Autoregulation of *hpdR* and its effect on CDA biosynthesis in *Streptomyces coelicolor*

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HpdR, an IclR-family regulator in *Streptomyces coelicolor*, is a substrate-dependent repressor for the tyrosine catabolic gene *hpdD*. In this study, S1 nuclease protection assays revealed that *hpdR* is subject to a negative autoregulation. Purified HpdR showed specific DNA-binding activity for the promoter region of *hpdR*, indicating that the autoregulation of *hpdR* is performed directly. The disruption of *hpdR* led to reduced production of CDA by *S. coelicolor* J1501, suggesting a positive effect of *hpdR* on CDA biosynthesis. Electrophoretic mobility shift assays showed that HpdR specifically bound to the promoter region of *hmaS* (SCO3229 in the CDA gene cluster), encoding 4-hydroxymandelic acid synthase. Disruption of *hmaS* in J1501 abolished CDA production. It is possible that *hpdR* regulates CDA biosynthesis by controlling the transcription of *hmaS*.

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INTRODUCTION

Streptomycetes are high-G + C Gram-positive, filamentous soil bacteria that have become a major focus for the study of microbial development and differentiation, antibiotic biosynthesis, and the mechanism of transcriptional regulation (Chater, 1993; Chater & Chandra, 2008). The genetically well-studied strain *Streptomyces coelicolor* A3(2) produces at least four chemically distinct classes of antibiotics: the blue-pigmented polyketide actinorhodin, the red-pigmented undecylprodigiosins, the lipopeptide calcium-dependent antibiotic (CDA) and the cyclopentanone antibiotic methylenomycin. The triggering of antibiotic biosynthesis in *Streptomyces* species is generally tightly linked with the initiation of morphological differentiation, and both processes are subject to genetic modulation via a hierarchical regulatory network, integrating various physiological and environmental signals (Li *et al.*, 2006).

Transcriptional regulators have been found to play important roles in development and differentiation in *Streptomyces* species (Liu *et al.*, 2005; Tian *et al.*, 2007; Wang *et al.*, 2009; Li *et al.*, 2009). IclR-family regulators contain a helix–turn–helix (HTH) motif in their N-terminal domain and bind their target promoters as dimer or tetramers. They play significant roles in diverse biological processes in *Streptomyces* species, such as regulation of drug resistance, degradation of aromatic

compounds, oxidative catabolism of tyrosine, and the inactivation of quorum-sensing signals (Yang *et al.*, 2007, 2009, Santamarta *et al.*, 2007). *ssgR* activates the transcription of *ssgA* in *S. coelicolor*, and is developmentally regulated at the transcriptional level (Traag *et al.*, 2004). NadR plays a global regulatory role in amino acid metabolism, quorum sensing, morphological changes, antibiotic production and expression of chaperones in *S. coelicolor* (Yang *et al.*, 2009). AreB from *Streptomyces clavuligerus* modulates leucine biosynthesis and cephamycin C and clavulanic acid production (Santamarta *et al.*, 2007). In our previous work, HpdR, an IclR-type regulatory protein in *S. coelicolor*, was shown to be a negative regulator of the expression of *hpdD* in tyrosine catabolism, in a substrate-dependent manner (Yang *et al.*, 2007). It bound a 90 bp region spanning the promoter of *hpdD*, which also overlapped the promoter of the adjacent *hpdA*, by which HpdR negatively regulated the transcription of *hpdD* and *hpdA*. These studies were performed in *S. coelicolor* M145. When the development and differentiation of *S. coelicolor* strain J1501 was studied, we found some phenomena different from those of strain M145. In this study, we describe the autoregulation of *hpdR* and the effect of *hpdR* disruption on the transcription of *hmaS*, involved in CDA biosynthesis in *S. coelicolor* J1501.

