for synthesizing and degrading ppGpp [24]. However, since the proteins involved in (p)ppGpp metabolism were originally named without any rationale concerning their activity, a new systematic nomenclature was proposed based on the comparative genomics of the genes encoding the proteins [34]: first (p)ppGpp synthetase I (represented by RelA in *E. coli*), second (p)ppGpp synthetase II (represented by SpoT in *E. coli*), and third (p)ppGpp synthetase III (originally named Rel or Rsh). The comparative analysis revealed that the *relA* and *spoT* genes were found in the β- and γ-subdivisions of proteobacteria, respectively, whereas the *spoT* gene was related to *rel* and *rsh* encoding the Rel and Rsh proteins,

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respectively, in the Actinobacteria and *Bacillus/Clostridium* groups [24].

It is believed that the stringent factor (p)ppGpp plays an important role in the secondary metabolism and morphological differentiation of *Streptomyces* spp. [1, 10, 12, 16, 25, 36, 26, 31]. The two genes, *relA* and *rshA*, involved in (p)ppGpp metabolism were already identified in *Streptomyces coelicolor* [5, 10, 32, 33], where a *ΔrelA* mutant of *S. coelicolor* was deprived of antibiotic production and morphological differentiation under nitrogen-limited culture conditions, yet not under phosphate-limited culture conditions [10]. However, no experimental evidence is available to indicate whether RshA is a bifunctional enzyme like RelA [31]. More recently, the two genes, *relA* and *rshA*, were also identified in *Streptomyces clavuligerus* [15], in which case it was reported that the *relA* gene was essential for morphological and physiological differentiation under nitrogen-limited culture conditions, whereas the *rsh* gene responded more apparently in a phosphate-limited culture of *S. clavuligerus* [13, 31].

Accordingly, the current study attempted to differentiate the molecular responses of the *relA* and *rshA* genes in *S. coelicolor* M600 to the starvation of a specific nutrient, such as amino acid, glucose, and phosphate. Additionally, to demonstrate the involvement of (p)ppGpp in the development of the phenotypic characteristics of *S. coelicolor*, the (p)ppGpp production profiles were compared after a forced or *in situ* downshift of a specific nutrient during a batch cultivation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Maintenance

Streptomyces coelicolor M600, a prototrophic plasmid-free strain, and two derivatives of M600, *ΔrelA*, and *ΔrshA* mutant strains, were used in the experiments. To maintain the *Streptomyces* strains, spores formed on a mineral salt agar medium (MS) were harvested and suspended in 20% (v/v) glycerol, and the spore suspension stored at −70°C [11]. *E. coli* DH5α was used as the host cell for the propagation of cosmids and plasmids, *E. coli* BW25113/pIJ790 was used as the host for the PCR-targeted disruption of the target genes [8, 20], and *E. coli* ET12567 [21] was used to obtain methylation-negative DNA. The *E. coli* strains were all grown in a Luria-Bertani medium containing appropriate antibiotics for maintaining plasmids and cosmids. The plasmid pIJ773 was used to amplify the apramycin-resistance cassette to disrupt the target genes, *relA* and *rshA*, and pUZ8002, a helper plasmid, was used for the conjugation in *E. coli*.

Deletion of *relA* and *rshA* in *S. coelicolor*

The genes for *relA* (SCO1513) and *rshA* (SCO5794) were disrupted using a PCR-targeting method, as described in Gust *et al.* [8, 20]. The synthetic oligonucleotides, *relA*-F (5'-TGACCGGGGGACCCGGATCGCGCACGAGGAC-TCTTGATTCCGGGGATCCGTCGACC-3'), *relA*-R (5'-CGGGAGCCCCTCTAGGTGCGCTGTGCGGCCCGCT-ATGTAGGCTAGAGCTTC-3'), *rshA*-F (5'-TTCCCCGT-CCGCCGGTCCGCGCAGCCGGAGCTCCCCATGATT-CCGGGGATCCGTCC-3'), and *rshA*-R (5'-CGGGAGCC-CCTCTAGGTGCGCCTGTGCGGCCCGCTATGTAGGC-TAGAGCTTC-3') were used to amplify the apramycin-resistance cassette, which was then introduced to *E. coli* BW25113/pIJ790 containing cosmid StL2 and St4H2, respectively. Pre-induced l RED functions with the addition of L-arabinose to obtain a target-gene-disrupted version of the mutant cosmid.

