RESEARCH LETTER

hmgA, transcriptionally activated by HpdA, influences the biosynthesis of actinorhodin in *Streptomyces coelicolor*

Yue Zhang[1,2], Linqi Wang[1,2], Shuli Zhang[1,2], Haihua Yang[1] & Huarong Tan[1]

[1]State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; and [2]Graduate School of Chinese Academy of Sciences, Beijing, China

Correspondence: Haihua Yang, State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China. Tel.: +86 01064807467; fax: +86 01064807461; e-mail: yanghh@sun.im.ac.cn

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Abstract

Homogentisate 1,2-dioxygenase (HmgA) converts homogentisate to maleylacetoacetate in the pathway of tyrosine catabolism in living organisms. Studies published to date have not elucidated the transcriptional regulation of *hmgA* and its biological function in *Streptomyces* spp. Here it is shown that the transcription of *hmgA* in *Streptomyces coelicolor* was activated by HpdA, an AsnC-type activator, in the presence of tyrosine. Disruption of *hmgA* led to enhanced production of diffusible brown pigment, suggesting that *hmgA* is required for tyrosine catabolism in *Streptomyces coelicolor*. The production of actinorhodin in the *hmgA* disruption mutant was several fold higher than that in the wild-type strain (M145), indicating that *hmgA* of tyrosine catabolism correlates with actinorhodin biosynthesis. Furthermore, S1 mapping showed that the transcription of *act*II-ORF4, a pathway-specific activator gene of actinorhodin biosynthesis, was increased in the *hmgA* disruption mutant. These results reveal the transcriptional regulation of *hmgA* and the positive contribution of *hmgA* disruption to the actinorhodin biosynthesis in *Streptomyces coelicolor*.

Introduction

As soil-dwelling and mycelial sporulating bacteria, *Streptomyces* must compete for scarce nutrients in the soil and adapt their metabolism (including primary and secondary metabolisms) to the nutritional conditions in order to persist. Catabolism and metabolism in the genus are usually transcriptionally controlled in response to environmental nutrition signals (Yang *et al*., 2007). Many compounds can be utilized by *Streptomyces* spp. For example, L-tyrosine can be used as either the carbon or the nitrogen source by *Streptomyces* spp. in nutrient-limited conditions (Pometto & Crawford, 1985). Recently, the genetically well-studied *Streptomyces coelicolor* M145 has been shown to utilize tyrosine as sole carbon and nitrogen source for its normal growth and development on agar plates (Yang *et al*., 2007).

The tyrosine catabolic pathway consists of five enzymatic reactions and is found in all living organisms (Yang *et al*., 2002) (Fig. 1). 4-Hydroxyphenylpyruvate dioxygenase encoded by *hppD* is involved in the second step of tyrosine catabolism; disruption of *hppD* was shown to affect the growth and brown pigment production of *Streptomyces coelicolor*. The transcriptional regulation of *hppD* has been intensely studied (Yang *et al*., 2007). Homogentisate 1,2-dioxygenase (HmgA) converts homogentisate (HG) to maleylacetoacetate (MAA) as the third step of tyrosine catabolism (Fig. 1). HmgA deficiency led to the accumulation of homogentisate hydroquinone, which undergoes facile nonenzymatic oxidation and polymerization to yield an intense brown pigment (Amaya *et al*., 2004). In addition, *hmgA* was shown to be essential for the development and growth of humans and animals, and *hmgA* deficiency can lead to a severe human disease known as alkaptonuria (Amaya *et al*., 2004). Disruption of *hmgA* in *Sinorhizobium meliloti* resulted in poor growth of the mutant (Milcamps & de Bruijn, 1999), and the *hmgA* disruption mutant of *Aspergillus nidulans* was unable to grow on media containing either phenylalanine or phenylacetate as sole carbon sources (Fernandez-Canon & Penalva, 1995) because both of the amino acids are catabolized through the tyrosine catabolic pathway. Members of the genus *Stretomyces*, such as *Streptomyces coelicolor* and *Streptomyces avermitilis*, have only a single gene coding for HmgA in their genomes. The transcriptional regulation and the biological function of *hmgA* in *Streptomyces* spp. have not been studied thus far. Whether *hmgA* is transcriptionally regulated by a similar

