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bldA Dependence of Undecylprodigiosin Production in *Streptomyces coelicolor* A3(2) Involves a Pathway-Specific Regulatory Cascade

JANET WHITE AND MERVYN BIBB*

Department of Genetics, John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, United Kingdom

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The production of the red-pigmented tripyrrole antibiotic undecylprodigiosin (Red) by *Streptomyces coelicolor* A3(2) depends on two pathway-specific regulatory genes, *redD* and *redZ*. RedD is homologous to several other proteins that regulate antibiotic production in streptomycetes; RedZ is a member of the response regulator family. *redZ* transcripts were detected during exponential growth and increased in amount during transition and stationary phases; transcription of *redD* was confined to the two latter stages of growth. Whereas mutation of *redD* had no effect on *redZ* transcription, transcription of *redD* was highly dependent on *redZ*, suggesting that RedZ is a transcriptional activator of *redD*. *bldA*, which encodes the only tRNA of *S. coelicolor* that can efficiently translate the rare leucine codon UUA, is required for Red production at higher phosphate concentrations. While the *redD* transcript contains no UUA codons, the *redZ* mRNA contains one. Transcription of *redZ* appeared to be unaffected in a *bldA* mutant; in contrast, *redD* transcription was undetectable, consistent with the translational dependence of *redZ* on *bldA* and the transcriptional dependence of *redD* on *redZ*. Red production in a *bldA* mutant was restored by multiple copies of *redZ*, presumably reflecting a low level of mistranslation of the *redZ* UUA codon, while multiple copies of *redD* had no effect, presumably a consequence of the severe dependence of *redD* transcription on RedZ. Transcription of *redZ* appears to be negatively autoregulated.

Members of the genus *Streptomyces* produce a wide variety of secondary metabolites that include about half of all known microbial antibiotics (1, 37). Many of these compounds have important applications in human medicine as antibacterial, antitumor, and antifungal agents and in agriculture as growth promoters, fungicides, antiparasitic agents, and herbicides. Antibiotic production in surface-grown cultures of streptomycetes generally coincides with the onset of morphological differentiation, which is characterized by the development of aerial hyphae and spores (11). The isolation of *bld* mutants that are deficient in both processes suggests at least some common elements of genetic control (7). *Streptomyces coelicolor* A3(2) is a particularly appropriate strain for studying the regulation of antibiotic production (reviewed in references 9, 10, and 25). It is genetically manipulable and produces at least four antibiotics, permitting the analysis of pathway-specific and pleiotropic regulation of antibiotic production. In addition to its fundamental interest, a thorough knowledge of how antibiotic biosynthetic pathways are regulated will inevitably allow for the development of more rational approaches to strain improvement (8).

The red-pigmented antibiotic undecylprodigiosin (Red) (Fig. 1) is the major component of a mixture of at least three prodigionines made by *S. coelicolor* (48). This highly nonpolar tripyrrole compound is chemically similar to the antibiotic prodigiosin produced by *Serratia marcescens* and is made by a similar convergent biosynthetic pathway (15, 16, 48, 49). Red production usually occurs in a growth phase-dependent manner, occurring in the transition and stationary phases of liquid-grown cultures (47) and coinciding with the onset of morphological development in surface-grown cultures. At least 18 genes are required for Red synthesis (13, 16, 43). Of these, *redD* appears to be a pathway-specific activator gene. This conclusion is based on the inability of *redD* mutants to cosynthesize Red with any of the other *red* classes (16, 43), the lack of *O*-methyltransferase activity (encoded by *redE* and/or *redF*) and reduced levels of *redE* and *redBF* transcripts in *redD* mutants (16, 39), and the ability of extra cloned copies of *redD* to elicit overproduction of Red (36, 39). The stimulatory effects of overexpression of *redD* on the transcription of at least one *red* biosynthetic gene and on the timing of Red production also support this conclusion (47). Furthermore, RedD shows extensive amino acid sequence similarity to the products of *act*II-ORF4 (18) and *dnrI* (46) of *S. coelicolor* and *Streptomyces peucetius*, respectively, both of which also appear to encode pathway-specific activator proteins.