The disrupted cosmids were isolated and transferred via *E. coli* ET12567/pUZ8002 to *S. coelicolor* by conjugation. The exconjugants were then selected on an MS agar containing apramycin (50 mg/ml), and the products of the double crossovers selected by screening for sensitivity to kanamycin (50 mg/ml). The disruption mutants were confirmed by Southern hybridization. The synthetic oligonucleotides *relA*P-F (5'-ACAGGCGCACACACGAGAGGGGCT-3'), *relA*P-R (5'-AGCAGTCGGAGAACC TCAAGCTCA-3'), *rshA*P-F (5'-ACAGGCCACACGAGAGGGGCT-3'), and *rshA*P-R (5'-AGCAGT CGGAGAACCTCAAGCTCA-3') were all used to amplify the probe DNA, and the DNA fragments purified from an agarose gel using a Gel Extraction Kit (QIAGEN Co.). The genomic DNA was purified from *S. coelciolor* using a salting-out procedure [30], whereas the transformation of *Streptomyces* with the plasmid and cosmid DNA was carried out as described by Kieser *et al.* [19]. A DIG DNA Labeling and Detection Kit (Roche Co.) was used to label the probe DNA.

Analysis of Intracellular (p)ppGpp Levels

The stored spores (10^8) were inoculated into a rich medium (GYM) containing 0.4% (w/v) glucose, 0.4% yeast extract, 1.0% malt extract, 0.1% NZ-amine, 0.2% NaCl, and a 0.1% (v/v) inorganic salts mixture. The inorganic salts mixture consisted of 0.0025% $MgSO_4 \cdot 7H_2O$, 0.0008% $CuSO_4 \cdot 5H_2O$, 0.0015% $FeSO_4 \cdot 7H_2O$, 0.0008% $MnSO_4 \cdot 4H_2O$, 0.003% $CaCl_2 \cdot 2H_2O$, and 0.002% $ZnSO_4 \cdot 7H_2O$. The culture grown in the seed culture medium was inoculated into the rich medium and cultured at 30°C on a rotary shaker (150 rpm). The seed cultures (2.5 ml) were also inoculated into 100 ml of a chemically defined (CD) medium supplemented with 1% casamino acid. The CD medium consisted of 2% (w/v) glucose, 0.3% $(NH_4)_2SO_4$, 0.52% K_2HPO_4, 0.24%, NaH_2PO_4, 0.05% NaCl, 0.005% KCl, 0.005% $MgSO_4 \cdot 7H_2O$, and 0.0001% $ZnSO_4 \cdot 7H_2O$ (pH was adjusted to 7.0 before steam sterilization). The cultures growing in the CD medium containing 1% casamino acid were harvested during the exponential growth phase by filtration (Whatman GF/C filters), washed rapidly with

the CD medium, resuspended in the CD medium without a specific element (glucose, casamino acid, or phosphate), and quickly returned to the shaking incubator. Following the downshift, half of the culture samples were harvested after 0, 5, 10, 15, 30, and 60 min, and then filtered. The other half was used for calculating dry cell weight (DCW). The filter cakes were transferred to 10 ml of formic acid, incubated at 4°C for 1 h, and centrifuged for 10 min at 6,000 ×g. The supernatants (formic acid-extractable fractions) were clarified by filtration using a 0.45-mm filter, and freeze-dried. The amount of ppGpp, pppGpp, and GTP was determined by HPLC using a Partisil-10 SAX (4.6× 250 mm) column (Whatman), as described previously [26, 27]. GTP (Sigma Co.), which were used as the standard for measuring the concentration of (p)ppGpp.