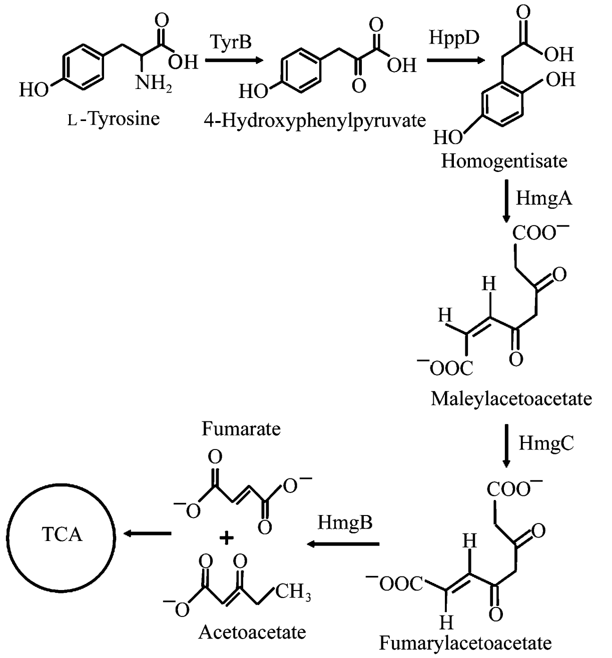

Fig. 1. The catabolic tyrosine pathway. The enzymes of the catabolic pathway are indicated. TyrB, tyrosine aminotransferase; HppD, 4-hydroxyphenylpyruvate dioxygenase; HmgA, homogentisate dioxygenase; HmgC, maleylacetoacetate isomerrase; HmgB, fumarylacetoacetate hydrolase. Arrows indicate the main flux of tyrosine catabolism. TCA, tricarboxylic acid cycle.

mechanism as that for *hppD*, and whether *hmgA* plays important roles in the morphological or physiological differentiation of *Streptomyces*, are important questions.

Actinorhodin, a blue-pigmented polyketide, is a well-characterized secondary antibiotic of *Streptomyces coelicolor* (Kim *et al.*, 2007). The production of actinorhodin is affected by a variety of physiological and environmental factors, including growth state, signal molecules, metabolism imbalances and various physiological stresses (Bibb, 1996). In the course of phenotypic analysis of an *Streptomyces coelicolor hmgA* mutant, we found that the *hmgA* disruption dramatically promoted the production of actinorhodin. It is inferred that tyrosine catabolism may have some correlation with the production of actinorhodin.

We report here for the first time that *hmgA* is transcriptionally activated by HpdA in the presence of tyrosine, and that *hmgA* disruption can enhance the transcription of *act*II-ORF4 and Act production.

Materials and methods

Bacterial strains, media and culture conditions

Streptomyces coelicolor M145 and its derived strains were grown at 28 °C on yeast extract malt extract (YEME), minimal medium (MM), minimal salts (MS), R2YE and supplemented minimal medium solid (SMMS) media as described by Kieser *et al.* (2000). To select strains, apramycin and kanamycin were added at concentrations of 10 $\mu g\,mL^{-1}$ for MM and YEME, and 50 $\mu g\,mL^{-1}$ for MS. The agar-based medium Tyr-PM was prepared as described by Yang *et al.* (2007). *Escherichia coli* JM109 was used to propagate plasmids (Sambrook *et al.*, 1989). *Escherichia coli* ET12567 (pUZ8002) was used for conjugal transfer of DNA from *E. coli* to *Streptomyces* (Kieser *et al.*, 2000). All *E. coli* strains were incubated at 37 °C in Luria–Bertani medium.

Plasmids and DNA manipulation

T-vector from Takara Biotechnology Corporation (Dalian, China) was used for cloning of the PCR-generated DNA fragment. pUC119::*aphII* was used to isolate the kanamycin resistance gene, *aphII*. The *E. coli–Streptomyces* shuttle vector pKC1132 (Kuhstoss & Rao, 1991) was used for gene disruption in *Streptomyces* and pSET152 (MacNeil *et al.*, 1992) was used to introduce a single copy of *hmgA* into *Streptomyces coelicolor*.

