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RraAS1 inhibits the ribonucleolytic activity of RNase ES by interacting with its catalytic domain in *Streptomyces coelicolor*

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RraA is a protein inhibitor of RNase E, which degrades and processes numerous RNAs in *Escherichia coli*. *Streptomyces coelicolor* also contains homologs of RNase E and RraA, RNase ES and RraAS1/RraAS2, respectively. Here, we report that, unlike other RraA homologs, RraAS1 directly interacts with the catalytic domain of RNase ES to exert its inhibitory effect. We further show that *rraAS1* gene deletion in *S. coelicolor* results in a higher growth rate and increased production of actinorhodin and undecylprodigiosin, compared with the wild-type strain, suggesting that RraAS1-mediated regulation of RNase ES activity contributes to modulating the cellular physiology of *S. coelicolor*.

Keywords: *Streptomyces coelicolor*, RNase E, RNase ES, RraA, RraAS1

Introduction

Bacterial RNA degradation and processing are controlled by numerous factors that include RNA structural determinants, RNA binding factors, and ribonucleases. Among them, an endoribonuclease, RNase E (Rne), has been shown to play a major role in the degradation and processing of RNA transcripts in *Escherichia coli* (Ghora and Apirion, 1978; Hagege and Cohen, 1997; Ow *et al.*, 2000; Lee *et al.*, 2002; Li and Deutscher, 2002; Lee and Cohen, 2003; Gao *et al.*, 2006). Rne is a large 1061-amino acid protein that consists of two distinct halves. The conserved N-terminal half contains catalytic activity essential for cell viability, whereas the unstructured C-terminal half serves as a scaffold region for multiprotein complex assembly, known as the degradosome (Kido *et al.*, 1996; McDowall and Cohen, 1996; Vanzo *et al.*, 1998; Callaghan *et al.*, 2004).

The expression level and enzymatic activity of RNase E are tightly controlled through several mechanisms in *E. coli*. First, RNase E cleaves the 5′ untranslated region of its own transcript when RNase E activity exceeds cellular needs (Mudd and Higgins, 1993; Jain and Belasco, 1995; Sousa *et al.*, 2001). Second, the 5′-monophosphorylated RNA serves as an allosteric RNase E activator (Mackie, 1998; Jiang and Belasco, 2004; Celesnik *et al.*, 2007; Kime *et al.*, 2010). Third, RNase E activity is regulated by protein inhibitors, regulator of ribonuclease activity A and B (RraA and B) (Lee *et al.*, 2003; Gao *et al.*, 2006; Gorna *et al.*, 2010), and L4 ribosomal protein (Singh *et al.*, 2009). RraA and B inhibit endonucleolytic cleavage of subgroups of transcripts, interacting with different RNase E C-terminal domain regions and altering the composition of the degradosome (Lee *et al.*, 2003; Gao *et al.*, 2006). It has been shown that RraA modulates RNA-binding and helicase activity by interacting with DEAD-box helicase (Ghora and Apirion, 1978; Pietras *et al.*, 2013).

Previous studies have demonstrated that a *Streptomyces coelicolor* endoribonuclease, named RNase ES (Rns), which bears RNase E-like activity, can functionally complement Rne in *E. coli* (Hagege and Cohen, 1997; Lee and Cohen, 2003). RNase ES is a 1,340-amino acid protein whose central portion (amino acids 563–973) has a 58.0% amino acid sequence similarity with the N-terminal catalytic region of *E. coli* RNase E (Lee and Cohen, 2003). The segments in both RNase ES termini (amino acids 1–562 and 974–1340) contain discrete putative acidic, arginine-rich, and proline-rich regions, which are similar to motifs located in the C-terminal half of the RNase E protein (Lee and Cohen, 2003). Like RNase E, RNase ES also interacts with degradosome components (Lee and Cohen, 2003; Kim *et al.*, 2007). In addition, *E. coli* RraA and B can physically interact with RNase ES scaffold domains to inhibit its enzymatic activity *in vivo* and *in vitro* (Yeom *et al.*, 2008a, 2008b).

