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*jadR** and *jadR2* act synergistically to repress jadomycin biosynthesis

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The biosynthesis of antibiotics is controlled by cascade regulation involving cluster-situated regulators (CSRs) and pleiotropic regulators. Three CSRs have been identified in the jadomycin biosynthetic gene cluster, including one OmpR-type activator (JadR1) and two TetR-like repressors (JadR* and JadR2). To examine their interactions in jadomycin biosynthesis, a series of mutants were generated and tested for jadomycin production. We noticed that jadomycin production in the *jadR*-jadR2* double mutant was increased dramatically compared with either single mutant. Transcriptional analysis showed that *jadR** and *jadR2* act synergistically to repress jadomycin production by inhibiting the transcription of *jadR1*. Furthermore, *jadR** and *jadR2* reciprocally inhibit each other. The complex interactions among these three CSRs may provide clues for the activation of the jadomycin gene cluster, which would otherwise remain silent without stimulation from stress signals.

***jadR**, *jadR2*, jadomycin biosynthesis, cluster-situated regulators (CSRs)**

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Streptomycetes are well-known for their ability to produce a wide variety of antibiotics and other bioactive compounds. The biosynthesis of antibiotics is subject to hierarchical regulatory control, which involves cluster-situated regulators (CSRs) and pleiotropic regulators [1]. In contrast to pleiotropic regulators, CSRs can directly activate or repress the transcription of neighboring regulatory or biosynthetic genes within a gene cluster.

In most cases, at least one CSR can be found within a specific antibiotic gene cluster. In the model actinomycete *Streptomyces coelicolor*, ActII-ORF4, a member of the *Streptomyces* antibiotic regulatory proteins (SARPs), is the only CSR required for the transcription of all five transcriptional units in the actinorhodin biosynthetic gene cluster [2]. Another member of the SARPs, SanG, was found to be the only activator of nikkomycin biosynthesis in *Streptomyces ansochromogenes* [3]. Multiple CSRs have also been reported within some antibiotic gene clusters. Two CSRs (PolY and PolR) were identified in the polyoxin biosynthetic gene cluster in *Streptomyces cacaoi* subsp. *asoensis* [4,5]. PolR is the direct activator of the polyoxin biosynthetic genes [5], while *polR* itself is activated by PolY, the product of the adjacent gene [4]. Daunorubicin (DNR) biosynthesis in *Streptomyces peucetius* is controlled by three CSRs (DnrO, DnrN and DnrI) [6]. DnrO, a TetR-like transcriptional regulator, activates *dnrN*, which is transcribed in the opposite direction to *dnrO*. DnrN, an atypical response regulator (ARR), initiates transcription of *dnrI*, the SARP product of which turns on DNR biosynthesis [6]. Five CSRs (TylP, TylQ, TylS, TylU and TylR) were characterized in the tylosin biosynthetic gene cluster in *Streptomyces fradiae* [7], and a circuit model of complex regulation was proposed [1,7].

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Jadomycins, a group of polyketide-derived angucycline antibiotics, are produced by *Streptomyces venezuelae* ISP5230 upon induction by heat shock, ethanol stress or phage infection [8,9]. Jadomycins possess broad-spectrum antimicrobial and tumor suppressive activities [10–12]. The *jad* gene cluster includes three transcriptional regulatory genes (*jadR1*, *jadR2* and *jadR**) and the determinants for putative γ-butyrolactone signal biosynthesis (*jadW1*, *jadW2* and *jadW3*), indicating that a complex regulatory system exists in jadomycin biosynthesis. JadR1 is an OmpR-type ARR that activates jadomycin biosynthesis by activating the first biosynthetic gene of the cluster, *jadJ* [13]. JadR1 also acts as a repressor of its own gene at the initial time [13]. JadR2 has a TetR-like N-terminal helix-turn-helix domain and belongs to a subfamily of "Pseudo" γ-butyrolactone (GBL) receptors. It represses jadomycin biosynthesis by directly inhibiting the transcription of *jadR1* [14]. *jadR** encodes a TetR family regulator and was also identified as a repressor of jadomycin biosynthesis (unpublished data). Though their roles in jadomycin biosynthesis have been investigated extensively, nothing is known about the interactions among these three CSRs. To address this issue, we constructed a series of mutants and evaluated jadomycin production in different mutant backgrounds. In addition, we performed transcriptional analysis of the three CSRs in different mutants. The results showed that *jadR** and *jadR2* can synergistically repress the transcription of *jadR1* to repress the biosynthesis of jadomycin in *S. venezuelae* ISP5230.

