The endonuclease activity of RNase III is required for the regulation of antibiotic production by *Streptomyces coelicolor*

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Received 9 July 2008

Revised 11 August 2008

Accepted 20 August 2008

The double strand-specific endoRNase RNase III globally regulates the production of antibiotics by *Streptomyces coelicolor*. We have undertaken studies to determine whether the endoRNase activity of *S. coelicolor* RNase III or its RNA binding activity is responsible for its regulatory function. We show that an *rnc* null mutant of *S. coelicolor* M145 does not produce actinorhodin or undecylprodigiosin. Restoring a wild-type copy of *rnc* to that mutant also restored antibiotic production. We constructed an *rnc* point mutant, D70A, in which an aspartic acid residue which is essential for the catalytic activity of RNase III was changed to alanine. The D70A mutation abolished the catalytic activity of the protein but not its ability to bind to RNA substrates. Introduction of a copy of the D70A gene into the *rnc* null mutant did not restore antibiotic production. This result suggests that the endoRNase activity of RNase III is required for the regulation of antibiotic production in *S. coelicolor*. We also reconstructed the C120 point mutation that was originally described in 1992. Although that mutation diminished antibiotic production by *S. coelicolor*, we confirm here that the C120 protein retains some RNase III activity.

INTRODUCTION

RNase III is a double strand-specific endonuclease that is found in bacteria and eukaryotes (Drider & Condon, 2004; Ji, 2006; Nicholson, 1999). In addition to its general role in RNA processing, RNase III is involved in the regulation of gene expression in *Escherichia coli* and other bacteria. Thus, in *E. coli* RNase III autoregulates its own expression by cleaving an untranslated leader in its mRNA. This cleavage leads to an increased rate of decay of the RNase III mRNA, downregulating expression of the RNase III gene *rnc* (Bardwell *et al.*, 1989; Matsunaga *et al.*, 1996, 1997). RNase III is also involved in regulation of the expression of the polynucleotide phosphorylase (PNPase) gene *pnp* in bacteria. RNase III cleaves a stem-loop structure in the 5' leader of the *pnp* transcripts in *E. coli*. The 3' ends produced by this cleavage then serve as targets for polyadenylation by poly(A) polymerase I, and the polyadenylated 3' ends are degraded by PNPase itself (Jarrige *et al.*, 2001; Robert-Le Meur & Portier, 1994). Thus, RNase III cleavage, polyadenylation and PNPase action on its own mRNA lead to instability of that mRNA, resulting in decreased synthesis of PNPase (Jarrige *et al.*, 2001; Robert-Le Meur & Portier, 1992, 1994). There are a number of other examples of the regulation of gene expression by RNase III in *E. coli* and other bacteria (reviewed by Drider & Condon, 2004; Ji, 2006; Nicholson, 1999).

RNase III also plays an important role in the regulation of antibiotic synthesis in *Streptomyces coelicolor*. Some years ago, Champness and co-workers identified an *S. coelicolor* locus, which they designated *absB* (Adamidis & Champness, 1992). The synthesis of all four antibiotics normally produced by *S. coelicolor* is severely reduced in an *absB* mutant, C120. Specifically, they reported that the production of actinorhodin (act) by the C120 mutant is reduced to only 2 % of the normally observed level and that the level of undecylprodigiosin (red) is only 15 % of that normally observed (Adamidis & Champness, 1992). Production of the calcium-dependent antibiotic and of methylenomycin is also reduced. The same group subsequently demonstrated that the *absB* locus encodes a homologue of RNase III, the double strand-specific endoRNase discussed above, and the C120 *absB* mutant was shown to contain a point mutation that results in a change of leucine to proline in the RNase III amino acid sequence (Price *et al.*, 1999). They demonstrated further that antibiotic levels are even lower in an RNase III disruptant than in C120 (Price *et al.*, 1999), a result that was recently confirmed (Sello & Buttner, 2008). Moreover, Aceti & Champness (1998) demonstrated decreased levels of the *actII-orf4* and *redD* transcripts, which encode the pathway-specific regulators or SARPs (*Streptomyces* antibiotic regulatory proteins) for act and red biosynthesis, respectively, in the *absB* mutant. They showed further that *absB* mutant strains are deficient in the processing of rRNA precursors compared with the wild-type strain (Price *et al.*,

Abbreviations: act, actinorhodin; red, undecylprodigiosin.

1999). These data strongly suggest that the *absB* locus does encode an RNase, and that in some fashion that locus functions as a global regulator of antibiotic production in *S. coelicolor*. These observations marked the first evidence for the regulation of antibiotic production at the level of RNA stability.