METHODS

Bacterial strains, plasmids and growth conditions. *Streptomyces coelicolor* J1501 (*hisA1 uraA1 strA1 pgl-1 SCP1⁺ SCP2⁺*; differentiation phenotype as wild-type strain) (Chater *et al.*, 1982) was used as host

Abbreviations: CDA, calcium-dependent antibiotic; EMSA, electrophoretic mobility shift assay; 4HPP, 4-hydroxyphenylpyruvate; tsp, transcription start point.

strain for gene propagation and gene disruption. Other *S. coelicolor* strains used in this work were all derived from J1501 (Table 1). *Escherichia coli* JM109 was used as standard host for plasmid replication. The non-methylating *E. coli* ET12567 (*dam dcm hsdS*; MacNeil *et al.*, 1992) was used to propagate DNA when it was to be introduced into *S. coelicolor* derivatives.

pKC1132 and pSET152 are bifunctional plasmids with autonomous replication in *E. coli* and capable of integrating into the *Streptomyces* chromosome, and harbour the apramycin-resistance gene *aac(3)IV* (Bierman *et al.*, 1992). The *Streptomyces/E. coli* shuttle vector pKC1132 was used to construct the disruption derivatives of *hpdR* and *hmaS*. pSET152 (Bierman *et al.*, 1992), which integrates into the *S. coelicolor* chromosome by site-specific recombination at the bacteriophage ϕ C31 attachment site, *attB* (Kuhstoss & Rao, 1991), was used to introduce single copies of genes into the *S. coelicolor* chromosome. pGEX-4T-3 (Pharmacia Biotech, 27-4583-01), containing an ampicillin-resistance gene, was used for overexpression of *hpdR* in *E. coli*. Media, culture conditions, protoplast transformations and chromosomal DNA isolation for *S. coelicolor* strains were carried out as described by Kieser *et al.* (2000).

When necessary, antibiotics were used in media as follows, at the concentrations given in parentheses: for *E. coli*, ampicillin (100 μ g ml⁻¹), kanamycin (100 μ g ml⁻¹), apramycin (50 μ g ml⁻¹) or hygromycin (50 μ g ml⁻¹) in LB; for *Streptomyces*, apramycin (100 μ g ml⁻¹), kanamycin (100 μ g ml⁻¹) or hygromycin (40 μ g ml⁻¹) in solid R2YE medium, or kanamycin (10 μ g ml⁻¹) or hygromycin (10 μ g ml⁻¹) in liquid YEME medium. The stock concentrations of apramycin, kanamycin and hygromycin stored at -25 °C were 100, 50 and 50 mg ml⁻¹, respectively.

DNA manipulation and sequencing analysis. Isolation of plasmid and chromosomal DNA, procedures for Southern blotting, and transformation of *Streptomyces* protoplasts were as described by Liu *et al.* (2005). All other DNA techniques, including transformation of competent *E. coli* cells, and blunt-ending of DNA by Klenow fragment and T4 polymerase, were as described by Sambrook *et al.* (1989), except that *hpdR* and *hmaS* probes used in Southern blot analysis were labelled non-radioactively (digoxigenin-11-UTP kit; Roche). For database searches, the programs TFASTA (Lipman & Pearson, 1985) and BLAST (Altschul *et al.*, 1990) were employed. Sequence analysis and homologue comparisons were performed with the programs of NCBI BLASTX.

Construction of an *hpdR* null mutant. The *hpdR* disruption mutant, hpdRDM¹, was constructed as described previously (Yang *et al.*, 2007), except that the host strain was J1501. In brief, the upstream region and partial 5'-end sequence encoding the α -helix-turn- α -helix domain of *hpdR* was replaced by the kanamycin-resistance gene (*aphII*) in J1501. To study the transcriptional level of the *hpdR* promoter without the influence of HpdR protein, plasmid pSET152::P_{hpdR}, containing the 0.4 kb *KpnI*-*HincII* fragment of the *hpdR* promoter region, was introduced into strain hpdRDM¹ to generate strain hpdRDM¹-P. The *hpdR* disruption mutant (hpdRDM¹) was selected

by its Kan^R Apr^S phenotype and confirmed by Southern blot hybridization.

Complementation of the *hpdR* mutant. To complement hpdRDM¹, a 1.4 kb *KpnI* fragment containing the complete promoter region and the coding region of *hpdR* was purified, filled in by T4 polymerase, and then subcloned into the site-directed recombinant plasmid pSET152 digested with *EcoRV*. The resulting plasmid pSET152::hpdR was introduced into hpdRDM¹, from which complemented colonies were selected by their Kan^R Apr^R phenotype. The complemented strain, hpdRCS, was further confirmed by Southern hybridization.