The pleiotropically acting *bldA* gene (9, 25), which encodes the only tRNA of *S. coelicolor* that can efficiently translate the rare leucine codon UUA (30, 32), is needed for antibiotic production by *S. coelicolor* under most conditions. In the case of actinorhodin (Act), this is explained by the presence of a UUA codon in the mRNA for ActII-ORF4, the pathway-specific activator protein (18). In contrast, the *redD* transcript contains no UUA codons (39). Nevertheless, Red biosynthesis does not occur in *bldA* mutants grown on most laboratory media, and transcription of at least one *red* biosynthetic gene (*redX*) is impaired in a *bldA* mutant (22). However, at lower phosphate concentrations, transcription of *redX* and Red production are restored to wild-type levels without any effect on Act synthesis or aerial mycelium formation (22). Since the expression of several TTA-containing genes remains *bldA*-dependent under these conditions (31), it seems that there are no TTA codons in the Red biosynthetic structural genes. Thus, the *bldA* dependence of Red production must reflect the existence of at least one unidentified TTA-containing gene that is required for activation of the *red* pathway at higher phosphate concentrations.

Attempts to identify suppressor mutations of *bldA* in which Red production, but not Act synthesis or morphological dif-

* Corresponding author. Phone: 44 1603 452571. Fax: 44 1603 456884. E-mail: bibb@bbsrc.ac.uk.

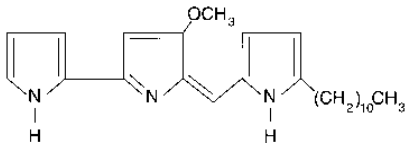

FIG. 1. Structure of undecylprodigiosin.

ferentiation, was restored led to the isolation of the Pwb (pigmented while bald) mutants (23). Analysis of three independently isolated Pwb mutants localized the suppressor mutations near the right-hand end of the *red* biosynthetic cluster, one being more precisely mapped to a position 4 to 5 kb 3′ of *redD*. Determination of the sequence of this region led to the identification of an open reading frame, *redZ*, that is likely to be responsible for the Pwb phenotype (19). *redZ* encodes a homolog of the response regulator family of proteins yet shows interesting differences that appear to preclude activation by phosphorylation by a cognate sensory histidine kinase. Significantly, *redZ* contains a single TTA codon.

The aim of this study was to determine whether *redZ* played an essential role in Red production and, if it did, to analyze the relationship between *bldA*, *redD*, and *redZ*.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and microbiological procedures. *Escherichia coli* K-12 strains JM101 (44) and ET12567 (34) were used for routine subcloning and were grown and transformed by standard procedures (44). *S. coelicolor* A3(2) strains were M145 (SCP1$^-$ SCP2$^-$) (24) and its derivatives M510 (Δ*redD*) (20), M512 (Δ*redD* Δ*act*II-ORF4) (20)), M550 (Δ*redZ*) (this work), and M554 (*redD*::KC899) (this work); and J1501 (*hisA1 uraA1 strA1 pgl* SCP1$^-$ SCP2$^-$) (11a) and it derivative J1681 (Δ*bldA*) (31). The *Streptomyces lividans* 66 strain was the wild-type isolate 1326 (22). Transformation and transfection were done as described previously (24). SFM medium (20) was used to make spore suspensions. R2YE (24), SMMS (a solidified version of SMM [20, 47]), and MM (24) containing different levels of phosphate were used to assess Red production. RNA was isolated from liquid cultures grown in SMM (47).

