

Characterization of *rrdA*, a TetR Family Protein Gene Involved in the Regulation of Secondary Metabolism in *Streptomyces coelicolor*[∇]

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Streptomyces not only exhibits complex morphological differentiation but also produces a plethora of secondary metabolites, particularly antibiotics. To improve our general understanding of the complex network of undecylprodigiosin (Red) biosynthesis regulation, we used an in vivo transposition system to identify novel regulators that influence Red production in *Streptomyces coelicolor* M145. Using this screening system, we obtained 25 Red-deficient mutants. Twenty-four of these mutants had a transposon inserted in the previously described Red biosynthetic gene cluster and produced different amounts of another secondary metabolite, actinorhodin (Act). One mutant was shown to have an insertion in a different region of the chromosome upstream of the previously uncharacterized gene *rrdA* (regulator of *redD*, *sco1104*), which encodes a putative TetR family transcription factor. Compared with wild-type strain M145, the *rrdA* null mutant exhibited increased Red production and decreased Act production. A high level of *rrdA* expression resulted in a severe reduction in Red production and Act overproduction. Reverse transcription-PCR analysis showed that RrdA negatively regulated Red production by controlling *redD* mRNA abundance, while no change was observed at the transcript level of the Act-specific activator gene, *actIII-orf4*. The effects on Act biosynthesis might arise from competition for precursors that are common to both pathways.

In addition to its complex morphological differentiation, the gram-positive genus *Streptomyces* is notable for its ability to produce a wide range of secondary metabolites during its life cycle. These metabolites include the majority of known pharmaceutically important secondary metabolites that exhibit antibacterial, anticancer, and immunosuppressive activities (6, 7). The genes responsible for the biosynthesis of secondary metabolites are often physically clustered in the genome and coordinately regulated by pathway-specific transcriptional activators (1, 4, 10, 12, 29, 33). These specific regulators are controlled by various higher-level pleiotropic regulators, and their expression is typically affected by a variety of environmental and physiological cues, including the nature and levels of carbon and nitrogen sources and the availability of phosphate and small signaling molecules, such as ppGpp and γ -butyrolactone (5).

Streptomyces coelicolor A3 (2) has been used for many years as a model organism in morphological and physiological differentiation studies, particularly in studies of the regulation of antibiotic biosynthesis (7). *S. coelicolor* produces four antibiotics: actinorhodin (Act), undecylprodigiosin (Red), methyl-enomycin, and calcium-dependent antibiotic. It has been shown that certain regulators are involved in the pleiotropic control of antibiotic production in *S. coelicolor*, including AbsA1/A2, AfsR/K, AtrA, and PhoR/P (18, 24, 31, 32). Re-

cently, mutational analysis and adventitious overexpression of key regulators in *S. coelicolor* revealed cross-regulation at the transcriptional level among disparate antibiotic biosynthetic pathways (15). The diversity of these regulatory elements suggests that the regulation of antibiotic production is a complicated process, and many genes remain to be identified.

Red is one of the prodiginine secondary metabolites, and this group of compounds is attracting increasing interest due to its immunosuppressive and anticancer activities (34). In this study, we used an insertion mutagenesis system to identify new genes that regulate Red biosynthesis in *S. coelicolor* M145. We discovered a novel TetR-like transcription factor gene, *rrdA* (regulator of *redD*, *sco1104*), that was involved in the regulation of secondary metabolism. In this study, we also report a link between Red production and Act production that probably occurs at the level of the metabolites utilized that are common to both pathways in *S. coelicolor*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5 α (22) was used for plasmid propagation. *E. coli* Rosetta-gami (Novagen) was used to express recombinant proteins. Mannitol soy flour (MS) (16) agar was used to generate spores and select *Streptomyces* exconjugants. YBP agar (2 g yeast extract, 2 g beef extract, 4 g Bacto peptone, 1 g MgSO₄, 15 g NaCl, 15 g agar, and 10 g glucose in 1 liter [final volume] H₂O) was used for phenotype screening and RNA preparation. YBP liquid medium was used for quantitative antibiotic assays. YEME (16) was used to cultivate mycelia for genomic DNA preparation. Conjugation of *E. coli* ET12567/pUZ8002 with *Streptomyces* was performed as described previously (16). Depending on the requirements, antibiotics were added at the following final concentrations: ampicillin, 50 μ g ml⁻¹; chloramphenicol, 33 μ g ml⁻¹; kanamycin, 30 μ g ml⁻¹; tetracycline, 12.5 μ g ml⁻¹; thio-strepton, 25 μ g ml⁻¹; and apramycin, 20 μ g ml⁻¹.

