Angucyclines as signals modulate the behaviors of *Streptomyces coelicolor*

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The angucycline antibiotic jadomycin B (JdB) produced by *Streptomyces venezuelae* has been found here to induce complex survival responses in *Streptomyces coelicolor* at subinhibitory concentration. The receptor for JdB was identified as a "pseudo" gamma-butyrolactone receptor, ScbR2, which was shown to bind two previously unidentified target promoters, those of *redD* (*redD*p) and *adpA* (*adpA*p), thus directly regulating undecylprodigiosin (Red) production and morphological differentiation, respectively. Because AdpA also directly regulates the expression of *redD*, ScbR2, AdpA, and RedD together form a feed-forward loop controlling both differentiation and Red production phenotypes. Different signal strengths (i.e., JdB concentrations) were shown to induce the two different phenotypes by modulating the relative transcription levels of *adpA* vs. *redD*. The induction of morphological differentiation and endogenous antibiotic production by exogenous antibiotic exemplifies an important survival strategy more sophisticated than the induction of antibiotic resistance.

Antibiotics are well known and important for their ability to inhibit microbial growth by highly specific interactions with such key targets as ribosomes, cell-wall biosynthetic machinery, etc. However, even at concentrations too low to affect the growth of target organisms, exposure to antibiotics can bring about changes in global gene expression, as first visually displayed by a promoter–reporter fusion method (1). Such effects gave rise to the concept of antibiotics as signaling molecules (2–4). However, only in a few cases could the receptors for antibiotic signals be identified (5–8), and in most cases, the signaling mechanisms remain poorly understood. Many known antibiotics are produced by streptomycetes, soil bacteria that engage in active reciprocal interactions in natural communities (9). There is evidence to suggest that many of these interactions are mediated by antibiotics (4, 10, 11). Thus, antibiotics offer great tools to probe the responses of cellular and ecological networks at the systems level; antibiotic–cell interactions could be used as models to dissect the molecular mechanisms underlying cellular responses.

In our previous works, an important type of antibiotic receptors was defined and designated "pseudo" gamma-butyrolactone (GBL) receptors, due to the fact that they are homologous to GBL receptors, but bind antibiotics instead of GBL molecules (12). The pseudo GBL receptor, ScbR2—situated in the coelimycin gene cluster in *Streptomyces coelicolor*—was shown to bind two structurally distinct endogenous antibiotics, actinorhodin (Act) and undecylprodigiosin (Red). Thus, it was implicated in cross-talk between different antibiotic biosynthetic pathways (12, 13). In this study, we describe a previously unidentified interaction between an exogenous antibiotic, jadomycin B (JdB) and *S. coelicolor*. At subinhibitory concentrations, the *Streptomyces venezuelae* angucycline antibiotic JdB induces *S. coelicolor* to undergo premature differentiation (formation of sporulating aerial mycelium) and early production of the red-pigmented antibiotic Red. This effect involves the binding of JdB by ScbR2, thereby relieving ScbR2-mediated repression of important activators of differentiation and Red production. Other angucyclines also elicited similar phenotypes, suggesting that they also triggered this signal transduction system as signals.

Results

Discovery of JdB as an Antibiotic Signal, Inducing Complex Survival Responses in *S. coelicolor*. GBLs are growth cycle-related signaling molecules produced by streptomycetes (13, 14). Previously, a bioassay method based on the pigment production by *S. coelicolor* was developed as a convenient assay for some GBL molecules (15). When we applied *S. venezuelae* culture extracts harvested at different times to *S. coelicolor* indicator plates, several fractions (12, 24, 30, 36, and 42 h) were observed to induce pigment production (Fig. S1). The early (12-h) fraction could have contained a GBL molecule, but the later fractions were deduced to contain a substance different from GBL molecules, because GBL production should have stopped at this stage (13). Upon further fractionation on HPLC, the active molecule was identified as JdB, an atypical angucycline antibiotic produced by *S. venezuelae* (16). JdB is active against Gram-positive bacteria and human cancer cell lines (17, 18), but its cellular targets in bacteria and human cells are not known. To demonstrate the responses of *S. coelicolor* to JdB, a lawn of *S. coelicolor* mycelium grown on supplemented minimal medium (SMM) agar was spotted with JdB, and a pink zone surrounding the spot of antibiotic addition was observed (Fig. 1*A*). Interestingly, early aerial growth was observed internal to the pigmented zone (Fig. 1*A*). Pigment production is a specific response to JdB, because three other antibiotics—ampicillin, erythromycin, and kanamycin—showed growth inhibition but did not induce obvious phenotypes. The ability of subinhibitory concentrations of JdB (1–5 μM) to induce pink pigment production was also observed in liquid cultures (Fig. 1*B*), and when analyzed by HPLC, the pigment showed a retention time matching that of a Red standard (Fig. S2*A*). Mass spectrometry

Significance

This work addresses the molecular basis for interspecies signaling effects of antibiotics, which have been a controversial but potentially significant emerging topic over the last few years. The "pseudo" gamma-butyrolactone (GBL) receptor (i.e., those GBL receptor homologues often found in *Streptomyces* genomes, but apparently not binding or responding to GBLs), ScbR2, was identified as the receptor of JdB. It has an extraordinary ability to bind and respond to exogenous angucyclines, as well as to be able to directly regulate the biosynthesis of different endogenous antibiotics and the morphological development of *Streptomyces*. Our findings significantly extend understanding of antibiotic-mediated signaling mechanisms and the ecological impact of antibiotics.