1 Materials and methods

1.1 Bacterial strains and growth conditions

S. venezuelae ISP5230 (wild-type strain, WT), Δ*jadR** (*jadR** disruption mutant), and their derivatives were grown on maltose-yeast extract-malt extract (MYM) agar medium [15]. The culture conditions for the production of jadomycin B (JdB) were as described previously [16]. *E. coli* DH5α was used as a host strain for propagating plasmids. *E. coli* ET12567 (pUZ8002) was used for transferring DNA from *E. coli* to *Streptomyces* by conjugation [13].

1.2 DNA manipulation

pBluescript KS(+) was used for routine DNA cloning. The *E. coli-Streptomyces* shuttle plasmid pKC1139, which contains a temperature-sensitive origin of replication from pSG5 [17], was used to construct recombinant plasmids for gene disruption. Isolation of plasmids and genomic DNA were performed according to standard procedures [18]. Conjugal transfer of recombinant plasmids from *E. coli* ET12567 (pUZ8002) into *S. venezuelae* was carried out as described previously [18].

1.3 Construction of mutants

All mutants were generated via double-crossover homologous recombination. For the construction of the *jadR1* mutant, two fragments corresponding to approximately 1.5 kb of the upstream and downstream sequences of *jadR1* were generated by PCR using the primer pairs R1D-LF/R and R1D-RF/R. The upstream fragment was digested with *Hin*d III and *Xba* I, the downstream fragment was digested with *Eco*R V and *Xba* I, and then the two fragments were inserted into *Hin*d III/*Eco*R V double digested pKC1139 to generate pKC1139::Δ*jadR1*. The recombinant plasmid was introduced into *E. coli* ET12567 (pUZ8002) and transferred into *S. venezuelae* ISP5230 via conjugation to give the *jadR1* disruption mutant (Δ*jadR1*). A similar procedure was used to construct pKC1139::Δ*jadR2* and the *jadR2* disruption mutant (Δ*jadR2*). For the construction of double disruption mutants, the recombinant plasmids pKC1139::Δ*jadR1* and pKC1139::Δ*jadR2* were transferred into Δ*jadR** via conjugation to obtain *jadR*-jadR1* (Δ*jadR*R1*) and *jadR*-jadR2* (Δ*jadR*R2*) disruption mutants, respectively.

1.4 HPLC analysis of JdB

HPLC conditions for the detection of JdB were the same as described previously [14].

1.5 RNA isolation and real-time RT-PCR

RNAs were isolated from *S. venezuelae* grown at 28°C at different time points (24, 48, and 72 h) in the absence of ethanol stress; The detailed steps for RNA extraction were described previously [13,14]. To exclude the possibility of genomic DNA contamination, each RNA sample was treated with DNase I (Promega, Wisconsin, USA). The quality and quantity of RNAs were examined by UV spectroscopy and agarose gel electrophoresis.

For quantitative real time RT-PCR, first-strand cDNA synthesis was carried out with the Superscript III first-strand Synthesis System (Invitrogen, California, USA) using 500 ng total RNAs following the manufacturer's instructions. All cDNA synthesis reactions included a replicate reaction without reverse transcriptase to ensure the complete removal of contaminating DNA from the RNA samples. Oligonucleotides were designed to amplify fragments of 60–150 bp (Table 1). The PCR procedures were as follows: reactions were performed in 72-well plates using a Rotor-Gene Q (Qiagen, Hilden, Germany). Each 20 μL reaction contained 10 μL of 2×SuperReal PreMix (SYBR Green I included), 6 pmol of each primer and 1 μL 5-fold diluted cDNA. The reaction parameters were as follows: 95°C for 10 min, followed by 40 three-step amplification cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 20 s and extension at 72°C for 30 s. A final dissociation stage