Construction of an *hmaS* null mutant in J1501. An 11 kb *KpnI*/*EcoRI*-digested fragment containing *hmaS* and its flanking sequence from SupercosE63 (provided by Professor Keith Chater, John Innes Centre, Norwich, UK) was inserted into the same sites of pIJ2925. The resulting plasmid pIJ2925::hmaS was digested with *EcoRI* and *NotI*, from which a 3.36 kb fragment containing *hmaS* was isolated and inserted into the same sites of pBluescript M13⁻ to generate pM13⁻::hmaS, which was then further digested with *BglII* and ligated with the 1.7 kb *BglII* DNA fragment, from pIJ963, containing the hygromycin-resistance gene (*hyg*) to give pM13⁻::hmaS::hyg. This plasmid was subsequently digested with *PstI* and *HindIII*, from which a 3.2 kb DNA fragment containing the 1.5 kb left flanking arm and the 1.7 kb *hyg* (Arm^L::hyg^R) was isolated, and end-blunted with mung bean nuclease (Takara). pM13⁻::hmaS was also digested with *HincII*, from which a 4.0 kb DNA fragment consisting of M13⁻ and 1 kb right flanking sequence of *hmaS* was isolated. This fragment was ligated with the end-blunted 3.2 kb fragment (Arm^L::hyg^R) to generate plasmid pM13⁻::Arm^L::Hyg^R::Arm^R, which was further digested with *XhoI* and *BstXI* to give a 4.2 kb fragment containing Arm^L::Hyg^R::Arm^R; this fragment was blunted by mung bean nuclease and then ligated with pKC1132 digested with *EcoRV*. Finally, the *BglII*-*HincII* fragment in *hmaS* was replaced by a 1.7 kb hygromycin-resistance gene to give pKC1132:: Δ hmaS::hyg, which was subsequently passed through *E. coli* ET12567 to remove possible Dam/Dcm methylation and then introduced into J1501 protoplasts using the PEG-mediated transformation method (Kieser *et al.*, 2000). Colonies which were potential disruptants of *hmaS* were selected by their Hyg^R Apr^S phenotype and identified by Southern blot hybridization. For Southern blotting assay, genomic DNA isolated from strains J1501 and hmaSDM was digested with *NcoI* and *BglII*, and the DIG-labelled 0.4 kb fragment upstream of the non-coding region was used as a probe.

Overexpression and purification of GST-HpdR. In order to produce HpdR in *E. coli*, the complete coding region of the *hpdR* gene was amplified by PCR using chromosomal DNA as template and oligonucleotides corresponding to the start and stop codons as forward and reverse primers: *hpdR*-primer1, 5'-ATGGATCCGTGACCGCGAGACCT-3' (*Bam*HI-recognized site underlined), and *hpdR*-primer2, 5'-ATACTCGAGCTACCGCAGCGCCTCCGCG-3' (*Xho*I-recognized site underlined). The *hpdR* PCR product digested

Table 1. *S. coelicolor* strains used in this study

Strain	Genotype	Source or reference
J1501	<i>his ura</i> SCP1 ⁻ SCP2 ⁻	Chater <i>et al.</i> (1982)
hpdRDM ¹	J1501/ Δ hpdR::aphII	This study
hpdRCS	hpdRDM ¹ /pSET152::hpdR	This study
hpdRDM ¹ -P	hpdRDM ¹ /pSET152::promoter ^{hpdR}	This study
hmaSDM	J1501/ Δ hmaS::hyg	This study

