Regulation of a Novel Gene Cluster Involved in Secondary Metabolite Production in Streptomyces coelicolor

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Antibiotic biosynthesis in the streptomycetes is a complex and highly regulated process. Here, we provide evidence for the contribution of a novel genetic locus to antibiotic production in Streptomyces coelicolor. The overexpression of a gene cluster comprising four protein-encoding genes (abeABCD) and an antisense RNA-encoding gene (α-abeA) stimulated the production of the blue-pigmented metabolite actinorhodin on solid medium. Actinorhodin production also was enhanced by the overexpression of an adjacent gene (abeR) encoding a predicted Streptomyces antibiotic regulatory protein (SARP), while the deletion of this gene impaired actinorhodin production. We found the α-abe genes to be differentially regulated and controlled at multiple levels. Upstream of α-abeA was a promoter that directed the transcription of α-abeABCD at a low but constitutive level. The expression of α-abeBCD was, however, significantly upregulated at a time that coincided with the initiation of aerial development and the onset of secondary metabolism; this expression was activated by the binding of AbeR to two heptameric repeats upstream of a promoter within α-abeA. Expressed divergently to the α-abeBCD promoter was α-abeA, whose expression mirrored that of α-abeBCD but did not require activation by AbeR. Instead, α-abeA transcript levels were subject to negative control by the double-strand-specific RNase, RNase III.

The streptomycetes are filamentous, soil-dwelling bacteria with a complex developmental life cycle. They also have prodigious secondary metabolite production capabilities and synthesize the majority of antibiotics currently in clinical use. Secondary metabolism can be correlated with changes in morphological development, as a shift from vegetative growth to the formation of reproductive aerial structures coincides with the initiation of antibiotic production. This is coordinated at a genetic level, with mutations in many characterized developmental regulators not only affecting aerial development but also reducing or eliminating antibiotic production (10).

Streptomyces coelicolor is the best-studied streptomycete, and it serves as a model system for investigating the regulation of development and secondary metabolism (3). Conveniently, it produces two pigmented antibiotics: the red, cell-associated undecylprodigiosin and the blue, secreted actinorhodin. These pigmented metabolites have greatly facilitated genetic studies into antibiotic regulation, as changes in antibiotic levels can be readily detected by visually screening colonies on a plate (4). Antibiotic production is a tightly controlled process with many regulatory inputs, such as metabolic and nutritional status (37), small signaling molecules (γ-butyrolactone) concentrations (47), and the proposed coupling of antibiotic synthesis and resistance (27, 46). It also is subject to multiple levels of genetic regulation, including the pleiotropic regulators that affect both development and antibiotic production, global antibiotic regulators that influence the production of multiple antibiotics but do not affect development, and pathway-specific regulators that control the synthesis of a single antibiotic and often are clustered together with their target biosynthetic genes (4, 29).

Pathway-specific regulators fall into two broad classes (5): the LAL (for large ATP-binding regulators of the LuxR family) (40, 55) and the SARP (for Streptomyces antibiotic regulatory protein) family regulators. SARPs appear to have roles in modulating antibiotic production, they are not limited to functioning solely within specific pathways. In fact, one of the best understood SARPs is AfsR, which is encoded at a genetic locus distinct from any secondary metabolic gene cluster, and it affects the production of at least two antibiotics in S. coelicolor (15). The DNA binding of AfsR has been well studied: it interacts specifically with direct, heptameric repeats (49), activating the expression of the neighboring afsS gene, which encodes a small, sigma-factor-like protein that affects antibiotic production via an unknown mechanism (53), and repressing the expression of genes involved in phosphate control (phoRP and pstS) (39).

In addition to transcription factor control, antibiotic production also is affected by the activity of the double-stranded RNA nuclease, RNase III. The mechanism underlying this effect is not yet understood; however, point mutations in the RNase III coding sequence, and the deletion of the gene itself (known both as mrc and as absB), abrogate antibiotic production (16, 35, 42), at least in part through the reduced expression of the pathway-specific regulators ActII-ORF4 and RedD (1).

Here, we identify a novel gene cluster that specifically affects
actinorhodin production when overexpressed. We show the genes within this cluster to be differentially regulated, displaying differential expression profiles that are affected by a previously uncharacterized SARP and by RNase III.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *S. coelicolor* strains, Escherichia coli strains, and all plasmids/cosmids used in this study are summarized in Table 1. *Streptomyces* strains were grown at 30°C on solid R2YE (rich), MS (soy flour-mannitol), or SMMS (supplemented minimal) agar medium, or in liquid culture in a 1:1 trypotone soya broth (TSB)-yeast extract-malt extract (YEME) mixture, as described by Kieser et al. (24). For phenotypic comparisons of different *S. coelicolor* strains, 10^5 to 10^6 spores were streaked for single colonies on the different solid media.

