

Cross-regulation among disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*

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Summary

A complex programme of regulation governs gene expression during development of the morphologically and biochemically complex eubacterial genus *Streptomyces*. Earlier work has suggested a model in which ‘higher level’ pleiotropic regulators activate ‘pathway-specific’ regulators located within chromosomal gene clusters encoding biosynthesis of individual antibiotics. We used mutational analysis and adventitious overexpression of key *Streptomyces coelicolor* regulators to investigate functional interactions among them. We report here that cluster-situated regulators (CSRs) thought to be pathway-specific can also control other antibiotic biosynthetic gene clusters, and thus have pleiotropic actions. Surprisingly, we also find that CSRs exhibit growth-phase-dependent control over *afsR2/afsS*, a ‘higher level’ pleiotropic regulatory locus not located within any of the chromosomal gene clusters it targets, and further demonstrate that cross-regulation by CSRs is modulated globally and differentially during the *S. coelicolor* growth cycle by the RNaseIII homologue AbsB. Our results, which reveal a network of functional interactions among regulators that govern production of antibiotics and other secondary metabolites in *S. coelicolor*, suggest that revision of the currently prevalent view of higher-level versus path-

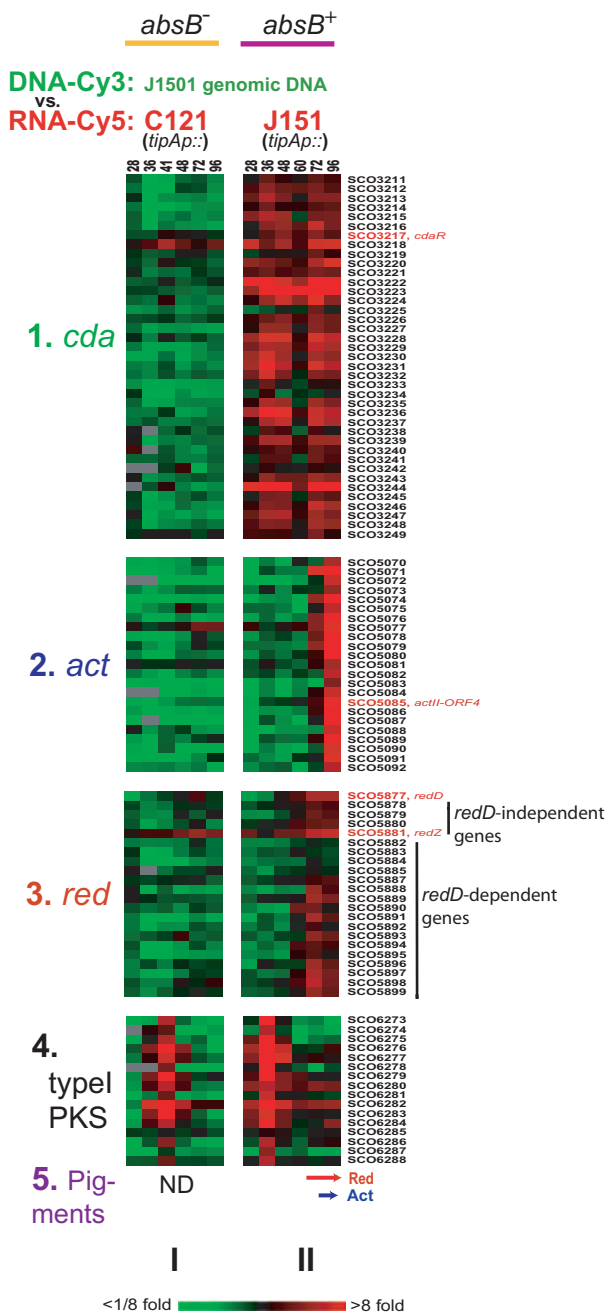
way-specific regulation of secondary metabolism in *Streptomyces* species is warranted.

Introduction

Members of the Gram-positive eubacterial genus *Streptomyces* are notable for their ability to produce a wide variety of pharmaceutically useful compounds as secondary metabolites; these include antitumour agents, immunosuppressants and nearly two-thirds of currently available natural antibiotics (Chater and Bibb, 1997; Challis and Hopwood, 2003). When cultured on solid media, members of the *Streptomyces* genus undergo a series of genetically programmed morphological and biochemical changes. Germination of spores initiates vegetative growth of *Streptomyces* as a tangle of multinucleate hyphae that extends into the medium. Later, aerial hyphae that reach away from the substrate appear, develop cross-walls and generate spores; concurrent with this process is a biochemical transition from primary to secondary metabolism (Hopwood, 1988; Chater, 1993).

Like most other streptomycetes, *Streptomyces coelicolor* is known to synthesize a variety of chemically diverse secondary metabolites (Hopwood *et al.*, 1995). Completion of the genome sequence led to the prediction of about two dozen pathways for secondary metabolites in this organism: antibiotics, pigments, siderophores, signalling molecules and complex lipids, including hopanoids (Bentley *et al.*, 2002). Earlier investigations indicate that genes encoding the products of individual pathways of secondary metabolism, commonly grouped together on the chromosome in physically distinct clusters, are subject to multiple levels of regulation (Arias *et al.*, 1999). Some regulators are located within the group of chromosomally clustered genes that they control, and the actions of these ‘low level’ regulatory genes commonly are referred to as ‘pathway-specific’ (Bibb, 1996); such *S. coelicolor* genes include *actII-ORF4* of the actinorhodin (Act) biosynthetic gene cluster (Fernandez-Moreno *et al.*, 1991), the *redD* and *redZ* genes of the undecylprodigiosin (Red) cluster (Takano *et al.*, 1992; White and Bibb, 1997), and *cdaR* of the calcium-dependent antibiotic (CDA) cluster (Chouayekh and Virolle, 2002; Ryding *et al.*, 2002). ‘Higher level’ regulatory genes largely situated outside of biosynthetic gene clusters exert pleiotropic effects on the production of multiple secondary metabolites, or on both