S. coelicolor contains genes that encode homologs of *E. coli* RraA, designated as RraAS1 and RraAS2 (41.6% and 36.0% amino acid sequence similarity, respectively, with RraA) (Ahn *et al.*, 2008). A recent study showed that RraAS2 requires both scaffold domains of RNase ES for high-affinity binding and inhibitory action on the ribonucleolytic activity (Heo *et al.*, 2016a). In this study, we investigated the ability of RraAS1 to regulate RNase ES ribonucleolytic activity, its mechanism of its action, and its functional role in the modulation of cellular physiology in *S. coelicolor*.

Materials and Methods

Strains and plasmids

The construction of *rne*-deleted *E. coli* strains that express

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full-length Rns (KSL2005) or its truncated form (KSL2006, KSL2007, or KSL2008) has been described previously (Lee and Cohen, 2003). pKAN6-RraAS1 and pKAN6-RraAS1-Myc were constructed by ligating polymerase chain reaction (PCR) DNA digested with *Nde*I and *Xba*I restriction enzymes into the same pKAN6 site. We synthesized PCR DNA fragments containing RraAS1 and RraAS1-Myc coding regions using primers RraAs1-Nde1-F (5′-GGAATTCCATATGTTCATTGCTGCGGCGAC-3′) and RraAs1-Xba1-R (5′-GCTCTAGATCATCGGGCCACCACCGCGC-3′) for RraAS1 and primers RraAS1-NdeI-F and RraAS1-XbaI-R (5′-GCTCTAGATCACAGGTCCTCCTCTGAGATCAGCTTCTGCTCCATTCGGGCCACCACCGCGCG-3′) for RraAS1-Myc, with *S. coelicolor* genomic DNA as a template.

Using a PCR-targeted system, an *rraAS1*-deleted *S. coelicolor* strain was constructed. The *rraAS1* coding sequence was replaced by an apramycin resistance cassette, which was amplified by PCR from PIJ773. The PCR primers used were ScRraS1-DEL5′ (5′-TCATTTAAGATGTGCATCATGAGAGGGGAGTGTCATCGTGATTCCGGGGATCCGTCGACC-3′) and ScRraS1-DEL3′ (5′-CGCAGTTCCGCGATCCGGGAGGCCGGCGGGGTGGCGATCATGTAGGCTGGAGCTGCTTC-3′). PCR products were transformed into BW25113/pIJ790, obtaining supercosmid (stH71) with the cloned target gene (*rraAS1*). The potent methyl-specific restriction *S. coelicolor* system was escaped by introducing the recombinant into a methylation-deficient *E. coli* host ET-12567/pUZ8002. The cosmid was moved by pUZ8002 to *Streptomyces* by conjugation. Exconjugants were screened for kanamycin and apramycin resistance. Double cross-over allelic exchange was confirmed by Southern blot analysis.

Measurement of plasmid copy number

Measurement of plasmid copy number has been described previously (Yeom and Lee, 2006; Yeom *et al.*, 2008b; Lee *et al.*, 2009; Kim *et al.*, 2016). Plasmid copy number was calculated relative to a concurrently present pSC101 derivative