Table 1 Primers used in this study[a)]

Primer	Sequence
For disruption	Protein200
R1D-LF	CCCAAGCTTCGGCTACATCGCGACGGAC
R1D-LR	GCTCTAGACGTGGGGCAGGTATGTGGG
R2D-LF	GCTCTAGAGAGGCGGACCGTCGACACCCAT
R2D-LR	GATATCTCGGCGGCGTGAACTCGGT
For real-time RT-PCR analysis	
real-R*F	ATCCGGGGCAACGTCGAG
real-R*R	GCGGCCATGGAGTACAGGG
real-R1F	CGACACCCATGTGAGCAGCC
real-R1R	GCCGAAGCGGAAACCCAC
real-JF	CACACGCGGTGGATCGAGAC
real-JR	CGGCAGGGAGACCTCAAGACG
real-R2F	CCTGCTCGCCTCGGACAT
real-R2R	CAGCCATTCGCCGTTGTC
real-hrdBF	CTCATCGAGCGGGGAAAGG
real-hrdBR	CCACTGGGTTGGCGGAATC
For EMSA	
PjadR*F	CGCGAGCCGAAGGTCGTGTACA
PjadR*R	GGGACGGTGCTGCCGATGATGA
PjadR2F	CGTAGCCGTGCTCGTCGAATTCTC
PjadR2R	GACCACGGGCACTCTTGACCACTTC
For overexpression of JadR* and JadR2 in *E. coli*	
R*EPF	CATATGGCCACCAGGAAGTACGAACAGC
R*EPR	CTCGAGGCGGCAGAGGGCGTGTTCG
R2EPF	ACATATGACCAAACAAGAGCGGGCCAC
R2EPR	ACTCGAGGGCGACCGACGTGTACGCCC
For confirmation of disrupted mutants	
conR*F	TCTCCGCCGTGGTCTGGGTG
conR*R	TGCCGCAAGCTGGAGGAACT
conR1F	ATGTCGCCCCAAGAAACGGAC
conR1R	CGCAAGCAGCGAGTCCCACG
conR2F	CTTCGTTCAAGACTCACCGATTCAGGG
conR2R	GGCGACCGACGTGTACGCCC

a) Underlined sequences indicate restriction enzyme recognition sites.

was run to generate a melting curve and consequently verify the specificity of the amplification products. After the PCR amplifications, the data were analyzed with the Rotor-Gene Q Series software. All samples were run in triplicate. The transcriptional level of target genes was normalized internally to the level to *hrdB* transcription according to Livak's method [19].

1.6 Over-expression and purification of JadR* and JadR2

*jadR** and *jadR2* were amplified by PCR using *S. venezuelae* ISP5230 genomic DNA as a template. The primers used were as follows: R*EPF and R*EPR for *jadR**, and R2EPF and R2EPR for *jadR2* (Table 1). The amplified fragments were digested with *Nde* I and *Xho* I and inserted into the same sites of pET-23b to generate pET23b::*jadR** and pET23b::*jadR2*, respectively. The recombinant plasmids were confirmed by DNA sequencing and then introduced into *E. coli* BL21 (DE3) for protein over-expression. Purification and concentration of proteins were performed as described previously [20].

1.7 Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as described previously [13]. The promoter regions of *jadR** and *jadR2* were obtained by PCR from genomic DNA of *S. venezuelae* ISP5230 with the primer pairs PjadR*F/PjadR*R and PjadR2F/PjadR2R (Table 1). Prior to PCR amplification, PjadR*R and PjadR2R were radiolabeled at the 5′-ends with [γ-P^{32}]-ATP and T4 polynucleotide kinase.