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three known chromosomal clusters of antibiotic biosynthetic genes of *S. coelicolor* at single gene resolution, the Cy5-dCTP red fluorescence-labelled cDNA from the *absB*⁻ and *absB*⁺ control strains [C121 (C120, *tipAp*::) and J151 (J1501, *tipAp*::) respectively] was separately hybridized on DNA microarrays with Cy3-dCTP green fluorescence-labelled genomic J1501 DNA (Fig. 2 and Fig. S1, also see web site <http://sncohenlab.stanford.edu/streptomyces2> or <http://www-genome.stanford.edu/microarray> for raw data). From these experiments, we obtained gene expression signatures for mycelium at different stages of the bacterial growth cycle in the presence

Fig. 2. Transcriptional effects of *absB* on secondary metabolite pathways. RNA samples from the control strains C121 (*absB*⁻, *tipAp*::) and J151 (*absB*⁺, *tipAp*::) were isolated at the indicated time points. For microarray experiments, Cy3-dCTP (green)-labelled J1501 genomic DNA was hybridized with Cy5-dCTP (red)-labelled cDNA corresponding to total RNA of C121 (Panel I) and J151 (Panel II) (indicated in figure as 'DNA vs. RNA'). Each individual secondary metabolite locus is shown in order of its position on the chromosome (panels 1–4). Rows correspond to individual genes and columns to different time points in hours after inoculation, as indicated. The change in transcript abundance for each gene is displayed by means of a colour scale. Brighter red shades represent higher transcript abundance and brighter green shades represent lower transcript abundance. Black indicates an equal amount of cDNA and genomic DNA, and grey represents the absence of data. Red and blue arrows in panel 5 indicate the onset of Act or Red production, respectively, and ND indicates no detectable Act or Red production. All original data can be downloaded from the Stanford Microarray Database (<http://www-genome.stanford.edu/microarray>).

or absence of *absB* function. We observed that there are about 200 genes having at least a sixfold difference in RNA abundance between *absB*⁻ and *absB*⁺ strains (Table S5), including genes involved in secondary metabolism, differentiation (e.g. *ramR*) and response to stress (e.g. the superoxide dismutase genes, *sodF1/2*). While the abundance of transcripts involved in secondary metabolism was largely decreased by the *absB* mutation, upregulation of transcript abundance in *absB*⁻ relative to *absB*⁺ bacteria was also observed (e.g. a gene cluster of unknown function, SCO4358–4363) (Fig. 2 and Fig. S1).

The abundance of mRNA encoded by *actII-ORF4* or *redD* and by previously demonstrated targets of these genes within the *act* and *red* clusters was increased

Table 1. Strains used in this study.

Strain	Relevant characteristic(s)	Reference
<i>S. coelicolor</i> A3(2)		
J1501	<i>hisA1 uraA1 strA1</i> SCP1 ⁻ SCP2 ⁻ Pgl ⁻	Kieser <i>et al.</i> (2000)
C120	J1501 <i>absB120</i>	Price <i>et al.</i> (1999)
C121	C120 <i>tipAp</i> ::	This study
C122	C120 <i>tipAp</i> :: <i>cdaR</i>	This study
C123	C120 <i>tipAp</i> :: <i>actII-ORF4</i>	This study
C124	C120 <i>tipAp</i> :: <i>redD</i>	This study
C125	C120 <i>tipAp</i> :: <i>redZ</i>	This study
J151	J1501 <i>tipAp</i> ::	This study
J152	J1501 <i>tipAp</i> :: <i>cdaR</i>	This study
J153	J1501 <i>tipAp</i> :: <i>actII-ORF4</i>	This study
J154	J1501 <i>tipAp</i> :: <i>redD</i>	This study
J155	J1501 <i>tipAp</i> :: <i>redZ</i>	This study
M145	Prototroph, SCP1 ⁻ SCP2 ⁻	Kieser <i>et al.</i> (2000)
M512	M145 Δ <i>redD</i> Δ <i>actII-ORF4</i>	Floriano and Bibb (1996)
M550	M145 Δ <i>redZ</i>	White and Bibb (1997)
<i>E. coli</i>		
DH5 α		Invitrogen
ET12567/pUZ8002	<i>dam dcm</i>	Kieser <i>et al.</i> (2000)

sharply in *absB*⁻ bacteria (Fig. 2). Similar effects were observed for *actII-ORF4*-regulated and *redD*-regulated genes located external to these clusters [e.g. the *eca* (expression co-ordinated with *act*) and *ecr* (expression co-ordinated with *red*) genes (Huang *et al.*, 2001); see Fig. S3]. These observations are consistent with earlier evidence that the *actII-ORF4* and *redD* regulatory genes are subject to *absB* control (Aceti and Champness, 1998). The *cdaR* gene and others in the *cda* cluster also were upregulated in *absB*⁺ mycelium (Fig. 2, panel II); however, whereas *cda* genes were activated by *absB* throughout the *S. coelicolor* growth cycle, *absB* expression resulted in upregulation of *act* and *red* cluster genes, and in production of the antibiotics actinorhodin and undecylprodigiosin (Fig. 2, panel 5-II) only at later time points, suggesting that additional factors whose actions are growth-phase-dependent can differentially modulate the effects of *absB* on different biosynthetic pathways. Additionally, the extent of *absB*-dependent upregulation of transcript abundance was greater for certain genes within individual biosynthetic clusters [for example, the *red* pathway transcriptional regulator RedZ (SCO5881) (White and Bibb, 1997) and a gene encoding a putative small hypothetical protein (SCO3218) in the *cda* cluster (Fig. 2, panels 3 and 1)], although lower transcript abundance throughout the cluster was observed in the *absB* mutant versus *absB*⁺ bacteria. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of *redZ*, SCO3218 and seven and eight other randomly selected genes from each of the *red* and *cda* clusters confirmed the quantitative difference in *absB* effects among genes of these clusters (see Table S2). Notwithstanding the production in *absB* mutant bacteria of transcripts of *redZ*, which is believed to be a transcriptional activator of *redD* (White and Bibb, 1997), the expression of *redD* and *redD*-dependent genes (Huang *et al.*, 2001) and the biosynthesis of undecylprodigiosin were not observed in the *absB* mutant C121 (Fig. 2, panels 3 and 5). This finding suggests that either AbsB itself or a separate *absB*-regulated function is required along with *redZ* for *redD* activation and undecylprodigiosin production. Transcripts of the type I polyketide gene cluster showed increased abundance at certain time points in both strains (Fig. 2, panel 4). Exempted from regulation by *absB* were some genes in the hopanoid and coelichelin biosynthetic pathways (Fig. S1).