**Construction of high-copy-number vector overexpression strains.** Overexpression strains used in this study were constructed by cloning the gene(s) of interest into pWHM3 (a high-copy-number shuttle plasmid) (Table 1) and introducing the resulting recombinant plasmid into *S. coelicolor* M600 through protoplasting transformation (24). The *SCO3287-SCO3291* (*abeABC* and *abeR*) gene cluster was excised from cosmid E15 (Table 1) using a rapid DNA ligation kit (Roche) prior to transformation into subcloning-efficiency DH5α *E. coli* cells (Invitrogen). The recombinant plasmid (pMC123) was isolated and digested with BglII before the resulting *SCO3287-SCO3291*-containing fragment was subcloned into the BamHI site of pWHM3, generating pMC120. pMC120 was passed through the nonmethylating *E. coli* strain ET12567/pUZ8002 (Table 1) prior to introduction into *S. coelicolor*. To overexpress *SCO3287-SCO3290* (*abeABC*), this gene cluster was excised from pMC123 using BamHI, and the resulting ~4.5-kb fragment was subcloned into the BamHI site of pWHM3. The resulting construct was designated pMC116. For *α*-287 (α*-abeA*) and *SCO3291* (*abeR*) overexpression, the corresponding genes were PCR amplified with *Pfu* DNA polymerase (Stratagene) using primers OE3291–1/OE3291–2, respectively (see Table S1 in the supplemental material). The PCR products were phosphorylated before ligation into the Smal site of pIJ2925; the resulting clones were verified by sequencing. *α*-287 (α*-abeA*) was excised from pIJ2925 using BamHI and EcoRI, while *SCO3291* (*abeR*) was removed using BglII and EcoRI. Both fragments subsequently were cloned into BamHI and EcoRI sites of pWHM3 to give pMC117 and pMC118 as α*-abeA* and α*-abeR* overexpression plasmids, respectively.

**Secondary metabolite assays.** For the actinorhodin and undecylenylproginosin quantification of *abe*-overexpression strains (relative to levels for empty-plasmid-
containing control strains), pregerminated spores were inoculated into R5 liquid medium (containing thiotrepton) to an optical density at 450 nm (OD_{450}) of 0.04 to 0.08. Cultures were shaken at 30°C, and samples were removed after 40, 64, 72, and 96 h. Actinorhodin and undecylprodigiosin levels then were quantified as described by Kang et al. (23). To assess the production of the calcium-dependent antibiotic (CDA), minor modifications were made to the method described by Kiefer et al. (24). S. coelicolor spores were spread on nutrient agar plates, and these were incubated at 30°C for 24 or 48 h. Agar plugs then were excised from regions of confluent growth and were transferred from these plates into empty plugs of an equivalent size on fresh nutrient agar plates, both with and without 12 mM CaCl₂. These plates then were overlaid with overnight cultures of S. aureus ATCC 29213 diluted 1/100 in soft nutrient agar. The plates were incubated overnight at 37°C, and zones of growth inhibition were determined for strains grown on agar plates left unsupplemented or supplemented with calcium.

**RNA isolation and transcript analysis.** RNA isolation and Northern blot analyses were carried out as outlined by Swierz et al. (45). Primers used for Northern blot hybridization are shown in Table S1 in the supplemental material. S1 nuclease mapping was conducted as described by Elliot et al. (14), using primers M13F and 3288R (Table S1) together with pMC125 (Table 1) as the template, to generate the probe used for mapping. For semiquantitative reverse transcription-PCR (RT-PCR), RT reactions were conducted using the SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer’s instructions, with minor modifications. Briefly, 5 µg of total RNA was mixed with RNase-free water to a final volume of 8 µL. Deoxyribonuclease triphosphates (dNTPs) and gene-specific oligonucleotides were added, and the resulting mixture was incubated at 95°C for 10 min before being chilled on ice. The remaining reaction components (RNase inhibitor, reverse transcriptase, buffer, and dithiothreitol [DTT]) then were added, and reverse transcription was performed at 42°C for 50 min. The reaction was terminated by being heated at 70°C for 15 min.

The resulting RT products were used as the template for subsequent PCR amplification using the following program: initial denaturation (95°C, 7 min); 15 to 28 cycles of 95°C for 45 s, 57 to 67°C for 30 s (the temperature was dependent on oligonucleotide composition, while the number of cycles was optimized to ensure amplification in the linear range of the reaction), and 72°C for 30 to 45 s (extra time on expected product sizes); and a final cycle of 5 min at 72°C. Negative controls (using RNA as the template) and positive controls (using chromosomal DNA or appropriate plasmid DNA as the template) were included for each PCR. PCR products were separated on a 2% agarose gel for transcript profile analyses.