Plasmids and phages. pIJ2925 (26) was used to clone the *redD* PCR (14) product for the construction of KC899, and pSET151 (2) was used to make pIJ4162. pIJ6014 (47) and pIJ68 (42) were used to provide multiple copies (50 to 100) (28) of *redD* and *act*II-ORF4, respectively; pIJ6013, which contains a 2.2-kb *Bam*HI-*Pst*I fragment encoding *redD* (Fig. 2, sites 11 to 14), is described in reference 47; pIJ4083 is described in reference 12; pHJL401 is described in reference 29. pIJ2034 (5) was used as a source of *hrdB*. To construct pIJ4099, the primers 5′ CATGGATCCGCAGCCCATGATGACAATGTGCAC and 5′ CAT GGATCCCATCCCCCGAAGTTGTACAGGCTG, whose 3′ ends correspond to nucleotide positions −300 and 174 with respect to the *redD* transcriptional start site (47), respectively, were used in the PCR to isolate a 524-bp fragment containing the *redD* promoter region. The PCR mix contained 1× PCR buffer (Boehringer Mannheim), 0.2 mM each deoxynucleoside triphosphate, 25 to 50 pmol of each primer, 20 ng of pIJ6013, 5 U of *Taq* polymerase (Boehringer Mannheim), and 5% glycerol in a total volume of 100 μl and was overlaid with mineral oil. Samples were subjected to 30 cycles of 50 s at 94°C, 40 s at 60°C, and 40 s at 72°C. The PCR product was cut with *Bam*HI and inserted in *Bam*HI-cleaved pIJ2925 to give pIJ4096; the *redD* promoter fragment was excised from pIJ4096 by using flanking *Eco*RI and *Hin*dIII sites and cloned in *Eco*RI-plus-*Hin*dIII-cleaved pIJ4083 to give pIJ4099. KC861 (4) was used to make KC899 for disruption of *redD*.

Construction of the *redZ* and *redD* mutants. To create a *redZ* null mutant, a 1.2-kb *Bam*HI-*Eco*RI fragment (Fig. 2, sites 1 to 4) that includes the first 54 bp of the *redZ* coding region and a 2-kb *Bam*HI fragment (Fig. 2, sites 5 to 10) that includes the last 104 bp of *redZ* were cloned, via a multistep procedure, in their native orientations either side of a 1.7-kb fragment containing the hygromycin resistance (Hyg^r) gene (*hyg*) of *Streptomyces hygroscopicus* in pSET151 to yield pIJ4162. Five micrograms of pIJ4162 DNA was denatured by adding NaOH to a final concentration of 1 M, the solution was neutralized by adding HCl, and the resulting single-stranded DNA was used to transform M145 protoplasts (40). Transformants were selected by adding thiostrepton to R2YE regeneration plates to give a final concentration of 10 μg ml^{-1} (pIJ4162 carries *tsr* conferring thiostrepton resistance [Thio^r]). Integration of pIJ4162 at the *redZ* locus in both of the Red$^+$ Thio^r Hyg^r (50 μg ml^{-1} in R2YE) transformants examined was confirmed by Southern analysis. By using a nick-translated 2-kb *Bam*HI fragment (Fig. 2, sites 5 to 10) as the probe, hybridizing bands corresponding to *redZ* (a 3-kb *Not*I band) and to the deleted version of the gene (a 1.7-kb *Not*I band) were observed, confirming the occurrence of a single crossover event. After three rounds of sporulation on nonselective medium (SFM), 15 Thio Hyg^r Red$^-$ colonies were obtained from a total of approximately 2,000 screened. Southern analysis of three of the Red$^-$ isolates confirmed the replacement of most of the *redZ* coding region by *hyg* (Fig. 2). One of the *redZ* mutants was designated M550. To create a *redD* mutant, the primers 5′ CATGGATCCATCCGCTCA TGGGAGTGCGGAGAAC and 5′ CATGGATCCCCCCAGTTCGTCGTCG AGCCGCTTC, whose 3′ ends correspond to nucleotide positions 110 and 868 of the *redD* mRNA (47), were used in the PCR to isolate a 806-bp DNA fragment internal to the *redD* transcription unit. PCR conditions were as described above. The PCR product was cut with *Bam*HI, inserted in *Bam*HI-cleaved pIJ2925, excised by using flanking *Bgl*II sites, and cloned in the *Bgl*II site of the Δ*attP c*$^+$ ϕC31 phage vector KC861 by using *S. lividans* as the host, yielding KC899. The structure of KC899 was confirmed by restriction endonuclease and Southern analysis. Red$^-$ M145 lysogens containing KC899 were obtained by spotting a suspension of the phage on a lawn of M145 on R2YE, harvesting the resulting spores from the spot, and plating the spores on R2YE containing 50 μg of thiostrepton ml^{-1} (KC899 carries *tsr* conferring Thio^r). Disruption of the *redD* transcription unit was confirmed by Southern analysis of total DNA isolated from one of the putative lysogens by using a nick-translated 2.2-kb *Bam*HI-*Pst*I fragment (Fig. 2, sites 11 to 14) as the probe; hybridizing M145 *Pst*I and *Bam*HI-*Pst*I bands of 16 and 4.3 kb, respectively, were replaced with the expected bands of 13 kb plus 5 kb and 4 kb plus 1.3 kb, respectively. The *redD* mutant, in which the coding region of *redD* was no longer transcribed from the *redD* promoter, was designated M554.