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description	Reference or source
<i>S. coelicolor</i> strains		
M145	Prototroph SCP1 ⁻ SCP2 ⁻	16
$\Delta rrdA$	M145 with <i>rrdA</i> gene disrupted	This study
$\Delta rrdA/pFDZ16$	$\Delta rrdA$ carrying integrative plasmid pFDZ16	This study
$\Delta rrdA/pFDZ16^*-rrdA$	$\Delta rrdA$ carrying integrative plasmid pFDZ16 [*] -rrdA	This study
WT/pFDZ16	M145 carrying integrative plasmid pFDZ16	This study
WT/pFDZ16-rrdA	M145 carrying integrative plasmid pFDZ16-rrdA	This study
<i>E. coli</i> strains		
DH5 α	F ⁻ <i>recA lacZ</i> Δ M15	22
ET12567	<i>dam dcm hsdS</i>	16
Rosetta-gami	$\Delta ara-leu7697 \Delta lacX74 \Delta phoA$ (PvuII) <i>phoR araD139 ahpC galE galK rpsL</i> F ⁻ [<i>lac</i> ⁺ (<i>lacI</i> ^q) <i>pro</i>] <i>gor522::Tn10</i> (Tc ^r) <i>trxB::Kan</i> (DE3)/pRARE (Cm ^r)	Novagen
Plasmids		
pFDZ315 (Tn315)	Conjugative plasmid that does not replicate in <i>Streptomyces</i> strains harboring transposon Tn315, which uses the transposase of insertion element IS204 as a <i>trans</i> factor and contains a kanamycin resistance gene, origin of pUC plasmids, and <i>ermE</i> promoter in the terminal inverted repeats; Kan ^r Amp ^{su}	X. Zhang et al., unpublished
pFDZ15	<i>E. coli-Streptomyces</i> integrative shuttle vector; vector pRT802 was cut with SmaI and EcoRV, resulting in a 3.8-kb segment that was subsequently inserted into the NaeI site of pBluescript II KS+ (Stratagene), yielding pFDZ15; Kan ^r Amp ^{su}	This study
pFDZ16	<i>E. coli-Streptomyces</i> integrative shuttle vector containing <i>tipA</i> promoter; the 5.4-kb ApaI/BclI fragment of pFDZ15 was ligated with the 5.0-kb BamHI/ApaI segment of pJ6021 to obtain pFDZ16; Kan ^r Thio ^r Amp ^{su}	This study
pFDZ16 [*] -rrdA	Derivative obtained from pFDZ16 depletion of <i>P_{tipA}</i> , containing the <i>rrdA</i> gene and its promoter; Kan ^r Amp ^r Thio ^r	This study
pFDZ16-rrdA	Derivative obtained from pFDZ16, containing the <i>rrdA</i> gene located downstream of the <i>tipA</i> promoter; Kan ^r Amp ^r Thio ^r	This study
pUCm-T	2.7-kb cloning vector; Amp ^r	Shenggong Company
pBC-AM	Donor of <i>aac(3)-IV</i> ; the 1.5-kb HindIII/EcoRI fragment of pULVK2A was inserted between the corresponding sites in vector pBC-SK(-) (Stratagene) to produce pBC-AM; Apra ^r Cm ^{td}	This study
pUC-LR	DNA fragment containing the <i>rrdA</i> gene, cloned in pUCm-T; Amp ^r	This study
pUC-LAR	DNA fragment <i>aac(3)-IV</i> containing apramycin resistance gene, cloned in pUC-LR; Amp ^r	This study
pHZ1358	<i>E. coli-Streptomyces</i> shuttle vector; Thio ^r Apra ^r	26
pHZ1358-LAR	Derivative obtained from pHZ1358, containing the <i>rrdA</i> gene replacement cassette; Amp ^r Thio ^r Apra ^r	This study
pET28a	Expression vector; Kan ^r	Novagen
pET28-RrdA	RrdA overexpression vector resulting in an N-terminal His ₆ oligopeptide fusion; Kan ^r	This study
pET22a	Expression vector; Amp ^r	Novagen
pET22-RrdA	RrdA overexpression vector resulting in an N-terminal His ₆ oligopeptide fusion; Amp ^r Cm ^r Tc ^r Kan ^r	This study
Primers		
Oxj130	5' ATCCATGGCGCATATGTCGCCGCGCAGCGCC 3'	
Oxj131	5' ATAAGCTTACCCGCGCAAGCGGTCC 3'	
Oxj144	5' TCTAGACCGCCGTTCTGTTGACTT 3'	
Oxj145	5' GGATCCACATCACCGTCTGCCCTC 3'	
Oxj161	5' TCTGCCCTCTGACCGCGG 3'	
Oxj162	5' GCCGTCCTCGCGGTTCT 3'	
Oxj163	5' CTGGCTCCTGGGCGGTCT 3'	
Oxj164	5' ACCAACCCTGGCGTCTCC 3'	
Oxj185	5' CGCCGAAGGAGGAACCGA 3'	
Oxj186	5' CCCGTCATCCACCGAACG 3'	
Oxj187	5' CCCAGGCGCTGGTAGACCT 3'	
Oxj188	5' CCGTCGAGCCGAAAGAGGA 3'	
Oxj201	5' CCGGAGCCAGCCAAAGATC 3'	
Oxj202	5' GGAGGGCGTTGAGGACGTT 3'	
Oxj203	5' TGCTGACCAAGCCGAGAA 3'	
Oxj204	5' CGGTGTACGTGGGACCTGAC 3'	
Oxj205	5' TGGTGCTGCTGCTCCTCAG 3'	
Oxj206	5' ATCCAGTCCCGCGTCAA 3'	
Oxj211	5' GCGCCTCGGTCAATGAAGA 3'	
Oxj212x	5' CGCTTTCGGGGAAAGTAGTAC 3'	
Oxj237	5' CTCTGTCATGGCGCTATTGA 3'	
Oxj238	5' TTCGCTCGACGCTCTTT 3'	

^a See reference 35.

^b See reference 11.

^c See reference 30.

^d See reference 17.

Mutagenesis of *S. coelicolor* M145. Insertional mutagenesis of M145 was conducted by *in vivo* transposition using Tn315 (Table 1) (X. Zhang, Y. Bao, X. Ou, P. Zhou, G. Zhao, and X. Ding, unpublished data) harbored by pFDZ315, which is a conjugative plasmid that does not replicate in *Streptomyces*. Tn315, which was derived from insertion element IS204 of *Nocardia asteroides* YP21 (35), contains a kanamycin resistance gene, the replication origin of pUC plasmids, and an *ermE* promoter in the terminal inverted

repeats. After plasmid pFDZ315 was introduced into *S. coelicolor* M145 by conjugation, a single copy of transposon Tn315 was inserted into the chromosome, and exconjugants were selected by growth on MS media flooded with kanamycin (30 μ g ml⁻¹). The chromosomal locations of the Tn315 insertions were determined by sequencing the transposon-flanking DNA by plasmid rescue. Briefly, genomic DNA from the mutants was isolated and digested with ApaI, a site for which is not present in Tn315. The DNA was

then self-ligated and transformed in *E. coli* DH5 α using a standard protocol (16). Colonies were selected for kanamycin resistance, and the rescued plasmids were sequenced.