We have shown recently that the *absB* gene identified by Champness and co-workers does indeed encode a double strand-specific endoRNase, i.e. an RNase III (Chang *et al.*, 2005). There are two general classes of mechanisms by which RNase III might regulate antibiotic production in *S. coelicolor*. In the first, the endoRNase activity of RNase III would be required. There is some evidence, however, that RNase III can regulate gene expression in other systems by binding to dsRNA without cleaving it (reviewed by Drider & Condon, 2004; Ji, 2006; Nicholson, 1999) (and see further below). Thus, a second class of mechanisms for the regulation of antibiotic synthesis would involve binding of RNase III to appropriate RNA targets in ways that affect the expression of genes that are critical for antibiotic production, but without cleaving those RNAs.

To distinguish between these possibilities, it seemed prudent to determine whether the endoRNase activity of RNase III is actually required for its regulation of antibiotic production in *S. coelicolor*. We show below that the activity is required for the regulation of act and red production. We show further that the RNase III protein bearing the C120 mutation retains some enzymic activity and cleaves a model RNA transcript identically to the wild-type protein.

METHODS

Bacterial strains, plasmids and growth media. *E. coli* strains DH5 α and BL21(DE3)pLysS were used as hosts for plasmid manipulation and protein expression, respectively. *E. coli* strains BW25113 and ET12567 (*dam*, *dcm*, *hsdS*; Flett *et al.*, 1997) were used

for gene disruption and conjugation purposes. *E. coli* strains were grown on Difco Nutrient Agar or Luria-Bertani medium supplemented with carbenicillin (100 μ g ml⁻¹) and/or kanamycin (50 μ g ml⁻¹), as necessary. *S. coelicolor* M145 (parental strain), JSE1880, JSE1851 and JSE1855 were grown on soya flour-mannitol (SFM) agar or on R2YE agar or in liquid media (Kieser *et al.*, 2000) with viomycin (30 μ g ml⁻¹), nalidixic acid (25 μ g ml⁻¹) or apramycin (50 μ g ml⁻¹), as necessary. For the experiments shown in Fig. 4, strains were grown on R2YE agar containing nystatin (315 U ml⁻¹) and appropriate antibiotics.

Construction of the *rnc* disruptant JSE1880. The *rnc* (*absB*) gene was replaced by a viomycin-resistance cassette using the λ red-mediated recombination system for *Streptomyces* (Gust *et al.*, 2003). Cosmid SC7A1, which bears the *rnc* (*absB*) gene SCO5572, was introduced by electroporation into *E. coli* strain BW25113 containing the λ red plasmid. The viomycin-resistance cassette was excised from pIJ780 (Gust *et al.*, 2003; Kieser *et al.*, 2000) with *EcoRI* and *HindIII*. The cassette was used as a template for PCR and amplified using primers AbsB.red.fwd2 and AbsB.red.rev2 (Table 1). The strain carrying both the *S. coelicolor* cosmid and the λ red plasmid was electro-transformed with the extended viomycin-resistance cassette and transformants were plated on Difco Nutrient Agar containing carbenicillin (100 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹) and viomycin (30 μ g ml⁻¹). Larger colonies were restreaked on plates with selective antibiotics (carbenicillin, kanamycin, viomycin) to enrich the population with cells bearing the mutant cosmid. Colonies were screened by PCR with primers ABSB OUT1 and ABSB OUT2 (Table 1). The 5' ends of these primers are situated approximately 100 bp upstream and downstream of the deleted region of *rnc*. These primers produced an 1124 bp fragment with the wild-type cosmid as template and an 1800 bp fragment with the cosmid bearing the disrupted *rnc* gene. DNA was prepared from a positive transformant and introduced into the methylation-deficient *E. coli* host ET12567 (Flett *et al.*, 1997) containing the non-transmissible plasmid pUZ8002 (Flett *et al.*, 1997; Gust *et al.*, 2003; Kieser *et al.*, 2000). After conjugation using spores of *S. coelicolor* M145, desired exconjugants (*rnc*⁻, *vio*^r *kan*^s) were selected by restreaking single colonies on R2YE plates with and without kanamycin, and the identity of positive exconjugants was verified by PCR using primers ABSB OUT1 and ABSB OUT2 (Table 1). The strain bearing the disrupted *rnc* gene was designated JSE1880.

Table 1. Primers used in this study

The bases underlined for the AbsB.red.fwd2 and AbsB.red.rev2 primers correspond to sequences in the viomycin cassette used for λ red-mediated recombination. The remaining bases flank the *rnc* (*absB*) gene. The underlined sequences represent the *NdeI* sites in primers SCABSBF1 and SCABSBF2, and the *BamHI* sites in primers SCABSBR1 and C120BH1Rev.