** lux reporter assays for measuring actII-orf4 and actIII expression.** Streptomyces strains (10⁵ to 10⁶ spores) were grown on 0.2 ml R2YE agar plugs in 96-well flat-bottomed plates (Microthor white plate; Thermo Scientific). Following growth for 16, 24, 32, 40, 48, 56, and 72 h, cultures were exposure to 1% decanal (luciferase substrate), and luminescence levels were determined. For each time point, 12 readings were taken (corresponding to 12 different wells/samples), and the results of these independent time courses were analyzed using the nonparametric module (Mann-Whitney test) of the SPSS v13.0 statistical analysis package.

**DNase I footprinting.** DNase I footprinting assays were conducted using the same binding conditions as those for the EMAS experiments. Probes were prepared by PCR amplification using 3287–2 and 327MSAF primers (Table S1), with either 327MSAF or 3287–2 being end labeled with [γ-³²P]dATP. Twenty-microliter reaction mixtures contained 0.5 µM labeled probe together with 0 to 200 nM purified protein in a buffer consisting of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 1 µg poly[d(dC-dC)], and 10% glycerol. Samples were incubated at room temperature for 25 to 30 min prior to loading. Electrophoresis was performed in 1× Tris-borate-EDTA buffer at 100 V for 50 to 60 min. Gels were exposed to Kodak Biomax XAR film at room temperature for 1 h.

**RESULTS**

**Bioinformatic analysis of the SCO3287-SCO3291 genetic region.** Our interest in this genetic region originally was stimulated by the presence of an antisense RNA, encoded by α-3287 on the strand opposite SCO3287 (45). SCO3287 is the first gene of an apparent four-gene operon that is, to date, unique to S. coelicolor and its very close relative, S. lividans (Fig. 1). SCO3287 encodes a highly charged (arginine-rich) cytoplasmic protein of 172 amino acids (aa). It is devoid of obvious functional motifs and bears no sequence similarity to any known protein. It is followed by two predicted membrane protein-encoding genes, SCO3288 and SCO3289, whose products are 243 and 536 aa, respectively. Neither of these genes is similar to any characterized gene, although SCO3289 encodes a product with homologues in other Streptomyces species, notably S. avermitilis, where it is cotsynthesized with a smaller downstream 30-kDa cutoff. Protein concentrations were determined using a Bradford assay (Bio-Rad) (6).

Electrophoretic mobility shift assays (EMSAs) were conducted using native 5% polyacrylamide gel electrophoresis. The probe was generated by PCR amplification using primers 3287–2 and 3287–3 (see Table S1 in the supplemental material), with the resulting PCR product being 5’ end labeled with [γ-³²P]dATP. Twenty-microliter reaction mixtures contained 0.5 µM labeled probe together with 0 to 200 nM purified protein in a buffer consisting of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 1 µg poly[d(dC-dC)], and 10% glycerol. Samples were incubated at room temperature for 25 to 30 min prior to loading. Electrophoresis was performed in 1× Tris-borate-EDTA buffer at 100 V for 50 to 60 min. Gels were exposed to Kodak Biomax XAR film at room temperature for 1 h.

DNase I footprinting assays were conducted using the same binding conditions as those for the EMAS experiments. Probes were prepared by PCR amplification using 3287–2 and 327MSAF primers (Table S1), with either 327MSAF or 3287–2 being end labeled with [γ-³²P]dATP, to create probes corresponding to the coding (non-template) or template strands, respectively. Purified AbeR (0 to 1.6 µM) was mixed with ~6 nM probe before adding 2.5 U DNase I (Roche) for 1 min, after which Pn buffer (Qiagen) was added to stop the DNase I reaction. The resulting cleavage products were purified using Qiagen MinElute columns and were eluted in 30 µl distilled H₂O. The elution products were vacuum dried for 30 to 45 min and resuspended in loading dye (100% [vol/vol] formamide; 1 mM EDTA, pH 8.0; 10 mM NaOH; 0.1% [wt/vol] bromophenol blue; 0.1% [wt/vol] xylene cyanol FF). Sequencing ladders were generated using the Sequenase v2.0 DNA sequencing kit (USB Corporation) per the manufacturer’s instructions, with minor modifications. Briefly, alkaline-denatured pMC123 (Table 1) was mixed together with 5 µM the appropriate primer (327MSAF//3287–2; see Table S1 in the supplemental material) in a total volume of 10 µL. These mixtures then were boiled for 3 min and placed immediately on ice for 5 min before being centrifuged for 30 s. To each tube, buffer, DTT, [γ-³²P]dATP, and Sequenase were added per the manufacturer’s instructions, and the resulting reaction mixtures were mixed with the dGTP labeling mix and the dA/G/A/T/C termination mix, which previously had been warmed to 37°C. These mixtures were incubated at 37°C for 2 min before stop solution was added (USB Corporation). All samples (reaction and sequencing) were heated at 90°C for 3 to 5 min prior to being loaded onto a 10% polyacrylamide gel, which was run at 45 W for either 45 (template strand) or 75 min (coding/non-template strand).