Gene disruption, complementation, and overexpression. Targeted gene replacement mediated by homologous recombination was used to generate an *rrdA* null mutant. A 2.2-kb DNA fragment containing the *rrdA* gene was amplified by PCR using primers OXj144 and OXj145 (Table 1) and cloned into pUCm-T (Shenggong Company, Shanghai, China) by T/A cloning to form pUC-LR. The 1.5-kb *Sma*I fragment containing the *aac(3)-IV* gene from plasmid pBC-AM (Table 1) was subsequently inserted into EcoNI-linearized pUC-LR, and the *rrdA* gene was separated into 1.1-kb left and 1.1-kb right arms for homologous recombination to produce pUC-LAR. pUC-LAR was further cut with *Xba*I/*Sca*I to generate the 3.7-kb gene replacement cassette LAR. LAR was then inserted between the corresponding sites in the *Streptomyces-E. coli* shuttle vector pHZ1358 (26), which is a very unstable vector in streptomycetes, yielding the inactivation construct pHZ1358-LAR. This construct was introduced by conjugation from the donor *E. coli* ET12567/pUZ8002 into the recipient *S. coelicolor* M145 (16). The Apra^r Thio^s double-crossover colonies were screened as possible mutant candidates in which the *rrdA* gene was disrupted by *aac(3)-IV* through homologous recombination. The mutants were confirmed by PCR using primers OXj163 and OXj164 (Table 1), which are located outside the homologous recombination regions, and this was followed by DNA sequencing.

The DNA fragment encompassing the complete *rrdA* gene and its possible promoter was amplified by PCR using primers OXj144 and OXj145. The product was cut with *Sac*I/*Xho*I to produce a 1.4-kb fragment that was then inserted between the corresponding sites in vector pFDZ16 (Table 1), which is a *Streptomyces-E. coli* single-copy integration shuttle vector. This resulted in pFDZ16**rrdA*. This plasmid was subsequently conjugated with the *rrdA* null mutant for genetic complementation from the donor *E. coli* ET12567/pUZ8002. Exconjugants were selected by growth on MS media flooded with thiostrepton (25 μ g ml⁻¹).

The *rrdA* gene was amplified by PCR using primers OXj130 and OXj131 (Table 1), cut with *Nde*I/*Hind*III, and inserted between the corresponding sites in vector pFDZ16, which is a *Streptomyces-E. coli* single-copy integration shuttle vector carrying the *tipA* promoter. This resulted in pFDZ16-*rrdA*. This plasmid was then conjugated from the donor *E. coli* ET12567/pUZ8002 into wild-type strain M145 to overexpress *rrdA*. The exconjugants were selected by growth on MS media flooded with thiostrepton (25 μ g ml⁻¹).

Quantification of antibiotics. Act and Red were assayed as previously described (16). Briefly, a culture grown in 40 ml YBP liquid medium was filtered, and the supernatant and pellet were separated. For Act, KOH was added to the supernatant to a final concentration of 1 M, and the optical density at 640 nm was determined. For Red, the mycelial pellet was dried under a vacuum and extracted with 10 ml methanol (adjusted to pH 2) overnight at room temperature. The optical density at 530 nm was then determined. Measurements were always obtained for three independent cultures.

RT-PCR analysis. Mycelia grown on cellophane disks in YBP medium at 30°C for different time periods were scraped, and the RNA was isolated using a modified Kirby mixture. The isolated RNA was subjected to phenol-chloroform extraction and DNase I treatment, as described previously (16). Reverse transcription (RT) was performed with a high-fidelity RNA PCR kit (Takara, Japan) used according to the manufacturer's instructions. The primers used for RT-PCR are shown in Table 1. The following primers were used: for *redZ*, OXj203 and OXj204; for *redD*, OXj201 and OXj202; for *actII-orf4*, OXj205 and OXj206; for *hrdB*, OXj237 and OXj238; and for *rrdA*, OXj211 and OXj212x. The PCR conditions were as follows: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The numbers of PCR cycles used were 27 for *redZ*, 29 for *redD*, and 26 for *actII-orf4*, *rrdA*, and *hrdB*. Two independent cultures were used for each condition, and the results were found to be consistent. RNA that was not reverse transcribed was used as a control, and the results obtained with this RNA were negative.

Production and purification of recombinant RrdA and electrophoretic mobility shift assays. A DNA fragment encoding the predicted 233-amino-acid sequence of RrdA was generated using primers OXj130 and OXj131. The PCR fragment was cut with *Nde*I and *Hind*III and inserted between the corresponding sites in the expression vector pET28a (Novagen) to generate pET28-RrdA. pET28-RrdA was cut with *Xba*I/*Hind*III to produce an 855-bp segment, and this segment was inserted between the corresponding sites in pET22a (Novagen) to obtain the final RrdA expression vector pET22-RrdA. The recombinant RrdA protein was tagged at the N terminus with a His₆ oligopeptide and was purified on a Ni-nitrilotriacetic acid spin column used according to the vendor's instructions (Qiagen). Electrophoretic mobility shift assays were performed using the method of Uguru et al. (31). DNA and protein were mixed to obtain a final volume of 20 μ l in TGEK buffer (10 mM Tris-Cl [pH 7.9], 10% [vol/vol] glycerol, 0.1 mM EDTA, 50 mM KCl) at 30°C for 20 min. Samples were run on native 5%

acrylamide-bisacrylamide (80:1) gels at 120 V for 1 h and subsequently visualized. The primer pairs used to generate the various *redD* DNA probes were OXj161/OXj162 (402 bp), OXj185/OXj186 (408 bp), and OXj187/OXj188 (410 bp).