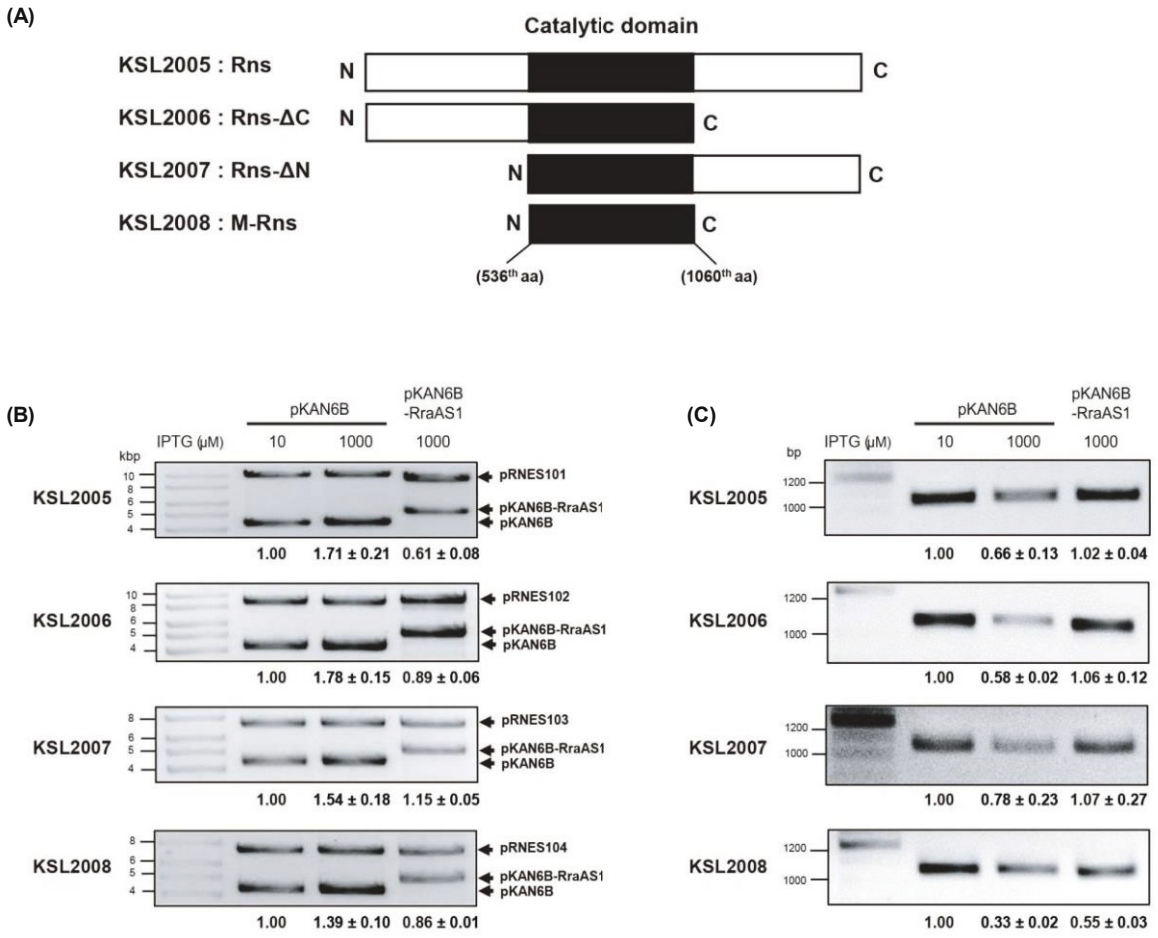

Fig. 1. Effects of coexpressed RraAS1 on the ribonucleolytic activity of RNase ES in *E. coli* cells. (A) Rns protein derivations used in this study. Regions containing highly conserved Rns catalytic domain sequences are shown as gray bars. (B) Effects of overproduced RNase ES or its derivations and coexpressed RraAS1 on ColE1-type plasmid copy number are presented. Plasmid DNA was isolated from cultures used in the KSL2005-8 growth curve and plasmid copy number was calculated (see 'Materials and Methods' section). Band densitometric measurements corresponding to each plasmid were converted to actual ratios after values were normalized according to ColE1-type plasmid size and are shown at the bottom of the gel. (C) Effects of overproduced RNase ES or its derivations and coexpressed RraAS1 on the steady-state level of *rpsO* mRNA. Total RNA was isolated from KSL2005-8 cells grown to an OD_{600} of 0.6 and RT-PCR was performed. Products were electrophoresed in 1.0% agarose gel, and band intensity was measured (see 'Materials and Methods' section). The results (mean ± SEM) are from three independent experiments performed in triplicate.

(pRNES101), whose replication was Rns-independent, by measuring the molar ratio of the pRNES101-104 plasmid to the ColE1-type plasmid (pKAN6, pKAN6-RraAS1).