**FIG. 1.** Genetic organization of SCO3287-SCO3291 (the abe gene cluster) and the associated antisense RNA-encoding gene (α-3287/α-abe4). Transcription start sites are indicated by vertical lines, with the line widths (and associated arrows) approximating relative transcript abundance.
gene, and *S. griseus*, where it exists as a single genetic unit. In *S. coelicolor*, SCO3289 appears to be translationally coupled with SCO3290, which encodes a 249-aa cytoplasmic protein with a predicted nucleotide binding domain similar to the Toll/interleukin-1 receptor (TIR-like) domain, suggesting a possible role in signal transduction (30) or protein-protein interactions (44).

Downstream of this four-gene cluster, oriented in the same direction, is a predicted transcription factor-encoding gene. Its product, SCO3291, is 477 aa, with a multidomain architecture. The C terminus of SCO3291 shares similarity with conserved hypothetical proteins from a wide variety of bacteria, although these are largely single-domain proteins. The function of this C-terminal region is unknown; it is worth noting that proteins with this domain usually are encoded adjacent to genes whose products have TIR-like domains, such as that found in SCO3290. The N terminus of SCO3291 contains an OmpR-like DNA binding domain (31) and a so-called BTAD, or bacterial transcription activator domain (58). These two domains are common to all SARP family regulators (54, 58) and suggest that SCO3291 has DNA binding properties. While many SARPs are encoded within antibiotic biosynthetic gene clusters and act as pathway-specific antibiotic regulators, the target genes for SCO3291 are not immediately obvious, as the nearest secondary metabolic gene cluster is 38 kb away (the calcium-dependent antibiotic cluster).

**Actinorhodin production increases upon gene/gene cluster overexpression.** To address the biological role of the SCO3287-SCO3290 gene products and the function of SCO3291, we first replaced the four-gene cluster with an antibiotic resistance gene and compared its phenotype to that of wild-type *S. coelicolor*. Both colony development and antibiotic production by the mutant strain closely resembled that of its wild-type parent when grown on a variety of solid culture media (data not shown). In contrast, the overexpression of SCO3287-SCO3290 (on a multicopy plasmid) significantly increased actinorhodin production during growth on solid rich (R2YE) (Fig. 2A) and supplemented minimal (SMMS) media (data not shown); CDA production was unaffected (data not shown). Increased antibiotic production could be due to the overexpression of the four-gene cluster itself or could result from the increased expression of the antisense RNA, α-3287, encoded on the strand opposite SCO3287 (Fig. 1). To differentiate between these two possibilities, we created an α-3287 overexpression strain by introducing α-3287 on a multicopy plasmid vector into wild-type *S. coelicolor*, and we found that this strain produced antibiotics at a level equivalent to that of an empty-plasmid control strain (data not shown). This suggested that the enhanced actinorhodin production seen for the SCO3287-SCO3290 overexpression strain was due to the overexpression of one or more of the four protein-encoding genes and not α-3287. Given the ability of these gene products to modulate antibiotic levels, we propose that SCO3287-SCO3290 be renamed *abeABCD* for antibiotic enhancement upon overexpression, and that α-3287 be renamed *α-abeA*.

We used a similar approach to investigate the role of SCO3291 in *S. coelicolor* development and metabolism, creating both SCO3291 deletion and overexpression strains. The replacement of SCO3291 with an apramycin resistance gene resulted in decreased actinorhodin production relative to that of its wild-type parent (Fig. 2B); this defect could be reversed upon complementation with a wild-type copy of SCO3291 on the integrating plasmid vector pMS82 (Fig. 2B). In contrast, overexpressing SCO3291 either on its own or with *abeABCD* resulted in increased actinorhodin production compared to that of empty-plasmid-containing control strains (Fig. 2A). We
FIG. 3. Expression of actII-ORF4 as measured using the lux reporter system during a 72-h time course. Luminescence levels are shown as relative light units (y-axis). Black bar, wild-type S. coelicolor containing the high-copy-number plasmid pWHM3; gray bar, wild-type strain containing pWHM3 + abeR; white bar, wild-type strain containing pWHM3 + abeABCD. All strains carried a chromosomally integrated pMU1 reporter plasmid containing the actII-orf4 promoter cloned upstream of the lux genes. Strains were grown on R2YE agar plugs in 96-well plates. Luminescence levels reflect the means (± standard errors) from three independent experiments following the subtraction of background levels (luminescence of each strain carrying pMU1 alone). Asterisks indicate those samples in which the mean luminescence levels were significantly different (at a 95% confidence level) between abe overexpression and plasmid-alone (pWHM3)-containing strains.

therefore propose that SCO3291 be renamed abeR, given the effect of its overexpression on antibiotic production.