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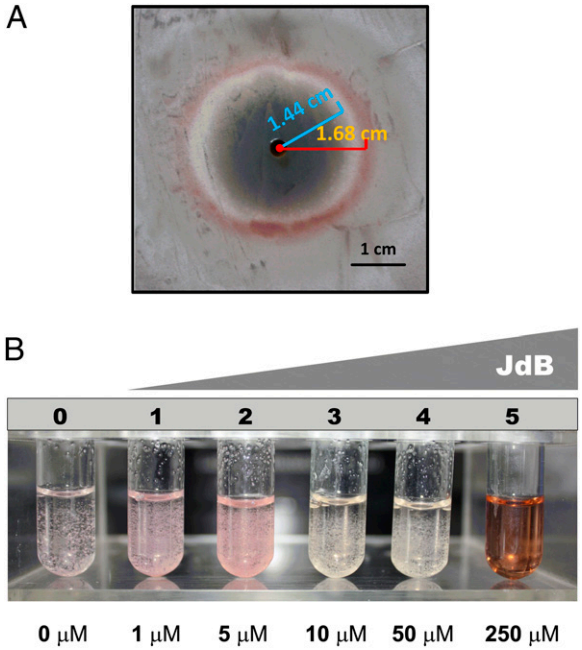

Fig. 1. The phenotypic responses of *S. coelicolor* M145 to JdB. (*A*) A plate assay of the response to JdB on SMM agar. JdB was dissolved in 100% DMSO, and 10 μL (10 mM) was placed in a well cut in SMM agar covering a lawn of *S. coelicolor*. The photograph was taken from the top of the plate. A red line shows the radius of the Red production zone; a blue line shows the radius of the aerial hyphal zone. (*B*) The responses of *S. coelicolor* M145 to increasing concentrations of JdB in liquid SMM, highlighting the production of a pink pigment at 1–5 μM JdB.

(MS) of the collected peaks confirmed that the pigment was indeed Red (Fig. S2*B*).

Identification of ScbR2 as the Receptor of JdB in *S. coelicolor*. To identify the receptor of JdB in *S. coelicolor*, we focused our attention on the pseudo GBL receptor, ScbR2. ScbR2 is a close homolog of JadR2 in *S. venezuelae*, which is known to recognize JdB as a ligand (12). We have previously reported that ScbR2 mediates cross-talk between different antibiotic biosynthetic pathways within *S. coelicolor*, because its interaction with target promoters is relieved by promiscuously binding to diverse pathway end products (12). The responses of ScbR2 to JdB and other antibiotics were analyzed by electrophoretic mobility shift assays (EMSAs) using two previously identified DNA targets of ScbR2 (P*kas*O and P*scbA*) that contained the promoter regions of *kasO* and *scbA*, respectively (12, 13). The ScbR2–DNA complexes were dissociated by JdB, but not by ampicillin, kanamycin, erythromycin, or tetracycline (Fig. 2*A*). To characterize the pattern of interaction between ScbR2 and JdB, the two molecules were purified and subjected to isothermal titration calorimetry (ITC) analysis, which measures the heat released or absorbed in the process of molecular interactions (19). The binding between ScbR2 and JdB was further verified, and the titration curve suggested that ScbR2 and JdB interact at a ratio near 1:1 (Fig. 2*B*). We then designed a reporter system in *Escherichia coli* to investigate whether JdB could relieve the repression of the *kasO* promoter (*kasO*p) by ScbR2 in vivo (Fig. 3*A*). The reporter system involved two plasmids: pScbR2, expressing ScbR2; and pO*kasO*lux, expressing the *lux* reporter genes under the control of *kasO*p. In the absence of JdB, ScbR2 repressed *kasO*p, thus turning off the expression of *lux* genes and bioluminescence (Fig. 3*B*). When JdB was added, bioluminescence was turned on in a dose-dependent manner (Fig. 3*B*). These results suggested that ScbR2 could sense and respond to JdB. The conclusion was supported by in vivo experiments in *S. coelicolor*, showing that the *scbR2* mutant (ΔscbR2) lost the JdB-dependent induction of pink pigment, whereas the parental strain (M145) and the complemented *scbR2* mutant (ΔscbR2::scbR2) both responded to JdB by producing pink zones (Fig. 3*C*).