Primer	Sequence
AbsB.red.fwd2	5'-AGGTCCTCGAGGTCTGAGCGGCTGGTGAAGGCACTGTGTGTAGG CTGGAGCTGCTTC-3'
AbsB.red.rev2	5'-GATGAGCGACGGTCCGAACGCCCGCCCTTTGCCGGGGCATTCCG GGGATCCGTCGACC-3'
ABSB OUT1	5'-AAGCGGAAGATGTCTCGCGCAGCAA-3'
ABSB OUT2	5'-TCGGGCAACTCGGGCATGGCTT-3'
MGabsBF1	5'-TCGGCTATCAGCTCGAGTCCGCC-3'
MGabsBR1	5'-GTGCCGGTGCCGTACGAGACG-3'
SCD70AF1	5'-TTCCTCGGGGCCCTCCGTG-3'
SCABSBF1	5'-CTGGTGAGAGGCCATATGTCAGTC-3'
SCABSBF2	5'-AGGTCTGAGCGGCATATGAGAGCC-3'
SCABSBR1	5'-GACGGGTCAGGCGGATCCGGACGC-3'
SC120LPF1	5'-CTCCAGGAACCCACCGCAACC-3'
C120BH1Rev	5'-GTTCTGGATCCTGCGGTGGACGGGTCA-3'

Construction of expression plasmids bearing wild-type and mutant *rnc* genes. To construct a plasmid for expression of the wild-type *rnc* gene, the 819 bp *rnc* (*absB*) ORF was released from pJSE1801 (Chang *et al.*, 2005) with *Bam*HI and *Nde*I. The resulting fragment was cloned into pIJ8600 (Kieser *et al.*, 2000; Sun *et al.*, 1999) digested with the same two enzymes, producing plasmid pJSE1851. This plasmid was transferred to *E. coli* ET12567/pUZ8002 by electrotransformation and conjugated into *S. coelicolor* JSE1880. Integration of pJSE1851 into the JSE1880 chromosome was confirmed by selecting for viomycin- and apramycin-resistant exconjugants, and via PCR using primers ABSB OUT1 and ABSB OUT2 (Table 1). To construct the plasmid that contained a point mutation in the *rnc* (*absB*) gene, a 627 bp fragment of the *absB* region was amplified in an initial PCR. This was done with a 5' primer (SCD70AF1, Table 1) that introduced an A to C base change in the second position of the codon for amino acid 70 in the RNase III amino acid sequence. This alteration changed the aspartic acid residue at position 70 to an alanine residue, creating the D70A mutation. The 3' primer used in these experiments contained a *Bam*HI site (SCABSBF1, Table 1). To amplify the full-length *rnc* product, the fragment obtained from the initial PCR was gel-purified and used as a 3' primer in a second PCR, together with a 5' primer that introduced an *Nde*I site into the product (SCABSBF2, Table 1). Sequencing of the resulting D70A gene showed that it contained an additional single base change but that change did not affect the amino acid sequence of the resulting protein. The PCR fragment was cloned into pCR2.1-TOPO (Invitrogen) following the manufacturer's protocol and the construct was designated pJSE1853. The *rnc* fragment was released from pJSE1853 by digestion with *Bam*HI and *Nde*I and was cloned into pET19B (Novagen), digested with the same enzymes, to yield pJSE1854. The mutant gene was also cloned into pIJ8600 to yield pJSE1855. *E. coli* BL21(DE3)pLysS was transformed with pJSE1854 for overexpression of the D70A protein and ET12567/pUZ8002 was transformed with pJSE1855 for conjugation into JSE1880, as described above. PCR analysis with appropriate primers of chromosomal DNA isolated from the parent organism *S. coelicolor* M145, from JSE1880, and from JSE1880 containing pIJ8600, pJSE1851 and pJSE1855, verified the identity of each of the relevant strains (data not shown).

Construction of the C120 mutant. For the reconstruction of C120, the mutant originally isolated by Adamidis & Champness (1992), which contained a single leucine to proline change at position 188 in the RNase III amino acid sequence, we again performed two rounds of PCR. The first round involved the amplification of a 300 bp fragment of the *absB* gene with a forward primer (SC120LPF1, Table 1) that introduced a single T to C change in the nucleotide sequence, and a reverse primer (C120BH1Rev) that introduced a *Bam*HI restriction site. The fragment obtained from the first PCR was then used as a 3' primer in the second round of PCR together with a 5' primer (SCABSBF1, Table 1) that contained an *Nde*I site. The resulting *rnc* fragment was cloned into pCR2.1-TOPO (Invitrogen), yielding plasmid pJSE1950. After sequencing to confirm the identity of the cloned insert, the mutated *rnc* fragment was removed from pJSE1950 with *Bam*HI and *Nde*I and ligated to pET19B which had also been digested with those enzymes. The resulting vector, pJSE1952, was used to transform chemically competent BL21(DE3)pLysS, which was then used to overexpress a hexahistidine-tagged version of the C120 protein. pJSE1811 (Chang *et al.*, 2005) was utilized for the production of wild-type RNase III. Overexpressing strains were grown and proteins (wild-type RNase III, and the D70A and C120 mutant proteins) were purified as described previously (Chang *et al.*, 2005).