2 Results

2.1 Organization of *jadR1*, *jadR2* and *jadR** in the *jad* gene cluster

Three CSRs (*jadR1*, *jadR2* and *jadR**) were identified in the *jad* gene cluster (Figure 1). Both *jadR1* and *jadR2* are located on the left side of the gene cluster. *jadR1* is located immediately upstream of *jadJ*, the first biosynthetic gene of the cluster. *jadR2* is located further upstream and is transcribed in the opposite direction to *jadR1*. *jadR** is situated on the right side of the gene cluster adjacent to *jadY*.

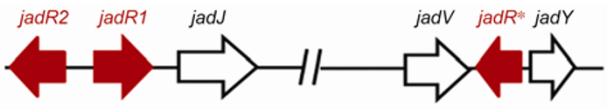

Figure 1 Organization of *jadR1*, *jadR2* and *jadR** in the *jad* cluster. The three CSRs are indicated with red arrows and the neighboring biosynthetic genes are indicated with black arrows.

2.2 Enhancement of jadomycin production in the *jadR*-jadR2* double mutant

A previous study showed that disruption of *jadR2* led to production of JdB independent of ethanol stress [14], and a similar finding was observed in the *jadR** disruption mutant (unpublished data). Because both JadR2 and JadR* are repressors of jadomycin biosynthesis, we were interested to know whether there was an interaction between these two CSRs. For this purpose, disruptions of *jadR1*, *jadR2*, *jadR*-jadR1* and *jadR*-jadR2* were taken place (Figure 2A), and subsequently the resulting disruption mutants (Δ*jadR1*, Δ*jadR2*, Δ*jadR*R1* and Δ*jadR*R2*) were obtaind and further verified by PCR (Figure 2B–D). Δ*jadR2*, Δ*jadR**, Δ*jadR*R2* and WT strains were fermented at different time points (24, 48 and 72 h) and tested for jadomycin production (Figure 3). In comparison with Δ*jadR** and Δ*jadR2*, production of JdB in Δ*jadR*R2* was increased dramatically at the three different time points. This result indicated that there was indeed an interaction between these two repressors.