RNA preparation and reverse transcriptase (RT)-PCR

The procedure for RT-PCR analysis has been described previously (Yeom and Lee, 2006; Kim *et al.*, 2016). Total RNA was prepared from *S. coelicolor* cells grown in R5 medium in a late log-phase as described previously (Jeong *et al.*, 2016). The primers used were rpsO 5'RT (5′-GTACACTGGGAT CGCTGAATT-3′) and rpsO 3'RT (5′-GGCCCCCTTTTCT GAAACTCG-3′) for *rpsO*, and rcsB 5′RT (5′-ATGAACAA TATGAACGTA-3′) and rcsB 3′RT (5′-TTAGTCTTTATC TGCCGG-3′) for *rcsB*, actII-ORF4 5′RT (5′-TCAGGCGG CACGAGGTGGTGG-3′) and actII-ORF4 3′RT (5′-ACCC GGTGCTCCCCCAGCAG-3′) for actII-ORF, redZ 5'RT (5′-TGCTGACCAAGCCCGAGAA-3′) and redZ 3'RT (5′-GC GGAGGTTGGCCTCCAGCA-3′) for redZ, and rpoB 5'RT (5′-TTCATGGACCAGAACAACC-3′) and rpoB 3′RT (5′-CGTAGTTGTGACCCTCCC-3′) for rpoB.

Co-immunoprecipitation

The procedure for co-immunoprecipitation has been described previously (Heo *et al.*, 2016b). Cells were cultured in LB medium containing 10 μM IPTG. Then, 10 mM IPTG and 0.2% arabinose were added when cell culture reached an OD_{600} of 0.1. They were further grown to an OD_{600} of 0.8 and harvested for immunoprecipitation studies.

Protein purification and Western blot assay

The procedures for protein purification and Western blot analysis have been described previously (Kim *et al.*, 2016). Hexahistidine-tagged M-RNase ES and RraAS1 were purified from KSL2008 and BL21 (DE3)-pET15b-rraAS1, respectively.

An *in vitro* cleavage assay

The procedures for BR10+hpT radiolabeling and the *in vitro* RNase E cleavage assay have been described previously (Kim *et al.*, 2016).

Antibiotics assay

The strains of *S. coelicolor* A3(2) used were M145 (prototrophic wild-type strain) and its derivative Δ*rraAS1*. Fresh spore preparation was pregerminated at 50°C for 10 min as described previously (Gust *et al.*, 2002). R5-liquid medium was used for actinorhodin (ACT) and undecylprodigiosin (RED) production (Huang *et al.*, 2001). ACT and RED production was determined according to the methods described by Kieser *et al.* (2000).

Results

Effects of coexpression of RraAS1 on ribonucleolytic activity of RNase ES *in vivo*

To investigate whether RraAS1 can modulate the ribonucleolytic activity of RNase ES, we utilized *E. coli* strains (KSL2005–2008) in which a chromosomal deletion in *rne* was complemented by a plasmid-borne *rns* gene or its truncated form encoding Rns (pRNES101), Rns-ΔC (pRNES102), Rns-ΔN (pRNES103), or M-Rns (pRNES104) under the control of an isopropylthiogalactoside (IPTG)-inducible lacUV5 promoter (Yeom *et al.*, 2008a, 2008b) (Fig. 1A). Addition of 10 μM IPTG to KSL2005–2008 cultures allowed the synthesis of C-terminally hexahistidine-tagged full-length or truncated forms of RNase ES that support survival and growth of this *rne* deletion mutant, as has been shown previously (data not shown) (Lee and Cohen, 2003).

Next, to test the effect of RraAS1 on RNase ES activity *in vivo*, a compatible Km^r plasmid expressing RraAS1 under the control of the arabinose-inducible promoter (pKAN6B-RraAS1) was introduced into KSL2005–2008 strains. Two known RNase E substrates in these strains were analyzed for their steady-state levels to examine whether RraAS1 could modulate the ribonucleolytic activity of RNase ES and its truncated forms (Rns-ΔC, Rns-ΔN, and M-Rns). The first substrate tested was RNA I, an antisense repressor of ColE1-type plasmid replication. The *in vivo* activity of RNase ES against RNA I was assessed by measuring the relative copy number of a ColE1 origin plasmid (pKAN6B or pKAN6B-RraAS1) compared to a pSC101 origin plasmid (pRNES101) in *E. coli* KSL2005 cells when RraAS1 was conditionally expressed by arabinose. Rns overexpression in the presence of 1 mM IPTG in KSL2005 cells increased pKAN6B copy number by approximately 1.7-fold relative to that observed in cells expressing Rns in the presence of 10 μM IPTG (Fig. 1B). Increased ColE1-type plasmid copy number under Rns overexpression was reduced by approximately 2.8-fold when RraAS1 was coexpressed in the presence of 0.2% arabinose. RraAS1 coexpression showed similar effects, albeit at different degrees, on ColE1-type plasmid copy number in KSL2006–2008 cells overproducing truncated Rns proteins (Fig. 1B). These results indicate that RraAS1 inhibited the ribonucleolytic activity of Rns proteins on RNA I *in vivo*. We further investigated the effects of RraAS1 coexpression on the ribonucleolytic activity of Rns proteins on *rpsO* mRNA abundance using semi-quantitative RT-PCR. We obtained analogous results (Fig. 1C), suggesting that RraAS1 can inhibit the ribonucleolytic activity of RNase ES *in vivo*, and its action does not require RNase ES scaffold domains.