Isolation, purification of plasmids and DNA fragments, and DNA transformation of *E. coli* were performed via established techniques (Kieser *et al.*, 2000). Restriction enzymes, T4 DNA ligase and T4 DNA polymerase were from Takara Biotechnology Corporation (Dalian, China). *Taq* DNA polymerase was purchased from Sino-American Biotechnology Company (SABC) and Pfu high-fidelity DNA polymerase was from Shanghai Sangon Biological Engineering and Technology Services Corporation (China).

Genomic DNA was isolated from *Streptomyces* strains as described by Kieser *et al.* (2000). Southern hybridization was carried out using the nonradioactive digoxigenin labeling and detection kit (Roche Molecular Biochemicals).

RNA isolation from surface-grown cultures of *Streptomyces coelicolor* and S1 mapping

Total RNAs were isolated from cultures on cellophane-overlaid MM, Tyr-PM, SMMS and R2YE plates as described by Liu *et al.* (2005). The quality and quantity of RNAs were examined by UV spectroscopy and also checked by agarose gel electrophoresis. *hmgA* probe was prepared by PCR using chromosomal DNA of M145 as template with a 5′-end-labeled primer hmgA-P1 and an unlabeled primer hmgA-P2 (Table 1). The *act*II-ORF4 probe was similarly prepared by PCR amplification using the unlabeled direct primer *act*II-ORF4-P1 and 5′-end-labeled reverse primer *act*II-ORF4-P2 (Table 1). The *hrdB* probe used as an internal control was prepared similarly by PCR using the unlabeled primer hrdB-P1 and labeled primer hrdB-P2 (Table 1). Purification of probes and S1 nuclease (Promega) digestions were performed as described by Liu *et al.* (2005).

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′)
hmgA-P1	GCCCTCGGGCAGCGCGCCCGCCA
hmgA-P2	CAGGACACAGACCGCGAGCAC
hrdB-P1	ACTCGGGCCACGCGGATTG
hrdB-P2	AGCCTTTCCCCGCTCAAT
hmgA-P3	GGGTCTGGTTCCGGTCATCCTCGTCATC
hmgA-P4	TGGTCGGCTCGCCCGTCTTCTCGTC
aphII-P1	ACGCGTCGACTCCCCTGGATACCGCTCG
aphII-P2	TGAACTGCAGGAACCCCAGAGTCCCGC
hmgA-P5	CGCCGATCCCGTCAACAGCAGGAC
hmgA-P6	CGGCAGCTTGCGGTTCAGGACGATG
actIIORF4-P1	TTGATTCGGAAGCCTCGACCACTG
actIIORF4-P2	CGATCCCACCACCTCGTGCC

Construction of the *hmgA* disruption mutant

Primer sequences used in this study are shown in Table 1. A 1964-bp fragment including the coding sequence of *hmgA* (SCO1715) and its flanking sequences was amplified with primers hmgA-P3 and hmgA-P4, and this was then inserted into the T-vector to give T∷*hmgA*, which was subsequently linearized with BstXI (an internal restriction site in *hmgA*) and blunted with T4 DNA polymerase. The kanamycin-resistance gene (*aphII*) was amplified by PCR using pUC-119∷*aphII* as template and aphII-P1 and aphII-P2 as primers. This *aphII* fragment was inserted into the end-blunted BstX I site of *hmgA* to generate plasmid T∷*hmgA*∷*aphII*, from which a 3.0-kb HindIII–EcoRI fragment containing *hmgA*∷*aphII* was isolated and inserted into the same sites of pKC1132 to give pKC1132∷*hmgA*∷*aphII*. The sequence fidelity of *hmgA* and *aphII* in pKC1132∷*hmgA*∷*aphII* was verified by sequencing and the confirmed recombinant plasmid was introduced into *Streptomyces coelicolor* M145 by conjugation from *E. coli* ET 12567 (pUZ8002) (Kieser *et al*. 2000). A mutant exhibiting the double-crossover phenotype (kanamycin resistance and apramycin sensitivity) was confirmed by Southern hybridization (using BamHI/KpnI-digested chromosomal DNAs from the mutant and wild-type strains) and designated the *hmgA* disruption mutant (hmgADM).

Construction of plasmid for gene complementation

In order to complement the *hmgA* disruption mutant, a 1676-bp DNA fragment containing the *hmgA* gene and its own promoter region was amplified by PCR with primers hmgA-P5 and hmgA-P6 (Table 1). The PCR product was ligated into the EcoRV site of pSET152 to give pSET152∷*hmgA*, which was then introduced into hmgADM by conjugation from *E. coli* ET12567 (pUZ8002).