RNA cleavage and electrophoretic mobility shift assays. The plasmid pJSE5600 (Chang *et al.*, 2008), which contains the cloned *rpsO-pnp* intergenic region from *S. coelicolor*, was linearized with *Spe*I.

The linearized plasmid was used as the DNA template for transcription with T7 RNA polymerase. Transcription reactions were performed as previously described (Chang *et al.*, 2005), with [³²P]CTP as the labelled precursor, except that no EDTA was added to reaction mixtures.

RNA cleavage assays, using the internally labelled *rpsO-pnp* transcript as substrate, were performed as described previously (Chang *et al.*, 2005). Immediately before the assay, RNA was heated at 100 °C for 30 s then placed on ice. Reaction mixtures for RNA cleavage contained 2 µg transcript (~10 000 c.p.m.) and 0–750 ng enzyme, either wild-type RNase III, or the D70A and C120 mutant proteins. Cleavage products were fractionated on 7 M urea/5 % polyacrylamide gels and were visualized by autoradiography. Kinetic assays of wild-type RNase III and the C120 mutant protein were performed as described previously (Chang *et al.*, 2005).

Electrophoretic mobility shift assays were performed in 20 µl reaction mixtures, incubated at 37 °C for 10 min. Mixtures contained 160 mM NaCl, 30 mM Tris-HCl (pH 8.0), 10 mM CaCl₂, 0.1 mM Na₂EDTA, 0.1 mM DTT, 5 %, v/v, glycerol, 10 ng µl⁻¹ *E. coli* tRNA, 100 ng RNA (32 nM, ~120 000 c.p.m., pre-heated at 100 °C for 30 s, then placed on ice), and 20–100 ng (30–160 nM) enzyme. Following incubation, the samples were immediately placed on ice for 20 min. The 6 % polyacrylamide gel was run at 75 V for 20 min and 4 °C in 0.5 × Tris-borate-EDTA buffer supplemented with 10 mM CaCl₂ prior to loading samples. Samples were then loaded on the gel and run at 85 V for ~1.5 h. Gels were dried and the results were visualized by autoradiography.

RT-PCR. To examine expression of the wild-type and D70A mutant *rnc* genes in the relevant strains, RT-PCR was performed. RNA was isolated and reactions were carried out using 10 µg total RNA, as described previously (Bralley & Jones, 2003). Random hexamers (Invitrogen) were used for the reverse transcription. The cDNA was amplified using primers MGabsBF1 and MGabsBR1 (Table 1), which are specific for *S. coelicolor rnc*, and products were displayed on agarose gels.

Miscellaneous methods. Protein was quantified with the Bio-Rad Protein Assay, using BSA as a standard. SDS-PAGE was performed on 12.5 % gels as described by Laemmli (1970), and gels were stained with Coomassie brilliant blue. PCR was performed using the Expand High Fidelity PCR System (Roche). Amplifications were generally done at an annealing temperature of 58 °C. A list of plasmids used or constructed in this study is presented in Table 2.

RESULTS

Construction and properties of JSE1880, pJSE1851/JSE1880 and pJSE1855/JSE1880

As indicated above, the C120 mutant isolated by Champness and co-workers contained a point mutation in the RNase III gene *rnc* (Price *et al.*, 1999). Those workers also constructed a disruptant by mutational cloning which contained two partial copies of *rnc*. For our studies, we felt it necessary to construct a true *absB* null mutant. To that end, the RNase III gene was replaced with a viomycin-resistance cassette in cosmid SC7A1 via PCR-targeted mutagenesis (Gust *et al.*, 2003). The cosmid containing the disrupted gene was transferred to *S. coelicolor* M145 by conjugation from *E. coli*. Several exconjugants were examined by PCR and one such strain, containing a