Effects of CSRs on expression of antibiotic pathways in the absence or presence of higher-level regulation

Our identification of genes in all three known *S. coelicolor* antibiotic biosynthetic pathways that produce little or no mRNA in *absB*⁻ bacteria enabled us to compare the effects of the cluster-situated regulatory genes *cdaR*,

actII-ORF4, *redD* and *redZ* in the absence or presence of extra-cluster control by *absB*. In these experiments (Fig. 3), gene expression in an *absB*⁻ strain that constitutively expressed *cdaR*, *actII-ORF4*, *redD* or *redZ* from the *tipA* promoter [C122–C125 (*tipAp::cdaR/actII-ORF4/redD/redZ*)] was compared through the growth-cycle with expression in the control strain C121 (*tipAp::*) (Fig. 3, panels A–D) and in *absB*⁺ bacteria [J152–J155 (*tipAp::cdaR/actII-ORF4/redD/redZ*) versus J151 (*tipAp::*)] (Fig. 3, panels E–H). Transcripts whose abundance showed a statistically significant change relative to either C121 or J151 control strain (> 75% genes in each cluster with *P*-value < 0.05; see Table S4) are highlighted with red or green triangles in Fig. 3. The relative increase (red triangles) or decrease (green triangles) in transcript abundance in the highlighted groupings was confirmed by repeats of array experiments and by quantitative real-time RT-PCR analysis of randomly selected transcripts (see Table S3).

As seen in Fig. 3, in the absence of *absB* function, constitutive expression of the *cdaR*, *actII-ORF4* and *redD* regulatory genes under control of the *tipA* promoter increased the abundance of transcripts encoded by genes in their own biosynthetic clusters, but decreased the abundance of transcripts from the type I polyketide gene cluster (Fig. 3, panels 1A, 2B, 3C and 4A–C). The genes most prominently affected by *redD* include the previously identified *redD*-dependent genes (Huang *et al.*, 2001) (Fig. 3, panel 3C) and *ecr* genes (Fig. S3). In contrast, while constitutive expression of *redZ* in the absence of *absB* resulted in increased expression of *redD*-independent genes, it failed to increase the transcripts regulated by *redD*, despite elevation of the *redD* transcript itself, arguing that *absB* acts directly or indirectly in concert with *redZ* for the production of functional RedD protein to activate the *redD*-dependent genes (Fig. 3, panel 3D). The absence of detectable undecylprodigiosin in extracts of mycelium obtained from *redZ* overproducing bacteria (Fig. 3, panel 5D) is consistent with these findings. Importantly, *redZ* overexpression additionally increased the abundance of transcripts from the *cda* gene cluster (Fig. 3, panel 1D), indicating that the actions of this CSR extend beyond the *red* pathway.

In the presence of *absB* function, transcripts of the *cda*, *act*, and *red* clusters and of the type I polyketide gene cluster were increased in abundance during certain periods of the *S. coelicolor* growth cycle (see Fig. 2, panel II). Constitutive expression of *cdaR*, *actII-ORF4* and *redD*, which activated expression of genes in their respective pathways, surprisingly also affected the abundance of transcripts from genes that function in disparate biosynthetic pathways. For example, constitutive expression of *cdaR* increased the relative abundance (compared with the control strain, J151) of transcripts from genes in the *cda* and *act* pathways at times when these pathway genes