Isolation and manipulation of DNA and RNA. Plasmid DNA was isolated from *E. coli* derivatives as described previously (6), and phage, plasmid, and total DNAs were isolated from KC899 and *S. coelicolor* strains as described in reference 24. Southern analyses (45) were performed with nick-translated ^{32}P-labelled probes (44). RNA was isolated as described in reference 24.

S1 nuclease protection assays. The 1.2-kb *Bam*HI-*Eco*RI fragment (Fig. 2, sites 1 to 4), uniquely labelled at the 5′ end of the *Eco*RI site by using [γ-^{32}P]ATP and T4 polynucleotide kinase (44), was used to detect *redZ* transcripts. Two different *redD* probes were used, one of which was obtained by PCR. The primer 5′ ACAGTTCGTCCACCAGGTCCGCGA, whose 5′ end is located 346 nucleotides 3′ of the *redD* transcriptional start site (47), was labelled with [γ-^{32}P]ATP

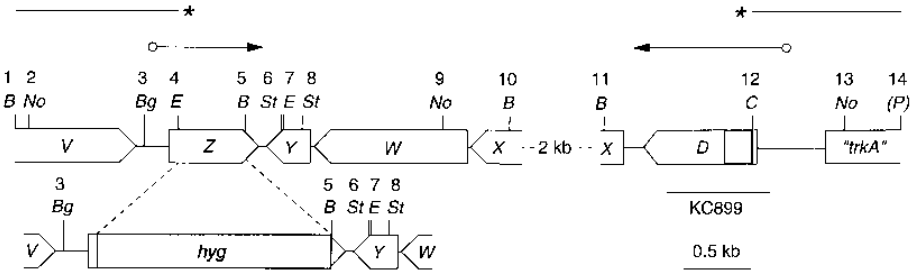

FIG. 2. Restriction map of the *redZ-redD* region of the *red* gene cluster. Broad arrows indicate protein-coding regions and their orientations. The *hyg* gene used to replace most of the *redZ* coding region in M550 and the extent of the in-frame deletion in *redD* in M510 are shaded; the DNA segment used to disrupt the *redD* transcription unit in M554 is indicated by KC899. The locations of *redD*p and *redZ*p, and their orientations, are shown by arrows. The uniquely end-labelled probes used to detect *redZ* and *redD* transcripts are shown above the restriction map; asterisks indicate the labelled ends. *Pst*I site 14 is derived from the cloning vector and is not present in the chromosomal sequence. "*trkA*" is a homolog of *trkA* of *E. coli*, and its role, if any, in Red production is unknown. Single *Bam*HI and *Stu*I sites lie in the 2-kb segment of *redX* that is not shown. The map was derived from references 19, 23a, and 39. B, *Bam*HI; No, *Not*I; Bg, *Bgl*II; E, *Eco*RI; St, *Stu*I; C, *Cla*I; (P), *Pst*I.