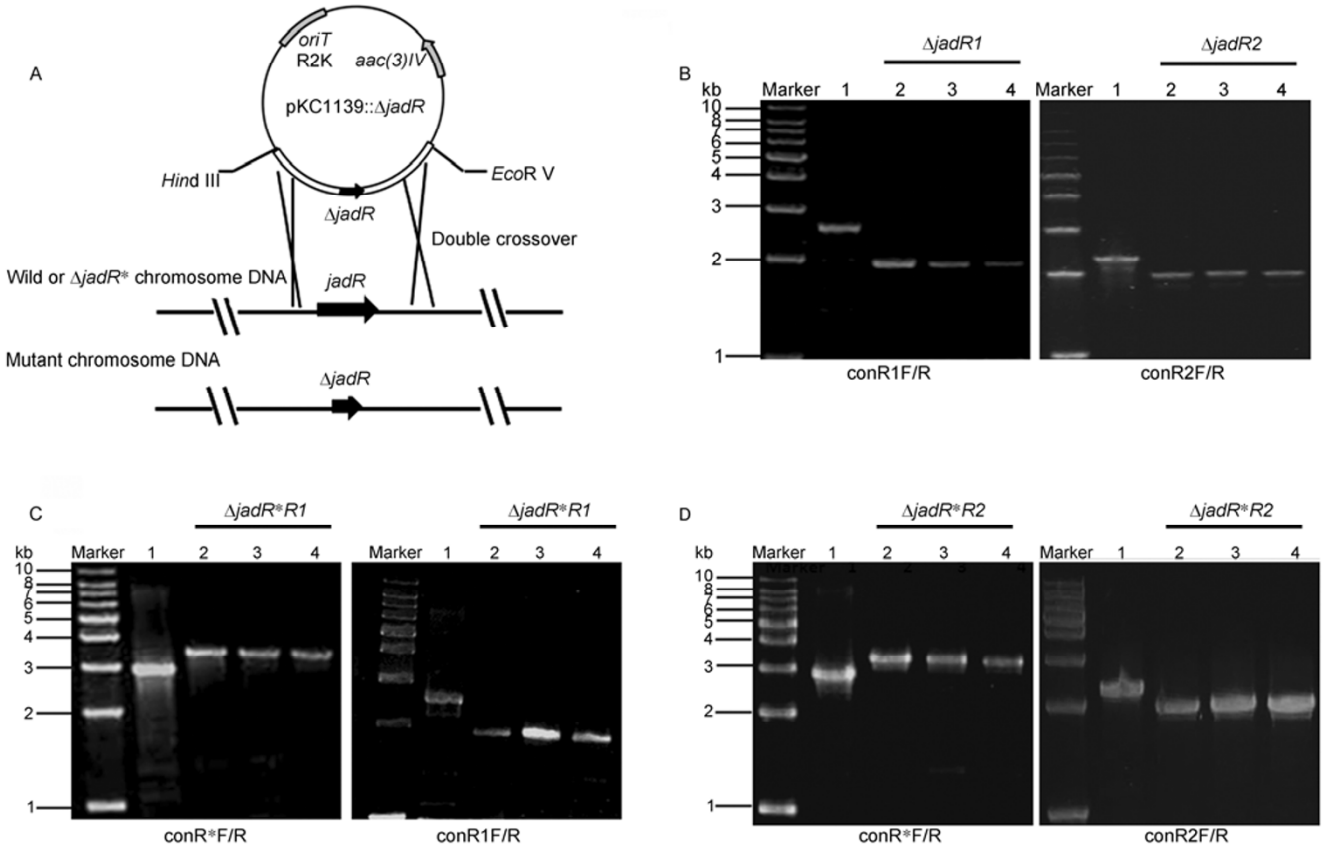

Figure 2 Disruption of *jadR1* or *jadR2* in WT *S. venezuelae* ISP5230 and Δ*jadR**. A, Diagram showing *jadR1* or *jadR2* disruption in the WT and Δ*jadR** disruption mutant via double crossover. *jadR*: *jadR1* or *jadR2*. B–D, Confirmation of *jadR1*, *jadR2* and *jadR** disruption by PCR amplification. PCR templates were genomic DNAs from the WT (lane 1) and the three independent mutants as indicated (lanes 2–4). The primer pairs used are also shown.

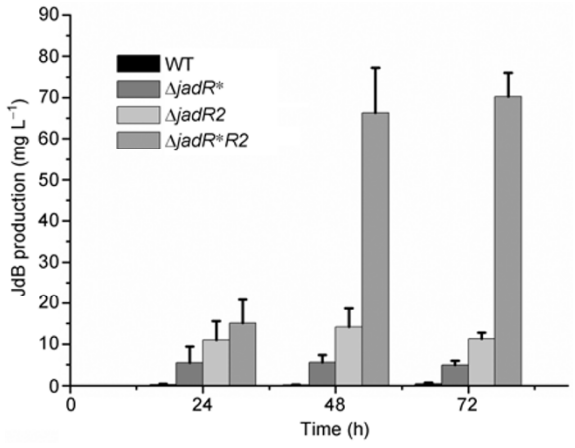

Figure 3 Effects of *jadR** and *jadR2* disruption on JdB biosynthesis. JdB production from different strains was evaluated by HPLC after organic extraction of the entire culture. Data are presented as the averages of the results of three independent experiments. Error bars show standard deviations.

2.3 Transcriptional analysis of *jadR1* and *jadJ*

JadR2 was shown to repress jadomycin production via direct repression of *jadR1* [14], and *jadR1* is also one of the binding targets of JadR* (unpublished data). To evaluate the effects of JadR2 and JadR* on *jadR1* transcription, quantitative real-time RT-PCR analysis was carried out. The results showed that the *jadR1* transcripts in Δ*jadR*R2* were higher than in Δ*jadR** or Δ*jadR2* at all tested time points (Figure 4A). Considering that JadR1 represses its own gene expression, the transcription of *jadR1* in Δ*jadR1* and Δ*jadR*R1* strains was analyzed, results showed that *jadR1* transcripts in Δ*jadR*R1* were also increased obviously in comparison with Δ*jadR** and Δ*jadR1* strains at all tested time points (Figure 4A). JadR1 was shown to activate jadomycin biosynthesis by activating *jadJ* [13]. The transcriptional level of *jadJ* was also analyzed by quantitative real-time RT-PCR (Figure 4B), and it showed a similar profile as that of *jadR1* in the Δ*jadR**, Δ*jadR2* and Δ*jadR*R2* strains, while its abundance was kept at low levels in the Δ*jadR1* and Δ*jadR*R1* strains. These results further confirmed that JadR1 is a key activator of *jadJ* and that JadR1, JadR2 and JadR* synergistically repress *jadR1*.