by *Bam*HI and *Xho*I was inserted into pGEX4T-3 at the same restriction sites. The resulting pGEX-4T-3:: *hpdR* was transformed into *E. coli* BL21(DE3) and the strain was cultured in 50 ml LB with 100 µg ampicillin ml⁻¹ at 37 °C. This culture was grown to mid-exponential phase (OD₆₀₀ 0.6), then IPTG was added to a final concentration of 0.1 mM and incubation continued for an additional 2 h at 30 °C. Cells were harvested by centrifugation at 7700 g, 4 °C for 10 min, washed twice with 50 ml buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) and then resuspended in 4 ml of the same buffer. The cell suspension was broken by sonification on ice. Triton X-100 was added to the cell lysate at a final concentration of 1%, and the mixture was gently shaken at 4 °C for 30 min to get more soluble protein. After centrifugation at 12 000 g for 10 min at 4 °C, the supernatant was mixed with 0.4 ml glutathione Sepharose 4B solution (Amersham Pharmacia, 17-0756-01) and gently shaken in an ice bath for another 30 min, and then the mixture was loaded onto a mini-column (USB). After washing with 5 ml 1 × PBS for three times, the GST-HpdR protein was specifically eluted with 1 ml elution buffer (10 mM reduced glutathione in 50 mM Tris/HCl, pH 8.0) and concentrated to about 3 µg µl⁻¹ by ultrafiltration (Millipore membrane, 10 kDa cut-off). As a control for protein purification and protein-DNA binding assay, GST was purified by the same strategy and used at the same concentration in the EMSA (see below).

Preparation of DNA probes for electrophoretic mobility shift assays (EMSAs). A 20 pmol sample of the downstream primer corresponding to the *hpdR* promoter, *hpdR*-primer4 (5'-CGTATC-GGCCAGCAGTTTGAGGA-3'), was end-labelled with [γ -³²P]ATP and 10 U T4 polynucleotide kinase (Promega) as described previously (Yang *et al.*, 2007). It was used with the unlabelled *hpdR*-primer3 (5'-CGCTACGTCCACCAGAGCG-3') to generate the 518 bp fragment containing the *hpdR* promoter region by PCR. The labelled DNA probe containing the *hpdR* promoter region was purified using the PCR Purification kit UniQ10 spin columns from Shanon (Shanghai Sangon Biological Engineering & Technology Co.). Similarly, the probe containing the promoter region of *hmaS* was prepared using primers *hmaS*-up (5'-ACCTGCTCCTCACAATGC-3') and *hmaS*-reverse (5'-GCGATGTCACTGGGCGGCAT-3').

EMSAs. For EMSAs of HpdR binding to target DNA, the reaction (20 µl) was carried out with about 0.2 pmol radiolabelled probe in buffer [125 mM HEPES pH 7.5, 2 mM DTT, 200 mM KCl, 10 mM MgCl₂, 0.16 µg calf BSA µl⁻¹, 10% glycerol and 1 µg poly(dI-dC)]. Poly(dI-dC) from Sigma was included in each reaction to eliminate non-specific protein binding. The mixture was incubated with purified GST-HpdR or GST at 30 °C for 20 min. For competition gel shift assays, a 30-fold excess of unlabelled probe (specific competitor) was added to the reaction system with 900 ng GST-HpdR and incubated for 20 min at 30 °C, after which the corresponding labelled probe was added and incubated for a further 20 min at 30 °C. DNA-protein complexes were separated from free probe on a 5% glycerol-containing non-denaturing 4.5% acrylamide gel in 1 × TBE buffer. The gels were dried and exposed to Kodak Biomax radiographic film.

S1 nuclease protection assays of *hpdR* transcription. RNAs for S1 nuclease mapping were isolated from *S. coelicolor* J1501 and hpdRDM¹-P after incubation for different periods on R2YE medium covered with cellophane (Yang *et al.*, 2007). S1 protection assays were performed using the *hrdB* gene (AY628703) as a control as before (Yang *et al.*, 2007). The probe used for detecting *hpdR* transcript was generated by PCR using the unlabelled *hpdR*-primer4 and radiolabelled *hpdR*-primer 3. High-resolution S1 nuclease mapping was carried out to identify the transcription start point and gene expression pattern of *hpdR* in the wild-type and mutant strains. S1 nuclease mapping was performed as described previously (Kieser *et al.*, 2000;

Liu *et al.*, 2005). When examining the expression level of *hpdR* in the wide-type and mutant strains, the probes for *hpdR* and *hrdB* (positive control for RNA integrity and loading) were added together to each RNA sample. The sequence ladder was made using an *fmol* DNA cycle sequencing kit (Promega) with the same labelled primer.