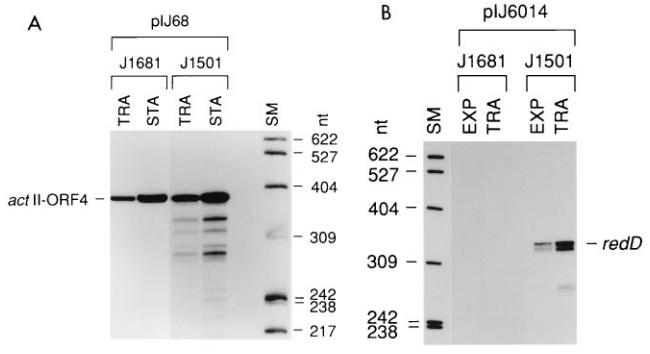

FIG. 3. Transcription of *act*II-ORF4 (A) and *redD* (B) in derivatives of J1501 (*bldA*$^+$) and J1681 (Δ*bldA*). RNA was isolated from liquid (SMM)-grown cultures of J1501 and J1681 containing pIJ68 (*act*II-ORF4) or pIJ6014 (*redD*) during exponential growth (EXP), transition phase (TRA), and stationary phase (STA) and used in S1 nuclease protection studies with uniquely end-labelled probes that should generate protected fragments of 390 and 346 nucleotides (nt) for *act*II-ORF4 and *redD* transcripts, respectively. SM, end-labelled *Hpa*II-digested pBR322 size markers.

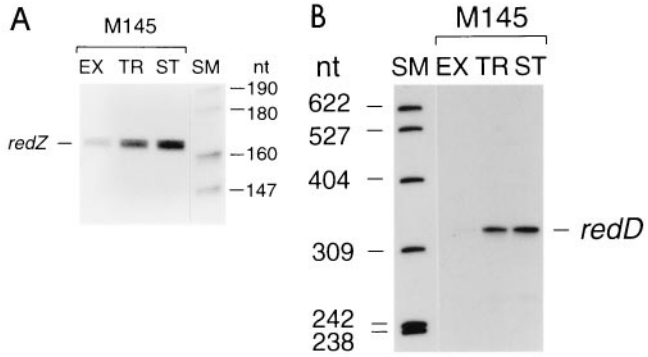

FIG. 4. Growth phase-dependent transcription of *redZ* and *redD* in M145. RNA was isolated from a liquid (SMM)-grown culture during exponential growth (EX), transition phase (TR), and stationary phase (ST) and used in S1 nuclease protection studies with uniquely end-labelled probes specific for *redZ* (A) and *redD* (B) transcripts, which should yield protected fragments of 166 and 346 nucleotides (nt), respectively. SM, end-labelled *Hpa*II-digested pBR322 size markers.

by using T4 polynucleotide kinase (44) and used in the PCR with the unlabelled primer 5′ CATGGATCCTGCTTCGTTTGCGTCGTTCAGTTC, whose 3′ end corresponds to nucleotide position −126 with respect to the *redD* transcriptional start site (47). PCR conditions were as described above. The other *redD* probe was the 1.1-kb *Cla*I-*Pst*I fragment (Fig. 2, sites 12 to 14), uniquely labelled (44) at the 5′ end of the *Cla*I site. To detect *act*II-ORF4 transcripts, a 465-bp *Xho*I-*Ase*I fragment uniquely labelled at the 5′ end of the *Xho*I site within the *act*II-ORF4 coding region was used (18). To determine *hrdB* transcript levels, a 520-bp *Hin*dIII-*Lsp*I fragment uniquely labelled at the 5′ end of the *Lsp*I site was used (5). For each S1 nuclease protection assay, about 0.02 pmol (approximately 10^4 Cerenkov counts min^{-1}) of labelled probe was hybridized to 60 μg of RNA in NaTCA buffer (38) at 45°C overnight after denaturation at 65°C for 15 min. All subsequent steps were as described in reference 27. *redZ* mRNA levels in transition-phase cultures of M145, M550, and M145(pIJ6014) were quantitated with a Fujix BAS 1000 phosphorimager; *hrdB* transcripts present in the same samples were used as internal standards. All of the S1 nuclease protection experiments were carried out at least twice, using RNA isolated from independently grown cultures, and the results presented were shown to be reproducible.