Identification of Two Targets of ScbR2 in *S. coelicolor*. To identify the JdB signal-transduction pathways, we used phenotypes on plates as a guide. Because the most readily observed phenotype observed on plates is the production of Red (Fig. 1*A*), we first focused on the *red* gene cluster (20). By scanning the promoter regions in front of *redD*, *redQ, redV*, and *redZ* with ScbR2 using EMSAs, we identified the promoter of *redD* (*redD*p) as the only target of ScbR2 in the *red* cluster (Fig. 4*A*). The result is logical because RedD is known to be the transcriptional activator of the *red* biosynthetic genes (21).

In addition, based on the observed early aerial hyphal growth internal to Red production zones (Fig. 1*A*) and the fact that ScbR2 shows ∼31% identity to ArpA of *Streptomyces griseus*—which was known to bind the promoter region of *adpA* to repress its transcription (22)—we also tested the binding of ScbR2 to the promoter of *adpA* (*adpA*p), the product of which is the master regulator of aerial hyphal production in *S. coelicolor* (23–25). Remarkably, binding between ScbR2 and *adpA*p was observed (Fig. 4*B*).

To confirm these findings, we performed chromosome immunoprecipitation-quantitative PCR (ChIP-qPCR) experiments using a monoclonal antibody against ScbR2 to measure its binding with *redD*p and *adpA*p at different times in vivo. Samples were taken from 30-, 51-, and 60-h liquid cultures (in SMM). Compared with the control, higher relative amounts of *adpA*p and *redD*p were observed in samples immunoprecipitated at 30 and 51 h (Fig. 4*C*), confirming direct binding between ScbR2 and these target promoters at those time points in vivo. However, the binding disappeared at 60 h, suggesting a dynamic temporal binding of ScbR2 with these targets.

Characterization of the Interactions Between ScbR2 and its Targets in Response to JdB. Our results showed that ScbR2 could directly bind *adpA*p and *redD*p, in addition to two previously identified targets, the *scbA*/*R* intergenic promoter (*scbA*/*R*p) and *kasO*p (12, 26). To characterize the interactions between ScbR2 and these four targets in response to JdB, we examined the dissociation of ScbR2 from these promoters by EMSAs and observed a growing degree of dissociation from all four target promoters

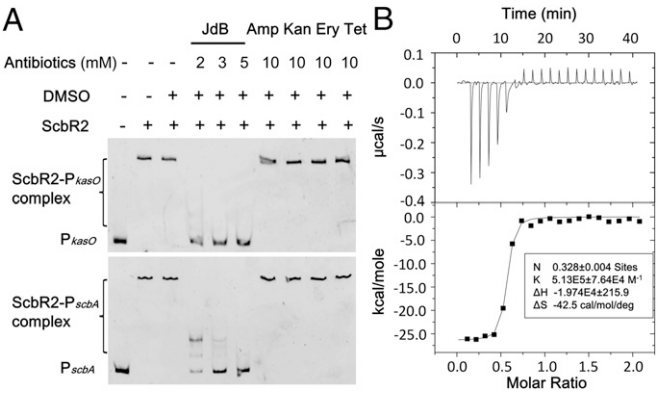

Fig. 2. Specific binding between the receptor ScbR2 and JdB in vitro. (*A*) Gel mobility shift assays of the DNA-binding activity of ScbR2 in the presence of various antibiotics. The assays were performed in 0.3 μM ScbR2, with 6.6 ng (0.13 nM) of P*scbA* or 6.6 ng (0.08 nM) of P*kasO* probes. Amp, ampicillin; Ery, erythromycin; Kan, kanamycin; Tet, tetracycline. (*B*) ITC analysis of the binding of ScbR2 to JdB. ScbR2 (100 μM) was injected into a solution of 20 μM JdB. (*Upper*) Heat changes measured as a function of time at 25 °C are shown. (*Lower*) Normalized heat changes (black square) and the best-fit curve (solid line) and calculated parameters are shown. (*Inset*) The estimated binding stoichiometry constant and enthalpy are shown.

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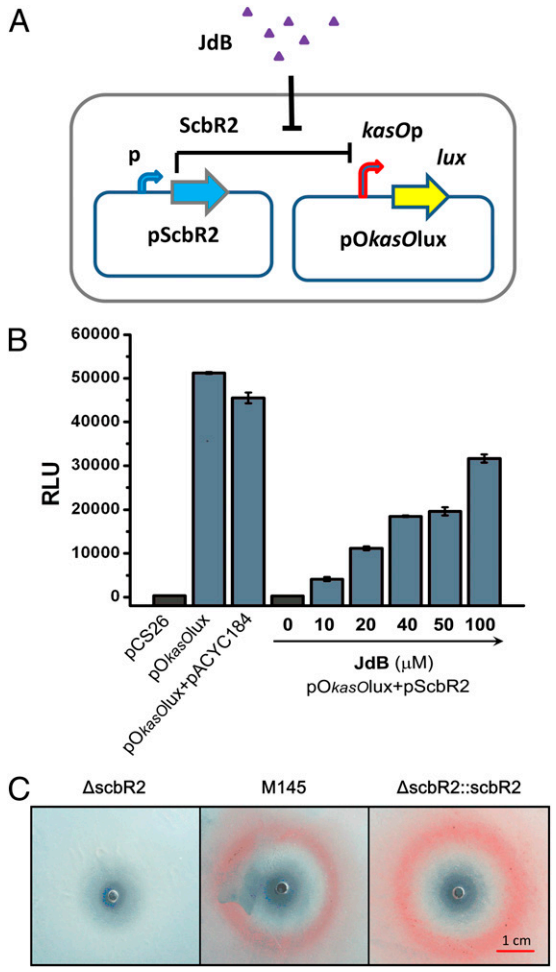