Culture Conditions for Antibiotic Production

The stocked spore suspension (10^8) was inoculated into 25 ml of a GG1 medium contained in a baffled flask (250 ml) and cultured at 30°C for 2 days using a shaking incubator. The GG1 medium consisted of 15 g glucose, 15 g glycerol, 15 g soya peptone (Difco), 3 g NaCl, and 1 g $CaCO_3$ per 1 l of distilled water. Next, 4 ml of the first seed culture was inoculated into 50 ml of a GYB medium in 500-ml flasks and cultured at 30°C for 2 days using a shaking incubator. The GYB medium consisted of 37.5 g

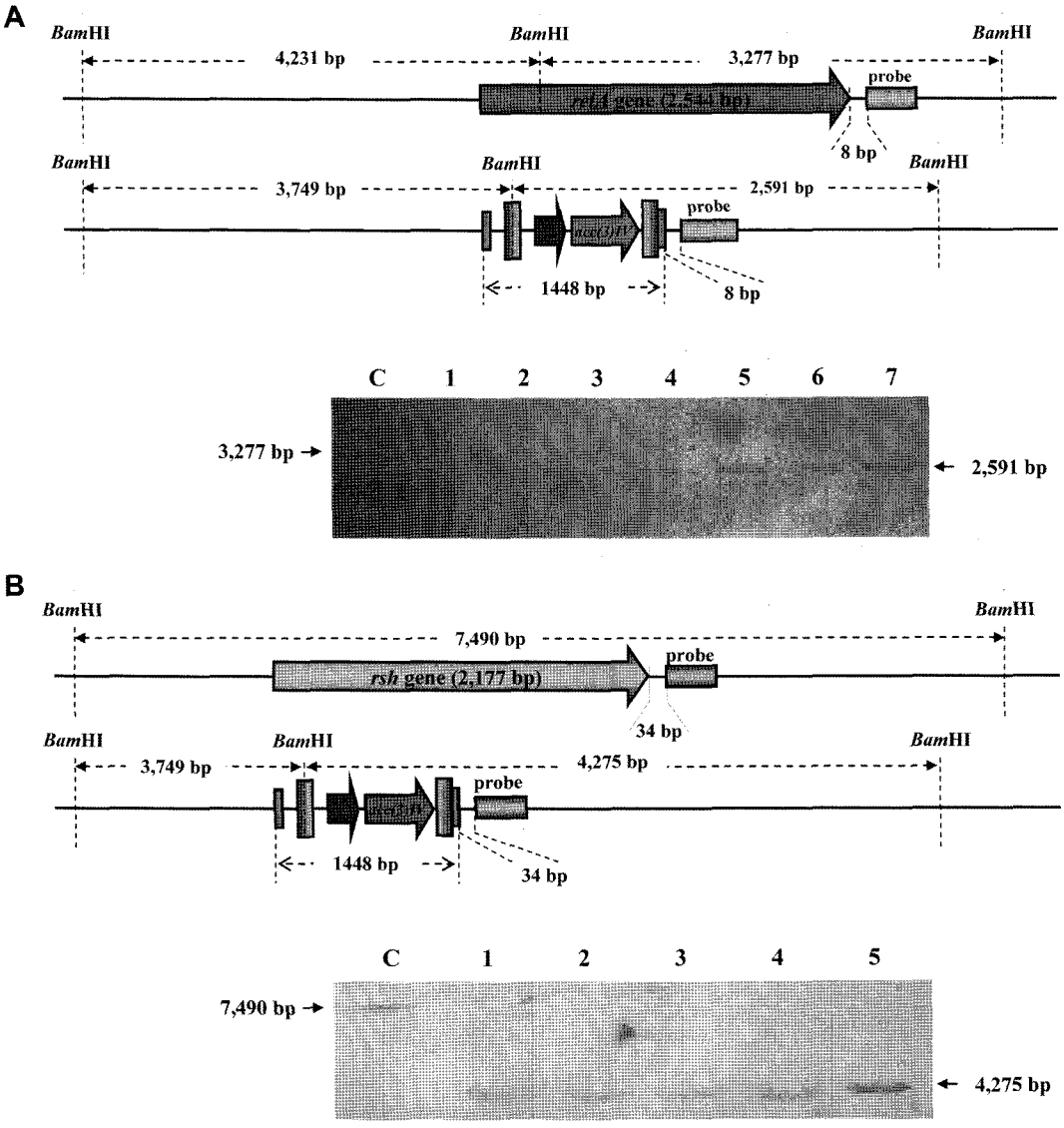

Fig. 1. Southern hybridization verifying disruption of *relA* and *rshA* in *S. coelicolor* M600.