Measurement of biomass and Act production

Spores of M145 and hmgADM grown on MM were collected in sterilized water, diluted and counted with a hemocytometer. Spore suspensions containing 10^9 spores were spread on cellophane-covered agar plates containing 30 mL media, these plates were incubated at 28 °C for different lengths of time (24, 48, 72, 96, 120, 144 h), and the mycelia were collected and weighed at 24-h intervals. Determination of fresh weight was always performed by using triplicated culture plates, and the mean values of the three samples are presented below in Fig. 4(b). Actinorhodin production was determined spectrophotometrically as described by Bystrykh *et al*. (1996). Cell cultures (20 mg) were treated by KOH (final concentration, 1 N) and centrifuged (8000 ***g***, 3 min), after which the $OD_{640\,nm}$ of the supernatants was determined. The absorbance values at $OD_{640\,nm}$ in Fig. 4(c) below are displayed for a 10-mg cell mass. Reproducibility of the results was confirmed by at least three separate experiments.

Results

Transcription of *hmgA* is activated by HpdA in the presence of tyrosine

Blast searches in the GenBank database revealed that the deduced product of SCO1715 showed high similarities to HmgA in *Pseudomonas putida*, *Sinorhizobium meliloti* and *Aspergillus nidulans*. It has been shown that *hmgA* of *P. putida* is transcriptionally regulated by an IclR-type regulator, HmgR (Arias-Barrau *et al*., 2004). In recent studies, we have shown that *hppD*, whose product converts 4-hydroxyphenylpyruvate to homogentisate in tyrosine catabolism of *Streptomyces coelicolor*, is transcriptionally regulated by an AsnC-type activator, HpdA (SCO2928) and an IclR-type repressor, HpdR (SCO2935) (Yang *et al*., 2007). To study whether *hmgA* in *Streptomyces ceolicolor* is regulated by a similar mechanism as that for *hppD*, the transcription of *hmgA* was determined by S1 mapping in the wild-type strain (M145), *hpdA* mutant and *hpdR* mutant grown either in the absence (MM) or in the presence (Tyr-PM) of tyrosine. As shown in Fig. 2(a), *hmgA* transcript was not detected in M145 incubated on MM, whereas *hmgA* transcript was readily detected in M145 grown on Tyr-PM with tyrosine, indicating that *hmgA* transcription was tyrosine-induced in M145. In the *hpdA* mutant, *hmgA* transcript was likewise undetectable in the absence of tyrosine, but it was about one-quarter that in M145 in the presence of tyrosine. This result suggested that *hpdA* is necessary for the higher-level transcription of *hmgA* on tyrosine-containing medium. In the absence or presence of tyrosine, levels of *hmgA* transcription in the *hpdR* mutant were the same as those of the parental strain, indicating that *hmgA* transcription was not