Fig. 3. Demonstration of the response of ScbR2 to JdB. (*A*) A schematic representation of the reporter system used to investigate the response of ScbR2 to JdB. (*B*) Investigation of the interactions of JdB with ScbR2 using a Lux reporter system in vivo. pCS26 contains a promoterless *lux* operon, and pACYC184 was used to express *scbR2*. They were used as controls. Relative light units (RLU) are represented as the average of at least three independent readings; error bars indicate ±SDs. (*C*) Plate assay of the responses of ΔscbR2 (*Left*), M145 (*Center*), and ΔscbR2::scbR2 (*Right*) to JdB on SMM agar. JdB was dissolved in DMSO, and 10 μL (10 mM) was spotted onto a lawn of *S. coelicolor*. The photographs were from the bottom of the plates.

in response to increasing concentrations of JdB (Fig. S3). As summarized in Fig. 5*A*, when the concentrations of ScbR2 and probes were kept constant, the sequence of dissociation is *redDP* > *adpAP* > *kasOP* > *scbA*/*R*p with increasing concentrations of JdB. From these results, we deduced that ScbR2 functions as a repressor of *redD*p and *adpA*p, with low concentrations of JdB being able to induce dissociation from *redD*p, leading to the production of Red; conversely, at higher JdB concentration, expression of the *adpA* regulon should be induced. These are exactly what we observed during earlier phenotype experiments on plates—i.e., the aerial hyphal zone showed a shorter radius than the Red pigment zone (Fig. 1*A*). To accurately determine the concentration range of JdB capable of inducing Red production, we repeated the JdB induction experiment in liquid SMM and carefully quantified Red production levels at different JdB concentrations. We again found that Red is only produced in the 1- to 5-μM JdB concentration range but is not produced at higher JdB concentrations (Fig. S4). To rule out the possibility that Red production was due to a lower growth rate, the growth of M145 at different JdB concentrations was also measured simultaneously: In the 1- to 10-μM range, JdB did not cause apparent growth inhibition (Fig. S5). Although the in vitro and in vivo results were basically consistent with the phenotypes of M145 in response to JdB, they did not explain the phenotype of ΔscbR2 and the lack of Red production by M145 at high concentrations (>5 μM) of JdB. If ScbR2 indeed functions as a repressor of both *redD*p and *adpA*p, its mutant ΔscbR2 would be expected to overproduce Red and to differentiate earlier. The ΔscbR2 mutant did show early aerial hyphae production and sporulation compared with M145 (Fig. S6*A*), but it did not overproduce Red; in fact, it completely lost the ability to produce Red. In contrast, the *scbR2*-overexpressing strain (M145::scbR2) lost the ability to differentiate on SMM plates (Fig. S6*A*).