The Southern hybridization indicates different patterns of digestion by BamHI after disruption of the *relA* and *rshA* genes. **A.** The 3,277-bp DNA fragment contains a partial *relA* gene from the wild-type, whereas the 2,591-bp DNA fragment represents an aparamycin-resistance cassette in replacement of *relA*. **B.** The 7,490-bp DNA fragment contains a partial *rshA* gene from the wild-type, whereas the 4,275-bp DNA fragment represents an aparamycin-resistance cassette in replacement of *rshA*.

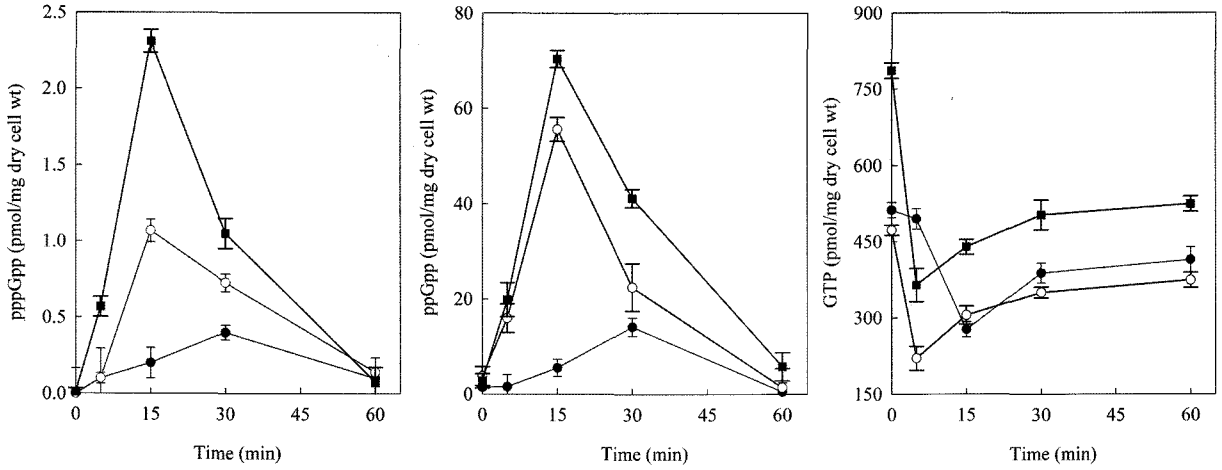

Fig. 2. Detection of pppGpp, ppGpp, and GTP from *S. coelicolor* M600 under glucose-limitation (●), amino acid-limitation (○), and phosphate-limitation (■) conditions.
After the downshift from CD to the medium without specific elements (1% glucose, 1% casamino acid, or 0.05 M phosphate), culture samples taken after 0, 5, 15, 30, and 60 min were analyzed for the amount of intracellular pppGpp, ppGpp, and GTP, using HPLC.

glucose and 15 g yeast extract (Difco) per liter of distilled water. Thereafter, 200 ml of the second seed culture was inoculated into 2 l of a supplemented minimal medium (SMM) contained in a jar fermentor (2 l, KoBioTech Co.), where the SMM consisted of 10 g glucose, 2 g casamino acid (Difco), 0.060 g NaH_2PO_4, 0.088 g K_2HPO_4, 0.6 g $MgSO_4$, 0.0001 g $ZnCl_2 \cdot 7H_2O$, 0.0001 g $FeSO_4 \cdot 7H_2O$, 0.0001 g $MnCl_2 \cdot 4H_2O$, 0.0001 g $CaCl_2 \cdot 6H_2O$, 0.0001 g NaCl, 5.73 g TES (Sigma), and 15 g of a Bacto agar (Difco) per liter of distilled water. The initial pH of all the media was adjusted to 7.2 before sterilization. Each medium was sterilized at 121°C for 15 min, plus the glucose, $(NH_4)_2SO_4$, KH_2PO_4, NaH_2PO_4, and other trace elements were sterilized separately and added aseptically. The culture temperature was maintained at 30°C and the pH adjusted to 7.2 by the automatic addition of 1 N HCl or 1 N NaOH. Agitation was fixed at 250 rpm and the aeration controlled to 1 volume of air per volume of medium per min (vvm).

