Regulation of morphological differentiation in S. coelicolor by RNase III (AbsB) cleavage of mRNA encoding the AdpA transcription factor

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Summary

RNase III family enzymes, which are perhaps the most widely conserved of all ribonucleases, are known primarily for their role in the processing and maturation of small RNAs. The RNase III gene of Streptomyces coelicolor, which was discovered initially as a global regulator of antibiotic production in this developmentally complex bacterial species and named absB (antibiotic biosynthesis gene B), has subsequently also been found to modulate the cellular abundance of multiple messenger RNAs implicated in morphological differentiation. We report here that regulation of differentiation-related mRNAs by the S. coelicolor AbsB/RNase III enzyme occurs largely by ribonucleolytic cleavage of transcripts encoding the pleiotropic transcription factor, AdpA, and that AdpA and AbsB participate in a novel feedback-control loop that reciprocally regulates the cellular levels of both proteins. Our results reveal a previously unsuspected mechanism for global ribonuclease-mediated control of gene expression in streptomycetes.

Introduction

Streptomyces species are prokaryotic soil-dwelling organisms that have a complex life cycle involving mycelial growth and spore formation. In addition to producing more than two-thirds of the antibiotics currently in clinical and veterinary use, Streptomyces are the source of multiple other types of pharmaceutically useful compounds (Challis and Hopwood, 2003). For more than 40 years, Streptomyces coelicolor has been widely employed as a model organism in studies of morphological development and antibiotic regulation in streptomycetes. These studies (reviewed in Bibb, 1996; Chater, 2006; Flardh and Buttner, 2009) plus more recent microarray-based investigations (Huang et al., 2001; Huang et al., 2005) have shown that antibiotic biosynthesis in S. coelicolor is controlled by a complex multifaceted regulatory network.

The absB gene was discovered in a screen for S. coelicolor mutants that are defective in antibiotic biosynthesis but which do not interfere with the morphological development associated with the production of antibiotics and other secondary metabolites in this micro-organism (Adamidis and Champness, 1992; Aceti and Champness, 1998). DNA sequence analysis suggested that the protein encoded by absB is a member of the RNase III family of endoribonucleases (Price et al., 1999) – which commonly recognize double-strand segments of stem-loop structures and in bacteria carry out the processing of pre-rRNA, tRNA and polycistronic mRNA (Conrad and Rauhut, 2002; Drider and Condon, 2004). Cells containing a mis-sense point mutation in the absB gene were found to accumulate 30s rRNA precursors (Price et al., 1999).

Subsequent studies have shown purified AbsB protein can carry out site-specific RNA cleavages in vitro (Chang et al., 2005), and have provided direct evidence that the AbsB protein functions as a ribonuclease in vivo (Gravenbeck and Jones, 2008; Xu et al., 2008). Additionally, microarray analysis of gene expression has shown that mutation of absB can dramatically and broadly affect the abundance of individual mRNAs in S. coelicolor, elevating the cellular levels of mRNAs implicated in sporulation while decreasing the abundance of mRNAs encoding activators of the biosynthesis of antibiotics and other secondary metabolites (Huang et al., 2005).

The decrease in antibiotic biosynthesis-related transcripts (e.g. redD, cdaR, actII-ORF4, or redZ) observed in the absB mutant strain (Huang et al., 2005), which is consistent with the defective antibiotic production phenotype associated with mutation of absB (Adamidis and Champness, 1992), implies that these absB-regulated transcripts are not substrates for the ribonucleolytic activity of AbsB/RNase III. In contrast, the increased abundance of sporulation gene transcripts (Huang et al., 2005)
raises the prospect that some or all of the sporulation gene transcripts elevated by absB mutation may be targets of ribonucleolytic digestion by the AbsB protein. Here we report the discovery that AbsB/RNase III regulation of S. coelicolor genes implicated in morphological development is mediated by endoribonucleolytic cleavage of mRNA encoding AdpA, initially identified in S. griseus as a pleiotropic AraC/XylS family transcription factor (Ohnishi et al., 2005). We further show that AbsB and AdpA participate in a post-transcriptional regulatory loop that modulates the cellular levels of the proteins encoded by both genes.