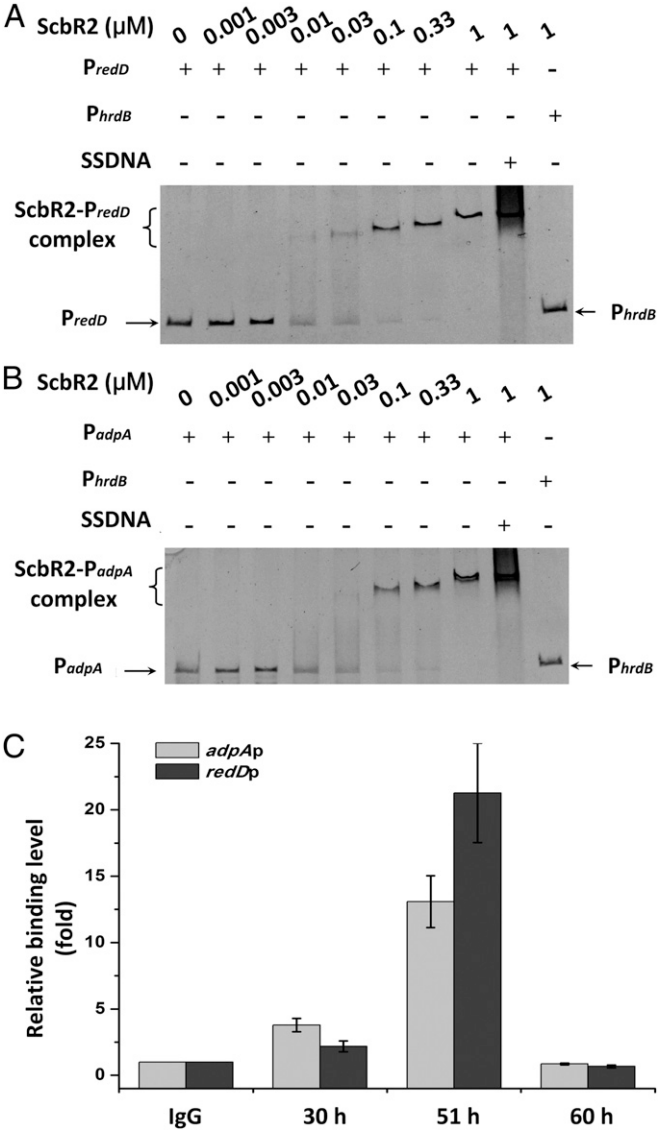

Fig. 4. Binding of ScbR2 with P*redD* and P*adpA* in vitro and in vivo. (*A* and *B*) Gel mobility shift assays of the binding of ScbR2 to P*redD* (*A*) and P*adpA* (*B*). The three probes (P*redD*, P*adpA*, and P*hrdB*) were used at a concentration of 0.12 nM in the assays. SSDNA, random fragments of salmon sperm DNA. P*hrdB* was used as negative control. (*C*) ChIP-qPCR assays in vivo. Anti-ScbR2 antibodies were used to immunoprecipitate ScbR2–DNA complexes from 30-, 51-, and 60-h cultures treated with formaldehyde. IgG-immunoprecipitated complexes were used as the negative control. The *y* axis represents the relative enrichment of P*redD* and P*adpA* compared with the control. The relative values are means ± SD from three independent experiments.

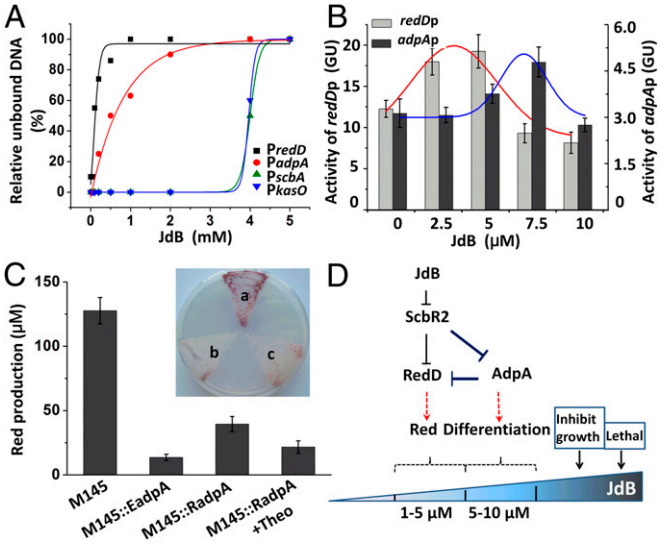

Fig. 5. The feed-forward loop controlling different behaviors of *S. coelicolor* M145 in respond to different concentrations of JdB. (*A*) Dissociation curves of ScbR2–DNA complexes in response to JdB. The concentrations of JdB used were 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 4, and 5 mM. Black squares, red closed circles, green triangles, and blue triangles represent the estimated relative unbound DNA of the P*redD*, P*adpA*, P*scbA*, and P*kasO* probes, respectively. (*B*) The activities of *redD*p and *adpA*p at different concentrations of JdB reported by the *gusA* reporter gene. Values are means and SDs from triplicate cultures. The trends of expression are fitted by Gaussian function above the columns of expression levels at different concentrations of JdB. (*C*) Red production of different *adpA*-overexpression strains. A concentration of 4 mM theophylline (Theo) was used to induce M145::RadpA. The values represent the average of at least three independent cultures plus SDs. (*Inset*) The Red production phenotypes of the *adpA*-overexpression strains on SMM plate: a, M145; b, M145::EadpA; c, M145::RadpA. (*D*) The signal transduction circuit controlling survival responses to JdB in *S. coelicolor* M145.

To understand why Red production is turned off at high JdB concentration, we designed an in vivo experiment to monitor the expression levels of *redD* and *adpA* in M145, ΔscbR2, and M145::scbR2 using the *gusA* reporter gene. Two reporter plasmids capable of monitoring *redD*p or *adpA*p were introduced into M145, ΔscbR2, and M145::scbR2, respectively, and the expression levels of *redD*p and *adpA*p in response to different JdB concentrations in liquid SMM were measured. As shown in Fig. 5*B*, the expression levels of *redD*p and *adpA*p (measured as GusA activities) displayed two different trends: Higher levels of *redD* expression were observed at a lower range of JdB concentrations (2.5–5 μM), but, in contrast, higher levels of *adpA* expression were detected at 7.5 μM JdB in the M145 transformants. Thus, interestingly, when the expression of *adpA* rose, the expression of *redD*p quickly declined (Fig. 5*B*). In contrast, in ΔscbR2, the expression of *redD* and *adpA* showed no response to JdB (Fig. S7), again indicating that the dynamic pattern of the expression of *redD* and *adpA* was controlled by ScbR2. Importantly, in ΔscbR2, the expression of *redD* constantly remained at a low level, whereas *adpA* was continuously expressed at a high level (Figs. S6*B* and S7), which are similar to the *adpA* and *redD* expression profiles of M145 transformants to 7.5 μM JdB. The low expression level of *redD* under these conditions provides a molecular explanation for the lack of Red production in ΔscbR2.