RESULTS AND DISCUSSION

Mutagenesis of *S. coelicolor* to identify genes that affect Red production. The genes involved in Red production reside in a single cluster (*sco5877* [*redD*] to *sco5899* [*redE*]) that is organized into four transcriptional units (*redDXWY*, *redZ*, *redQRSTUV*, and *redPONMLKJIHGFE*) (Fig. 1A) (34). Production begins with the synthesis of 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC) and 2-undecyl-pyrrole, and condensation of the monopyrrole with MBC leads to the formation of the tripyrrole pigments of Red (Fig. 1B).

We used an in vivo transposition system to generate a new collection of mutants that were defective in Red production (see Materials and Methods for details). We succeeded in obtaining 25 insertion mutants that did not produce red-pigmented antibiotics. In each of these mutants, we sequenced the DNA flanking the transposon Tn315 and compared the sequences with the complete *S. coelicolor* genome (<http://strepdb.streptomyces.org.uk>) by BLAST analysis. This led to identification of genes that were disrupted or influenced. Of the 25 mutations, 24 were localized in 11 genes and two gene spacers within the Red biosynthetic gene cluster (Fig. 1A), while only 1 insertion mutation in K74 was outside this cluster. The phenotypes of 15 representative insertion mutants are shown in Fig. 1C and 1D. Compared with wild-type strain M145, insertion mutants K29, K69, K89, K90, K3, K72, K93, K17, K35, R19, and R26 (with mutations in the *redX*, *redW*, *redV*, *redU*, *redI*, and *redH* genes) showed no apparent change in blue-pigmented antibiotics, while mutants K68, K77, R7, K88, R11, R6, K78, R46, K1, K2, K7, K15, and K25 (with mutations in *redD*, the *redQ-redP* gene spacer, *redO*, *redN*, the *redN-redM* gene spacer, and *redL*) produced large amounts of blue-pigmented Act but no Red (Fig. 1C and 1D). The genes that had little effect on Act production (*redX*, *redW*, *redV*, *redU*, *redI*, and *redH*) encoded proteins that synthesize MBC or condense MBC and 2-undecyl-pyrrole into Red; however, these genes do not participate in the biosynthesis of 2-undecyl-pyrrole. Of the genes that significantly influence Act production, *redD* encodes the direct regulator that activates the transcription of Red biosynthetic genes, while *redP*, *redQ*, and *redL* encode proteins that participate in the biosynthesis of 2-undecyl-pyrrole. The *redO*, *redN*, and *redM* genes are located upstream of *redL* but are in the same transcriptional unit (Fig. 1A).

Analysis of mutant K74 led to identification of *rrdA* (*sco1104*), a *tetR* family gene. The insertion in mutant K74 was located 40 bp upstream of the translation initiation codon of *rrdA* (*sco1104*) (Fig. 2A). Since the IS204-based transposon Tn315 contains an internal *ermE* promoter, it is likely that the loss of Red production is due to a high level of *rrdA* expression rather than a polar effect caused by Tn315 integration in the K74 mutant. The predicted 233-amino-acid protein encoded by the *rrdA* gene, which is not clustered with either the Red biosynthesis or Act biosynthesis genes (it is separated from the Red biosynthesis and Act biosynthesis genes by approximately 5,270 kb and 4,360 kb, respectively) exhibited sequence simi-