**Results**

**Role of AdpA in AbsB-controlled gene expression**

The transcript most dramatically elevated in absB mutant bacteria in microarray studies was sti1 (SCO0762) (Huang et al., 2005), which encodes a member of the subtilisin inhibitor (SSI) family of protease inhibitors found in multiple streptomycetes and which is known to be implicated in the formation of aerial mycelia and spores in *Streptomyces albogriseolus* (Taguchi et al., 1995; Kato et al., 2005). Direct assay in the absB point mutant strain C120, which replaces leucine by proline at position 120 of the absB open reading frame (Adamidis and Champness, 1992), showed increased sporulation (Fig. S1), and RT-PCR studies in the same strain as well as in an absB null mutant strain (J-5572) constructed in our lab (Fig. 1A) confirmed both the absB dependence and the temporally regulated elevation of sti1 mRNA abundance relative to parental bacteria (J1501). This increase was most prominent 48 h after plating of spores – the time in the S. coelicolor life cycle associated with the formation of aerial mycelium. However, notwithstanding the dramatic increase in sti1 transcript abundance observed in bacteria defective in absB (both in C120 and The absB null mutant strain J-5572), we did not detect cleavage of sti1 transcripts by purified AbsB/RNase III protein (Fig. S2A) under conditions where the ribonuclease cleaved its own mRNA (Fig. S2B). These observations suggest that the regulation of at least sti1 by AbsB is accomplished indirectly, rather than by AbsB/RNase III digestion of sti1 transcripts. Potentially, such indirect regulation could be mediated by either an AbsB-targeted regulatory RNA or an AbsB-targeted mRNA encoding a transcription-activating protein.

**Sti1** is only one of many sporulation-gene-associated genes whose transcripts are elevated in absB mutant bacteria (Huang et al., 2005). As an approach to identifying possible AbsB-targeted intermediates that may upregulate multiple sporulation-associated transcripts, we searched for features in common among AbsB-controlled genes. Expression of sti1 (SCO0762) is TTA-dependent and is regulated in S. coelicolor by AdpA, an AraC/XylS family transcription factor (Kim et al., 2005; Kim et al., 2008). Our microarray experiments (Huang et al., 2005) and confirmatory RT-PCR experiments (Fig. 1B) had shown that *ramR*, another sporulation-associated gene (Ma and Kendall, 1994) known to be transcriptionally controlled by AdpA (Nguyen et al., 2003), is also upregulated in absB mutant bacteria. Moreover, as we had observed for sti1 transcripts, *ramR* mRNA was also not targeted by the ribonucleolytic action of purified AbsB/RNase III protein (Fig. S2C).

AdpA was discovered initially because of its effects on antibiotic biosynthesis and sporulation in *S. griseus* (Ohnishi et al., 1999) and subsequently found to similarly affect these processes in *S. coelicolor* (Nguyen et al., 2003; Takano et al., 2003, Park et al., 2009). The S. coelicolor adpA gene has also been called bldH, and production of the AdpA protein is controlled by *bldA* (Merrick, 1976; Lawlor et al., 1987) – a sporulation-regulating gene encoding a trNA that enables the translation of AdpA and
other genes containing TTA codons, which are rare in streptomycetes (Takano et al., 2003). Interestingly, the TTA codons of the adpA genes of all sequenced Streptomyces species are located at precisely the same position in the protein coding sequence, suggesting that their remarkably conserved location may have biological relevance.

The above observations and considerations raised the possibility that upregulation of sporulation-associated genes in absB mutant bacteria may be mediated by AdpA. Consistent with this notion, review of our microarray data (Huang et al., 2005) and the S. coelicolor genome sequence (Bentley et al., 2002) indicated that 20 S. coelicolor genes whose expression was increased or decreased more than sixfold by mutation of absB contain, 5′ to the ORF, a sequence showing 100% correspondence to the AdpA-binding motif (5′-TGCCSNWY-3′) (S: G or C; W: A or T; Y: T or C) that has been found in the promoter regions of multiple S. griseus AdpA target genes (Yamazaki et al., 2004). These genes and the positions of the AdpA-binding motifs relative to the start codon of the ORF are shown in Table S1.