A Feed-Forward Loop Controlling Two Distinct Phenotypes. Based on previous reports, the pleiotropic regulatory protein AdpA could directly bind to and negatively regulate *redD*p expression (24, 27, 28). Overexpression of *adpA* reduced Red production, whereas disruption of *adpA* increased Red production (24, 27). The direct binding of AdpA with *redD* promoter was demonstrated by EMSA (28). To prove that the decline of *redD* expression level at 7.5 μM JdB is due to the induced overexpression of AdpA, strains in which *adpA* overexpressed at different levels were constructed. M145::EadpA contains an additional copy of *adpA* expressed from the constitutive promoter *ermE*p*, and M145::RadpA contains an additional copy of *adpA* expressed from a riboswitch-controlled ermEp1-E* promoter (29). As expected, Red production was severely reduced in the *adpA*-overexpressing strains both in liquid medium and on plate (Fig. 5*C*): The riboswitch-controlled *adpA* could be induced to overexpress by the corresponding signal (theophylline), and a reduction of Red production in response to this signal could be observed (Fig. 5*C*). These results from in vivo experiments, in which *adpA* levels were quantitatively adjusted, were fully consistent with *redD* expression being negatively modulated by AdpA. Together, our results reveal a clear mechanism by which different JdB concentrations could induce different phenotypes by controlling the relative levels of AdpA and RedD (Fig. 5*D*). This dynamic control is made possible by the differential dissociation of ScbR2 from the two targets: *adpA*p and *redD*p. When the concentration of external JdB is low (1–5 μM), JdB could only relieve the repression of ScbR2 on *redD*p so to trigger Red production; when JdB levels increase further (to ~7.5 μM), the transcription of *adpA* is de-repressed from ScbR2, thus turning on aerial hyphae differentiation, and at the same time turning off Red production due to the repression of *redD*p by AdpA.

Prevalence of Angucycline Signals. To find out whether angucyclines from different streptomycetes could function as signals and be sensed by ScbR2, the dissociation of ScbR2 from *scbA*/*R*p was evaluated. The experiment was first carried out in the presence of 2,3-dehydro-UWM6 (DHU; **2**) (Fig. 6*A*), a common intermediate in angucycline biosynthesis (30). DHU indeed inhibited the binding of ScbR2 to the target DNA (Fig. S8). We then tested the activity of a series of angucycline compounds to induce Red production in the plate assay: DHU; Jadomycin A (**4**) and Hse (**6**) from the jadomycin pathway; gaudimycin A (**8**) and C (**7**) from *Streptomyces* sp. PGA64 (31); and landomycin A (**10**) and D (**9**) found in *Streptomyces cyanogenus* S136 (32) (Fig. 6*A*). As shown in Fig. 6*B*, most compounds induced Red production, except **10** and **9**. The lack of signalling effects of **10** and **9** may be due to the long sugar chain attached to the landomycin core structure. ΔscbR2 lost such response, suggesting that these angucyclines were sensed by ScbR2.

Discussion

Angucyclines are the largest group of polycyclic aromatic polyketides identified in actinomycetes (30). Previously, they were mainly recognized for their diverse chemical scaffolds and biological activities. In this work, these molecules are discovered as agents capable of inducing complex survival phenotypes in a streptomycete. This finding implies they may act as signaling molecules in interspecies interactions.

The receptor of exogenous angucycline signals was firmly identified as ScbR2, which represents a recently characterized class of antibiotic signal receptors, widely distributed among streptomycetes (13). The fact that ScbR2 could recognize multiple structurally distinct antibiotics—including the endogenous antibiotics, Act and Red (12), and exogenous angucyclines—endows to it the property to integrate information from multiple signals; thus, ScbR2 could play important roles not only in intracellular communication but also in interspecies interaction and cellular adaptation to environmental signals. Hitherto, four targets (*adpA*, *redD*, *kasO*, and *scbA*/*R*) directly controlled by ScbR2 have been identified (Fig. S9). AdpA is the master regulator of differentiation and secondary metabolism that coordinates the expression of hundreds of genes involved in aerial hyphae production and sporulation (23–25). *scbA* and *scbR* (encoding GBL receptor and synthase, respectively) form a divergent gene pair controlling the temporal production of GBL signal in *S. coelicolor* (33). Both *redD* and *kasO* are cluster-situated regulatory genes,