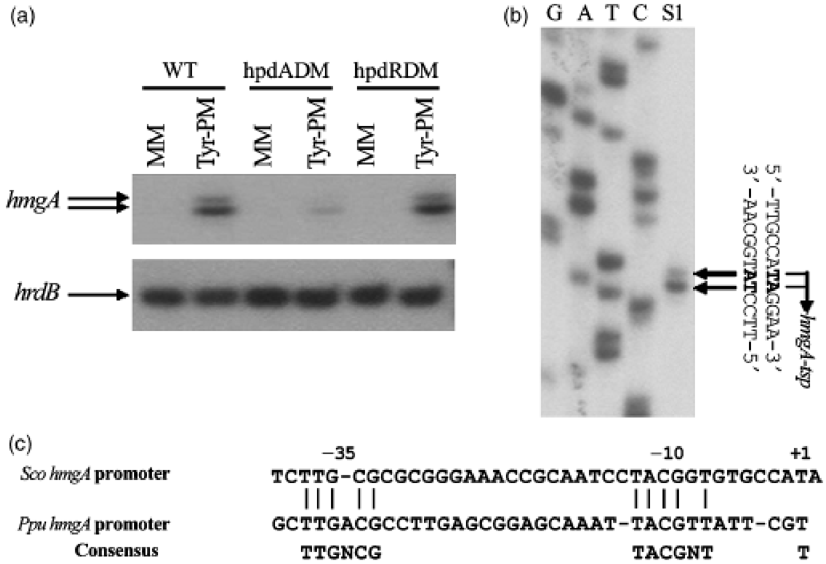

Fig. 2. Transcriptional assay of *hmgA*. (a) Transcription of *hmgA* in the wild-type (WT), *hpdA* disruption mutant (hpdADM) and *hpdR* disruption mutant (hpdRDM) strains was detected by S1 mapping assay. Total RNAs from WT, hpdADM and hpdRDM on MM or Tyr-PM plates for 48 h were hybridized with *hmgA* probe. S1 mapping assay of *hrdB* with the same RNA samples was used as control. (b) The determination of transcription start point of *hmgA*. Arrowheads indicate the transcripts of *hmgA*. The curved arrow shows the transcriptional orientation. G, A, T and C show the nucleotide sequence ladder of *hmgA*. (c) Similarities of the − 10 and − 35 regions of *hmgA* promoters in *Streptomyces coelicolor* (*Sco*) and *Pseudomonas putida* (*Ppu*).

affected by *hpdR*. Taken together, these results indicate that the transcription of *hmgA* in *Streptomyces coelicolor* is activated by HpdA in a tyrosine-dependent manner, but is not affected by *hpdR*. The transcriptional regulation mechanism of *hmgA* is not the same as that of *hppD*. This phenomenon indicates that *hmgA* in *Streptomyces coelicolor* might be subject to different and complex transcriptional regulation.

Similar transcriptional results were obtained with RNA samples isolated at different time points (72, 96, 120 and 144 h) (data not shown).

The promoter region of *hmgA*

The transcription start point (tsp) of *hmgA* was determined by high-resolution S1 nuclease mapping (see 'Materials and methods') and located to A or T at positions 38 or 39 nt upstream from the potential translational start codon (ATG) of *hmgA* (Fig. 2b). The putative − 10 (TACGGT) and − 35 (TTGCGC) regions of *hmgA* in *Streptomyces coelicolor* showed high similarities with those of the *hmgA* promoter in *P. putida* (Fig. 2c), suggesting that these two homologous *hmgA* genes in different strains might be recognized and prompted to transcribe by similar σ factors.

hmgA disruption influences tyrosine catabolism in *Streptomyces coelicolor*

To analyze the biological function of *hmgA* in *Streptomyces coelicolor*, an *hmgA* disruption mutant was constructed via double crossover (Fig. 3a), and the allelic exchange of *hmgA* with *hmgA*::*aphII* in the transformant was verified by Southern hybridization (Fig. 3b). The hybridization signal in the mutant strain is about 1 kb longer than that in the wild-type strain, indicating that the 1-kb *aphII* gene is expectedly inserted into the central region of *hmgA*. The effect of *hmgA* on tyrosine catabolism in *Streptomyces coelicolor* was shown by the observation of brown pigment production in hmgADM and wild-type strains. On MM medium, neither hmgADM nor the wild-type strains produced visible brown pigment, indicating that tyrosine catabolism was not taking place or was undetectable (data not shown). On Tyr-PM, the wild-type strain produced slight diffusible brown pigment, whereas hmgADM produced abundant diffusible brown pigment, which is an oxidation product of homogentisate (Fig. 3c). Wild-type level of pigment production was restored by the introduction of wild-type *hmgA* into hmgADM (Fig. 3c), excluding potential polar effects on adjacent genes. These results showed that *hmgA* is required for tyrosine catabolism in *Streptomyces coelicolor*.

hmgA disruption facilitates the biosynthesis of Act

In the course of phenotypic analysis of *Streptomyces coelicolor hmgA* disruption mutants, we found that *hmgA* disruption did not affect morphological development but enhanced Act production when they were incubated on agar R2YE, SMMS and Tyr-PM plates (Fig. 4a). To quantify the production of actinorhodin, biomass and Act production of hmgADM and M145 grown on agar plates were determined at 24-h intervals. On R2YE medium, the biomass of hmgADM was less than that of M145 at nearly all the time points (Fig. 4b), but Act production of hmgADM was several fold higher than that of M145 from 48 to 144 h (Fig. 4c). On SMMS and Tyr-PM media, similar profiles were observed.