AdpA promoter activity has been shown by earlier studies to be developmentally controlled, peaking just prior to the formation of aerial hyphae (Nguyen et al., 2003). Our Northern blot analysis confirmed this finding, and also indicated that the steady state level of full-length adpA transcripts was increased in both the C120 absB point mutation strain and in absB null mutation strain J-5572 at all times tested (Fig. 2A). Moreover, expression of the absB ORF in C120 under control of the thiostrepton-inducible tipA promoter (C120 + absB) reversed the adpA transcript elevation otherwise seen in the absB mutant C120 (Fig. 2B, lanes 4 vs. 3). We also observed that the abundance of adventitiously overexpressed adpA mRNA (Fig. 2C) and protein (Fig. 2D) following induction of the adpA gene under control of the tipA promoter was greater in C120 absB mutant bacteria than in absB+ cells (Fig. 2C, lane 8 vs. lane 6; Fig. 2D, lane 4 vs. lane 2, lane 8 vs. lane 6).

The notion that the AbsB/RNase III protein targets adpA transcripts ribonucleolytically was confirmed directly in demonstrating the ability of purified AbsB/RNase III protein to cleave a 1600 nt segment of adpA mRNA synthesized in vitro by bacteriophage T7 RNA polymerase on a template consisting of a PCR-amplified adpA-encoding segment of S. coelicolor genomic DNA (see Experimental procedures). As shown in Fig. 3, treatment of adpA transcripts with increasing amounts of wild type AbsB/RNase III protein resulted in decreasing intensity of the 1600 nt band corresponding to adpA transcript and yielded multiple degradation products (lanes 2–6) – whereas treatment of this transcript with ribonucleolytically inactive nonsense mutant AbsB protein did not detectably affect the transcript's abundance or generate cleavage products (Fig. 3, lanes 7–11).

Global effects of AbsB cleavage of adpA mRNA on S. coelicolor gene expression

Taken together, the results described argue strongly that accumulation of adpA transcripts in absB mutant bacteria (C120) underlies at least some of the previously observed phenotypic effects of absB mutations (Huang et al., 2005). To learn more fully the extent to which cleavage of adpA mRNA by the AbsB/RNase III ribonuclease accounts for specific absB-mediated alterations in gene expression, we used DNA microarray analysis to investigate the effects of adpA mutations on gene expression in both parental (J1501) and absB mutant (C120) strains. Similar numbers of spores of J1501 (morphologically wild type), J-2792 (∆adpA), C120 (absB point mutant) and C-2792 (absB point mutant, ∆adpA) were spread onto R5 solid medium. The total mass of mycelia was collected from each strain and weighed as an indicator of cell growth and/or proliferation, which was similar in all four strains (Fig. S3). RNA was harvested at various times after germination of spores (36, 48, 60, 72 or 96 h), and Cy5-dCTP red fluorescence-labelled cDNA from the J-2792 (∆adpA) and C-2792 (absB, ∆adpA) strains was separately hybridized on DNA microarrays with Cy3-dCTP green fluorescence-labelled cDNA from strains J1501 and C120. Results from these experiments provided gene expression signatures for mycelium obtained at different stages of the bacterial growth cycle in the presence and absence of AdpA function (Fig. 4).