2.4 Interactions among *jadR1*, *jadR2* and *jadR**

To further examine the interactions among these three CSRs,

the transcriptions of *jadR** and *jadR2* were examined in different disruption mutants. The transcriptional level of *jadR** increased significantly in the Δ*jadR2*, Δ*jadR**, Δ*jadR*R1* and Δ*jadR*R2* strains compared with the WT strain (Figure 5A). JadR2 bound to the promoter region of *jadR** to form protein-DNA complexes in a concentration-dependent manner (Figure 5B), implying that JadR2 might exert direct repression on *jadR** transcription. The transcriptional level of *jadR2* increased obviously in Δ*jadR2*, Δ*jadR** and Δ*jadR*R2* (Figure 5C). Remarkably, the abun-

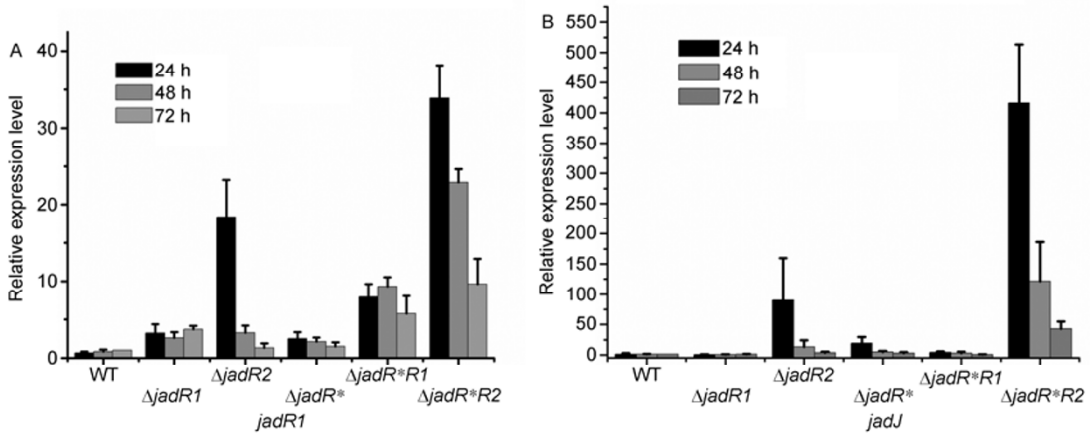

Figure 4 Effects of *jadR** and *jadR2* on *jadR1* and *jadJ* transcription. A, Transcriptional analysis of *jadR1* (A) and *jadJ* (B) in the WT, Δ*jadR1*, Δ*jadR*2, Δ*jadR**, Δ*jadR*R1* and Δ*jadR*R2*. The expression levels of *jadR1* and *jadJ* are presented relative to the wild type sample collected after incubation for 72 h, which was arbitrarily assigned a value of 1. Data are presented as the averages of the results of three independent experiments conducted in triplicate. The *hrdB* transcription was assayed as an internal control. Error bars show standard deviations.