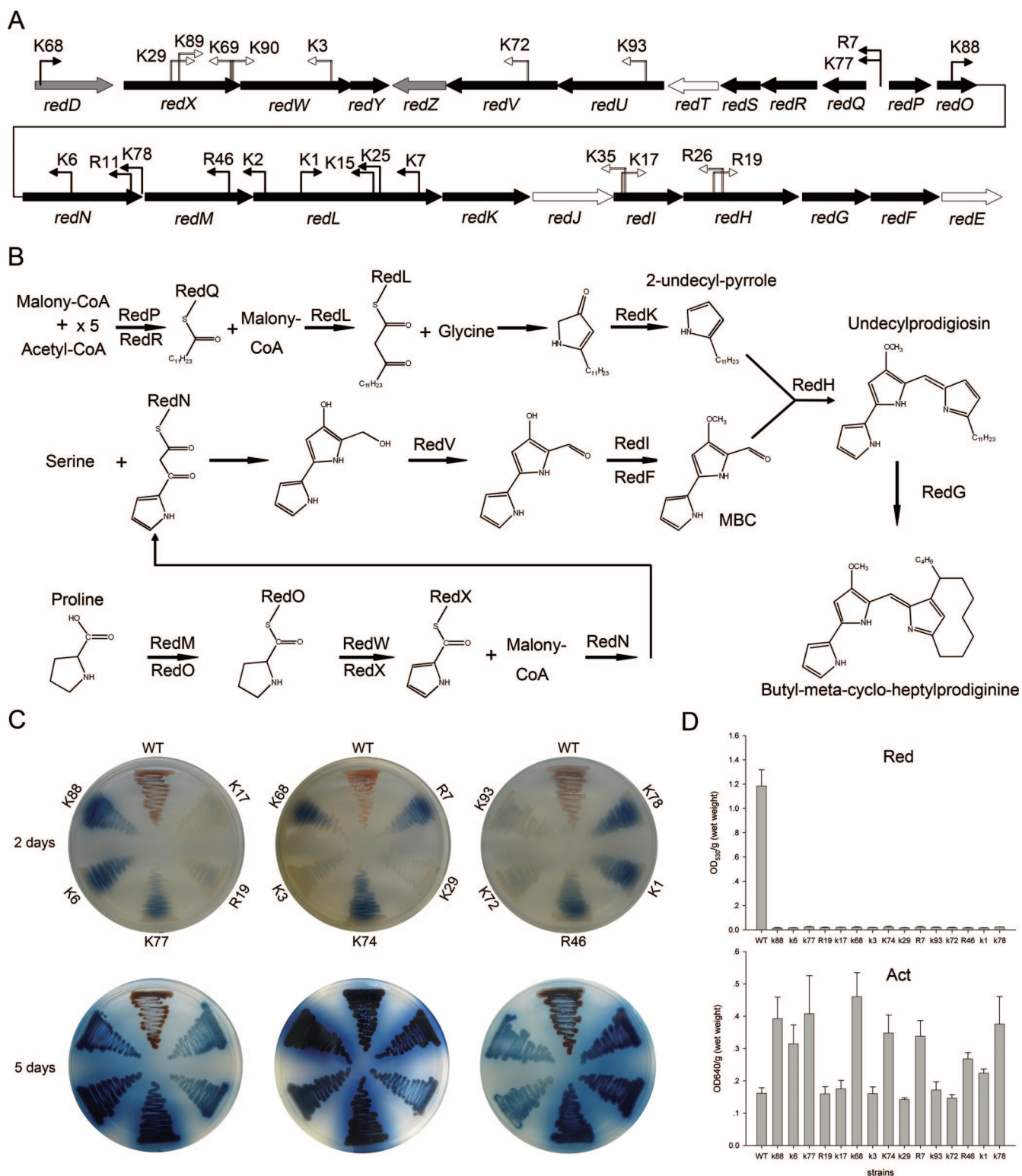


FIG. 1. Phenotypes of Red-deficient mutants and locations of the insertions. (A) Locations of the mutations in the Red biosynthetic gene cluster. The black arrows indicate genes that are required for Red biosynthesis, while the open arrows indicate genes that are not essential. The gray arrows indicate the *redD* and *redZ* regulatory genes. The bent open arrows indicate mutations that influence only Red production, while the filled bent arrows indicate mutations that affect both Red production and Act production. The directions of the bent arrows indicate the transcript orientation of *P_{ermE}* in Tn315 in the mutants. (B) Red biosynthetic pathway. RedU is required for activation of the acyl carrier protein domains of RedO. The function of RedY is unknown (34). (C) Mutants and wild-type strain M145 were grown on YBP agar at 30°C for 2 days and 5 days. The bottoms of the plates are shown. WT, wild type. (D) Antibiotic production assay. Act production and Red production by the mutants and wild-type strain M145 were assayed in cultures grown in YBP liquid medium at 30°C for 88 h. The bars indicate the averages of three independent determinations, and the error bars indicate the standard errors. OD₅₃₀, optical density at 530 nm; OD₆₄₀, optical density at 640 nm.

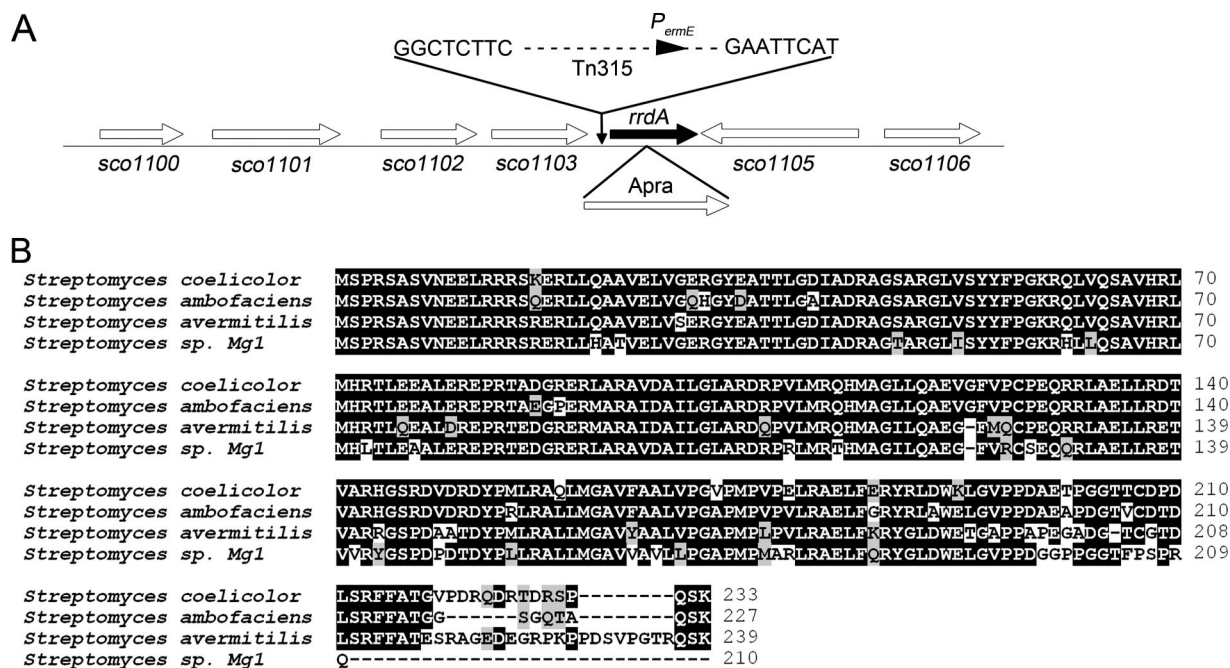


FIG. 2. RrdA is highly conserved in *Streptomyces*. (A) *rrdA* (*sco1104*) locus in the *S. coelicolor* genome. The insertion site of transposon Tn315 in mutant K74 is indicated. The *aac(3)-IV* fragment (1.5 kb, conferring apramycin resistance) used to disrupt the *rrdA* gene is indicated below the map. (B) Multiple-amino-acid-sequence alignment of RrdA from *S. coelicolor* and the homologous proteins from *S. avermitilis* MA-4680 (SAV1503), *S. ambofaciens* ATCC 23877 (accession number CAJ90078.1), and *Streptomyces sp.* strain Mg1 (accession number EDX26764.1) obtained using BioEdit 7.0.0 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

larity to the TetR family of transcriptional regulators, and its function has not been determined.