Analytical Methods

Triplicate culture samples (10 ml) were harvested from the culture vessels ascptically and used for the subsequent analyses. The mycelium of *S. coelicolor* A3(2) was collected by centrifugation (10,000 ×*g*, 10 min) and washed twice with a physiological saline solution and once with distilled water. The washed mycelium was then collected by vacuum filtration (Whatman GF/C paper), dried at 80°C for 24 h, and weighed. The glucose concentration was determined with the glucose-oxidation method [18] using

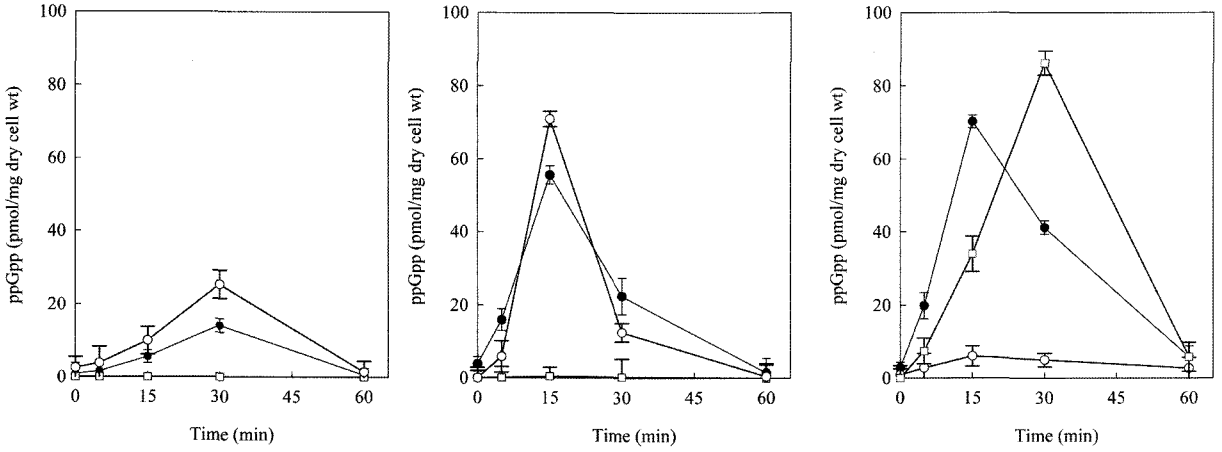

Fig. 3. Detection of (p)ppGpp from wild-type (M600) (●), *ΔrelA* (□), and *ΔrshA* (○) mutants under glucose-limitation (**A**), amino acid-limitation (**B**), and phosphate-limitation (**C**) conditions.
After the downshift from CD to the medium without specific elements (1% glucose, 1% casamino acid, or 0.05 M phosphate), culture samples taken after 0, 5, 15, 30, and 60 min were analyzed for the amount of intracellular pppGpp, ppGpp, and GTP, using HPLC.

a Trinder glucose assay kit (Sigma Co.). The concentrations of ammonia and phosphate were determined as reported previously [6, 30].

The actinorhodin (Act) concentration was collectively measured using the following method [2]: 0.5 ml of 3 N NaOH was added to 1 ml of a culture sample, mixed thoroughly, and centrifugation carried out at 1,000 $\times g$ for 2 min. The Act concentration was then determined by measuring the absorbance of the supernatant at 640 nm and calculated using the molar extinction coefficient (ε_{640}= 25,350 M^{-1} cm^{-1}). The undecyl-prodigiosin (Red) was extracted from a cell pellet harvested by centrifugation from 1-ml culture aliquots using the method in Williams *et al.* [35]. An equal volume of methanol (pH 1.0) was added to the cell pellet and mixed thoroughly for 2 min. After removing the cell debris by centrifugation, absorbance measurements were made at 530 nm and the Red concentration calculated using the molar extinction coefficient (ε_{530}= 100,150 M^{-1} cm^{-1}).