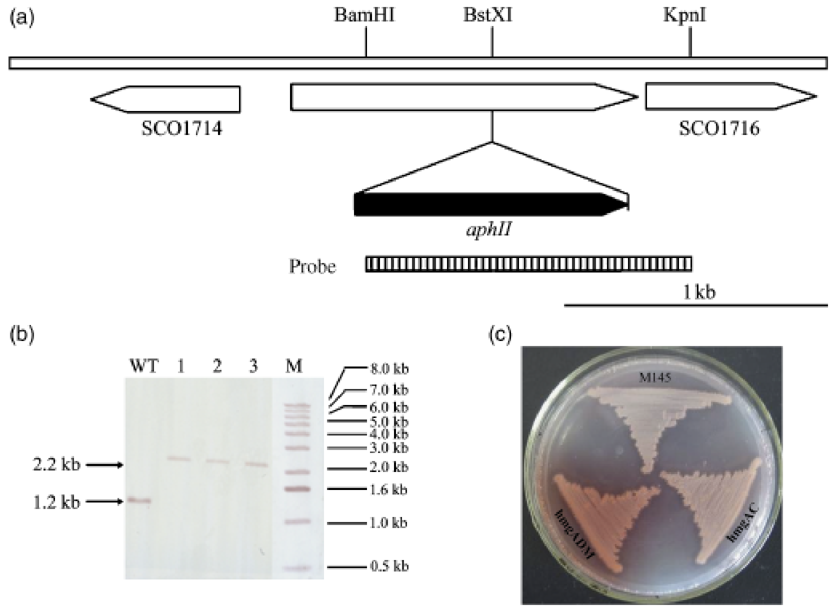

Fig. 3. Disruption of *hmgA* influences tyrosine catabolism in *Streptomyces coelicolor*. (a) Scheme of *hmgA* disruption. *aphII* (kanamycin resistance gene cassette), shown by a black arrow, was inserted into the BstXI site of *hmgA*. The DNA fragment used as probe is indicated by a grid bar. (b) Southern blotting of genomic DNA from wild-type and *hmgA* disruption mutants. DNA was digested with KpnI and BamHI. WT, *Streptomyces coelicolor* wild-type M145. Lanes 1–3, *hmgA* mutants. M, DNA molecular weight marker. (c) Production of brown pigment in hmgADM, M145 and hmgAC. hmgADM, *hmgA* disruption mutant; M145, wild-type strain; hmgAC, complementary strain of hmgADM.

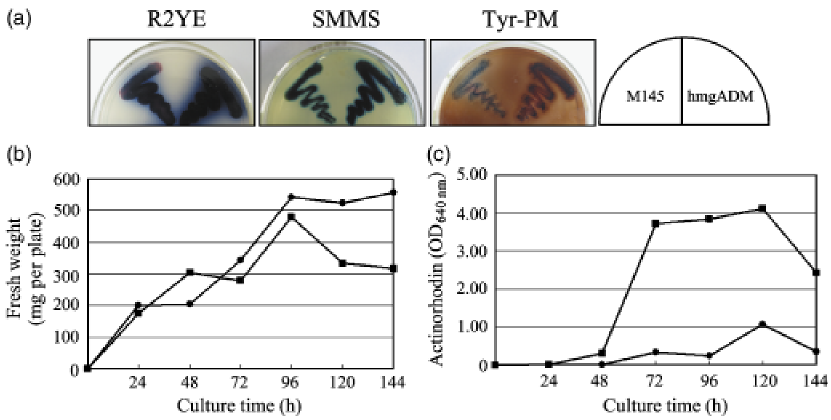

Fig. 4. Effect of *hmgA* disruption on actinorhodin production. (a) Act production of hmgADM and M145 on R2YE, SMMS and Tyr-PM plates for 6 days. KOH (1N) was added to the SMMS and Tyr-PM plates to show the blue pigment clearly. (b) Biomass assay of M145 (•) and hmgADM (■) on cellophane-covered R2YE medium. (c) Absorbance of intracellular blue-pigmented antibiotic Act. Cell cultures (20 mg) from the agar medium at each time point were treated with KOH (final concentration, 1 N) and the $OD_{640\,nm}$ corresponding to 10 mg of mycelium was determined.