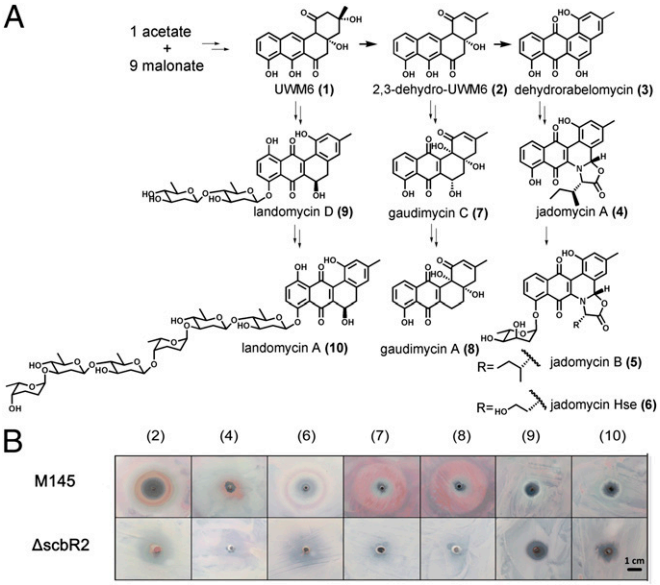

Fig. 6. A simplified biosynthetic route to representative angucyclines and the phenotypic responses of *S. coelicolor* to various angucyclines. (*A*) A simplified biosynthetic route to representative angucyclines; the key structures are numbered in brackets. (*B*) The phenotypic responses of *S. coelicolor* to various angucyclines on SMM plates. The angucyclines were dissolved in 100% DMSO, and 10 μL (10 mM) was spotted on a lawn of *S. coelicolor* grown on SMM.

controlling production of Red and the yellow-colored coelimycin, respectively (21, 26). All these genes governed by ScbR2 are key regulatory genes in the growth cycle of *S. coelicolor*, so ScbR2 is a pleiotropic regulator of many downstream genes. It is important to point out that the binding between ScbR2 and *adpA*p and *redD*p disappeared at 61 h during the stationary phase (Fig. 4*C*), probably because ScbR2 is released from these targets by the endogenous antibiotics, Act and Red, which are programmed to appear at the transition phase before the onset of differentiation (12). Therefore, these endogenous antibiotics may function as physiological signals to fine-tune the timing of cellular behaviors via ScbR2. These predictions underpin ScbR2 as one of the most important regulators of the growth cycle and as a key regulator of survival responses to antibiotic signals in *S. coelicolor*.

Understandably, the regulation of *adpA* is complex in *S. coelicolor* (34). It is repressed by its own product (27), as well as by ScbR2 and another pleiotropic regulator, BldD (35). In addition, *adpA* mRNA is a target of RNase III processing and contains a *bldA*-dependent UUA codon, so the level of AdpA is controlled by the abundance of bldA tRNA and mRNA stability (25, 36). Recent results showed that the expression of AdpA and *bldA* is highly mutually dependent. AdpA directly activates *bldA* expression, and the latter is needed for the functional translation of AdpA. Therefore, AdpA and *bldA* form a strong positive-feedback regulatory relationship that is biologically important in that it could trigger the irreversible onset of morphological differentiation and sporulation (37). AdpA also plays an important role in the regulation of secondary metabolism (34): There is evidence suggesting that it directly regulates both Red and Act production in *S. coelicolor* (24, 28). In this work, the finding that AdpA negatively regulates Red production when it is highly expressed is biologically relevant because it is important to shut down antibiotic production once the cells have made the commitment to differentiate, which will allow the allocation of limited resources to aerial growth. Because there is also ample evidence to suggest that AdpA could trigger antibiotic production (34), it is possible that AdpA plays dual roles in the regulation of endogenous antibiotic production—i.e., acting both as activators and repressors on the same target genes at different expression levels (38). A high level of AdpA kept *redD* at a low level, despite the fact that AdpA directly activates *bldA* expression, which is required for the translation of *redZ*, and RedZ is the activator of *redD* expression (39). Thus, in theory, a high level of AdpA should indirectly up-regulate *redD* expression via *bldA* and *redZ*; however, due to the fact that AdpA also directly regulates *redD*, the combined outcome is that a high level of AdpA will override the activation by RedZ and thus shut down *redD* expression.

ScbR2, AdpA, and RedD form an incoherent type II feed-forward loop (FFL) (40) controlling two distinct physiological behaviors (morphological differentiation and Red production) of M145 in response to an exogenous JdB signal (Fig. 5*D*). It is important to point out that, within this FFL, RedD is the bottom-level regulator regulated by both ScbR2 and AdpA, so the expression of *redD* is controlled by the dynamic levels of ScbR2 and AdpA. By such a design, the regulatory loop has gained the property of responding to signal concentrations. Thus, two distinct physiological behaviors (aerial hyphae differentiation and Red production) of M145 in response to exogenous JdB signal have been explained mechanistically at molecular levels. It is important to note that this FFL is part of a network consisting of two top-level regulators—ScbR and ScbR2—and three lower-level regulators—AdpA, KasO, and RedD—which also control their own regulons (Fig. S9). Based on this network, we could predict the behavior of *S. coelicolor* at higher (inhibitory) concentrations of JdB. At these concentrations, the *scbR/A* and *kasO* should be induced to express, so that GBL and coelimycin should be produced. Therefore, a concentration gradient of antibiotic could induce a series of different responses; such complex responses are highly relevant to the survival and effective competition of streptomycetes. These findings represent a significant advance in the understanding of the ecological and physiological consequences of antibiotic production.