RESULTS

Deletion of *relA* and *rshA* Genes in *S. coelicolor* M600

The *relA* and *rshA* genes were deleted in the genome of *S. coelicolor* M600 (parent-strain) using the PCR-targeting method [8, 20]. As such, the DNA fragment (3,277 bp) in Fig. 1A is a BamHI DNA fragment containing the *relA* gene from the chromosome of *S. coelicolor* M600, whereas the signal (2,591 bp) for the *ΔrelA* mutant indicates a BamHI DNA fragment containing a partial apramycin-resistance cassette integrated by homologous recombination into the chromosome in replacement of *relA* (Fig. 1A). The DNA fragment (7,490 bp) in Fig. 1B is a BamHI DNA fragment containing the intact *rshA* from the parent strain chromosome, whereas the signal (4,275 bp) for the *Δrsh* mutant represents a BamHI DNA fragment containing a partial apramycin-resistance cassette integrated by homologous recombination into the parent strain chromosome instead of *rshA*. Southern hybridization also confirmed that the *relA* and *rshA* genes were both completely deleted.

(p)ppGpp Production with Nutrient Downshift or *In Situ* Starvation During Batch Culture

The dynamics of the intracellular concentration of (p)ppGpp after a forced shift-down of a specific nutrient in a culture of *S. coelicolor* M600 are shown in Fig. 2. The concentration of both ppGpp and pppGpp increased instantly after the artificial depletion of glucose, amino acids, or phosphate. In addition, the concentration of GTP was inversely related to the concentrations of ppGpp and pppGpp, suggesting that GTP was used for the synthesis of ppGpp and thereafter pppGpp. The higher concentration of ppGpp than pppGpp indicated that ppGpp was the major component of the stringent factor in *S. coelicolor*. The concentration of (p)ppGpp reached the maximum level at 15 min after the shift-down, at which point it was clear that the (p)ppGpp production in the parent strain was triggered significantly by the limitation of phosphate and amino acids, yet sluggishly and weakly by the limitation of glucose.

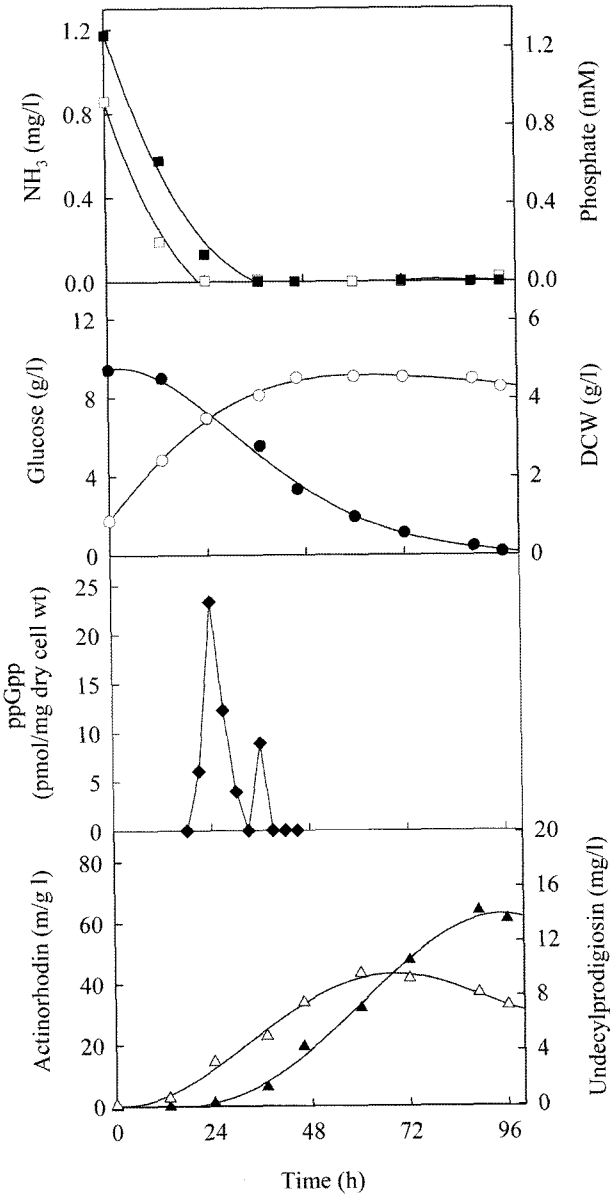

Fig. 4. Detection of ppGpp from batch culture of *S. coelicolor* M600.

