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 pathway-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 hopenoids (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 (Chouvayek 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...
primary metabolism and morphological development (e.g. afs, abs and bld genes). An important question in Streptomyces biology is how these multiple levels of control of antibiotic biosynthetic pathways are co-ordinated (Bibb, 1996). The task of co-ordination is made even more complex by the production of multiple antibiotics in a single organism (Hopwood et al., 1995) and the occurrence of multiple regulatory genes in some antibiotic gene clusters (White and Bibb, 1997; Bate et al., 1999; Ryding et al., 2002).

The availability of the ~8.7 Mb chromosomal DNA sequence of S. coelicolor has enabled global investigations of gene expression in this organism using DNA microarrays (Huang et al., 2001; Bentley et al., 2002; Karoonuthaisiri et al., 2005). Earlier microarray-based experiments have shown that genes located within the same biosynthetic gene cluster can be regulated differentially as well as co-ordinately during growth and development of S. coelicolor (Huang et al., 2001); these studies also have identified chromosomally distant genes whose expression is co-ordinated with genes in antibiotic biosynthetic gene clusters and whose promoter regions resemble those of the biosynthetic genes. While these observations argue that the actions of regulators located within biosynthetic gene clusters can extend beyond their own cluster, cluster-situated regulators (CSRs) continue to be viewed largely as ‘pathway-specific’. We report here the results of microarray experiments aimed at gaining a better understanding of functional interactions among the genes that regulate expression of antibiotics during S. coelicolor development. Our findings indicate that, contrary to current notions, there is extensive ‘cross-talk’ between CSRs of streptomycetes, that these ‘low level’ regulators can control expression of genes designated previously as ‘higher level’ regulators, and that the extent of cross-talk between different biosynthetic pathways is modulated by the pleiotropic regulator AbsB during the cell cycle growth. Our results indicate that a complex network of functional interactions exists among disparate biosynthetic pathways of S. coelicolor and suggest that revision of the currently prevalent paradigm for higher-level versus pathway-specific regulation of secondary metabolism in Streptomyces species is warranted.

Results

Effects of absB on expression of genes in antibiotic biosynthetic pathways

The absB gene of S. coelicolor is a pleiotropic regulator of antibiotic synthesis (Adamidis and Champness, 1992). Earlier work has shown that mutations in absB, which encodes an RNase III homologue, result in decreased abundance of transcripts encoded by the actII-ORF4 and redD genes, which affect the expression of genes in the

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Fig. 1. The integrative P
\(_{\text{tipA}}\) expression vector, pIJ6902. ter fd, major transcription terminator of phage fd; t\(_{\text{tipA}}\), transcription terminator from phage λ; P
\(_{\text{tipA}}\), the tipA promoter; tsr, thiostrepton-resistance gene; aac(3)I\(_{\text{IV}}\), apramycin-resistance gene selectable in E. coli and streptomycetes; ori pUC18, origin of replication from pUC18; oriT RK2, origin of transfer from plasmid RK2; int \(_{\text{C31}}\) and attP, the integrase gene and attachment site of the temperate phage \(_{\text{C31}}\) respectively. Unique sites are shown in bold. The EBI accession number of pIJ6902 is AJ937361.

Act and Red pathways respectively (Aceti and Champness, 1998). We sought to investigate the effects of CSRs on antibiotic biosynthetic pathways in bacteria devoid of AbsB-mediated ‘higher-level’ regulation; to this end, the chromosomally integrating P
\(_{\text{tipA}}\) expression vector, pIJ6902, was constructed (Fig. 1) and cdaR, actII-ORF4, redD and redZ were individually placed under control of the tipA promoter of this plasmid. These constructs or an ‘empty’ vector lacking a tipA promoter-regulated gene were then integrated site-specifically into the chromosomes of S. coelicolor hosts (absB\(^{-}\) J1501 and its absB missense mutant, C120). Similar quantities of spores of the C120 and J1501 derivatives (see Table 1, C121–125 and J151–J155) were spread onto modified rich R5 solid medium containing apramycin (to provide continued selection for the chromosomally inserted plasmid) and thiostrepton (to induce the tipA promoter). Mycelia from each strain were collected and weighed as a measurement of cell growth and/or proliferation, which were similar in C121–C125 and J151–J155 (Fig. S2). RNA harvested at various times (28–96 h) was used to prepare fluorescence-labelled cDNA for microarray analyses (Figs 2 and 3).