Materials and Methods

Bacterial Strains and Growth Conditions. Bacterial strains used in this study are listed in Table S1. *S. coelicolor* strains M145, ΔscbR2 (scbR2DM), M145::scbR2 (scbR2OE), and ΔscbR2::scbR2 (scbR2COM) were handled as described (12). *S. venezuelae* ISP5230 was grown in glucose–Mops (GM) medium (13). SMM agar was used to observe the phenotypic responses of *S. coelicolor* M145, ΔscbR2, M145::scbR2, and ΔscbR2::scbR2 to angucyclines. The effect of JdB to *S. coelicolor* was also tested in liquid SMM (41). The growth rates of *S. coelicolor* M145 were measured by the diphenylamine method (42). *E. coli* strains were grown in Luria–Bertani containing ampicillin (100 μg/mL), kanamycin (50 μg/mL), or chloramphenicol (50 μg/mL) when necessary.

Preparations of *S. venezuelae* ISP5230 Culture Extracts and Bioassay. Cultures of *S. venezuelae* ISP5230 grown in GM medium for different times were harvested and extracted by ethyl acetate. The extracts were dried, dissolved in DMSO, and then used in bioassay on a lawn of *S. coelicolor* M145. The plate indicator bioassay for GBL molecules was carried out as described by Takano et al. (15). JdB was isolated and purified from *S. venezuelae* ISP5230 cultures as described (43).

Liquid Culture Analysis of the Effects of JdB on *S. coelicolor* M145 and Measurement of Red Production. *S. coelicolor* M145 was grown in liquid SMM. Replicate cultures were inoculated with increasing amounts of JdB, and the cultures were incubated at 30 °C at 220 rpm for 48 h before observation.

The mycelia were harvested, treated, and used to measure Red production by HPLC; the peak corresponding to Red was analyzed by LC-MS. JdB and other angucyclines samples were purified to show a single peak on HPLC. Details of sample preparation, conditions to perform HPLC, and HPLC-MS are presented in *SI Materials and Methods*.

Gel Mobility Shift Assays (EMSAs). His_6-tagged ScbR2 was purified from *E. coli* BL21 (DE3) harboring pET23b::*scbR2*, as described (12). To identify target binding sites of ScbR2, the promoter regions of *adpA*, *kasO*, *scbA*, *redD*, *redQ*, *redV*, *redZ*, and *hrdB* were obtained by PCR from the genomic DNA of

S. coelicolor M145 using the primers listed in Table S2. The subsequent binding experiments were performed by using a modified gel mobility shift assay as described (12) (*SI Materials and Methods*).

Bioluminescence Detection in *E. coli* and β-Glucuronidase Activity Assay in *S. coelicolor*. The plasmids used are listed in Table S1, and the primers are listed in Table S2. The plasmid p*OkasO*plux containing *kasO*p controlled *lux* reporter genes, and ScbR2 expression vector pScbR2 were previously constructed in our laboratory (12). The bioluminescence of *E. coli* reporter cultures was measured after 12-h incubation by using a single-tube luminometer (Turner Biosystems 20/20n). The construction of *gusA* reporter plasmids and strains and β-glucuronidase activity assay in *S. coelicolor* are described in *SI Materials and Methods*.

ITC. Protein concentration was determined spectrophotometrically at 280 nm by using a molar extinction coefficient (ε280). Both ScbR2 and JdB were dissolved in the same buffer [5 mM NaCl, 2 mM Tris·HCl, pH 7.0, 1 mM DTT, 5% (vol/vol) glycerol]. The titration was initiated with a first injection of 2 μL of ScbR2, followed by 19 injections of 4 μL at 25 °C. The instrument software was used to calculate the normalized heats released from each injection.

ChIP-qPCR. The ChIP protocol was modified from Johnson et al. (44). Details are presented in *SI Materials and Methods*.

Promoter Dissociation Assays and Calculation of Dissociation Curves. By using the above-described EMSA conditions, small molecules were added at various concentrations. The relative abundance of bound probes was measured by grayscale. The dissociation curves of ScbR2–DNA complexes in respond to JdB were fitted by the software Origin (Version 8.0).

Construction of AdpA Overexpression Strains. The construction of different AdpA overexpression plasmids and strains is presented in *SI Materials and Methods*. The effects on Red production were observed in liquid SMM, and the levels of Red production were measured by HPLC.

Preparations of Jadomycin Pathway Intermediates and Other Angucyclines. Details are presented in *SI Materials and Methods*.

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