The TetR family is a common class of transcriptional factors that regulate a wide range of cellular activities in different bacteria. These activities include antibiotic production, multi-drug resistance, amino acid metabolism, pathogenicity, and development (3, 13). This family of proteins typically possesses an N-terminal helix-turn-helix DNA-binding domain and a C-terminal ligand-binding domain. These proteins activate or repress their target genes by binding to their promoters. Genome analysis revealed that *S. coelicolor* contains 151 genes encoding proteins that belong to the TetR family of transcriptional regulators (2, 21), but only a few of these genes have been characterized in detail. ActR regulates Act export by repressing the transcription of the efflux pump ActA (27). Pip and PqrA control pristinamycin and paraquat resistance genes by repressing the transcription of drug efflux pumps (8, 9). CprA and CprB, together with the products of unknown target genes, control secondary metabolite formation and sporulation (20). ScbR controls γ -butyrolactone synthesis by activating the transcription of ScbA (28), while AtrA controls Act biosynthesis by activating ActII-ORF4 transcription (31).

Functional domain analysis using the simple modular architecture research tool (SMART; <http://smart.embl-heidelberg.de/>) revealed that the region corresponding to 20 to 66 amino acid residues of RrdA is a helix-turn-helix DNA-binding domain, while the C terminus of RrdA does not appear to be similar to the C-terminal ligand-binding domain of most TetR family proteins. As shown in Fig. 2B, RrdA and proteins homologous to it are highly conserved, and so far, these proteins

have not been identified in any organism other than *Streptomyces*. SAV1503 from *Streptomyces avermitilis* MA-4680 and hypothetical proteins from *Streptomyces ambofaciens* ATCC 23877 (accession number CAJ90078.1) and *Streptomyces sp.* strain Mg1 (accession number EDX26764.1) have 83%, 90%, and 81% identity to RrdA, respectively. However, the genes surrounding *rrdA* are conserved in many divergent bacteria, including *Streptomyces*, *Saccharopolyspora*, *Salinispora*, *Solibacter*, *Erythrobacter*, and *Nocardia*. The products of the *sco1100*, *sco1101*, and *sco1102* genes showed amino acid sequence similarity to integral membrane proteins. The *sco1103*, *sco1105*, and *sco1106* genes encode a hydrolase, a secreted protein, and a lipoprotein, respectively. The specific functions of these genes have not been determined yet, and no obvious relationships among these predicted proteins have been identified.

RrdA influences Red and Act production in different ways. To assess the role of *rrdA* in *S. coelicolor* development, we attempted to knock out *rrdA* in *S. coelicolor* M145. We succeeded in constructing an *rrdA* mutant by partially replacing *rrdA* with the apramycin resistance cassette *aac(3)-IV* using a homologous recombination strategy. Four independent double recombinants were selected and confirmed by PCR and sequencing (data not shown). When the organisms were grown on solid YBP medium, Red production was significantly increased, while Act production was apparently unchanged in the *rrdA* mutant compared with wild-type strain M145 (Fig. 3A). The phenotype was complemented by pFDZ16*-*rrdA*, an integrating plasmid containing only *rrdA* and its probable 0.7-kb upstream promoter DNA fragment. We also tried to

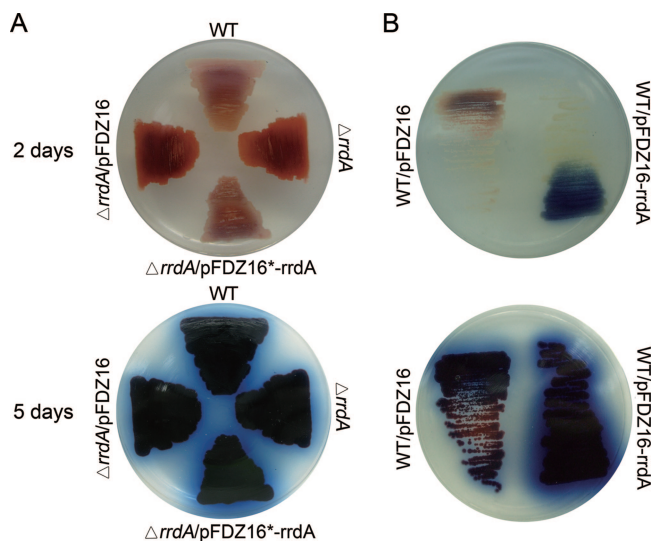


FIG. 3. Phenotypes of the *rrdA* null mutant and high-expression strain. The bottoms of the plates are shown. (A) Colonies of the parent strain *S. coelicolor* M145 (WT), the *rrdA* null mutant ($\Delta rrdA$), the *rrdA* mutant harboring the empty vector pFDZ16 ($\Delta rrdA/pFDZ16$), and the complemented strain ($\Delta rrdA/pFDZ16^{*}-rrdA$) were grown on YBP medium at 30°C for 2 days and 5 days. (B) Colonies of the parent strain M145 harboring the empty vector pFDZ16 (WT/pFDZ16) and the *rrdA* high-expression strain (WT/pFDZ16-*rrdA*) were grown on YBP medium at 30°C for 2 days and 5 days.

overexpress *rrdA* in wild-type strain M145 by introducing pFDZ16-*rrdA*, a plasmid containing the P_{tipA} promoter and *rrdA* coding sequence. Previous studies have reported that genes cloned with the *tipA* promoter appear to be expressed at basal levels even in the absence of thiostrepton induction (23, 30). Transformants of *S. coelicolor* containing pFDZ16-*rrdA* produced no red-pigmented antibiotics either with or without thiostrepton induction, so observation of the phenotype and

RT-PCR analysis (see below) of the *rrdA* high-expression strain were performed in the absence of thiostrepton induction. As shown in Fig. 3B, a high level of *rrdA* expression resulted in loss of Red production and production of amounts of Act larger than those in M145; thus, the behavior of this mutant was the same as that of the K74 mutant.