Table 2. Plasmids used or constructed in this study

Plasmid	Properties	Source or reference
pIJ780	Template plasmid; amp ^r , vio ^r	Gust <i>et al.</i> (2003)
pUZ8002	Non-transmissible <i>oriT</i> mobilizing plasmid; kan ^r	Kieser <i>et al.</i> (2000)
pIJ8600	Streptomycete integrative expression vector; thio ^r , apra ^r	Kieser <i>et al.</i> (2000); Sun <i>et al.</i> (1999)
pJSE1801	<i>S. coelicolor rnc</i> cloned into pET11A (Novagen)	Chang <i>et al.</i> (2005)
pJSE1811	<i>S. coelicolor rnc</i> cloned into pET19B (Novagen)	Chang <i>et al.</i> (2005)
pJSE1851	<i>S. coelicolor rnc</i> cloned into pIJ8600	This study
pJSE1853	<i>S. coelicolor rnc</i> D70A cloned into pCR2.1 TOPO (Invitrogen)	This study
pJSE1854	<i>S. coelicolor rnc</i> D70A cloned into pET19B (Novagen)	This study
pJSE1855	<i>S. coelicolor rnc</i> D70A cloned into pIJ8600	This study
pJSE1950	<i>S. coelicolor rnc</i> C120 cloned into pCR2.1 TOPO (Invitrogen)	This study
pJSE1952	<i>S. coelicolor rnc</i> C120 cloned into pET19B (Novagen)	This study
pJSE5600	<i>S. coelicolor</i> 459 bp <i>rpsO-pnp</i> intergenic region cloned into pCR2.1 TOPO (Invitrogen)	Chang <i>et al.</i> (2005)

disrupted chromosomal copy of *rnc*, was designated JSE1880.

A copy of wild-type *rnc* was prepared via PCR and cloned into the streptomycete expression vector pIJ8600, as described in Methods. This vector contains the thiostrepton-inducible *tipA* promoter (Kieser *et al.*, 2000; Sun *et al.*, 1999), and the plasmid resulting from the insertion of *rnc*, designated pJSE1851, was transferred to JSE1880 by conjugation from *E. coli*.

To determine whether the endoRNase activity of *S. coelicolor* RNase III was required for the regulation of antibiotic production, it was necessary to construct a mutant gene whose product lacked that activity but retained other relevant biological functions. Nicholson and co-workers have described an *rnc* mutation in *E. coli* which appears to fulfil those criteria. In this mutant, designated D45A, an aspartic acid residue which is essential for the catalytic function of RNase III is changed to an alanine residue (Sun *et al.*, 2004). D45A is essentially unable to cleave model substrates; the catalytic activity of the enzyme is ~30 000-fold lower than that of the wild-type RNase III. Nevertheless, the D45A mutant is capable of binding an RNA substrate in a fashion that is comparable to that of the wild-type enzyme (Sun *et al.*, 2004).

The aspartic acid residue identified by Sun *et al.* (2004) is conserved in bacterial RNases III, as shown in Fig. 1. Indeed there is strong conservation of several amino acids in the region of the proteins that contains the critical Asp residue. This observation suggested that mutating that Asp residue in *S. coelicolor* RNase III would produce a protein with properties similar to those of the D45A mutant in *E. coli*. As described in Methods, we constructed a mutant of *S. coelicolor* RNase III in which the Asp residue at position 70 was changed to Ala (D70A).

The D70A protein lacks catalytic activity but retains the ability to bind an RNA substrate

In addition to the construction of pJSE1855, we cloned the D70A gene into pET19B. This cloning attached a hexahistidine tag to the N terminus of the RNase III ORF. We overexpressed the protein and purified it by immobilized metal ion affinity chromatography. We then examined the activity of wild-type *S. coelicolor* RNase III and the D70A protein in RNA cleavage assays using an RNA substrate derived from the *S. coelicolor rpsO-pnp* operon. This transcript, designated 5600, is similar to the one used in our original characterization of the catalytic activity of *S. coelicolor* RNase III (Chang *et al.*, 2005), and

Fig. 1. PILEUP comparison (Devereux *et al.*, 1984) of the region of bacterial RNases III containing the critical aspartic acid residue mutated in this study (arrow). Species shown are as follows: *S. coelicolor*, *Wollinella succinogenes*, *Aquifex aeolicus*, *Bacillus subtilis*, *Caulobacter crescentus*, *Corynebacterium diphtheriae*, *E. coli*, *Geobacter sulfurreducens*, *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Synechocystis*, *Thermotoga maritima*.

was also used by us in a kinetic analysis of PNPase (Chang *et al.*, 2008). The left panel of Fig. 2 shows the results of cleavage assays using the 5600 transcript labelled internally with [32 P]CTP and wild-type RNase III. It can be seen that the enzyme cleaves this substrate effectively, as we have demonstrated previously (Chang *et al.*, 2005). Cleavage produces two major bands, shown in Fig. 2 and representing cleavage on the 5' and 3' sides of an internal loop in the 5600 transcript (Chang *et al.*, 2005). A third band, representing the apex of the stem-loop in the transcript, ran off the bottom of the gel shown in Fig. 2. In contrast, even at the highest concentration of D70A protein employed, no cleavage of this substrate was observed, even after 20 min of incubation at 37 °C (Fig. 2, middle panel). Amounts of enzyme up to 1 µg did not lead to observable cleavage of the substrate (data not shown). Thus, as was the case for the D45A mutant in *E. coli*, our data indicate that the D70A change in *S. coelicolor* RNase III abolishes the catalytic activity of the enzyme.