Transcription of *act*II-ORF4 was increased in the *hmgA* mutant

Disruption of *hmgA* led to increased production of Act in *Streptomyces coelicolor*. To establish whether the influence of *hmgA* on Act production is at the level of transcriptional control of the Act biosynthetic cluster, or was through posttranscriptional effects such as precursor supply to the pathway, the transcription of a positive regulator gene (*act*II-ORF4) of Act biosynthesis was studied in hmgADM and M145, respectively. As shown in Fig. 5, the transcription level of *act*II-ORF4 in hmgADM was much higher than those in M145 on both R2YE and SMMS media, suggesting that *hmgA* disruption positively affects transcription of *act*II-ORF4.

Discussion

At least three transcriptional regulators of different families have been identified as participating in the transcriptional regulation of *hmgA* in bacteria: *Streptomyces coelicolor hmgA* is activated by an AsnC-type regulator HpdA, *P. putida hmgA* is repressed by an IclR-type regulator HmgR (Arias-Barrau *et al*., 2004) and *Sinorhizobium meliloti hmgA* is activated by an ArsR-type regulator (Milcamps *et al*., 2001). These results indicated that the transcriptional regulation of *hmgA* in different bacteria was complex and varied.

In *Streptomyces coelicolor*, two tyrosine catabolic genes, *hmgA* and *hppD*, have been studied. Both are activated by HpdA in the presence of tyrosine, whereas *hppD* is repressed by *hpdR* but *hmgA* is not. Furthermore, the dependency of *hppD* and *hmgA* to the HpdA activator is different: activation of *hppD* is completely dependent of the presence of HpdA, whereas transcription of *hmgA* is partially dependent on HpdA, which could be deduced from the transcript of *hppD* (Yang *et al*., 2007) and *hmgA* in the *hpdA* mutant when tyrosine was present (Fig. 2a). These findings indicate that although tyrosine catabolic genes of *Streptomyces coelicolor* are induced to express by the presence of tyrosine,

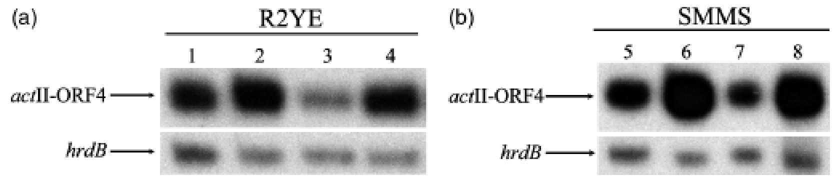

Fig. 5. Transcriptional analysis of the *act*II-ORF4. (a) S1 nuclease protection assays were performed using 40 μg of RNA from M145 and hmgADM strains grown on R2YE agar plates. RNAs were taken at 60 h from M145 (lane 1) and hmgADM (lane 2), or at 108 h from M145 (lane 3) and hmgADM (lane 4). Assays of *hrdB* transcript in the same samples were used as a control for RNA levels. (b) S1 nuclease protection assays were performed using 40 μg of RNA from M145 and hmgADM grown on SMMS agar plates. RNAs were taken at 60 h from M145 (lane 5) and hmgADM (lane 6), or at 108 h from M145 (lane 7) and hmgADM (lane 8). Assays of *hrdB* transcript in the same samples were used as a control for RNA levels.

they might undergo different transcriptional regulation in order to adapt to nutrient-starved conditions.

Disruption of *hmgA* in *Sinorhizobium meliloti* or *A. nidulans* resulted in the poor or no growth of the strains. In contrast to these bacteria, disruption of *hmgA* in *Streptomyces coelicolor* did not affect its growth, but led to a dramatic increase in Act production. The increased production of Act was not due to the increased biomass of the *hmgA* disruption mutant, but was caused by the increased expression of *act*II-ORF4 in the *hmgA* disruption mutant.

hmgA is necessary for tyrosine catabolism and the proper production of actinorhodin. This is the first report that *hmgA*, a key gene of primary catabolism, affects the production of Act polyketide, a secondary metabolite in *Streptomyces coelicolor*. Whether it is a specific characteristic of *Streptomyces coelicolor hmgA* or whether other *Streptomyces* homologs have similar effluences on secondary metabolites remain to be answered pending extensive studies of *hmgA* in other *Streptomyces* species.

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