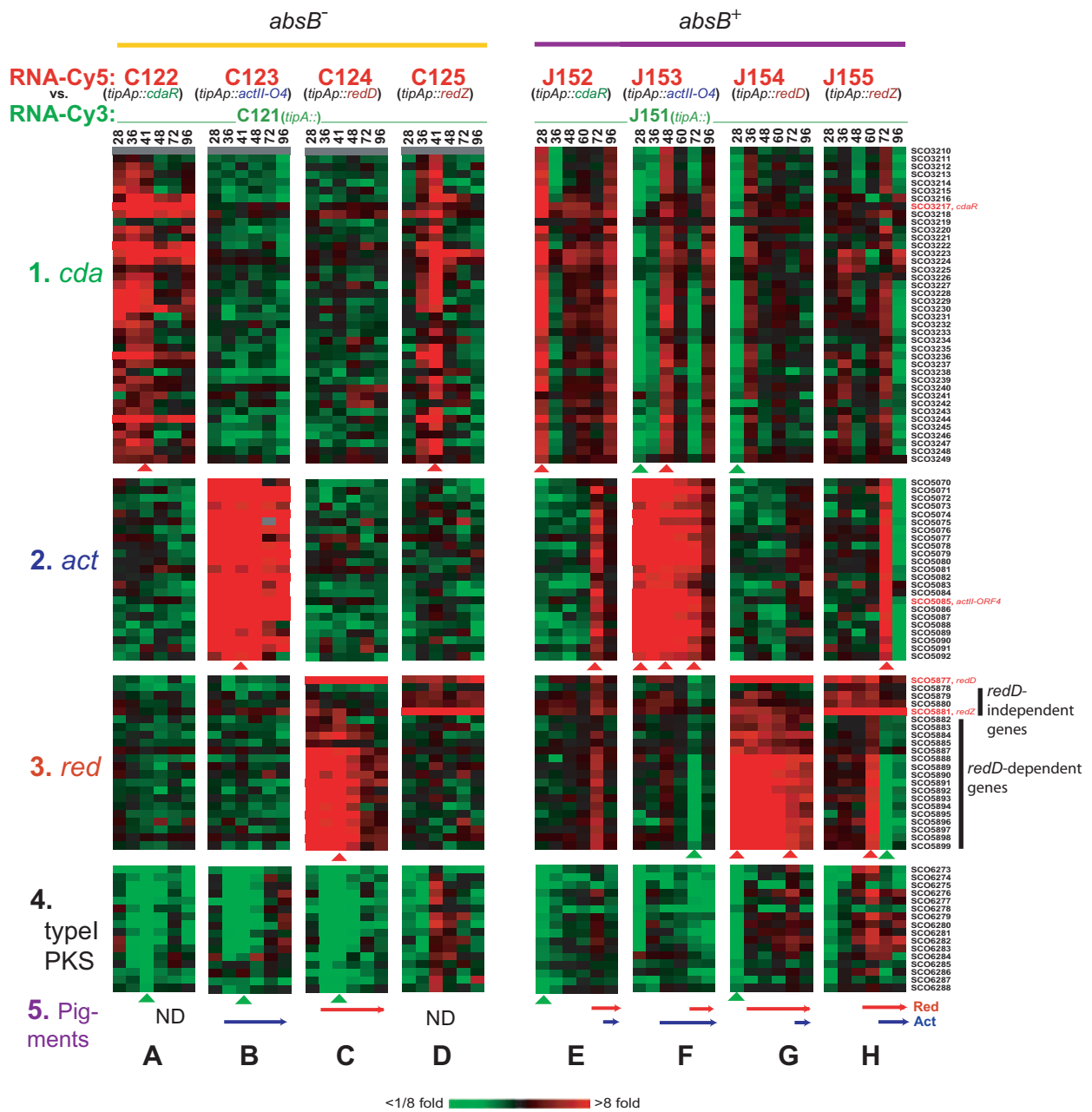


Fig. 3. Transcriptional effects on secondary metabolite pathways during induction of cluster-situated regulatory genes in the absence or presence of *absB*. RNA samples from *absB*⁻ (C121–C125) and *absB*⁺ (J151–J155) strains were isolated at the indicated time points during parallel growth on modified R5 media containing thioestrepton. For microarray experiments (indicated in figure as ‘RNA vs. RNA’), Cy5-dCTP (red)-labelled cDNA samples from *absB*⁻ strains (C122–C125, *tipAp*:: each of four regulatory genes) were hybridized with the same time point Cy3-dCTP (green)-labelled cDNA samples of the *absB*⁻ control strain (C121, *tipAp*::); Cy5-dCTP labelled cDNA samples from *absB*⁺ strains (J152–J155, *tipAp*:: each of four regulatory genes) were hybridized with the same time point Cy3-dCTP labelled cDNA sample of the *absB*⁻ control strain (J151, *tipAp*::). The change in transcript abundance for each gene is displayed by means of a colour scale, in which colour saturation represents the magnitude of the difference of RNA abundance between the detected strains (C122–C125 or J152–J155) and control strains (C121 or J151) at the same indicated time point. The brighter red shades represent higher transcript abundance and brighter green shades represent lower transcript abundance in detected strain comparing with the control strain. Black indicates an equal amount of RNA abundance between the two strains, and grey represents the absence of data. Ratios of genes with multiple spots on the array were averaged. Red and blue arrows in panel 5 indicate the onset of Act or Red production, respectively, and ND indicates no detectable Act or Red production. Triangles highlight instances of the increased (red) or decreased (green) expression relative to the control strain in clusters containing >75% genes having statistically significant changes (*P*-value <0.05) in expression. The relative increase or decrease in RNA abundance in the highlighted groupings was confirmed by repeat array experiments and quantitative real-time RT-PCR analysis.

were not normally expressed (i.e. at 28 h and 72 h respectively) (Fig. 3, panels 1E and 2E). Conversely, constitutive expression of *actII-ORF4* first decreased and then increased the abundance of *cda* transcripts relative to control strain (J151, *tipAp::*) at 28 and 48 h respectively (Fig. 3, panel 1F); it also resulted in a relative decrease in expression of *redD*-independent genes of the *red* cluster at 72 h, when these genes normally show elevated expression in the control strain J151 (Fig. 3, panel 3F; Fig. 2, panel 3-II). A parallel effect on the abundance of *ecr* transcripts was observed (Fig. S3).

Constitutive expression of *redZ* in *absB*⁺ bacteria resulted not only in the expected constitutive increase in *redD* and *redD*-independent transcripts, relative to the control strain (J151, *tipAp::*) containing the empty vector (Fig. 3, panel 3H), but also in a transient increase in the relative abundance of *redD*-dependent and *act* transcripts at times prior to the normal appearance of these transcripts in the control strain (Fig. 3, panels 3H and 2H). Accompanying these latter increases was the synthesis of undecylprodigiosin and actinorhodin at earlier than normal times (60 h and 72 h, respectively, versus 72 h and 96 h; Fig. 3, panel 5H, and Fig. 2, panel 5-II). Paralleling a relative decrease in expression of *redD*-dependent genes of the *red* cluster at 72 h in *absB*⁺ bacteria that constitutively express *actII-ORF4* (J153, *tipAp::actII-ORF4*; Fig. 3, panel 3F) were a relative decrease in expression of *redD*-dependent genes in mycelia that constitutively express *redZ* (J155, *tipAp::redZ*; Fig. 3, panel 3H) and a concurrent relative elevation of *act* gene expression in these cells (Fig. 3, panel 2H). Effects of *redZ* expression on other gene clusters outside of the Red and Act biosynthetic gene clusters were also observed, suggesting that this cluster-situated regulatory gene may have more general regulatory functions. Among the genes affected were a previously undefined 15-gene cluster (SCO6566–SCO6580) and genes of the *whiE* locus, which encodes a grey pigment present in *S. coelicolor* spores (Davis and Chater, 1990; Kelemen *et al.*, 1998) (Fig. S4). Interestingly, constitutive expression of any of these CSRs affected the abundance of mRNAs of the type I polyketide in both *absB* mutant and *absB*⁺ bacteria (Fig. 3, panel 4).

Effects of mutations in cluster-situated regulatory genes on disparate antibiotic biosynthetic pathways

Consistent with our finding that constitutive expression of *redZ* activates genes in other biosynthetic clusters, as well as genes in the *red* cluster (Fig. 3, panels D and H), we observed that deletion of *redZ* in *absB*⁺ bacteria (M550, $\Delta redZ$) resulted in delayed expression of genes of the *act*, *cda* and type I polyketide gene clusters for 6–12 h, and impaired expression of *redD* and other genes in the *red*