Physical interactions between RNase ES and RraAS1

The experimental results described above imply that, unlike other RraA homologs that require RNase E-like enzyme terminal scaffold domains for their binding, RraAS1 can exert its inhibitory effect on the enzymatic activity of RNase ES by interacting with the catalytic domain of the enzyme. This notion was tested by immunoprecipitating RraAS1 in KSL2005–2008 cells and analyzing Rns proteins with Western blot analysis. In these experiments, C-terminally Myc-tagged RraAS1 (RraAS1-Myc) was coexpressed in KSL2005–2008 cells and immunoprecipitated with an antibody to Myc-tag. It has been shown that RraAS1-Myc is as functional as RraAS1 *in vivo* (Heo *et al.*, 2016b). As shown in Fig. 2A, Rns and all of its truncated forms were co-immunoprecipitated with RraAS1-Myc, showing that the catalytic enzyme domain was suffi-

cient to bind to RraAS1-Myc.

Inhibition of the catalytic activity of RNase ES by RraAS1 *in vitro*

To investigate if RraAS1 exerted inhibitory effects on the ribonucleolytic activity of RNase ES by interacting with its catalytic domain, thus directly acting on the enzyme, we purified RraAS1 and M-Rns for *in vitro* cleavage assay. We used a 5′-32P-end-labeled BR10+hpT (p-BR10+hpT) oligonucleotide containing RNA I cleavage site for an RNA substrate (Kim *et al.*, 2016). The cleavage of p-BR10+hpT by M-Rns was reduced in a manner dependent on RraAS1 concentration (Fig. 2B), revealing that RraAS1 inhibited the ribonucleolytic activity of RNase ES via direct action on the enzyme's catalytic activity.

Effects of rraAS1 deletion on the physiology of *S. coelicolor*

We aimed to identify the physiological role of RraAS1 in *S. coelicolor*. For this purpose, an *rraAS1*-deleted *S. coelicolor* strain was constructed using the gene replacement proce-