To determine the effects of amino acid and phosphate on the synthesis of ppGpp, a 2-l batch culture of *S. coelicolor* M600 was carried out. A 50-ml sample was then harvested every 3 h after 12 h to 48 h of culturing. The changes in the concentration of ppGpp (◆), glucose (●), dry cell weight (DCW, ○), NH_3 (□), Phosphate (■), Actinorhodin (Act, ▲), and Undecyl-prodigiosin (Red, △) were all measured. The culture was carried out at 30°C, 200 rpm, 1 vvm.

The (p)ppGpp production in the *ΔrelA* mutant was completely eliminated with limited glucose or amino acids (Fig. 3A and 3B), yet not with limited phosphate (Fig. 3C). The (p)ppGpp production in the *ΔrshA* mutant was higher than that in the parent strain M600 under glucose- or amino acid-starved conditions (Fig. 3A and 3B), yet much lower under phosphate-starved conditions (Fig. 3C). However, the (p)ppGpp production in the *ΔrelA* and *ΔrshA* mutants declined rapidly to undetectable levels after 1 h.

The *in situ* dynamics of the ppGpp concentration during a batch culture of *S. coelicolor* M600 are shown in Fig. 4, where it is interesting to note that two peaks were detected for ppGpp at 24 h and 36 h when the nitrogen (represented as NH_3) and phosphate were completely depleted. The initiation of antibiotic production was followed by the appearance of ppGpp, even in the case of a high glucose concentration.

Antibiotic Production in Batch Cultures

The batch culture kinetics of the *S. coelicolor* strains in 2 l jar fermentors are shown in Fig. 5. The mycelium growth and glucose uptake rate for the *ΔrelA* mutant were much faster than those for the parent strain (M600). However, the mycelium growth entered a decline phase without a sustained stationary phase, even when the glucose concentration was still high. The glucose consumption and mycelium growth of the *ΔrshA* mutant were faster than those of the parent strain, yet slower than those of the *ΔrelA* mutant. It was clear that Act production was completely eliminated in both the *ΔrelA* and *ΔrshA* mutants. However, the Red production in the *ΔrshA* mutant was significantly enhanced compared with that in the parent strain (M600).

Discussion

It has been previously reported that a RelA protein encoded by *relA* in *S. coelicolor* A3(2) is a bifunctional enzyme involved in both synthesizing and degrading (p)ppGpp and that the *relA* gene is involved in a stringent response under limited nitrogen [5, 10]. Additionally, *relA*-mediated ppGpp synthesis is apparent under the conditions of limited glucose and amino acid, yet less crucial under the conditions of limited phosphate [17]. Thus, it has been suggested that an alternative gene, possibly the *rshA* gene, may be involved in the activation of antibiotic biosynthesis under limited phosphate conditions;