Analysis of the data using previously described methods (Huang et al., 2005) indicated that adpA inactivation prevented expression of multiple other sporulation-related genes in both absB+ (J-2792 vs. J1501) or absB mutant (C-2792 vs. C120) bacteria (Fig. 4A). As was observed for sti1, expression of the ramR gene cluster (SCO6681,SCO6682) (Keijser et al., 2002), and also of bldN (SCO3323) (Bibb et al., 2000), bldM (SCO4768) (Molle and Buttner, 2000), SCO4114 (a sporulation-associated protein) (Li et al., 2006) and whiH (SCO5819, a sporulation-related transcription factor) (Ryding et al., 1998) were dramatically decreased in adpA mutant bacteria (J-2792) relative to J1501 levels at all time points tested (Fig. 4A). These results which are consistent with earlier studies showing that adventitious overexpression of adpA activates sporulation (Nguyen et al., 2003), also show that AdpA is necessary for normal sporulation in S. coelicolor. Expression of the spore pigment-related whiE cluster (SCO5318-SCO5321) (Davis and Chater, 1990) was also reduced in adpA null mutant bacteria relative to the parental strain, although only at the 96 h time point.
Inactivation of adpA in strain J-2792, a J1501 derivative, resulted in premature upregulation of multiple antibiotic biosynthesis genes (Fig. 4B), as has been reported previously for this lineage (Nguyen et al., 2003). Whereas expression of Red and Act pathway-related genes normally is not activated until 60–72 h after the plating of J1501 spores onto R5 solid medium, and reaches a peak at 72–96 h (Huang et al., 2005), in J-2792 (ΔadpA) expression of the Red and Act biosynthetic gene clusters was prematurely increased, as indicated by comparison of mRNA abundance relative to the morphologically wild type parental strain at the 48 h time point. Consistent with
this finding, adpA inactivation resulted in premature production of pigmented antibiotics (Fig. 5A, upper panel). The premature turn on of the Red and Act genes in adpA null mutant bacteria suggests that the absence of expression of these genes early in the S. coelicolor life cycle is attributable in part to the action(s) of AdpA. The stimulatory effects of adpA mutation on antibiotic production in the J1501 lineage (Nguyen et al. 2003 and our current results) contrast with the inhibition of antibiotic production reported to result from adpA mutation in derivatives of S. coelicolor M145 (Takano et al., 2003).

In absB mutant bacteria (C120), adpA transcripts and protein accumulate (Figs 2 and 3) and antibiotic biosynthesis gene transcripts are sharply decreased in abundance (Huang et al., 2005). However, notwithstanding accumulation of AdpA in these bacteria, and the demonstrated ability of AdpA accumulation to downregulate antibiotic biosynthesis in this lineage (Nguyen et al., 2003), introduction of an adpA null mutation into a J1501-derived absB mutant did not restore antibiotic synthesis (strain C-2792 mutated both adpA and absB; Fig. 5B). We therefore conclude that a mechanism other than enhanced AdpA repression of antibiotic biosynthetic genes underlies the defective antibiotic synthesis observed in absB mutant bacteria.

AbsB and AdpA participate in a feedback-control regulatory loop

Previous studies from our lab have shown that the AbsB protein cleaves the transcript that encodes it and consequently that absB mRNA is elevated in absB missense mutant bacteria (i.e. strain C120; Xu et al., 2008). However, we observed paradoxically that despite elevation of mutant absB mRNA in strain C120, the abundance of the mutant AbsB protein was decreased at late times in the growth cycle (Mut AbsB, Fig. 6A, bottom panel), whereas the abundance of the wild type AbsB protein remained constant throughout the growth cycle (Fig. 6A, top panel). The decrease in mutant AbsB protein, which temporally correlates with the time of onset of enhanced AdpA synthesis (Nguyen et al. 2003 and Fig. 2A), was partially reversed by mutation of adpA (strain C-2792; absB, ΔadpA; Fig. 6A bottom panel). The notion that adpA expression can regulate the abundance of the AbsB protein was directly confirmed by experiments showing that adventitious overexpression of adpA under control of a thiostrepton-regulated tipA promoter incrementally decreased AbsB protein abundance (Fig. 6B).

adpA mutation results in decreased expression of multiple protease genes

Western blot analysis using anti-AbsB antibody showed the occurrence of an immuno-reactive band of lower molecular weight than full-length AbsB protein in bacteria that adventitiously express AdpA (Fig. 6B, lane 6), consistent with the occurrence of AdpA-induced degradation of AbsB protein. In S. griseus, AdpA has in fact been shown to be an inducer of both intracellular and secreted proteases (Ohnishi et al., 2005, Akanuma et al., 2009), and our microarray analysis showed that in S. coelicolor, the abundance of RNAs encoding multiple proteases or putative proteases (SCO6995 (putative intracellular serine protease), SCO2529 (putative intracellular metalloprotease), SCO2920 (putative secreted protease) and SCO5446 (probable intracellular neutral protease) significantly increased in J1501; C120 + adpA; overexpression of adpA in C120). hrdB transcripts were the internal control.