qRT-PCR assays of *hmaS*. Total RNA samples from J1501 and hpdRDM¹-P, isolated as mentioned above, were treated with Genomic DNA cleaning reagent (Applygen), and then with DNase (Promega) to which RiboLock RNase Inhibitor (Fermentas) was added. The DNA-free total RNA sample was used to synthesize cDNA first. Pre-assays were performed using serial dilutions of cDNA as PCR template to examine the efficiency of amplification. To determine the level of *hmaS* transcription, real-time RT-PCR was performed using primers *hmaS*-primer1 (5'-TCCTTTCCCTTCCTTCATTG-3') and *hmaS*-primer2 (5'-GGGACGAAACCGAGGCTGT-3') with the SYBR PrimeScript RT-PCR kit II (Takara). The relative level of amplified mRNA was normalized to mRNA expression of the housekeeping gene *S. coelicolor* *hrdB*, which was amplified as an internal control using primers *hrdB*-primer3 (5'-CGCACCATCCGTATCCC-3') and *hrdB*-primer4 (5'-TCACCGAACTCGCTGTCCG-3').

Well-based bioassay of CDA. An inoculum of 10⁸ spores of *S. coelicolor* was plated on Oxoid Nutrient Agar (25 ml) and incubated at 30 °C for 2 to 7 days. The plates were frozen at -70 °C for 10 h and then slowly thawed at room temperature. The cells and medium were removed by centrifugation (12 857 g, 10 min) and the resulting supernatant was collected for the assay of CDA production. The CDA-sensitive *Staphylococcus aureus* was used as indicator strain. A 50 µl aliquot of an overnight culture of *Staph. aureus* was added into 25 ml melted soft nutrient agar (per litre: 13 g nutrient broth, 7 g Bactoagar; temperature about 45 °C) in the presence of added Ca(NO₃)₂ (16 mM) and poured into a plate. Agar plugs were cut from the dried plates, creating wells into which the collected supernatant was added. Calcium-dependent bioactivity was observed as a zone of inhibition after overnight incubation at 37 °C. The calcium-dependent bioactivity was assayed three times for each strain. The diameter of the inhibition zone was measured and used to evaluate the quantity of CDA production. Inhibition zones did not appear on control plates lacking Ca(NO₃)₂.

RESULTS

Identification of the *hpdR* promoter region

For precise localization of the transcription start point of *hpdR*, high-resolution S1 mapping was performed using RNA isolated from *S. coelicolor* J1501 (see Methods). A single transcription start point (tsp) was identified as a G residue around 210 nt upstream of the translational start of *hpdR* (Fig. 1). This tsp is preceded by the typical -35 and -10 hexamers, TTGATA and ATTCTG, in the upstream region.

Transcription of *hpdR* is developmentally regulated

To study the transcription of *hpdR*, high-resolution S1 mapping was performed using RNA isolated from *S. coelicolor* J1501 grown on R2YE medium for different times. The transcription of *hpdR* could be readily detected throughout the life cycle, but with different levels at different time points. *hpdR* was transcribed at a higher level when the

Fig. 1. Determination of the transcription start point (tsp) of *hpdR*. (a) High-resolution S1 nuclease mapping of *hpdR* in *S. coelicolor* J1501. The tsp is indicated by an asterisk and an arrow. Lanes G, A, T, and C are a dideoxy sequencing ladder generated using the same oligonucleotide as was used to make the S1 mapping probe. (b) Sequence of the *hpdR* promoter region. The -35 and -10 regions are indicated by bold and underlined letters, the tsp is indicated by bent arrow, the potential Shine-Dalgarno sequence (RBS) is underlined, and the potential translational initiation codon (GTG) is indicated by bold letters.

strain was growing as substrate mycelia and forming the primary aerial hyphae (at the 24 and 48 h time points, respectively), and then transcription decreased and remained at a relatively low level during the subsequent growth of aerial hyphae and sporulation (Fig. 2a). These results indicated that *hpdR* was transcribed in an attenuated pattern during the life cycle in J1501. Therefore, its transcription may be developmentally regulated.