We also monitored the actinorhodin and undecylprodigiosin levels for abeABCD, abeR, and abeABCD and abeR overexpression strains relative to those for a control plasmid-containing strain during liquid culture growth. We observed no difference in the level of undecylprodigiosin produced by the overexpression and control strains (data not shown), and while we did observe increased actinorhodin production for the overexpression strains on some occasions, this effect was not consistently reproducible (data not shown), unlike the increased actinorhodin production that was observed consistently for overexpression strains grown on solid culture (Fig. 2).

Enhanced actinorhodin production by overexpression strains may be due to the extended expression of actII-orf4 but not to an overall increase in expression. Many characterized antibiotic regulators mediate their effects through the transcriptional control of pathway-specific activators (1, 33, 37). As actinorhodin levels were significantly increased in both abeABCD and abeR overexpression strains during growth on solid media, we sought to probe the effect of this overexpression on the transcription of the actinorhodin pathway-specific regulator actII-orf4, and the actinorhodin biosynthetic gene actIII, using luciferase transcriptional reporters (12). We introduced the reporter constructs and corresponding control plasmid into the abeABCD and abeR overexpression strains and monitored luminescence levels over time. We did not detect any consistent differences in actIII expression during the 3-day time course (data not shown). For actII-orf4, however, expression from 24 to 32 h actually was lower in the two overexpression strains than in the control strain, but by 72 h we observed a statistically significant increase in promoter activity in both overexpression strains compared to that of the wild type (Fig. 3), suggesting that the extended expression of actII-orf4 contributes to enhanced actinorhodin production.

Transcription analyses of abeABCD reveal a complex operon expression structure. To begin dissecting the function of the unique abe gene cluster, we examined the expression of these genes on rich (R2YE) medium, starting with abeA. Unexpectedly, we could not detect abeA transcripts at any point during a 3-day time course using semiquantitative RT-PCR (Fig. 4A). When we looked at the expression of abeBCD, however, transcripts were readily detectable; these genes were expressed first during the onset of secondary metabolism and aerial hyphae formation (at 31 h), and expression continued through at least 72 h (Fig. 4A). This suggested that despite being separated from abeA by a mere 8 bp, abeBCD was transcribed independently. To further explore this possibility, S1 nuclease mapping was conducted using a probe that encompassed the final 326 nucleotides (nt) of the abeA coding sequence and the first 64 nt of the abeR coding sequence. We identified a transcription start site ~77 nt upstream of the abeB translation start site within abeA (Fig. 4B) and 210 nt downstream of the α-abea transcription start site (Fig. 1 and 5E).

To further examine abeA expression and determine whether abeBCD expression was completely uncoupled from that of abeA, we investigated the transcription of these genes in our abeABCD overexpression strain, where the gene cluster was cloned into a high-copy-number plasmid. We found abeA to be constitutively expressed and determined that there was transcriptional read-through into abeB (and likely abeCD), as determined by RT-PCR using primers that flanked either side of the abeBCD-specific promoter within abeA (see Fig. S1 in the supplemental material). Taken together, these findings suggest that abeBCD is constitutively transcribed at a low level, while abeABCD is expressed at much higher levels and is induced at a time corresponding to the initiation of antibiotic production and aerial hyphae formation.

AbeR activates the expression of abeBCD. We also examined the expression of abeR and found the timing of its expression to be very similar to that of abeBCD in a wild-type background,
with transcripts detected from 31 to 72 h (Fig. 4A). Given this finding and considering that the overexpression of \( \text{abeR} \) had phenotypic consequences similar to those of the overexpression of \( \text{abeABCD} \), we wondered whether there might be a regulatory connection between \( \text{AbeR} \) and \( \text{abeBCD} \). To probe this possibility, we examined the transcript levels of \( \text{abeBCD} \) in the \( \text{abeR} \) deletion and overexpression strains described above. We found that \( \text{abeBCD} \) expression was barely detectable in the \( \text{abeR} \) deletion strain (Fig. 5A), while in the overexpression strain, transcript levels were increased considerably compared to those of an empty-plasmid-carrying control strain (Fig. 5B). We also tested whether \( \text{abeA} \) transcripts were detectable in the \( \text{abeR} \) overexpression strain, but as was the case for the wild type, no expression could be observed (data not shown).