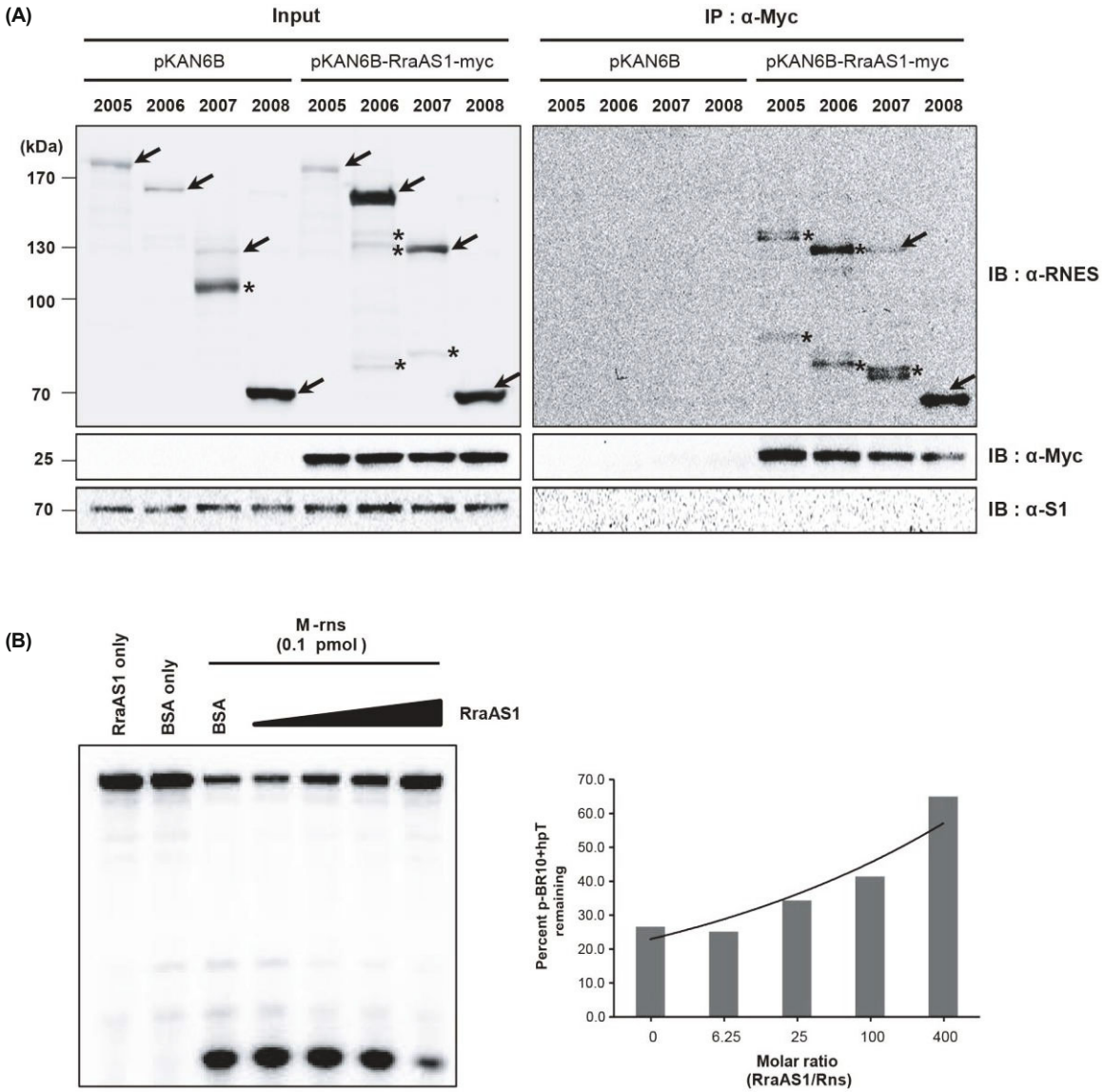

Fig. 2. Characterization of RraAS1 action on RNase ES. (A) Physical interactions of RNase ES with RraAS1 in *E. coli* cells. Immunoprecipitation of RNase ES or its derivatives and Myc-tagged RraAS1 are shown. Myc-tagged RraAS1 precipitated with RNase ES and its truncated forms. KSL2005-8 cell lysates overproducing RNase ES or its derivations and Myc-tagged RraAS1 were subjected to α-Myc IP and Western blotting using antibodies against α-RNES. S1 was used as a loading and IP specificity control. Full-length proteins expressed are indicated with arrows and the smaller bands in each lane, which are degradation products of the proteins, are indicated with asterisks. (B) RraAS1 inhibition of cleavage of p-BR10 + hpT by M-Rns *in vitro*. 2 pmol of 5′-end labeled p-BR10 + hpT RNA were incubated with 0.1 pmol of M-Rns with varying RraAS1 concentrations (0.625, 2.5, 10, and 40 pmol) or BSA (40 pmol) in 20 μl of 1 cleavage buffer at 37°C for 1 h. RraAS1 only or BSA only are used as controls. Samples were denatured with loading buffer at 65°C for 5 min and electrophoresed on 12% polyacrylamide gel including 8 M urea. The percentage of uncleaved p-BR10 + hpT in the gel was quantitated using a phosphorimager and Quantity one software.