RESULTS

Transcription of *redD*, but not of *act*II-ORF4, depends on *bldA*. The ability to bypass the requirement for *bldA* for Act production by replacing the single TTA codon present in the pathway-specific activator gene *act*II-ORF4 with the synonymous leucine codon TTG (18) indicates that transcription of *act*II-ORF4 does not depend on *bldA*. Although Red production is *bldA* dependent under most growth conditions, the pathway-specific activator gene for Red synthesis, *redD* (a homolog of *act*II-ORF4), does not contain a TTA codon (39). Since TTA codons do not appear to be present in the *red* biosynthetic structural genes (see above), this finding raised the possibility that transcription of *redD* depends on *bldA*. To address this issue directly, transcription of *redD* and of *act*II-ORF4 was assessed by S1 nuclease protection analysis using RNA isolated from *S. coelicolor* J1501 (*bldA*$^+$) and J1681 (Δ*bldA*) containing either pIJ6014, a multicopy plasmid carrying *redD*, or pIJ68, a multicopy plasmid carrying *act*II-ORF4 (Fig. 3). While transcription of *act*II-ORF4 was readily detected in transition- and stationary-phase cultures of both strains (Fig. 3A), transcription of *redD* was detected only in the *bldA*$^+$ strain (Fig. 3B). These results indicate the existence of a gene that is required for *redD* transcription, that depends on *bldA* for expression, and that may therefore contain a TTA codon.

***redZ*, a second pathway-specific regulatory gene for Red production.** The isolation of the Pwb mutants, the localization of the mutations in the *red* cluster, and the subsequent determination of the sequence of this region led to the identification of *redZ* as a likely candidate for the *bldA*-dependent gene required for *redD* transcription. To determine if *redZ* was required for Red production, most of the *redZ* coding region of *S. coelicolor* M145 was replaced by a *hyg* gene, yielding M550 (Fig. 2). Red production in the resulting *redZ* mutant was completely abolished, but there was no apparent effect on Act synthesis. To confirm that the loss of Red synthesis in M550 was due to mutation of *redZ* and not to a polar effect on the expression of a downstream gene(s), a 1-kb *Bgl*II-*Stu*I fragment (Fig. 2, sites 3 to 6) that contained the *redZ* coding region and promoter (see below) was cloned in pHJL401 (copy number of ca. 10 per genome) that had been cut with *Bam*HI and *Sma*I, yielding pIJ4159. Transformation of M550 with pIJ4159 restored Red production. Although *S. lividans* 66 possesses a functional *red* gene cluster, it does not make the antibiotic on most commonly used media. Transformation of *S. lividans* with either pIJ4159 (ca. 10 copies of *redZ*) or pIJ6014 (ca. 50 to 100 copies of *redD*) resulted in copious Red synthesis on R2YE and on SMMS. However, in *S. coelicolor* M145, only the higher-copy-number plasmid (pIJ6014) elicited marked overproduction of Red on the same media.

Transcription of *redZ* occurs in a growth phase-dependent manner and does not require *bldA*. Transcription of *redD* and Red production occur in the transition and stationary phases of liquid (SMM)-grown cultures of *S. coelicolor* M145 (47). To see if *redZ* was transcribed in a growth phase-dependent manner also, the approximate position of the *redZ* transcriptional start site was first located by S1 nuclease protection analysis about 110 nucleotides upstream of the predicted ATG translational start codon (19). RNA was then isolated from M145 at different stages of growth in SMM, and transcription of *redZ* was examined by using the same procedure. Transcripts of *redZ* were detectable during exponential growth, but their level increased markedly during the transition to stationary phase (Fig. 4A). In the same RNA samples, transcription of *redD* was found to be confined to the transition and stationary phases of growth (Fig. 4B).