To determine whether \( \text{AbeR} \) was directly involved in controlling \( \text{abeBCD} \) expression, we overexpressed \( \text{AbeR} \) as an

**FIG. 5.** Regulation of \( \text{abeBCD} \) transcription by \( \text{AbeR} \). Shown are transcription profiles of \( \text{abeBCD} \) in *S. coelicolor* wild-type and \( \text{abeR} \) deletion strains (A) and in \( \text{abeR} \) overexpression and plasmid (pWHM3)-alone control strains (B), as determined using semiquantitative RT-PCR. RNA samples used in the RT-PCR were harvested at time points indicated above each panel; the number of PCR amplification cycles was optimized for each experiment: 25 cycles (A) and 27 cycles (B) (note that strains carrying the high-copy-number plasmid pWHM3 appear to have reduced overall transcript levels compared to those of non-plasmid-containing strains [unpublished data]). For the no-RT control PCR, RNA (not subjected to reverse transcriptase) served as the template to ensure no DNA contamination. The 16S rRNA gene was amplified (15 cycles) as a control for RNA levels and RNA integrity. (C) EMSA of \( [\gamma^{32P}] \text{dATP-radiolabeled probe} \) (214 bp) containing the \( \text{abeBCD} \) promoter region together with \( \text{His6-AbeR} \). AbeR binding specificity was tested using specific (cold probe) and nonspecific competitor DNA (\( \text{chpD} \) coding sequence). (D) DNase I footprinting assay of \( \text{His}_{\text{xx}}{\text{AbeR}} \) binding to the \( \text{abeBCD} \) promoter region. Protected regions are indicated by the vertical lines to the right of each footprint. Increasing concentrations of purified AbeR (0 to 1.67 \( M \), with increasing concentrations of protein indicated by the black triangle) were incubated together with 6 nM singly end-labeled probe. Sequencing ladders are shown to the left of each footprint. (E) Coding sequence of \( \text{abeA} \) encompassing the approximate \(-10 \) and \(-35 \) promoter sequences of \( \alpha\text{-abeA} \) and \( \text{abeBCD} \). Mapped transcriptional start sites for both transcripts are indicated by dotface letters. DNA sequences protected by \( \text{AbeR} \) are indicated by horizontal lines, and the protected four direct heptameric repeats are boxed. Sites of DNase I hypersensitivity are indicated by vertical arrows, while a potential AdpA binding site is shaded in gray.
N-terminally His-tagged protein in E. coli for use in EMSAs, and we purified this protein using Ni affinity chromatography. EMSAs were performed using a DNA fragment that encompassed both the α-abeA (antisense gene) and abeBCD promoter regions as the probe. We found this fragment to be effectively bound by the AbeR fusion protein at a concentration of 93 nM. Competition experiments confirmed the specificity of this interaction, with cold probe effectively competing with the labeled probe for binding, but an unrelated DNA fragment (the S. coelicolor chpD coding sequence, where ChpD is associated with aerial development but not antibiotic production [14]) failed to compete (Fig. 5C). The analysis of the sequence contained within the specifically bound DNA fragment revealed there to be four tandemly arranged direct repeats of 7 nt, with the first three repeats each separated by 4 nt and the fourth located a further 15 nt downstream. DNase I footprinting experiments were conducted to determine whether these repeated regions were protected by AbeR binding. As seen in Fig. 5D and E, protection by AbeR covered the repeated regions, with sites of DNase I hypersensitivity seen on both strands within the 15-bp region separating the third and fourth repeats.

AbeR does not control α-abeA expression. As the binding site of AbeR was located between the promoter regions for abeBCD and α-abeA, we wanted to determine whether AbeR also affected the expression of α-abeA. Using Northern blotting, we examined α-abeA transcript levels in the abe overexpression and deletion strains and found its expression to be unaffected in each instance (data not shown), suggesting that the effect of AbeR binding was limited to the activation of abeBCD expression.