Fig. 3. AbsB cleavage of adpA transcripts. 2 μg of in vitro transcribed adpA transcript was incubated with 6.25, 12.5, 25, 50, 100 ng of purified His-tagged AbsB protein (lanes 2–6) and the same amounts of mutant AbsB protein (lanes 7–11) that had been similarly purified as described. The arrows indicate the positions of uncleaved RNA and the cleavage products.
zinc metalloprotease) was dramatically decreased in adpA null mutant strains J-2792 (ΔadpA) and C-2792 (absB, ΔadpA) (Fig. 4C). Moreover, the SCO6995 and SCO5446 genes contain a sequence similar to the AdpA-binding motif in their putative promoter regions (Table S2).

**Discussion**

Our results demonstrate that mRNA encoded by the multifunctional transcription factor AdpA is a target of the AbsB/RNase III protein of *S. coelicolor* and that the abundance of adpA mRNA and protein is modulated by AbsB.
cleavage of adpA transcripts. They further show that AdpA production is necessary for the stimulatory effects of absB mutations on sporulation-associated genes, and that AdpA in turn regulates AbsB actions by promoting degradation of the AbsB protein – possibly by initiating production of a still-unidentified protease. The AbsB/AdpA autoregulatory circuit (as depicted schematically in Fig. 7) thus constitutes a biologically complex pathway of transcriptional and translational regulation.

The discovery of AbsB resulted from the observation that absB mutant bacteria are defective in antibiotic synthesis (Adamidis and Champness, 1992; Aceti and Champness, 1998; Price et al., 1999). Mutation of absB was also found to dramatically increase the abundance of certain sporulation-associated transcripts (Huang B A 60 hours 120 hours J1501 J-2792 (ΔadpA) Duration of culture 60 hours 120 hours

C120 (absB) C-2792 (absB, ΔadpA)

120 hours

Fig. 5. A. Phenotype of J-2792 (ΔadpA) compared with J1501 (morphologically wild type). B. Phenotype of C-2792 (absB, ΔadpA) compared with C120 (absB mutant). Cultures were grown on R5 medium for the duration shown on the right.

Fig. 6. A. AbsB protein abundance in J1501 (morphologically wild type), J-2792 (ΔadpA), C120 (absB mutant), C-2792 (absB, ΔadpA) strains growing on R5 plates for 24–72 h as indicated. Western blotting was performed following the method in Experimental procedures using rabbit polyclonal antibody generated against purified AbsB protein. The protein abundances of S12 were used as loading control. The image shown is representative of three Western blotting experiments that were quantitatively analysed. B. Western blotting of AbsB protein abundance in J1501 and C120 strain expressing adpA adventitiously (J151: empty vector control in J1501; C121: empty vector control in C120 (absB mutant); J1501 + adpA: adpA overexpression in J1501; C120 + adpA: adpA overexpression in C120). Cultures were grown for 48 h on R5-aparamycin plates containing (+) or lacking (−) thiostrepton.

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Fig. 7. Proposed AbsB/AdpA autoregulatory loop. Arrows indicate positive control. Perpendicular lines indicate negative control.

et al., 2005). Although the phenotypic effect of absB mutation on sporulation is less dramatic than the effect on antibiotic biosynthesis, we observed that the absB mutant strain C120 generates thicker and greyer spores on rich R5 medium, and a larger fraction of these spores are biologically functional (Fig. S1). Our finding that introduction of an adpA mutation into absB mutant bacteria does not reverse the defect in antibiotic production (Fig. 5B), indicates that defective cleavage of adpA transcripts in absB mutant bacteria is not sufficient to account for the absence of antibiotic biosynthesis in these bacteria. This result, which contrasts with the effects of adpA mutation on the absB-dependent enhanced sporulation phenotype, suggests that separate mechanisms underlie the effects of AbsB on antibiotic synthesis and sporulation.