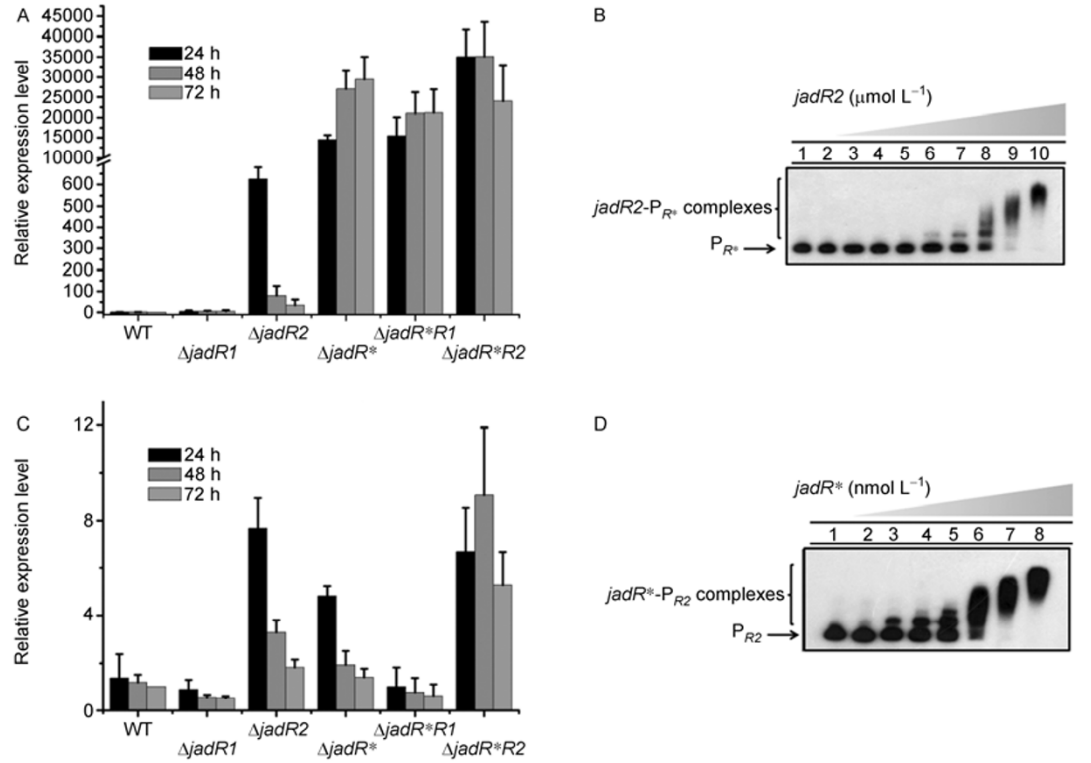

Figure 5 Interactions among *jadR**, *jadR2* and *jadR1*. A, Transcriptional analysis of *jadR**. B, EMSA of JadR2 with γ-P^{32} labeled P_{R*}. Each lane contains about 2 ng of labeled probe. Lanes 1–6 contain 0, 0.017, 0.0425, 0.085, 0.17, 0.425, 0.85, 1.7, 4.25 and 8.5 μmol L^{-1} purified JadR2, respectively. C, Transcriptional analysis of *jadR2* in the WT, Δ*jadR1*, Δ*jadR2*, Δ*jadR**, Δ*jadR*R1* and Δ*jadR*R2* strains. D, EMSA of JadR* with γ-P^{32} labeled P_{R2}. Each lane contains about 6 ng of labeled probe. Lanes 1–6 contain 0, 10, 20, 40, 80, 160, 320 and 640 nmol L^{-1} purified JadR*, respectively. Data are presented as the averages of the results of three independent experiments conducted in triplicate. The *hrdB* transcription was assayed as an internal control. Error bars show standard deviations.

dance of *jadR2* transcript in Δ*jadR*R2* was even higher than in Δ*jadR** and Δ*jadR2* at later time points (mRNAs isolated from samples incubated for 48 and 72 h), demonstrating the cooperative repression of *jadR** and *jadR2*. EMSA indicated binding of JadR* to the promoter region of *jadR2* (Figure 5D), suggesting that direct repression of *jadR2* by JadR* was taking place. In contrast, the *jadR2* transcripts in the Δ*jadR1* and Δ*jadR*R1* strains were lower than in the WT strain, suggesting that JadR1 can also activate the transcription of *jadR2*.