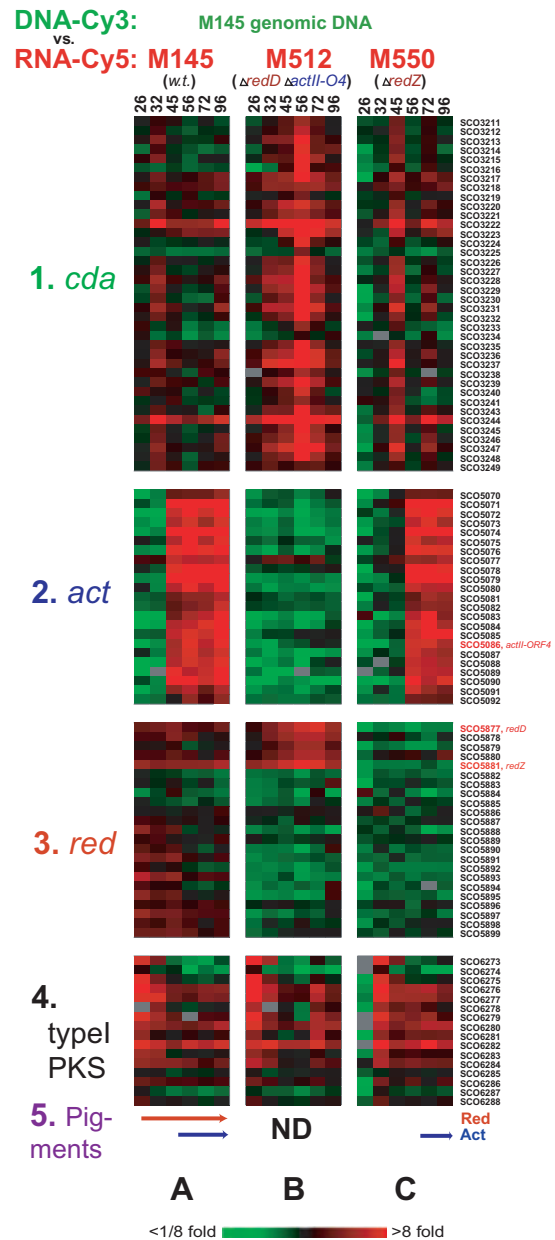


Fig. 4. Expression profiles of secondary metabolite loci genes in *S. coelicolor* M145, mutant M512 ($\Delta redD \Delta actII-ORF4$) and M550 ($\Delta redZ$). RNA samples isolated from M145 (wild type), M512 ($\Delta redD \Delta actII-ORF4$) and M550 ($\Delta redZ$) were isolated at the indicated time points on modified R5 medium. For microarray experiments, Cy5-dCTP labelled cDNA corresponding to total RNA of M145 (panel A), M512 (panel B) and M550 (panel C) was hybridized with Cy3-dCTP labelled M145 genomic DNA. Data were displayed as described in the Fig. 2 legend.

cluster (Fig. 4, panel C). Detection of Act was delayed for 16 h in M550 ($\Delta redZ$) versus the wild-type strain M145 (Fig. 4, panel 5). These findings provide further evidence for functional interaction between *redZ* and multiple biosynthetic gene pathways. Additionally, we observed that *red* pathway mRNAs were more abundant in cells blocked

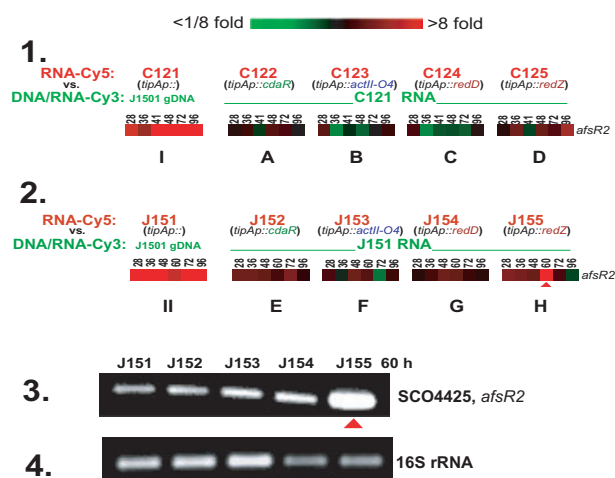


Fig. 5. Expression profiles for *afsR2* gene during the *S. coelicolor* growth cycle and during induction of CSRs. Panels 1-I and 2-II are from the same data set as Fig. 2 (panels I and II); panels A–H are from the same data set as Fig. 3 (panels A–H); panel 3, RT-PCR analysis of *afsR2* 60 h mRNA samples from strains J151–J155; panel 4, RT-PCR analysis of 60 h 16S rRNA samples from strains J151–J155. Red triangles highlight the higher expression observed in strain J155 (*tipAp::redZ*) compared with the control strain J151 (*tipAp::*) at 60 h.

in the Act pathway (Huang *et al.*, 2001). In our current experiments we observed that *cda* pathway transcripts were also more abundant in bacteria deleted in *actII-ORF4* and *redD* (Fig. 4, panel 1B), providing an additional demonstration of the effects of CSRs on disparate pathways.

Cross-regulation between *redZ* and higher level regulator *afsR2/afsS*

Overexpression of the higher level regulator AfsR2 (known also as AfsS) has long been known to stimulate the production of multiple antibiotics in *Streptomyces lividans* (Vogtli *et al.*, 1994) and *S. coelicolor* A3(2) (Floriano and Bibb, 1996). In our microarray experiments, we observed that *afsR2* was highly and constitutively expressed during the growth cycle of both of the *S. coelicolor* strains we examined (Fig. 5, panels 1-I and 2-II). In *S. coelicolor* mycelium expressing *redZ* constitutively under control of the *tipA* promoter (J155, *tipAp::redZ*), we surprisingly found that transcription of *afsR2* was increased dramatically over the control strain level specifically at 60 h (Fig. 5, panel 2-H), when Red pigment precociously appeared (Fig. 3, panel 5H). Twelve hours later (at 72 h), actinorhodin production by this strain was observed, whereas this antibiotic normally was not detectable until 96 h in control strain J151 (Fig. 3, panel 5H, and Fig. 2, panel 5-II). RT-PCR analysis confirmed the relative increase in *afsR2* mRNA abundance in bacteria

expressing *redZ* under the control of *tipA* (Fig. 5, panels 3–4).