Fig. 2. S1 nuclease mapping of *hpdR* in the parent strain J1501 (a) and *hpdRDM1-P* (b). The strains were incubated on solid R2YE medium. The incubation time points are indicated above each panel. *hrdB*, the major sigma factor of *S. coelicolor*, was used as a positive internal control.

hpdR is subject to negative autoregulation

To study whether *hpdR* is subject to autoregulation, like other members of the IclR family (Gui *et al.*, 1996), the transcription analysis was applied to the *hpdR* promoter region in an *hpdR* disruption mutant (*hpdRDM1-P*), which contains the intact promoter region of *hpdR* but lacks the complete coding region for HpdR. S1 mapping assay showed that the transcription of the *hpdR* promoter in *hpdRDM1-P* was at nearly the same level as that of J1501 at 24 h, but it remained at the same level from 24 h to 120 h, whereas the transcription of *hpdR* in J1501 decreased after 48 h (Fig. 2). These results indicated that the transcription of *hpdR* seems to be subtly autoregulated during the life cycle in J1501.

HpdR specifically binds to the promoter region of its own gene

To assess whether the autoregulation of *hpdR* was direct or not, an EMSA was performed. A 518 bp DNA fragment containing the promoter of *hpdR* was labelled and used as a probe. The EMSA showed that HpdR specifically bound to the radiolabelled fragment and formed different DNA-HpdR complexes at low or high concentrations of protein (Fig. 3). The retardation was inhibited by the addition of a 30-fold excess of unlabelled *hpdR* promoter DNA fragment; additionally, an equal amount of GST protein had no binding activity to the same DNA fragment (data not shown). These results suggested that HpdR in *S. coelicolor* can specifically bind to its own promoter region.

To determine the shortest fragment bound by HpdR, several DNA fragments were used in the EMSA experiments. A 159 bp probe (-125 to +34 relative to the tsp), containing the -35, -10 and tsp regions of the *hpdR* promoter, was bound by HpdR and exhibited a similar

Fig. 3. EMSA of HpdR binding to the *hpdR* promoter region (*P_{hpdR}*). Radioactively labelled *P_{hpdR}* DNA (5 nM in each lane) was incubated with increasing amounts of GST-HpdR (see Methods). Lane 1, free labelled DNA probe (518 bp) in the binding buffer. Lanes 2-7, labelled probe was incubated with 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 μM GST-HpdR, respectively. Lane 8, labelled probe was incubated with 0.6 μM GST-HpdR and a 30-fold excess of unlabelled probe.

profile (data not shown). This result indicated that the binding region of HpdR was located in the 159 bp DNA fragment.

hpdR disruption leads to decreased CDA production

hpdR was disrupted in *S. coelicolor* J1501 via double-crossover (Fig. 4a, b) and the resulting disruption mutant was designated *hpdRDM*¹. This mutant produced 10–30 % of the amount of CDA produced by J1501 (Fig. 4c), suggesting that *hpdR* may positively regulate CDA biosynthesis. The phenotype of *hpdRDM*¹ could be complemented by introducing a wild-type copy of *hpdR* on the integrating vector pSET152, and therefore was attributed to functional inactivation of *hpdR* rather than polar effects on the transcription of downstream genes or mutations in other loci.

Fig. 4. CDA production by *hpdRDM*¹. (a) Construction of *hpdRDM*¹ and *hpdRCS*. The DNA fragment containing the promoter and HTH coding region of *hpdR* was replaced by a kanamycin-resistance gene (*aphII*) in J1501 to generate the *hpdR* disruption mutant *hpdRDM*¹. A 1.4 kb *KpnI* fragment containing the promoter region and coding sequence of *hpdR* was blunted and inserted into the *EcoRV* site of pSET152 to generate pSET152:::*hpdR*, which was used to complement *hpdRDM*¹. (b) Southern hybridization analysis of *KpnI*-digested chromosomal DNA from wild-type (lane 1), the disruption mutant (lanes 2–3; duplicates) and the complemented strain (lanes 4–5; duplicates) to confirm the integration via homologous recombination. The DIG-labelled 0.3 kb DNA fragment containing the internal region of *hpdR* was used as a probe. The 1.4 kb signal (I) in the wild-type strain (J1501) is from a *KpnI* fragment corresponding to the complete *hpdR* gene. Strain *hpdRDM* shows a 2.0 kb signal (II) corresponding to a fragment with part of the *hpdR* gene and the 1.0 kb kanamycin-resistance gene. The complemented strain contains the 1.4 kb complete *hpdR* that was inserted into the chromosomal ϕ C31 *attB* site of *hpdRDM* by pSET152 mediation. (c) Assay for CDA production by J1501 (A), *hpdRDM*¹ (B) and *hpdRCS* (C) in the presence or absence of $\text{Ca}(\text{NO}_3)_2$.