RNase III contributes to the destabilization of α-abeA transcripts. Having established that AbeR played an important role in activating the expression of abeBCD but did not impact α-abeA expression, we wanted to identify factors that affected α-abeA expression, as its expression profile mirrored that of abeBCD and abeR (Fig. 6A) (45). The positioning of α-abeA suggested that the most likely interaction partner for α-abeA would be abeA-containing transcripts, and the resulting double-stranded RNA complexes would be reasonable targets for RNase III. We therefore examined the expression of α-abeA in wild-type and RNase III (rne [absB]) mutant strains carrying the abeBCD overexpression construct. We found that α-abeA transcripts were present in far greater abundance during later stages of growth (3 to 4 days) in the rne (absB) mutant strain than in the wild type (Fig. 6A), suggesting that α-abeA may be targeted for degradation by RNase III late in development, possibly in conjunction with its predicted target, abeA. However, when we examined abeA expression, expecting to see similarly increased levels in the rne (absB) mutant, we found this was not the case. After normalizing abeA transcript levels using the 16S rRNA transcript control, and accounting for plasmid copy number using transcript levels of the plasmid-containing transcripts, and the resulting dou-

FIG. 6. Expression of α-abeA, abeA, and abeBCD in S. coelicolor wild-type and mutant strains lacking RNase III. (A) Northern blot analysis of α-abeA expression in wild-type and rnc (absB) mutant strains carrying the abeBCD overexpression plasmid (pMC116; Table 1). 5S rRNA was examined as a control for RNA integrity and loading. (B) Levels of α-abeA, abeA, and abeBCD in the rnc (absB) mutant strain relative to its wild-type parent S. coelicolor M600. Transcript levels were normalized relative to 5S rRNA (for α-abeA) and 16S rRNA genes (for abeA and abeBCD) to account for differences in input template RNA and tsr levels to account for differences in plasmid copy number levels for wild-type and mutant strains. Transcript abundance was determined using the ImageJ analysis of Northern blotting (α-abeA) and RT-PCR (abeA, abeBCD, 16S rRNA gene, tsr) results. The data presented are the averages (± standard deviations) from four experiments (two experimental replicates of two independent RNA time courses).
for degradation by RNase III; the loss of RNase III leads to increased AdpA levels and, correspondingly, increased transcription of AdpA-activated genes (56). We examined the sequence upstream of \(\alpha-abeA\) and found a reasonable match to the AdpA-binding motif (TGGCCGGCC versus TGGC SNGWWY, where S is G/C, W is A/T, and Y is T/C [57]) located 134 nt upstream of the \(\alpha-abeA\) transcription start site (Fig. 5E). To determine whether the increased expression of \(\alpha-abeA\) in the \(rnc\) (\(absB\)) mutant was mediated through AdpA, we created an \(rnc\) \(adpA\)-\(abeA\)/H9251 double mutant and examined \(\alpha-abeA\) expression. We found that \(\alpha-abeA\) levels remained high in the double mutant strain (data not shown), suggesting that increased \(\alpha-abeA\) transcription in the \(rnc\) mutant was not due to increased activation by AdpA but was instead mediated through RNase III by another, yet-to-be-determined mechanism.

**DISCUSSION**

Here, we elucidate the regulation of a novel gene cluster having a role in antibiotic production in *S. coelicolor*. On solid media, we showed that the overexpression of \(abeABC\) and/or \(abeR\) resulted in the increased production of the blue-pigmented antibiotic actinorhodin, while the deletion of \(abeR\) resulted in decreased actinorhodin production. Unlike many genes that affect antibiotic production in *S. coelicolor* when overexpressed (e.g., *metK* [33] and *afsR* [15]), \(abe\) gene/cluster overexpression resulted in the maintenance of actII-orf4 expression at elevated levels later in development rather than stimulating expression at all stages of culture growth.

Antibiotic production is subject to multiple levels of regulatory control and is impacted by physiological factors, like metabolic precursor concentrations, and by environmental conditions, like nutrient availability and signaling molecule abundance. Given the putative membrane localization of AbeB and AbeC and the cytoplasmic positioning of the TIR-like domain-containing AbeD, we considered the possibility that these proteins may have a role in sensing and responding to environmental cues. *S. coelicolor* produces at least three chromosomally encoded \(\gamma\)-butyrolactone signaling molecules, SCB1, SCB2, and SCB3 (20, 47), along with the plasmid-encoded methylenomycin furans (MMFs) (11, 34). However, these molecules all are freely diffusible across cytoplasmic membranes and bind dedicated cytoplasmic receptor proteins, making it unlikely that the \(abe\) proteins have an intermediary role in sensing or transducing signals in response to these molecules, although we cannot exclude the possibility that they recognize a currently unknown signaling molecule(s). We also considered the possibility that the \(abe\) gene cluster responds to changes in the nutritional status of the colony and/or influences the switch from primary to secondary metabolism, given that \(abeABC\) and \(abeR\) expression initiated at a time consistent with this physiological transition. We also observed increased antibiotic production only on media where glucose was included as the primary carbon source (SMMS and R2YE), suggesting that the \(abe\) gene effect is subject to catabolite repression. Recent work has begun to illuminate the regulatory connections linking primary and secondary metabolism, with the regulators DasR and AtrA having central but antagonistic roles (37, 51). DasR negatively regulates actinorhodin and undecylylprodigiosin production through the repression of actII-orf4 and redZ (encoding a regulator of RedD expression); repression is relieved in the presence of N-acetylglucosamine (GlcNac), although only during growth on poor carbon sources (37). AtrA directly activates the expression of actII-orf4 and stimulates actinorhodin production but does not affect the production of undecylylprodigiosin (51). It also activates the expression of nageE2, which encodes the permease specific for the import of GlcNac into the cell (31a). There is no obvious connection between the \(abe\) genes and either DasR or AtrA: there are no binding sites for either protein upstream of \(abeR\), the effect of \(abe\) gene overexpression does not require a poor carbon source, and actII-orf4 expression during \(abe\) overexpression is not significantly altered, instead appearing to be extended during culture growth.