Discussion

Experiments carried out over the past four decades indicate that a complex regulatory programme has evolved in *S. coelicolor* to control the biosynthesis and flux of secondary metabolites. It has long been recognized that the production of multiple secondary metabolites by this organism is co-ordinated during the growth cycle, perhaps to facilitate its ability to compete against other biological species (Challis and Hopwood, 2003). Such co-ordination has been believed to be mediated by global regulators that activate expression of chromosomal clusters of disparate biosynthetic genes by turning on lower level regulators that highly specifically control transcription of other genes in their own cluster. Additionally, the supply of metabolites utilized in common by different pathways has been postulated to alter the actions of pathways whose protein products compete for the same precursor (Chater, 1990; Sun *et al.*, 2002). The investigations reported here indicate the existence of functional interactions among participants in the *S. coelicolor* regulatory programme: the ability of regulators situated within biosynthetic gene clusters to alter expression of other clusters as well as their own, and also to modulate the effects of regulators that act more globally. They further show that cross-pathway regulation is controlled temporally in *S. coelicolor* by *absB*. Functional interactions among transcriptional regulators have been observed also for *Escherichia coli* (Oshima *et al.*, 2002) and yeast (Kaniak *et al.*, 2004).

Our results indicate that CSRs that act pleiotropically can function pleiotropically and that their effects on disparate biosynthetic pathways can occur at the RNA level. Whether competition between pathways for pools of precursor metabolites used in common or actions of biosynthetic pathway products on other pathways have a role in these effects has not been determined.

Foremost perhaps among CSRs is the Red cluster gene *redZ*, which we show can upregulate the production of all three known antibiotics of *S. coelicolor*. Induction of *redZ* transiently increased expression of *cda*, *red* and *act* in either the absence or presence of expression of *absB*. Deletion of *redZ* abolished *red* expression and delayed expression of two other antibiotics and the type I polyketide. Interestingly, the sequence of the RedZ protein shows a helix–turn–helix motif, plus 26% end-to-end identity to AbsA2, the response regulator component of a two-component system encoded by a locus in the *cda* cluster (Guthrie *et al.*, 1998; Anderson *et al.*, 2001; Ryding *et al.*, 2002). AbsA2, together with the sensor kinase encoded by the neighbouring gene, AbsA1, can *negatively* affect the flux through other antibiotic production pathways in

addition to more prominently repressing its own pathway, paralleling the *positive* regulatory effects of RedZ on disparate pathways.

Our microarray analysis indicated that the abundance of mRNAs encoded by *afsR2* and several other genes that have been annotated in the *Streptomyces* database (ScoDB; <http://streptomyces.org.uk/>) as putative regulators (SCO5147, SCO4908 and SCO1699) were increased dramatically in J155 (*tipAp::redZ*) over the control strain level (J151 *tipAp::*) (Fig. 5 and Fig. S4), thus demonstrating unanticipated *redZ* control over the *afsR2* higher level regulator and providing a possible basis for RedZ activation of the Red and Act biosynthetic pathways. However, despite constitutive expression of *redZ* throughout the *S. coelicolor* growth cycle, the effects of *afsR2* were not prominently observed until the 60 h time point, implying that other cellular factors regulated along with *afsR2* are needed for activation of these pathways.

Our results suggest that *absB* regulation of production of undecylprodigiosin in *S. coelicolor* results from *absB* activation of *redD*, possibly through stimulation of the production of *afsR2* gene product, which we found can restore antibiotic production in the absence of *absB*. Similarly, *redZ* upregulation restored expression of CDA biosynthetic genes in the absence of *absB*, but required *absB* to activate *act* genes (Fig. 3, panels 1D and 2H). Whereas earlier work (Kim *et al.*, 2001; Lee *et al.*, 2002) and our unpublished studies indicate that *afsR2* overexpression is sufficient to activate all of these pathways, it seems unlikely that *afsR2* expression is required for this effect as antibiotic biosynthesis occurred in cultures that failed to produce *afsR2*.

Analysis of the *S. coelicolor* genomic DNA sequence suggests that there are more than 20 distinct pathways for the production of secondary metabolites in this microorganism (Bentley *et al.*, 2002). Effects of the CSRs we studied on other pathways were observed, the original data are available at <http://sncohenlab.stanford.edu/streptomyces2> or <http://www-genome.stanford.edu/microarray>. Among the genes altered by RedZ and ActII-ORF4 were the *whiE* cluster (SCO5314–5321) (Fig. S4) and the coelichelin non-ribosomal peptide synthetase (NRPS) gene locus (SCO489–499). A large gene locus of unknown function (SCO6566–6580) was also regulated by *redZ*, resulting in increased expression in both C125 (*tipAp::redZ*) and J155 (*tipAp::redZ*), and decreased expression in M550 ($\Delta redZ$), as compared with M145. For other gene groups affected by induction of *redZ* see Fig. S4.

Overexpression of *actII-ORF4* was found to increase transcripts of the catalase (*catA*, SCO0379) and superoxide dismutase genes (*sodF1/2*, SCO2633/SCO0999) (Fig. S5), which have been implicated in cellular resistance to the toxic effects of exposure to oxidants (Cho and

Roe, 1997; Chung *et al.*, 1999). This observation suggests that *S. coelicolor* may increase its anti-oxidative defence mechanisms during production of actinorhodin.

The extent and boundaries of the type I polyketide locus (from SCO6273 to SCO6288) were inferred from sequence analysis of *S. coelicolor* genomic DNA (Bentley *et al.*, 2002). During our studies, we observed that a gene SCO6272 (SC2C4.02, a putative secreted FAD-binding protein), adjacent to the inferred type I polyketide locus, showed an expression correlation coefficient of >0.9 with genes designated by Bentley *et al.* as components of the type I polyketide locus (Fig. S6). Moreover, expression of three other genes separated from SCO6272 by only 5 kb also showed a high correlation coefficient (>0.8), with expression of genes in the type I polyketide locus; two of these, *scbR* (SCO6265) and *scbA* (SCO6266), mediate the synthesis of gamma-butyrolactone SCB1 of *S. coelicolor* (Takano *et al.*, 2001). The third is a putative histidine kinase gene (SCO6268, SCAH10.33c). These findings raise the possibility that these genes collectively may have a role in regulating the type I polyketide locus (Fig. S6). Supporting this postulated connection between SCB1 and the type I polyketide is a recent report by Takano *et al.* (2005), which has identified a regulatory gene, *kasO*, in the type I polyketide cluster that is regulated directly by *scbR*.

absB mutant bacteria previously have been found to be deficient in antibiotic production in *S. coelicolor*, and the DNA sequence of this gene indicates that it encodes a *S. coelicolor* homologue of the *E. coli* endoribonuclease, RNase III (*rnc*) (Price *et al.*, 1999). RNase III proteins of bacteria are known to have a diverse role in the processing of double-stranded mRNA and rRNA substrates (Court, 1993), and RNase III homologues in eukaryotes can process double-stranded RNAs into small regulatory RNAs (Lee *et al.*, 2003; Carmell and Hannon, 2004; Tijsterman and Plasterk, 2004). Our finding that *absB*, which is widely conserved in streptomycetes (Price *et al.*, 1999), has extensive effects on the abundance of about 200 different *S. coelicolor* transcripts having a multitude of functions, suggests that ribonucleolytic processing of duplex RNA regions may modulate key aspects of gene expression in this organism (Fig. S1).