HpdR positively controls CDA biosynthesis via directly targeting *hmaS*

The positive effect of *hpdR* on CDA production led us to speculate that *hpdR* may control CDA biosynthesis via directly regulating the biosynthetic gene(s) involved. To find the potential targets regulated by HpdR in the CDA biosynthetic gene cluster, the genes and their upstream regulatory regions in this cluster were analysed. The sequence alignment showed that the upstream regulatory region of *hmaS*, which encodes an essential enzyme, HmaS (SCO3229), involved in CDA biosynthesis (Hojati *et al.*, 2002), has 55 % identity with the HpdR-bound region upstream of *hppD* (Fig. 5a). Both HmaS and HppD are non-haem, α -keto-acid-dependent iron oxygenases; they catalyse the conversion of 4-hydroxyphenylpyruvate (4HPP) to different products, 4-hydroxymandelate and homogentisate, respectively (Denoya, *et al.*, 1994; Hojati *et al.*, 2002; Gunsior *et al.*, 2004). Therefore, *hmaS* may be a potential target of HpdR.

To investigate this possibility, an EMSA was first performed. A 328 bp DNA fragment containing the intergenic region between *hmaS* and its upstream gene was used as a probe. HpdR showed specific binding with this probe (Fig. 5b), suggesting that *hmaS* may be regulated by HpdR in a direct manner. To further identify the role of HpdR in regulating *hmaS*, the transcriptional level of *hmaS* in both wild-type J1501 and *hpdRDM*¹ was studied by real-time PCR. The results showed that the transcription of *hmaS* in J1501 was activated by HpdR at 72 h (Fig. 5c), which is consistent with the production of CDA; no CDA production was detectable at 24 or 48 h (data not shown). The transcriptional level of *hmaS* in *hpdRDM* was decreased compared to that in J1501 (Fig. 5c), which is also consistent with the effects on CDA production. These results imply that the transcription of *hmaS* is partially controlled by *hpdR*.

When *hmaS* was disrupted in J1501 via double crossover (Fig. 6a), the production of CDA was completely abolished in the resulting disruption mutant, *hmaSDM* (Fig. 6b); this was similar to the phenotype of an *hmaS*-disrupted mutant of *S. coelicolor* MT1110 (Hojati *et al.*, 2002). All the above results (Figs 5 and 6) showed that *hmaS*, an essential gene in CDA biosynthesis, is a novel and direct target of HpdR.

DISCUSSION

In *S. coelicolor* J1501, the transcription level of *hpdR* was different at the early and late developmental stages (Fig. 2), indicating that its transcription may be related to the developmental and differentional regulation of *S. coelicolor* J1501. This strain showed substrate mycelium growth and growth of primary aerial hyphae at 24 h and 48 h, respectively, and showed abundant aerial hyphae formation and sporulation at the later time points. Our previous study showed that the disruption of *hpdR* resulted in a white phenotype in J1501, suggesting an important role of *hpdR* in the morphological differentiation of this

Fig. 5. *hmaS* is a potential target of HpdR. (a) Comparison of the upstream regulatory regions of *hmaS* and *hppD* in *S. coelicolor*. The upstream region of *hppD* (P_{hppD}) contains the complete HpdR-binding site, indicated by the overhead line. The boxed sequences indicate the -35 and -10 regions of the *hppD* promoter. (b) Specific binding of HpdR to the promoter region of *hmaS*. Lane 1, free labelled P_{hmaS} DNA (328 bp) in the binding buffer. Lanes 2–4, the labelled P_{hmaS} fragment was assayed in the presence of 0.1, 0.3 and 0.9 μ M purified HpdR–GST. Lane 5, labelled P_{hmaS} was assayed in the presence of 0.9 μ M HpdR–GST and 30-fold excess of unlabelled P_{hmaS} (as competitor DNA). Lane 6, labelled P_{hmaS} DNA was assayed in the presence of 0.9 μ M GST. (c) Transcription assay of *hmaS* in J1501 and hpdRDM at 72 h (left), and bioassay of CDA production by J1501, hmaSDM and hpdRDM (right). The qRT-PCR assays were performed as described in Methods; transcription is shown in arbitrary units.

strain (Tan *et al.*, 2002). The target genes regulated by HpdR and involved in the morphological differentiation of J1501 will be determined in future work.