Production of Act and Red by the *rrdA* high-expression strain, a null mutant, a complemented strain, and M145 was measured in YBP liquid media at fixed intervals. As shown in Fig. 4, the *rrdA* null mutant produced Red earlier and at much higher level than the parent strain did, but the level of Act production was lower than that in the parent strain. In the *rrdA* high-expression strain, Red production dramatically decreased to levels that could barely be detected, and the level of Act production was higher than that in M145 on days 3 and 4. On day 5, the difference in Act production between M145 and the *rrdA* high-expression strain did not appear to be as significant as the difference observed when the organisms were cultured on solid media (Fig. 1A). The slight but notable difference in Act production between liquid media and solid media might be explained by differences in the developmental stages of the *rrdA* high-expression strain under these two different culture conditions. These results underline the fact that RrdA is a negative regulator of Red production and has a positive effect on Act production.

RrdA negatively regulates Red production by controlling the abundance of RedD mRNA. The expression of antibiotic biosynthetic clusters is normally regulated by pathway-specific activators (4, 10, 29). In *S. coelicolor*, Act biosynthesis and Red biosynthesis have been shown to depend on the transcriptional activation of the Act and Red biosynthetic clusters by ActII-ORF4 and the RedD and RedZ proteins, respectively. RedD, which is the direct transcriptional activator for the Red biosynthetic cluster, is RedZ dependent (12, 33). The transcription of *rrdA*, *actII-orf4*, *redD*, and *redZ* in M145, an *rrdA* high-expression strain, and a null mutant was therefore analyzed by

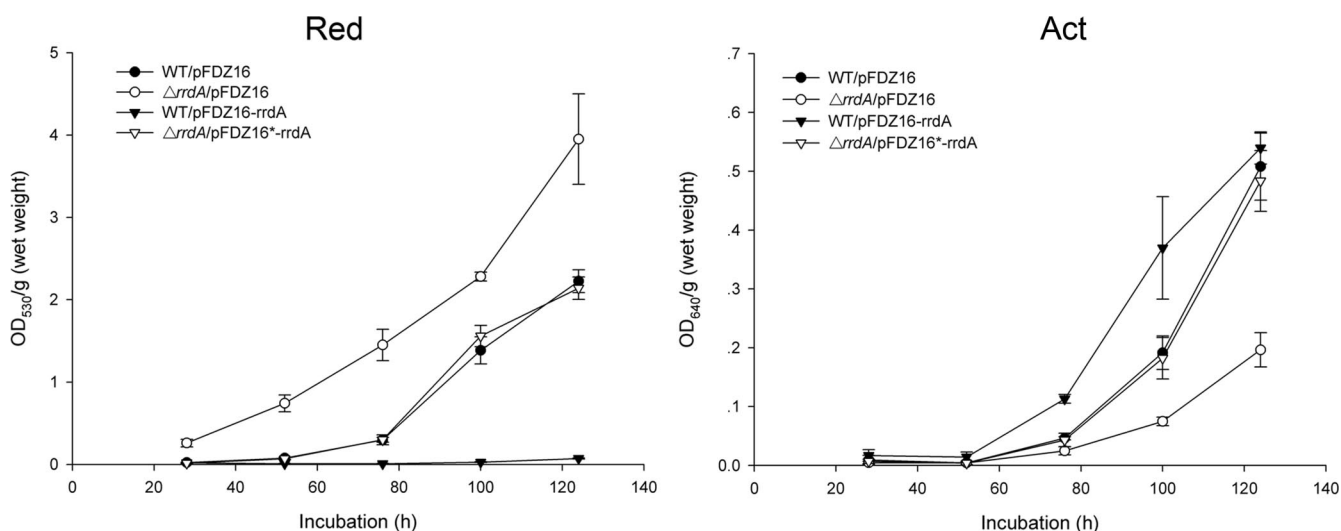


FIG. 4. Antibiotic production by the M145 strain harboring the empty vector pFDZ16, the *rrdA* null mutant harboring the empty vector pFDZ16, the complemented strain, and the *rrdA* high-expression strain. Incubation was carried out in the YBP liquid medium at 30°C. The symbols indicate the averages of three independent determinations, and the error bars indicate the standard errors. OD₅₃₀, optical density at 530 nm; OD₆₄₀, optical density at 640 nm.

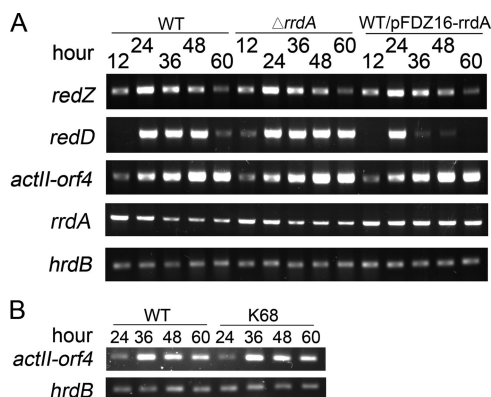


FIG. 5. RT-PCR results for wild-type strain M145, the *rrdA* null mutant, the *rrdA* high-expression strain, and *redD* null mutant K68. (A) Transcriptional levels of the pathway-specific activators, including *redD* (primers Oyj201 and Oyj202, 368 bp), *redZ* (primers Oyj203 and Oyj204, 322 bp), *actII-orf4* (primers Oyj205 and Oyj206, 320 bp), *rrdA* [primers Oyj211 and Oyj212x, 166 bp; the primer sequences are located in the upstream region of the insertion site of the *aac(3)-IV* fragment used for gene disruption], and *hrdB* (control) (primers Oyj237 and Oyj238, 207 bp). (B) RT-PCR results for the *redD* null mutant K68 and wild-type strain M145 for transcriptional detection of *actII-orf4*. WT, wild type.