dure (Gust *et al.*, 2002). *rraAS1* gene deletion was confirmed with Southern blot analysis (Fig. 3A). This *rraAS1* mutant and wild-type strains were tested for growth rates and ability to produce two major antibiotics, actinorhodin (ACT) and undecylprodigiosin (RED). The results show that the growth rates of the deletion mutant were higher than those of the wild-type (Fig. 3B). We also observed that the production levels of the antibiotics ACT and RED were approximately two-fold higher in the *rraAS1* mutant (Fig. 3C and D). These results imply that RraAS1-mediated regulation of the ribonucleolytic activity of RNase ES affects *S. coelicolor* growth and secondary metabolism. However, alterations in the production of ACT and RED do not appear to be associated with RraAS1-mediated regulation of expression of global regulators in secondary metabolite production such as *actII-ORF* and *redF* (Fig. 3E): steady-state levels of *actII-ORF* and *redZ* mRNA were not significantly changed in the *rraAS1* mutant.

Discussion

Genes encoding RraA homologs are widely found in Bacteria, Archaea, and plants. Biological activities of these homologs from *Vibrio vulnificus* (RraAV1 and RraAV2) and *S. coelicolor* (RraAS2) as inhibitors of RNase E-like enzymes have been shown (Yeom *et al.*, 2008a, 2008b; Lee *et al.*, 2009; Heo *et al.*, 2016b; Kim *et al.*, 2016). In this study, we also show that RraAS1 is a protein inhibitor of RNase ES. However, its mode of action appears to be different from other RraA homologs as indicated by its direct interaction with the catalytic domain of RNase ES to exert its inhibitory effect on the ribonucleolytic activity of the enzyme. Thus, it is less likely that RraAS1 interferes with binding of degradosome components to terminal scaffold domains of RNase ES compared to other RraA homologs that require terminal scaffold domains of RNase E-like enzyme for their high-affinity binding. In this respect, RraAS1 may well directly modulate the