Consistent with the observed phenotypic effects of adpA null mutations, adpA function was required for the increase of sporulation-related transcripts observed in the absB mutant strain during microarray analysis (Fig. 4A). However, while adpA mutation leads to premature antibiotic synthesis, this event did not occur in the absence of the AbsB/RNase III gene product (Fig. 4B), suggesting that the AdpA regulation of antibiotic biosynthesis that we and others (Nguyen et al., 2003) have observed in the J1501 lineage is dependent on AbsB. Together, these data lead us to suggest that the ability of AdpA excess to decrease antibiotic production may occur by AdpA-mediated downregulation of AbsB protein abundance (Fig. 6) occurring through the AbsB/AdpA regulatory loop described here (Fig. 7).

OrnA (SCO2793), the gene immediately 3’ to adpA encodes an orthologue of Escherichia coli oligonucleotidase, and in both S. griseus and S. coelicolor this gene has been reported to affect morphological differentiation and antibiotic generation (Ohnishi et al., 2000; Sello and Buttner, 2008). In S. coelicolor strain M145, adpA mutation has been reported to have a polar effect on expression of ornA (Takano et al., 2003), even though the two genes are separated by a 341 bp intergenic region (Bentley et al., 2002). Notwithstanding this reported polarity, recent nuclease S1 analysis of transcriptional start sites within the adpA/ornA region has led to the conclusion that the two genes are not co-transcribed in this Streptomyces strain (Sello and Buttner, 2008). However, RT-PCR experiments carried out in another S. coelicolor strain J1501 during the investigations reported here, detected a transcript >2000 nucleotides in length that includes sequences from both adpA and ornA (Fig. S4A). Additionally, the abundance of the bicistronic adpA-ornA transcript was greater in an absB derivative of J1501 strain at later times of culturing (from 60 h to 120 h) (Fig. S4A). Importantly, in adpA null mutant strains J-2792 (∆adpA) and C-2792 (absB, ∆adpA), we observed that the abundance of ornA-containing transcripts in strains deleted for adpA is dramatically elevated during the life cycle (Fig. S4B), suggesting that read-through transcription from the adpA promoter may suppress transcription initiation from the intercistronic ornA promoter, and that the increase of ornA expression may contribute to the antibiotic-deficiency phenotype of the double null mutation strain C-2792 (absB, ∆adpA).

Although the work reported here is not intended to address in detail the mechanism underlying feedback control of AbsB by AdpA, the data we have presented argue that control occurs post-transcriptionally at the level of protein abundance. We hypothesize that AdpA may activate the transcription of genes encoding intracellular proteases that attack the AbsB protein, and that mutation of absB, which stabilizes adpA mRNA and leads to increased AdpA production (Fig. 2), increases intracellular protease gene transcription – and consequently accelerates AbsB protein degradation. Consistent with this model, transcripts of two putative protease genes (SCO5446, SCO6995) were elevated by adventitious expression of adpA under control of a thiostrepton-induced tipA promoter. We also observed that adventitious expression of these two genes inhibited antibiotic production (data not shown). However, we were unable to detect a direct effect of the proteases on AbsB protein degradation in vivo, indicating that their ability to reduce antibiotic biosynthesis is mediated by mechanism(s) other than targeting of the AbsB protein. Introduction of the SCO2529 and SCO2920 genes in the plJ6902 vector into E. coli as a prelude to their transfer to and expression in S. coelicolor was not successful.