To determine if the lack of Red production in *bldA* mutants might reflect a lack of *redZ* transcription, S1 nuclease protection studies were conducted with RNA isolated from J1501 (*bldA*$^+$) and J1681 (Δ*bldA*) at different stages of growth in liquid culture. *redZ* transcripts were detected in the *bldA* mu-

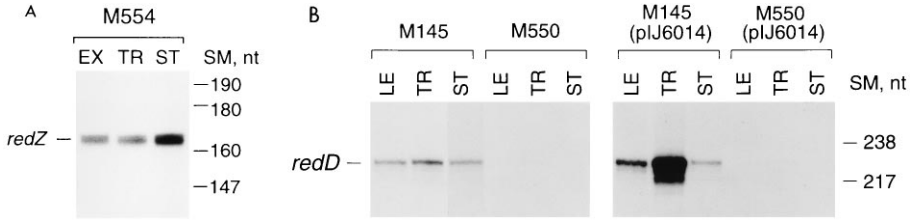

FIG. 5. Analysis of the transcriptional dependence between *redZ* and *redD*. (A) Transcription of *redZ* in the *redD* mutant M554. RNA was isolated from a liquid (SMM)-grown culture during exponential growth (EX), transition phase (TR), and stationary phase (ST) and used in S1 nuclease protection studies with a uniquely end-labelled probe specific for *redZ* transcripts that should yield a protected fragment of 166 nucleotides (nt). (B) Transcription of *redD* in M145, in the *redZ* mutant M550, and in derivatives of each containing pIJ6014. RNA was isolated from liquid (SMM)-grown cultures during late exponential growth (LE), transition phase (TR), and stationary phase (ST) and used in S1 nuclease protection studies with a uniquely end-labelled probe specific for *redD* transcripts that should yield a protected fragment of 229 nucleotides. SM, end-labelled *Hpa*II-digested pBR322 size markers.

tant throughout growth (since the growth characteristics of J1681 were markedly different from those of J1501 [the *bldA* mutant reached a final optical density at 450 nm which was about half of that attained by J1501 and exhibited an extended transition phase], quantitative comparisons of the levels of the *redZ* transcript in the two strains were deemed inappropriate).

Transcription of *redD* depends on *redZ*. To assess whether there was a transcriptional dependence between *redD* and *redZ*, a defined *redD* mutant of M145, M554, was constructed by gene disruption (Fig. 2) using KC899, a derivative of the Δ*attP* c^+ phage vector KC861. A series of S1 nuclease protection experiments was carried out with RNA samples isolated at different stages of growth from SMM-grown cultures of M145, M554 (*redD*::KC899), M550 (Δ*redZ*), M145(pIJ6014), and M550(pIJ6014), with each of the latter two strains containing 50 to 100 copies of *redD*. Disruption of *redD* had no effect on *redZ* transcription (Fig. 5A), but replacement of *redZ* with *hyg* resulted in a severe reduction in the level of the *redD* transcript, regardless of the whether *redD* was present at a single copy in the chromosome (M550) or in multiple copies in M550(pIJ6014) [Fig. 5B; prolonged exposure of the gel to X-ray film revealed a low level of the *redD* transcript in RNA isolated from transition-phase cultures of M550 and M550(pIJ6014)]. Thus, transcription of *redD* depends strongly on *redZ*.

Introduction of multiple copies of *redZ* in the form of pIJ4159 into the Δ*redD* mutant M510 failed to suppress the loss of Red production on either R2YE or SMMS, consistent with a regulatory hierarchy in which RedZ activates *redD* transcription, and RedD then activates some gene(s) needed for Red synthesis. Likewise, transformation of M550 (Δ*redZ*) with pIJ6014 (i.e., with 50 to 100 copies of *redD*) did not restore Red production; this may simply reflect a stringent requirement for RedZ for *redD* transcription (Fig. 5B) or may indicate that RedZ plays more than one role in Red synthesis.