Our results showed that *hpdR* is subject to a fine-tuning autoregulation. The transcription level of the *hpdR* promoter was almost the same at 24–48 h in either the presence or absence of HpdR protein, but it was greatly increased after 48 h without HpdR. Therefore, it is deduced that *hpdR* may start the autoregulation from 48 h in response to some developmental signal(s). Our previous study revealed that 4HPP is a specific signal from tyrosine catabolism, which can affect the binding of HpdR to the target promoter region of *hppD* (Yang *et al.*, 2007), but whether it also serves as the signal to regulate the autoregulation of HpdR remains unclear.

4HPP has been proved to be an intermediate in both the catabolic pathway of tyrosine and the biosynthetic pathway of CDA. In the main catabolic pathway of tyrosine, HppD catalyses the conversion of 4HPP to homogentisate (Brownlee *et al.*, 2004; Johnson-Winters *et al.*, 2003). In the CDA biosynthetic pathway, HmaS catalyses the conversion of 4HPP to L-4-hydroxymandelic acid (Hojati

et al., 2002). HpdR negatively regulates the transcription of *hppD* and positively regulates the transcription of *hmaS*, implying that this protein plays a significant regulatory role in both tyrosine catabolism and CDA biosynthesis. The repression of *hppD* by HpdR keeps HppD at a constant low level, which prevents the overconversion of 4HPP to homogentisate. HpdR also activates the transcription of *hmaS*, thus accelerating the conversion of 4HPP to L-4-hydroxymandelic acid and the final steps of CDA biosynthesis. Therefore, HpdR possibly co-regulates the transcription of genes involved in a primary catabolic pathway (tyrosine catabolism) and a secondary metabolic pathway (CDA biosynthesis). This co-regulation would lead to the inhibition of 4HPP being converted to homogentisate, and enhanced CDA biosynthesis.

Our results have shown that HpdR exhibits different regulatory functions in *S. coelicolor*. It might act as a repressor for *hppD* and *hpdA* (Yang *et al.*, 2007) or an activator for *hmaS*. *hpdR* appears to play a significant role in a hierarchical regulatory network. The HpdR-bound DNA sequence upstream of *hpdR* has comparatively low identity to the upstream regulatory regions of *hppD* and

Fig. 6. Disruption of *hmaS* abolishes CDA biosynthesis in J1501. (a) Southern hybridization analysis of *NcoI/BglII*-digested chromosomal DNA from wild-type strain J1501 (lane 1) and the *hmaS* disruption strain *hmaSDM* (lanes 2–5; samples from four colonies) to confirm correct integration by homologous recombination. A DIG-labelled 0.4 kb DNA fragment containing the upstream region of *hmaS* was used as a probe. The 1.38 kb band in the wild-type strain represents the *NcoI–BglII* fragment. The *hmaS* disruption mutant (*hmaSDM*) shows a 2.2 kb signal corresponding to the remaining partial *hmaS* and the inserted 1.7 kb hygromycin-resistance gene. (b) CDA production assay of J1501 and *hmaSDM* after growth at 28 °C for 5 days on Nutrient Agar. The CDA-sensitive *Staph. aureus* was used as indicator strain.

hmaS, suggesting that HpdR-bound motifs are not very well conserved. This also implies that HpdR may bind to other regulatory regions and regulate the transcription of the corresponding genes. Another IclR-type regulator, NadR, was proved to be involved in amino-acid-dependent growth, quorum sensing and antibiotic production in *S. coelicolor* (Yang *et al.*, 2009). Other studies have shown that IclR-type regulators usually play multiple regulatory roles in different biological pathways (see Introduction). Further work is needed to determine whether HpdR controls other target genes in *S. coelicolor* or *hpdR* is regulated by other unknown regulators.

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