To determine whether D70A retained the ability to bind RNAs, electrophoretic mobility shift assays were performed, as described in Methods. Results of such assays are shown in Fig. 3. The left panel of the figure shows that wild-type *S. coelicolor* RNase III binds the 5600 transcript effectively with 20–100 ng of enzyme in reaction mixtures. The right panel of the figure shows that D70A retains the ability to bind the substrate, at the same protein concentrations used for the wild-type enzyme. Although no attempt was made in these experiments to measure dissociation constants, the data of Fig. 3 suggest that D70A binds the 5600 transcript at least as well as does wild-type RNase III at comparable concentrations. Indeed, D70A appears in our hands to bind the substrate more effectively than the wild-type enzyme. Thus, our data indicate that like D45A, the D70A mutation abolishes the catalytic activity of *S. coelicolor* RNase III but not its ability to bind RNA substrates.

The endoRNase activity of *S. coelicolor* is required for the regulation of act and red synthesis

We tested the ability of JSE1880 and various derivatives thereof to produce two of the antibiotics normally elaborated by *S. coelicolor*, i.e. act and red. Fig. 4 shows the results of growth of the various strains on R2YE medium. It can be seen that M145 makes both act and red. The red antibiotic can be clearly seen after 3 days of incubation, and although both antibiotics are present after 5 days of incubation, the deep-blue colour of act prevents visualization of the red antibiotic at longer incubation times. As predicted, JSE1880 did not make detectable amounts of either antibiotic. The JSE1880 plate shown in Fig. 4 was incubated for 5 days. No act or red was detectable even after 7–10 days of incubation.

As shown on the top right in Fig. 4, a five-day incubation of pIJ8600/JSE1880 produced no detectable act. Interestingly, a small amount of red antibiotic was observed after 5 days of incubation, and the amount of antibiotic increased slightly in the presence of thiostrepton. We currently have no explanation for this observation. Restoring a wild-type copy of *rnc* (pJSE1851) to JSE1880 also restored the ability of the resulting strain to produce both the act and red antibiotics. The *tipA* promoter was somewhat leaky in our hands, and it can be seen that significant amounts of red and some act were produced in a five-day incubation in the absence of thiostrepton. Both 5 and 15 µg thiostrepton ml⁻¹ stimulated production of act and red. The most straightforward interpretation of this observation is that thiostrepton induces transcription of the *rnc* gene in pJSE1851, leading to the production of wild-type RNase III.

The bottom-right row of plates in Fig. 4 shows the results of the analysis of pJSE1855/JSE1880. Again, a small amount of red antibiotic was observed and the amount was highest

Fig. 2. RNA cleavage assays. Left panel, cleavage of the 5600 transcript with varying amounts of wild-type *S. coelicolor* RNase III; middle panel, cleavage of the transcript with the D70A mutant RNase III; right panel, cleavage of the transcript with the C120 mutant RNase III. Cleavage reactions were performed as described in Methods and the products were analysed on 5 % urea-polyacrylamide gels. Bands were visualized by autoradiography of dried gels.

Fig. 3. Electrophoretic mobility shift assays. Left panel, assay of the binding of ^{32}P -labelled 5600 transcript by wild-type *S. coelicolor* RNase III. Right panel, binding of the transcript by the D70A mutant RNase III. Binding assays and electrophoresis on 6% non-denaturing polyacrylamide gels were performed as described in Methods.

at $15\text{ }\mu\text{g thioestrepton ml}^{-1}$. However, the amount observed for pJSE1855/JSE1880 was essentially the same as that observed in the control strain containing pIJ8600. No additional red was produced when plates were incubated for up to 7 days and no act was observed at any time (data not shown). Again, the most straightforward interpretation of this observation is that pJSE1855/JSE1880 does not produce the active RNase III which is required for the production of act and red. Results similar to these were

observed when the relevant strains were grown on SFM medium.