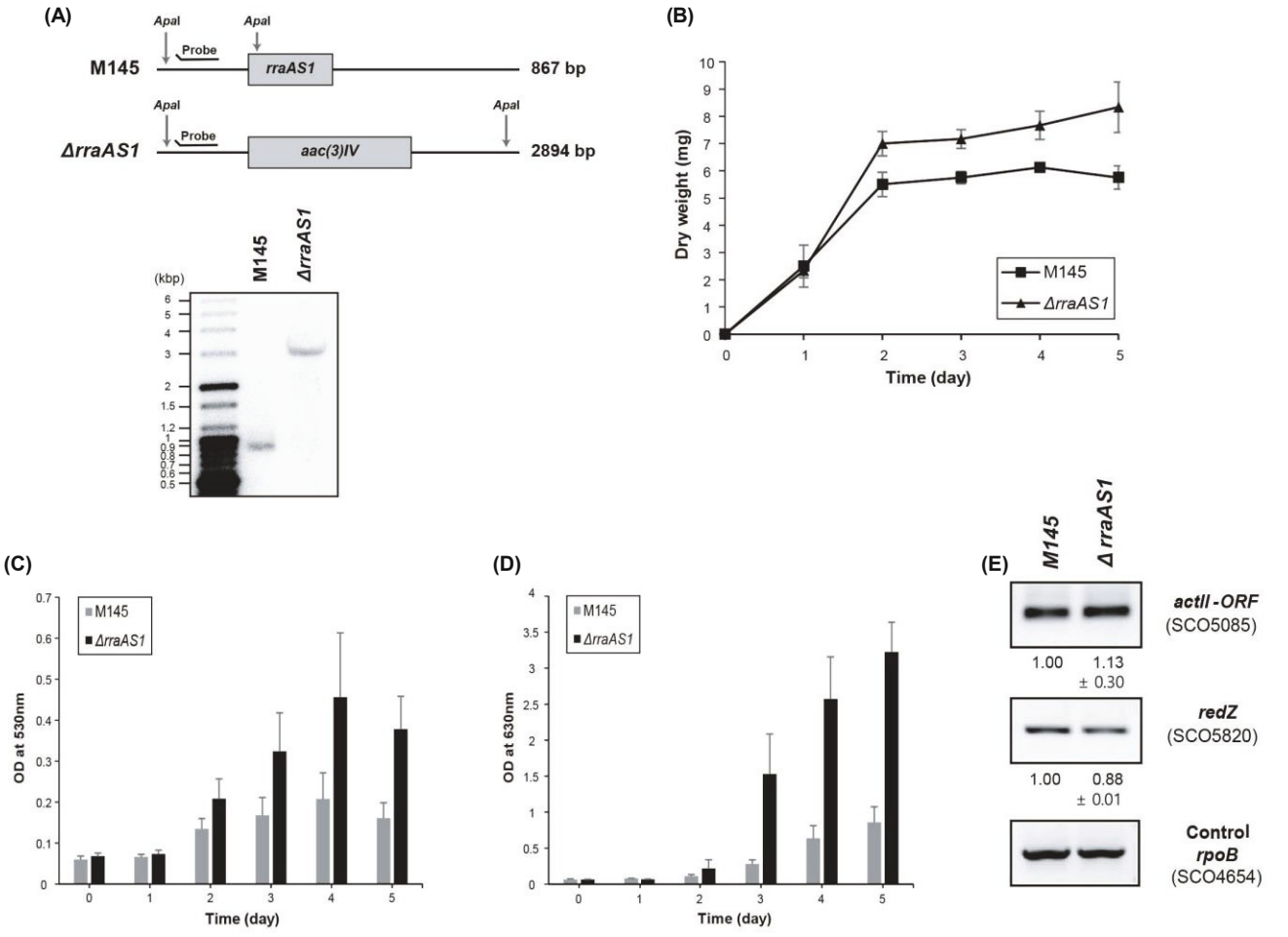

Fig. 3. Effects of *rraAS1* deletion on *S. coelicolor* physiology. (A) Southern blot analysis of an *rraAS1*-deleted strain. Genomic DNAs of *S. coelicolor* M145 and *S. coelicolor*Δ*rraAS1* were digested with *Apa*I. DNAs were loaded in a 0.8% agarose gel and stained with ethidiumbromide. (B) Growth rates of *S. coelicolor* M145 and *S. coelicolor*Δ*rraAS1* in liquid R5 medium. Growth rates were measured with dry weights. Cells were centrifuged and dried at 80°C overnight. (C, D) The levels of the antibiotics undecylprodigiosin (RED) (C) and actinorhodin (ACT) (D). Antibiotics were measured in *S. coelicolor* M145 and *S. coelicolor*Δ*rraAS1* cells grown in R5 medium using a spectrometric method described in 'Materials and Methods'. Data were normalized relative to dry weight biomass. (E) Effects of rraAS1 deletion on the steady-state level of *actII-ORF* and *redZ* mRNA. Total RNA was prepared from *S. coelicolor* M145 and *S. coelicolor*Δ*rraAS1* cells grown in R5 medium in a late mid-log phase and the abundance of *actII-ORF* and *redZ* mRNA was measured using semi-quantitative RT-PCR. Error bars are calculated from at least two independent experiments performed in duplicate.

catalytic activity of RNase ES rather than indirectly remodeling the degradosome composition, as has been proposed for RraA action on RNase E (Gao *et al.*, 2006).

RraA homologs can exert an inhibitory effect on the ribonucleolytic activity of structurally dissimilar RNase E-like enzymes found in distantly related bacteria (Yeom *et al.*, 2008a, 2008b; Lee *et al.*, 2009; Heo *et al.*, 2016b), implying that they have a conserved function in a common mechanism of global control of transcript abundance in bacteria. However, their physiological role has not been clearly characterized. In this respect, our findings implying involvement of RraAS1 in *S. coelicolor* growth and secondary metabolism provide evidence for the active role of these protein inhibitors in modulation of cellular physiology. A recent genome-wide study on *S. coelicolor* transcriptome shows that the mRNA abundance of *rraAS1* is approximately five times higher than that of *rraAS2* (Jeong *et al.*, 2016), indicating that RraAS1 is likely to play a more active role in regulating RNase ES activity *in vivo*. Further studies will unveil mechanisms underlying modulation of cellular physiology by *rraAS1*-mediated RNA metabolism.

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