3 Discussion

The onset and level of production of antibiotics is determined by many physiological and environmental factors. These factors normally converge on CSRs to control the timing and level of antibiotic production [1]. Typically, one or multiple CSRs can be found within a gene cluster. The presence of multiple CSRs may reflect complex regulation in antibiotic biosynthesis. In the model organism *S. coelicolor*, two repressors (ScbR and ScbR2) and one activator (CpkO) were identified in the *cpk* cluster required for production of a polyketide-derived antibiotic, cryptic polyketide (CPK) [14,21]. CpkO is the direct activator for CPK biosynthesis, while ScbR and ScbR2 can repress the transcription of *cpkO*. ScbR, an A-factor receptor protein (ArpA) homologue, can sense increasing levels of the γ-butyrolactone signaling molecule (SCB1) and relieve *cpkO* repression [21]. In contrast to ScbR, ScbR2 is a "pseudo" GBL receptor and is not able to recognize the endogenous SCB1. However, it can bind the endogenous antibiotics actinorhodin and undecylprodigiosin as ligands, leading to derepression of *cpkO* [14]. Likewise, the methylenomycin (MM) biosynthetic genes are regulated by a cascade involving two ArpA-like repressors (MmyR and MmfR) and one activator (MmyB). The repressive activities of MmyR and MmfR on MmyB are thought to be modulated by small autoregulator molecules (methylenomycin furans, MMFs), and MMF biosynthesis is also controlled by MmyR and MmfR [22]. Another well-studied regulatory circuit involving CSRs is the regulation of tylosin production in the non-model organism *S. fradiae* [7]. This gene cluster encodes five CSRs, including two repressors, TylP and TylQ, and three activators, TylS, TylU and TylR. TylR is the direct activator of the tylosin biosynthetic gene cluster. The expression of *tylR* requires the combined action of two SARP activators (TylS and TylU) and two ArpA-like repressors (TylP and TylQ) [7].

Control of jadomycin production in *S. venezuelae* is a complex process involving three CSRs (JadR1, JadR2 and JadR*). JadR1 is the direct activator of the jadomycin biosynthetic gene cluster [13]. Like ScbR in CPK biosynthesis, JadR2 in *S. venezuelae* can bind two endogenous antibiotics (chloramphenicol and jadomycin) and these interactions lead to derepression of *jadR1* [14]. JadR* was recently identified as another repressor of *jadR1* (unpublished data). In addition, the *jad* gene cluster contains three putative γ-butyrolactone biosynthetic genes (*jadW1*, *jadW2* and *jadW3*). Though it has not been identified yet, the biosynthesis of this putative γ-butyrolactone is regulated by JadR2 [23]. The identification of this small molecule and its targets in jadomycin biosynthesis will be extensively investigated.

It is conceivable that the presence of multiple CSRs simplifies the interactions among them. Both JadR2 and JadR* were identified as repressors of *jadR1*. In this study, we first examined whether there is an interaction between these two repressors. For this purpose, a *jadR*-jadR2* double mutant was generated. Evaluation of jadomycin production and transcriptional analysis indicated that JadR2 and JadR* can repress *jadR1* transcription in a synergistic fashion. Similar interactions were also found in the regulation of asukamycin production in *S. nodosus* subsp. *asukaensis*, in which four CSRs exert a synergistic activation on asukamycin production [24]. Furthermore, JadR2 and JadR* have a repressive effect on each other by directly binding to each other's promoter (Figure 5B and D). By generating more double mutants, we also found synergistic repression of *jadR1* transcription by JadR* and JadR1 (Figure 4A). Activation of *jadR2* by JadR1 was also indicated by lower *jadR2* transcript levels in Δ*jadR1* and Δ*jadR*R1* than in the WT (Figure 5B). However, in a previous study no direct binding of JadR1 to the promoter of *jadR2* was observed [14]. We speculate that the activation of *jadR2* by JadR1 was indirect.

The close interactions among CSRs not only provide possibilities for activation or enhancement of antibiotic production, but also demonstrate the adaptability of *Streptomyces* to complex and variable environmental changes.

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