The cross-regulation we've observed occurs in both the presence (Figs 2 and 3) or absence (Fig. 4) of antibiotics. However, our microarray analyses also indicated that the extent of dependence of biosynthetic pathway gene expression on *absB* was influenced by the addition of thiostrepton and apramycin in the medium (data not shown): the abundance of *red* and *act* transcripts was decreased, and of type I polyketide gene transcripts was increased, in the presence of these antibiotics, suggesting that control of secondary metabolism by regulatory genes of *S. coelicolor* can be affected by alterations in the cellu-

lar environment. The ability of extracellular agents to alter the actions of individual regulatory genes may account in part for the well-known dependence of antibiotic production on medium composition. Notwithstanding such observations, the differential expression that provides a basis for our conclusions cannot result from effects of antibiotics *per se*, as experimental cultures were compared with control cultures that included the same antibiotics (Fig. 3).

Experimental procedures

Construction of the integrative P_{tipA} expression vector, pJ6902, and its derivatives

A 0.625 kb fragment carrying a polylinker downstream of the thiostrepton-inducible P_{tipA} promoter, both ends flanked by transcriptional terminators, was amplified from the multicopy expression vector pJ6021 (Takano *et al.*, 1995) by PCR using *Pfu* DNA polymerase (Promega) and the primers (5'-GCCTCGTGATCACCAATAAAAAACGCCCGCGG-3' and 5'-CGCGTGATCAGCCCCGAAAAGCGCCTTTGAC-3'). The PCR product was cloned into EcoRI-XbaI-cut pSET152 (Takano *et al.*, 1995) for which both cleavage sites had been filled in to generate blunt ends (thereby destroying both sites). The sequence of the inserted fragment in the resulting construct, pJ6901, was confirmed. The *tsr* gene was amplified by PCR from pJ6021 using *Pfu* DNA polymerase and the primers THIONHE (5'-GAAATGTAGCTAGCAGGCGAATAC TT-3') and THIONHEREV (5'-GACGAATCGGCTAGCAGGA ACCGAGCGTCC-3'), both of which carry NheI sites. The resulting 1.06 kb PCR product was cut with NheI and cloned into the unique NheI of pJ6901 to create pJ6902, and the *tsr* sequence in the final construct was confirmed.

Open reading frames (ORFs) of *cdaR*, *actII-ORF4*, *redD* and *redZ* were amplified by PCR and cloned into the pCR2.1-TOPO vector (Invitrogen) with an NdeI site overlapping an ATG start codon. NdeI and EcoRI/BamHI were used to remove the inserted ORFs, which were cloned into the integrative vector pJ6902 under control of the *tipA* promoter. All cloned products were confirmed by DNA sequencing. The primers used for PCR amplification are as follows: *cdaR*-L, GAGCAT-ATGGATCTTCGGCTGATAGAACCG; *cdaR*-R, CAGCACTCGCTGTGGCCGTC; *redZ*-L, CAACAT-ATGACG ACCCGTGTCTGGTGTG; *redZ*-R, CCGCACGGAACGCG AAGC; *redD*-L, CGGCAT-ATGACGGTGGGGGAGTGCT TG; *redD*-R, GTTTCCGTGACGGTGGGTGTGTC; *actII-ORF4*-L, GCGCAT-ATGAGATTCAACTTATGGGACGTGTC CAT; *actII-ORF4*-R, CGGTGCTACACGAGCACCTTCTCAC.

Strains, growth conditions, RNA isolation and assay of antibiotics

Streptomyces coelicolor strains used in this study are listed in Table 1. pJ6902 and its derivatives containing insertions of the *cdaR*, *actII-ORF4*, *redD* and *redZ* genes were introduced by conjugation from *E. coli* ET12567/pUZ8002 into J1501 and C120 strains, where they integrated site-specifically into the chromosome at the ϕ C31 *attB* site. The ex-

conjugants (J151–J155 and C121–C125, Table 1) were checked by PCR.

We used cellophane membranes placed on plates for all total RNA sample isolation from surface-grown cells. RNA from M145 and its mutants grown on solid medium was obtained as described earlier (Huang *et al.*, 2001). Spores (10^5 per plate) of strains C121–C125 and J151–J155 were plated onto R5-medium containing 10 mM CaCl_2 , 50 $\mu\text{g ml}^{-1}$ apramycin and 50 $\mu\text{g ml}^{-1}$ thiostrepton as the final concentration. We detected growth of each strain by measuring dry cell weight (Miguelez *et al.*, 1999) and we combined the surface-grown cells harvested from 4 to 10 plates at each time point and used the modified Kirby-mix method (Kieser *et al.*, 2000) to extract total RNA. RNA samples were purified using the RNeasy Kit (Qiagen). Later experimental repeats of microarray and quantitative real-time RT-PCR experiments at certain time points (highlighted with the green or red triangles in Fig. 3) were performed with RNA samples isolated and purified using the RNeasy[®] Plant Kit.

Assays for Act and Red were as described previously (Huang *et al.*, 2001). However, CDA production on solid medium containing thiostrepton was impractical by bioassay of the indicator *Staphylococcus aureus* strain because this CDA-sensitive bacterium is also sensitive to thiostrepton.