Experimental procedures

Strains and growth conditions

The strains used in this study are cited and referenced in Table 1. S. coelicolor strain J1501 and absB point mutation
Strains used in this study.

| Strain          | Relevant Characteristic(s) | References        |
|-----------------|-----------------------------|-------------------|
| S. coelicolor A3(2) | hisA1 uraA1 strA1 SCP1– SCP2– Pgl– | Kieser et al. (2000) |
| J1501           | J1501 absB120               |                   |
| C120            | J1501 absB                 | Price et al. (1999) |
| J-5572          | J1501 ΔabsB                | This study        |
| J-2792          | J1501 ΔadpA                | This study        |
| C-2792          | C120 ΔadpA                 | This study        |
| J151            | J1501 tipAp::               | Huang et al. (2005) |
| C121            | C120 tipAp::adpA           | Huang et al. (2005) |
| J1501 + absB    | J1501 tipAp::absB          |                   |
| C1501 + absB    | C120 tipAp::absB           |                   |
| J1501 + adpA    | J1501 tipAp::adpA          |                   |
| C120 + adpA     | C120 tipAp::adpA           |                   |
| J1501 + (His)adpA | J1501 tipAp::(His)adpA |                   |
| C120 + (His)adpA | C120 tipAp::(His)adpA |                   |
| J1501 + adpA(His) | J1501 tipAp::adpA(His) |                   |
| C120 + adpA(His) | C120 tipAp::adpA(His) |                   |

E. coli

| DH5α           | dam dcm                    | Invitrogen        |
|----------------|-----------------------------|-------------------|
| ET12567/pIJ6902 | NdeI/BamHI                 | Kieser et al. (2000) |

strain C120 were used in these studies. Strains expressing absB or adpA adventitiously from the inducible tipA promoter [J1501 + absB; C120 + absB; J1501 + adpA; C120 + adpA; J1501 + (His)adpA; J1501 + adpA(His); C120 + (His)adpA; C120 + adpA(His)] were constructed by introducing the apramycin resistance plasmid pJ6902 containing the intact absB/adpA open reading frame (NdeI/BamHI) driven by the thioestrepton inducible tipA promoter into J1501 and C120 by conjugation (Huang et al., 2005). AbsB and adpA null mutation strains were constructed by the insertional inactivation via double crossing over method as follows: the mutant allele was created by introducing an apramycin resistance gene cassette into a plasmid containing a thioestrepton resistance gene as a replacement for a DNA fragment carrying part of the target gene. The construct was transferred by conjugation into strains J1501 and C120, and apramycin resistant, thioestrepton sensitive clones were selected. The null mutations were confirmed by genomic PCR, real-time PCR and Southern blotting. The primers used for the construction of the null mutation strains and overexpression strains are listed in the Table S3.

For the growth on solid media, 150 μl of spore suspensions were inoculated at a density of OD600 = 0.06 onto cellophane membranes placed on R5 plates, and mycelia were collected from the cellophane surface at indicated time points. The growth of each strain was detected by measuring dry cell weight. 100 μg ml⁻¹ apramycin and 50 μg ml⁻¹ thioestrepton were added to the medium for the growth of thioestrepton-inducible overexpression strains.

RNA extraction, Northern blotting, reverse transcription and quantitative PCR

Mycelia were collected from solid or liquid growth culture, immediately frozen by addition of liquid nitrogen, and ground into powder with mortar and pestle. Then 20 μg of total RNA isolated using the RNeasy® Plant Kit was treated with additional DNase I (10 Unit; QIAGEN) and electrophoresed on denaturing agarose gels (NorthernMax-Gly 10× Gel Prep/Running Buffer (Applied Biosystems). Following transfer to Zeta-probe (Bio-Rad) membranes, RNA was analysed by Northern blotting at 50°C in Ultrahyb hybridization buffer using randomly radiolabelled DNA containing the ORF sequence as probe (NorthernMax kit, Applied Biosystems). After one round of low-stringency wash (2XSSC, 0.1%SDS) at 25°C and three rounds of high-stringency wash (0.1XSSC, 0.1%SDS) at 50°C, membranes were exposed to phosphorimaging (Typhoon, GE) for signal detection and quantification. For quantitative real-time RT-PCR, first strand cDNA synthesis was carried out using 2 μg of total RNA and SuperScript III (Invitrogen), following the manufacturer’s instructions, and the Bio-Rad iCycler TM Real-Time PCR Detection System and IQTM SYBR Green Supermix Kit were used for the PCR amplification using 1–10 times diluted first strand reaction product as template. The PCR conditions were as follows: 94°C for 10 min, 40 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s. The target cDNA was normalized internally to hrdB levels or 16S rRNA (samples were diluted 10⁴ times) (all the primers used for RT-PCR are listed in Table S3). For reverse transcription of adpA-ornA readthrough transcript, Herculase II (Stratagene) was used (95°C for 3 min, 35 cycles of 94°C for 30 s, 67°C for 40 s and 72°C for 2 min).