The foregoing observations and their interpretation hinge on the assumption that both the wild-type *rnc* and the D70A gene are expressed in the relevant constructs described above. To test this assumption, RNA was isolated from wild-type *S. coelicolor* mycelium and from mycelium of strains pIJ8600/JSE1880, pJSE1851/JSE1880 and

Fig. 4. Antibiotic production by wild-type and mutant strains of *S. coelicolor*. Spores were streaked to R2YE medium. Plates bearing the wild-type strain, M145, were incubated for 3 or 5 days to permit visualization of both the red and act antibiotics. All other plates were incubated for 5 days. Plates streaked with strains bearing derivatives of pIJ8600 were incubated without thioestrepton or with 5 or $15\text{ }\mu\text{g thioestrepton ml}^{-1}$ to induce expression from the *tipA* promoter. The JSE1880 plate contained viomycin ($30\text{ }\mu\text{g ml}^{-1}$), while the plates bearing pIJ8600 derivatives contained apramycin ($50\text{ }\mu\text{g ml}^{-1}$). All plates contained nalidixic acid ($25\text{ }\mu\text{g ml}^{-1}$) and nystatin (315 U ml^{-1}).

JSE1855/JSE1880, and the RNA was analysed by RT-PCR using *rnc*-specific primers as described in Methods. Fig. 5 shows the results of this analysis. Lane 2 shows the product obtained by PCR with M145 chromosomal DNA alone. Lane 3 shows that a product of the same size is obtained by RT-PCR using RNA isolated from M145. No such product is seen in lane 4, which shows the results of RT-PCR using RNA isolated from pJSE8600/JSE1880. The *rnc*-specific product is observed in lanes 5 and 6, which show the results of RT-PCR using RNA isolated from pJSE1851/JSE1880 and pJSE1855/JSE1880, respectively. Thus, the *rnc* genes are expressed in those strains. Taken together, the plate assay and RT-PCR results lead to the conclusion that the catalytic activity of RNase III is required for its regulation of act and red production in *S. coelicolor*. In the absence of active RNase III, even when a form of the enzyme capable of RNA binding was present, no act or red was produced.

The C120 mutant RNase retains enzymic activity

The C120 mutant isolated in the pioneering studies of Champness and co-workers was not only used in their studies, it has also been used recently in a microarray study of the expression of genes involved in antibiotic synthesis (Huang *et al.*, 2005b) and in an analysis of *rnc* autoregulation in *S. coelicolor* (Xu *et al.*, 2008). Given the extensive use of this mutant and the fact that Champness and co-workers observed a more drastic effect on antibiotic production in their *rnc* disruptant, as compared with C120 (Price *et al.*, 1999), it was of interest to determine whether the C120 protein retained any enzymic activity in

Fig. 5. RT-PCR analysis of RNAs isolated from *S. coelicolor* strains. RT-PCR was performed as described in Methods using random hexamers for reverse transcription and the MGabsBF1 and MGabsBR1 primers (Table 1) for PCR. Products were displayed on an agarose gel. Lane 1, size standards. The asterisk indicates the 1 kb standard band. Lane 2, PCR only with DNA from *S. coelicolor* M145; lane 3, RT-PCR with RNA from M145; lane 4, RT-PCR with RNA from pJSE8600/JSE1880; lane 5, RT-PCR with RNA from pJSE1851/JSE1880; lane 6, RT-PCR with RNA from pJSE1855/JSE1880. The arrow indicates the position of the band expected from PCR using the MGabsBF1 and MGabsBR1 primers. The lower band shown in lanes 3 and 5 is a PCR artefact.

our assays. To this end, we reconstructed C120 as described in Methods and examined its activity using the 5600 transcript. Results of this analysis are shown in Fig. 2 (right panel). It can be seen that the C120 protein retains some enzymic activity, although it is not as active on a molar basis as our preparation of wild-type RNase III. We performed kinetic assays as described previously (Chang *et al.*, 2005) to allow a more detailed comparison of the activities of the wild-type and mutant enzymes. The k_{cat}/K_m value for the wild-type enzyme was $2.76 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, while the corresponding value for C120 was $3.26 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$. Thus, the catalytic activity of the wild-type enzyme is 85 times greater than that of C120. The electrophoretic analysis suggests that the same products obtained from cleavage assays containing wild-type RNase III are produced by the C120 mutant enzyme (Fig. 2).