Both \(abeR\) and \(abeABC\) overexpression enhanced actinorhodin production, but interestingly, the deletion of each did not have the same phenotypic consequences: the deletion of \(abeR\) significantly reduced actinorhodin production, while the loss of \(abeABC\) had little effect on antibiotic levels. This suggested that AbeR has additional regulatory targets in the cell, or that AbeR has other functions in the cell that are independent of its transcriptional activator role (possibly mediated through its uncharacterized C-terminal domain). We defined the AbeR binding site through EMSA and DNaseI footprinting experiments and found that, like other SARP[s], AbeR bound heptameric direct repeats separated by 4 or 15 nt positioned on the same face of the DNA helix upstream of \(abeBCD\). A search of the genome using the CGGAAG(G/C)C (\(n_{4/15}\))CGGAAN(G/C)C sequence as a query failed to identify other candidate binding sites. Thus, the only known AbeR binding sites are within the \(abeA\) coding sequence. It is interesting that most SARP[s] control the expression of genes that are located in close proximity on the chromosome.

The differential expression of genes within the \(abeABC\) operon is not unprecedented in *S. coelicolor* (9), but it also is not considered to be the norm. Microarray studies of *S. coelicolor* gene expression have shown that the first gene of an operon is typically the most highly expressed, with expression levels then decreasing throughout the length of the operon (26). This is obviously not the case for \(abeA\), which was expressed at far lower levels than any of the other \(abe\) genes. Comprehensive transcriptome analyses of *E. coli* (13, 41), *Helicobacter pylori* (43), *Listeria monocytogenes* (50), and *Mycoplasma pneumonia* (18) now are beginning to shed light on the transcriptional complexity that exists in bacteria. Recent studies of *H. pylori* and *M. pneumoniae* have revealed extensive intraoperon expression dynamics, with different genes within an operon having distinct induction/repression characteristics relative to other genes within the same operon. Our findings here are consistent with there being flexible operon expression in *S. coelicolor*. Additional complexity at the antisense transcript level also is appearing to be widespread, with antisense transcripts being detected throughout the *E. coli*, *H. pylori*, and *M. pneumoniae* genomes (13, 18, 41, 43). What role these antisense transcripts have in regulating gene expression or protein activity remains to be seen.

Unexpectedly, we found that \(\alpha-abeA\) did not appear to have a role in modulating the transcript stability of its sense counterpart, \(abeA\), as increased levels of \(\alpha-abeA\) in an \(rnc\) (\(absB\)
mutant could not be correlated with a subsequent change in \( \alpha\text{-}abeA \) transcript abundance. While we cannot exclude the possibility that \( \alpha\text{-}abeA \) controls \( \alpha\text{-}abeA \) expression via an RNase III-independent mechanism (e.g., \( \alpha\text{-}abeA \) translation), we did observe changes in the overall transcript abundance of both \( \alpha\text{-}abeA \) and \( \alpha\text{-}abeA \) when RNase III was absent. \( \alpha\text{-}abeA \) transcripts, as well as \( \alpha\text{-}abeB\text{-}CD \) transcripts, were consistently less abundant in an \( \alpha\text{-}nce(A) \) mutant than in its wild-type parent. This is reminiscent of the reduced expression seen for the antibiotic biosynthetic clusters of actinorhodin, undecylprodigiosin, CDA, and a cryptic polypektide in an \( \alpha\text{-}b\text{abs}(B) \) mutant (21). In contrast, \( \alpha\text{-}abeA \) levels were increased in the \( \alpha\text{-}abs(B) \) knockout strain. This effect was not mediated through AdpA, which is negatively regulated by RNase III, and may instead reflect either the direct targeting of \( \alpha\text{-}abeA \) by RNase III during later growth stages or control by some other RNase III-dependent factor.

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