In vitro cleavage assay

The N-terminal hexahistidine tagged wild type and mutant AbsB proteins were produced in the protease-deficient E. coli strain BL21 by cloning the absB ORF in the expression plasmid pET28a, and purified by Ni-column chromatography as described before (Xu et al., 2008). Protein concentration was determined by BCA assay (Pierce) and confirmed by electrophoresis of the purified samples on 12% SDS-polyacrylamide gel.

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DNA corresponding to the full length \textit{stt1}, \textit{ramR} and \textit{adpA} transcript was amplified from \textit{S. coelicolor} genomic DNA using 5’ primers that included the sequence of the bacterial ribophosphate T7 promoter, the primer sequences for \textit{stt1}, \textit{ramR}, and \textit{adpA} in \textit{vitro} transcription are listed in Table S3. RNA substrates were synthesized using MEGAscript T7 kit (Applied Biosystems) and purified by RNaseasy Mini Kit (QIAGEN). For \textit{stt1}, \textit{ramR} and \textit{absB} locus transcripts (Xu et al., 2008), 2 \(\mu\)g of RNA substrates were incubated with 25 ng of purified protein with RNase III buffer (Applied Biosystems) in 20 \(\mu\)l of reaction mixtures. Reaction mixtures were incubated at 37°C for 30 min then quenched by the addition of lysis buffer RLT from an RNaseasy Mini Kit (QIAGEN). Cleavage products were purified using RNaseasy Mini Kit (QIAGEN) and electrophoresed in a glyoxal-denatured 1.5% agarose gel using NorthernMax-Gly 10x Gel Prep/Running Buffer (Applied Biosystems). Visualization of the reaction was carried out by exposing gels to UV light. For \textit{adpA} transcripts, 2 \(\mu\)g of RNA substrates were incubated with 6–100 ng of purified protein in 20 \(\mu\)l of reactions (Applied Biosystems RNaIse III buffer).

\section*{Protein extraction and Western blotting}

Purified \textit{AbsB} protein was submitted to the company (Covance) to make rabbit polyclonal anti-\textit{AbsB} antibody. For Western blotting, mycelia were collected from solid or liquid growth culture, and destructed by sonication in NP-40 lysis buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1%NP-40] with protease inhibitor cocktail (Roche). After quantification with BCA protein assay kit (Pierce), the protein was electrophoresed on a 12% SDS-polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and probed with anti-\textit{AbsB} or monoclonal anti-His antibody (QIAGEN).

\section*{Microarray analysis}

10–15 \(\mu\)g of total RNA was denatured in the presence of 5–6 ng of 72%-GC-content hexamers (total 13 \(\mu\)l) at 75°C for 10–15 min and snap-cooled in ice water before addition of the remaining reaction components: 3 \(\mu\)l of Cy3–dCTP or Cy5–dCTP (Amersham Pharmacia Biotech) and 14 \(\mu\)l of a cocktail that included 6 \(\mu\)l of 5x Superscript II Buffer, 3 \(\mu\)l of DTT (0.1 M), 3 \(\mu\)l of dNTP (4 mM dATP, 4 mM dTTP, 10 mM dGTP and 0.5 mM dCTP) and 2 \(\mu\)l of Superscript II (Invitrogen). The reverse transcriptase reaction was carried out for 10 min at 25°C followed by 2 h at 42°C. After labelling, purification of cDNA, hybridization and washing were carried out as described previously (Huang et al., 2001). Built-in functions of the Stanford Microarray Database (http://smd.stanford.edu/) were used to normalize and analyse microarray data.

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