DISCUSSION

The autoregulation of *rnc* expression in *E. coli* (Bardwell *et al.*, 1989; Matsunaga *et al.*, 1996, 1997) and *Streptomyces* (Xu *et al.*, 2008) and the regulation of *pnp* expression by RNase III (Robert-Le Meur & Portier, 1992, 1994) in *E. coli* are examples of the involvement of the catalytic activity of RNase III in the control of gene expression. In a study of the structure of the complex between *Aquifex aeolicus* RNase III and a model dsRNA substrate, Blaszczyk *et al.* (2004) proposed that RNase III can assume two different RNA binding modes, one which supports RNA processing and one which allows dsRNA binding but not RNA cleavage. They argue that the latter binding mode allows RNase III to regulate gene expression without dsRNA processing. There is evidence to support this hypothesis. Thus, Oppenheim *et al.* (1993) have shown that translation of the cIII mRNA in cells infected by bacteriophage λ is strongly dependent on RNase III. They proposed that the cIII mRNA can adopt alternative structures, one of which (ON) can be translated efficiently by *E. coli* ribosomes and one of which (OFF) has the Shine–Dalgarno sequence and the AUG initiation codon sequestered in a base-paired region and cannot be efficiently translated. They argued that RNase III can bind to the ON structure without cleaving it, thereby stabilizing that structure and facilitating the translation of the cIII mRNA. Dasgupta *et al.* (1998) isolated a mutant RNase III, designated Rnc70, which retained the ability to bind to dsRNAs but did not cleave them. They showed that multicopy expression of *rnc70* suppressed the cold-sensitive phenotype in an *E. coli* strain bearing the *ssyA3* mutation. This result also indicates that RNase III can regulate gene expression, even when it cannot cleave RNA substrates, presumably via the ability of the enzyme to bind RNAs under these conditions.

The foregoing observations suggested to us the formal possibility that RNase III could regulate antibiotic production in *S. coelicolor* by binding to specific RNA targets without cleaving them. It was thus of critical importance, before exploring the mechanism of that

regulation further, to determine whether the catalytic activity of the enzyme was necessary for antibiotic regulation. The most straightforward interpretation of the results presented here is that the catalytic activity of RNase III is required for its regulation of act and red synthesis in *S. coelicolor*. Replacing the wild-type protein with D70A, which can bind RNA substrates but cannot cleave them, was not sufficient to restore antibiotic production to JSE1880. We did not examine the effects of D70A on the production of the calcium-dependent antibiotic or on methylenomycin production.

We have also demonstrated that one of the mutations originally isolated by Champness and co-workers, C120, does not completely abolish the activity of RNase III. Although the mutation changes leucine to proline (Price *et al.*, 1999), and would be expected to affect the tertiary structure of the protein in a significant fashion, we observed that the C120 protein was still able to cleave the 5600 transcript, albeit with an efficiency that was 85-fold lower than that of the wild-type enzyme. The C120 enzyme appears to be more active in our assays, with the *rspO-pnp* transcript as substrate, than in the experiments described by Xu *et al.* (2008) using the *absB* transcript as substrate. Our findings, coupled with the observation of Champness and co-workers that antibiotic production is detectably reduced in the C120 mutant (Adamidis & Champness, 1992), suggest that antibiotic synthesis in *S. coelicolor* is exquisitely sensitive to regulation by RNase III.

The question remains, what are the targets for RNase III regulation of antibiotic production in *S. coelicolor*? There are numerous studies which indicate that various antibiotic regulatory circuits are superimposed on each other in *S. coelicolor* (Bibb, 1996; Chater & Hopwood, 1989; Huang *et al.*, 2005a), so it is possible that RNase III is required for the processing of transcripts of regulatory genes that have already been identified. Champness and co-workers have shown, for example, that expression of the *actII-orf4* and *redD* genes is dependent on RNase III (Aceti & Champness, 1998), although there is currently no evidence that the transcripts of these genes are substrates for RNase III. It seems equally possible, however, that transcripts of other, as-yet-unidentified, genes are the actual targets for RNase III regulation of antibiotic production. Thus, RNase III might process transcripts for activators of antibiotic production. In the *absB* mutant, those activators would not be produced, at least not in their active forms, and no antibiotics would be made. Alternatively, RNase III might be required to degrade the transcripts for repressors of antibiotic synthesis. In the *absB* mutant, those transcripts and their protein products would persist. A third possibility would involve effects of RNase III action on phosphate and nitrogen levels in *S. coelicolor*. Normally, RNase III cleavage coupled with the subsequent decay of target RNAs might affect the levels of those two nutrients. Abolishing RNase III activity might lead to decreases in phosphate and nitrogen levels, both of which are well known to affect the production of streptomycete anti-

biotics (Bibb, 2005; Hillerich & Westpheling, 2008; Rodriguez-Garcia *et al.*, 2007). Experiments are in progress to examine these possibilities.

ACKNOWLEDGEMENTS

These studies were supported in part by a grant from the Emory University Research Committee.

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Edited by: M. Paget