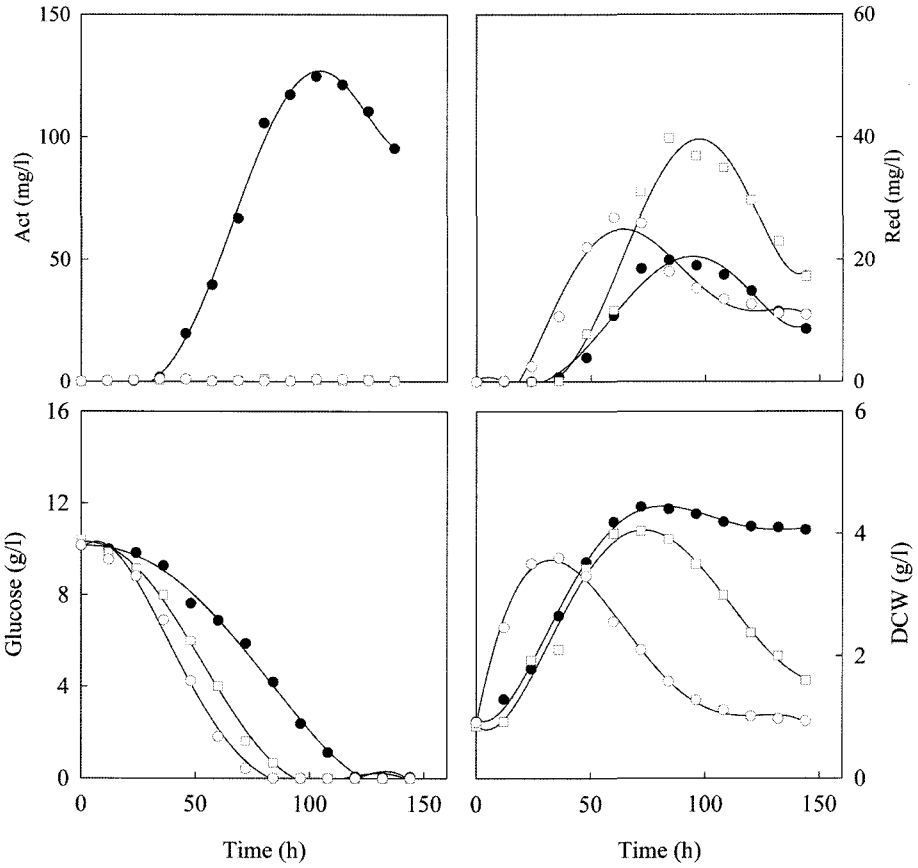

Fig. 5. Comparison of concentrations of glucose, DCW, Act, and Red in batch cultures of *S. coelicolor* M600 (●), and *ΔrelA* (○) and *ΔrshA* (□) mutants.

however, the role of the *rshA* gene has not yet been fully elucidated in *S. coelicolor*. The RelA and Rsh proteins encoded by the *relA* and *rshA* genes, respectively, in *S. clavuligerus* have also been found to be bifunctional enzymes [13, 15, 31]. Nonetheless, despite the identification of the *relA* and *rsh(A)* genes in *Streptomyces* spp., the involvement of these genes in ppGpp metabolism responding to nutrient limitation is not yet differentially understood.

Accordingly, based on the results of the current study, (p)ppGpp production in *S. coelicolor* M600 was clearly triggered in the case limited in phosphate or amino acids, yet weakly triggered by limited glucose (Fig. 2). (p)ppGpp production in the *ΔrelA* mutant was completely eliminated with limited amino acids or glucose, yet significantly enhanced with limited phosphate, whereas in the *ΔrshA* mutant, (p)ppGpp production was high with limited amino acids or glucose, yet very low with limited phosphate (Fig. 3). Therefore, the results indicate that the RelA protein encoded by the *relA* gene plays a major role in the production of (p)ppGpp under conditions of limited amino acids or glucose, yet a very weak role under limited phosphate. Conversely, the RshA protein encoded by *rshA* plays a major role in the production of (p)ppGpp under conditions of limited phosphate, yet not in the case of limited glucose or amino acids.

The concentration of ppGpp in the parent strain declined rapidly to form a very sharp peak without a sustained level, and then completely disappeared within 1 h after its appearance. In contrast, the maximum concentration of (p)ppGpp in the *ΔrelA* and *ΔrshA* mutants was higher than the corresponding concentration observed in the parent strain, suggesting that RelA and RshA also possess (p)ppGpp degradation activity.

Although actinorhodin production was completely abolished in both the *ΔrelA* and *ΔrshA* mutants, the undecylprodigiosin production was higher than that in the parent strain, M600 (Fig. 5). These results corresponded to the result from Hoyt and Jones [12]. The glucose uptake rate was also increased in both the *ΔrelA* and *ΔrshA* mutants, and neither mutant strain was able to sustain a stationary phase during the 2-l batch culture, in contrast to the wild-type strain, M600.

In conclusion, the *in situ* production of ppGpp in *S. coelicolor* was found to be selectively regulated by either the RelA or RshA proteins, which were differentially expressed in response to the specific nutrient limitation. Thus, it is believed that the RelA and RshA proteins are both bifunctional, possessing the ability to synthesize and hydrolyze (p)ppGpp, plus the *relA* and *rshA* genes are strongly related to the physiological changes in *S. coelicolor*.

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