RT-PCR. Total RNA was isolated both from M145 cultures at different stages of development and from mutants grown on cellophane placed on YBP solid medium. As shown in Fig. 5A, the results correlated well with the results of Red production in liquid media or on solid YBP medium. The level of transcription of *redD* was slightly higher in the *rrdA* null mutant than in M145 and significantly lower at 36 to 60 h in the *rrdA* high-expression strain than in M145. However, at 24 h, *redD* seemed to be transcribed at the same level in the *rrdA* high-expression strain and the wild type. The levels of transcription of *actII-orf4* and *redZ* appeared to be the same as those in M145. The level of the *rrdA* transcript peaked at 12 h and then decreased to a stable level after 36 h. At this point, M145 became pink, which indicates the onset of Red production. Since the primer sequences of *rrdA* are located in the upstream region of the insertion site of the *aac(3)-IV* fragment used for gene disruption, the *rrdA* transcription profile of the *rrdA* null mutant could also be detected, and it was very similar to that of M145, while in the *rrdA* high-expression strain the *rrdA* transcript level was higher in all distinct phases during development. These results indicated that RrdA negatively regulated Red production by controlling the abundance of RedD mRNA.

Since *rrdA* encodes a putative TetR family transcription factor, we performed electrophoretic mobility shift assays to examine the binding of the purified recombinant RrdA protein to three different DNA probes (nucleotides -296 to 115, -94 to 315, and 61 to 462 relative to the transcriptional start site of *redD*) (19) in TGEK buffer. The effects of Na^+ , Mg^{2+} , and Mn^{2+} on RrdA binding were also tested. Unfortunately, no specific binding of RrdA to the DNA probes was observed under any of the test conditions used (data not shown). It is possible that the activated binding of RrdA to the *redD* promoter requires additional factors or that RrdA indirectly regulates *redD* transcription and one or more as-yet-unidentified proteins may participate in this regulation.

Cross-regulation of Red and Act production might occur through a mechanism involving competition for common precursors. Huang et al. reported that deletion of the Red-specific regulator gene *redZ* caused delayed transcription of the *actII-orf4* and Act biosynthesis genes in *S. coelicolor* (15). RrdA, which upregulates Act production and downregulates Red production, affects transcription of only the Red-specific regulator gene *redD*. We hypothesized that another mechanism may be involved in the cross-regulation of Red biosynthesis and Act biosynthesis. Therefore, we determined the transcriptional level of *actII-orf4* in the *redD* null mutant K68. However, no difference was observed between K68 and wild-type strain M145 (Fig. 5B). The results indicated that the positive effect of the *redD* mutation on Act production was not due to any alteration at the transcriptional level of *actII-orf4*. Together with the observation that mutations in Red biosynthesis genes affect Act production, this suggests that Red production and Act production are interlinked at a nontranscriptional level.

It is known that Act production begins with the synthesis of a 16-carbon polyketide backbone by a type II polyketide synthase complex that uses malonyl coenzyme A (malonyl-CoA) and acetyl-CoA as the precursors (27). These precursors are also necessary for the production of 2-undecyl-pyrrole, which is an intermediate metabolite in the Red biosynthesis pathway (34). The *redD* mutation eliminated transcription of most biosynthetic gene clusters (14, 19), and mutations in the *redQ-redP* gene spacer, *redO*, *redN*, the *redN-redM* gene spacer, and *redL* disrupted or influenced, probably due to a polar effect, the expression of key enzymes (RedP, RedQ, and RedL) involved in the synthesis of 2-undecyl-pyrrole. This in turn diverted malonyl-CoA and acetyl-CoA from Red biosynthesis to Act biosynthesis. This hypothesis can be used to provide a simple explanation for the positive effect of RrdA on Act production. Thus, a decrease in Red production could result in an increase in Act production, and an increase in Red production could lead to a decrease in Act production due to the levels of the metabolic precursors utilized by both biosynthetic pathways (Fig. 4). A similar effect was also observed for nanchangmycin production by *Streptomyces nanchangensis*. A DNA fragment deletion in cluster C of *S. nanchangensis* resulted in at least a threefold increase in nanchangmycin production because biosynthesis of the compounds involved similar precursors (26). *redX*, *redW*, *redV*, *redU*, *redI*, and *redH* encode proteins that participate in the biosynthesis of MBC or in the final step of Red biosynthesis. Liquid chromatography-mass spectrometry analysis of the *redU* mutant showed that Red was eliminated and 2-undecyl-pyrrole accumulated (25). Therefore, disruption of these genes had little effect on the accumulation of malonyl-CoA and acetyl-CoA and had no obvious influence on production of blue-pigmented Act.

In conclusion, our study revealed that the nontranscriptional cross-regulation of Act biosynthesis and Red biosynthesis might involve competition for common precursors. We also partially characterized the TetR-like protein gene *rrdA* and found that it encodes a novel negative regulator of Red biosynthesis in *S. coelicolor*. These observations should be useful in fermentation engineering of undecylprodigiosin, which is a candidate drug for cancer therapy (34).

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