In order to assess the global effects of the absB mutation, including its effects on transcripts originating in the
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 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. *ramF*) and response to stress (e.g. the superoxide dismutase genes, *sodF*). 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 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristic(s)</th>
<th>Reference</th>
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<td></td>
<td></td>
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<tr>
<td>J1501</td>
<td>hisA1 uraA1 strA1 SCP1− SCP2− Pgi−</td>
<td>Kieser et al. (2000)</td>
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<tr>
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<td>J1501 absB120</td>
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<tr>
<td>C121</td>
<td>C120 tipAp::</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
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<tr>
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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 undecylandprodigiosin 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 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 Champaign, 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 undecylandprodigiosin (Fig. 2, panel 4) 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 undecylandprodigiosin 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 undecylandprodigiosin 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 hopenoid and coelichelin biosynthetic pathways (Fig. S1).
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 thiostrepton. 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, tipA::); 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, tipA::). 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, ΔredZ) 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 cluster (Fig. 4, panel C). Detection of Act was delayed for 16 h in M550 (ΔredZ) 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

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**Fig. 4.** Expression profiles of secondary metabolite loci genes in S. coelicolor M145, mutant M512 (ΔredD ΔactII-ORF4) and M550 (ΔredZ). RNA samples isolated from M145 (wild type), M512 (ΔredD ΔactII-ORF4) and M550 (ΔredZ) 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.
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 AfS-R2 (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* stains we examined (Fig. 5, panels 1-1 and 2-II). In *S. coelicolor* mycelium expressing redZ constitutively under control of the tipA promoter (J155, tipA::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) (Fig. 5 and Fig. S4), thus demonstrating unanticipated 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 (∆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 endonuclease, 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 tsp expression vector, pJ6902, and its derivatives*

A 0.625 kb fragment carrying a polyclinier downstream of the thiostrepton-inducible P tsp promoter, both ends flanked by transcriptional terminators, was amplified from the multicopy expression vector plp6021 (Takano et al., 1995) by PCR using Pfu DNA polymerase (Promega) and the primers (5′-GCCTCGTACACAAATAAAACGCCGGCGG-3′ and 5′-CGGGTATGACGACCGGCAAAGCGGCTTTGAC-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, plp6901, was confirmed. The tsp gene was amplified by PCR from plp6021 using Pfu DNA polymerase and the primers THIONHE (5′-GAATATGCTAGCAGGGCATAC TT-3′) and THIONHEREV (5′-AGCGCAT ATGAGATTCAACTTATTGGGACGTGTC; redD-R, CCGCACGGAACGCGCAGCACTCGCTGTGGCCGTC; redZ-R, CCGCACGGAACGCGCAGCACTCGCTGTGGCCGTC; and actII-ORF4-L, GCGCAT-ATGACGGGTGCTGCT). The PCR product was cloned into EcoRI-XbaI-cut pSET152 (Takano et al., 1995) by PCR amplification, RNA labelling and hybridization 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).

*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://sncohenlab.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× Klenow buffer, 6 µl 10× dNTP (4 mM dATP, 4 mM dTTP, 10 mM dGTP and 0.5mM 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 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 (10⁶ per plate) of strains C121–C125 and J151–J155 were plated onto R5-medium containing 10 mM CaCl₂, 50 µg ml⁻¹ apramycin and 50 µg 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 RNAeasy 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 RNAeasy® 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.
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 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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**References**


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://snohenlab.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 \( \sim 10^5 \) 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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