Streptomyces coelicolor microarray experiments and quantitative real time RT-PCR

The whole-genome DNA sequences with 7846 *S. coelicolor* M145 ORFs (ftp://ftp.sanger.ac.uk/pub/S_coelicolor/sequences/) were used for design of primer pairs that amplified 50–2400 bp internal fragments of putative ORFs (<http://snohenlab.Stanford.edu/streptomyces>). The arrays contain 97% of 7846 ORFs, and about 10% of the ORFs printed on arrays were duplicates or overlapping sequences. Primer design, PCR amplification, RNA labelling and hybridization were as described earlier (Huang *et al.*, 2001; Elliot *et al.*, 2003).

Genomic DNA was labelled with Cy3-dCTP as follows. Two micrograms of M145 or J1501 genomic DNA was fragmented by sonication to an average size of 500–1000 bp, mixed with 8 μg of high-GC (72%) hexamers (total 20 μl), and after incubation at 98°C for 5 min, transferred to ice water, 30 μl of labelling mix [5 μl 10 \times Klenow buffer, 6 μl 10 \times dNTP (4 mM dATP, 4 mM dTTP, 10 mM dGTP and 0.5 mM dCTP), 3 μl Cy3-dCTP (Amersham Pharmacia Biotech), 1 μl Klenow DNA polymerase (NEB 50 U μl^{-1}) and 15 μl ddH₂O] was added and the mixture was incubated for 5–6 h at 37°C in the dark. The Cy3-dCTP labelled genomic DNA probe was purified using Microcon-10 filters (Amicon), and hybridized with Cy5-dCTP labelled cDNA.

Quantitative real-time RT-PCR was applied for validation of gene expression changes observed in microarrays. First-strand cDNA synthesis was carried out using 2 μg total RNA and SuperScript II (Invitrogen), following the manufacturer's instructions (Cat. No. 18064-014). Quantitative real-time PCR of randomly selected genes from the antibiotic or type I polyketide cluster was performed using the Bio-Rad iCycler[™] Real-Time PCR Detection System and iQ[™] SYBR Green Supermix Kit (170-8880). Five per cent of the first-strand reaction was used as DNA template, real-time 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 16S rDNA levels (see Table S1 for all RT-PCR primers).

Microarray data normalization and analysis

Built-in functions of the Stanford Microarray Database (<http://www-genome.stanford.edu/microarray>) were used to normalize and analyse RNA-versus-genomic DNA data. The normalization assumes equal log average signal intensity in each fluorescence channel, and that RNA/gDNA ratios were equivalent to relative transcript abundances after normalization (Bernstein *et al.*, 2002). Data analysis of RNA-versus-RNA hybridization was performed as described earlier (Huang *et al.*, 2001; Elliot *et al.*, 2003), and a k-nearest neighbours (KNN) algorithm was applied for missing data (Troyanskaya *et al.*, 2001).

We determined the statistical significance of the changes in gene expression as follows. Using replicate microarrays for a subset of the conditions, we calculated the standard error of the expression values for each gene across the replicates and took the median standard error of all the genes as the fudge factor. We then estimated the standard error for each gene as the sum of the standard error of that gene in the replicates plus the fudge factor (Efron *et al.*, 2001). For those conditions for which we did not have replicates, we calculated an approximate *t*-statistic for each gene as the expression value of that gene divided by its estimated standard error. Because *redD*-dependent genes and *redD*-independent genes have different expression patterns, we separated them into two subclusters for *t*-score calculation. We estimated the *P*-value (two-tailed) for each gene using the *t*-statistic. Gene expression was considered to be significantly changed if its *P*-value was <0.05. Expression in an entire cluster was considered to be significantly changed if expression of at least 75% of the genes within it were significantly changed. Quantitative real-time PCR analysis and additional microarray experiments were carried out to further evaluate the alterations in gene expression (see *Supplementary materials* at <http://sncohenlab.stanford.edu/streptomyces2>).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Global effects of *absB* gene. In total, ~1500 genes that were highly expressed during the growth cycle were selected and hierarchically clustered according to transcript abundance. Panels I and II are from the same data set as Fig. 2 (panel I and II). Rows correspond to individual genes and columns to different time points, as indicated. The change in transcript abundance for each gene is displayed by means of a colour scale. Brighter red shades represent higher transcript abundance and brighter green shades represent lower transcript abundance. Black indicates an equal amount of cDNA relative to genomic DNA, and grey represents the absence of data. Arrows indicate examples of several known genes/gene clusters and a previously unidentified gene cluster. In order to compare the different gene expression between the *absB*⁻ and *absB*⁺ strain directly, transcript abundance for the *absB* mutant (panel I) was subtracted from transcript abundance of *absB*⁺ the strain (panel II) to obtain the net fold difference between them. In total, ~200 genes were observed to have at least a sixfold different abundance in at least one time point between the *absB* mutant and *absB*⁺ the strain (Table S5). All original data can be downloaded from <http://sncohenlab.stanford.edu/streptomyces2> or the Stanford Microarray Database (<http://www-genome.zstanford.edu/microarray>).

Fig. S2. Growth curves of strains in Figs 2 and 3. Growth of

mycelia was monitored as mg dry cell weight per plate after inoculation of ~10⁵ per plate spores on modified R5-solid medium.

Fig. S3. Expression of *eca/ecr* genes is similar to expression of the *act* and *red* biosynthetic genes respectively. Panels I and II are from the same data set as Fig. 2 (panel I and II); panels A–H are from the same data set as Fig. 3 (panels A–H). See Figs 2 and 3 legends in the main text for details.

Fig. S4. Additional targets regulated by *redZ*. Panels are from the same data sets shown in Figs 2–4 as indicated. See Figs 2–4 legends in the main text for details.

Fig. S5. Additional targets regulated by *actII-ORF4*. Panels I and II are from the same data sets as Fig. 2 (panel I and II); Panels A–H are from the same data set as Fig. 3 (panels A–H). See Figs 2 and 3 legends in the main text for details.

Fig. S6. Extent of the type I polyketide locus and similar expression patterns observed for *scbR/A*. Panel I is from the same data set as Fig. 2 (panel I); panels A–D are from the same data set as Fig. 3 (panels A–D). See Figs 2 and 3 legends in the main text for details.

Table S1. Primers used for quantitative real-time RT-PCR.

Table S2. Results of quantitative real-time RT-PCR in Fig. 2.

Table S3. Results of quantitative real-time RT-PCR in Fig. 3.

Table S4. *P*-values of the genes in Fig. 3.

Table S5. Ratio of ~1500 genes in Fig. S1.

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