Multiple copies of *redZ*, but not of *redD*, restore Red production in a *bldA* mutant. Multiple copies of *act*II-ORF4 (in the form of pIJ68) suppress the need for *bldA* for Act production on some solid media (e.g., the complex medium R2YE [42]) but not on others (e.g., the relatively simple SMMS [our unpublished results]), perhaps reflecting differences in translational accuracy under different nutritional conditions. To determine whether multiple copies of *redD* or *redZ* could suppress the dependence of Red production on *bldA*, J1501 (*bldA*$^+$), J1681 (Δ*bldA*), J1681(pIJ6014) (ca. 50 to 100 copies of *redD*), and J1681(pIJ4159) (ca. 10 copies of *redZ*) were grown on R2YE and SMMS, and Red production was assessed. While additional copies of *redZ* restored Red synthesis on R2YE, but not on SMMS, multiple copies of *redD* did not restore Red production on either medium.

Red production can be restored to *bldA* mutants by reducing the level of phosphate in the medium from 4 mM to 0.04 mM (22). To determine whether this nutritional suppression of the *bldA* phenotype required *redZ* or *redD*, Red production by M145, M550 (Δ*redZ*), M510 (Δ*redD*), and M512 (Δ*redD* Δ*act*II-ORF4) was assessed on MM containing 4 and 0.04 mM phosphate, together with the control strains J1501 (*bldA*$^+$) and J1681 (Δ*bldA*). While Red production was restored in J1681 at the lower phosphate concentration, it was not restored in M550, M510, or M512. Thus, *redZ* and *redD* are needed for *red* gene expression regardless of the phosphate concentration.

Transcription of *redZ* is negatively autoregulated. The foregoing data suggest that RedZ acts as a transcriptional activator of *redD*. If so, the presence of the *redD* promoter region on a high-copy-number plasmid might be expected to titrate RedZ and cause a reduction in Red synthesis. To assess this possibility, a 524-bp PCR product containing the *redD* promoter region was cloned in the high-copy-number (50 to 100 copies per genome) plasmid pIJ4083, yielding pIJ4099. The level of Red production by M145 transformants containing pIJ4099 or pIJ4083 was the same on both R2YE and SMMS and similar to that of M145. The apparent lack of titration might have reflected negative autoregulation of *redZ* transcription by RedZ. *redZ* transcript levels were compared in RNAs isolated at different times from M145, M550 (Δ*redZ*), and M145(pIJ6014) (50 to 100 copies of *redD*) (Fig. 6A). Quantitation of transition-phase RNA samples by using transcripts derived from the major and essential σ factor gene (*hrdB*) of *S. coelicolor* (3) as an internal standard revealed 14- and 6-fold increases in the

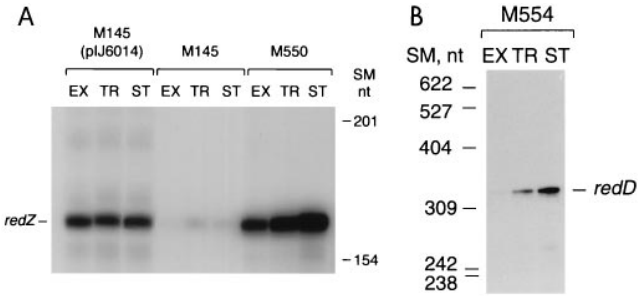

FIG. 6. Negative autoregulation of *redZ*, but not of *redD*, transcription. (A) Transcription of *redZ* in M145, M550 (Δ*redZ*), and M145(pIJ6014); (B) transcription of *redD* in M554 (*redD*::KC899). RNA was isolated from liquid (SMM)-grown cultures during exponential growth (EX), transition phase (TR), and stationary phase (ST) and used in S1 nuclease protection studies with uniquely end-labelled probes specific for *redZ* and *redD* transcripts that should yield protected fragments of 166 and 346 nucleotides (nt), respectively. SM, end-labelled *Hpa*II-digested pBR322 size markers.

levels of *redZ* mRNA in M550 (Δ*redZ*) and M145(pIJ6014) (50 to 100 copies of *redD*), respectively, compared to M145, consistent with negative autoregulation of *redZ* transcription (the inability of pIJ4159 [ca. 10 copies of *redZ*] to stimulate Red production in M145 [see above] is also consistent with negative autoregulation). In contrast, disruption of *redD* had no effect on the levels of its own transcript (compare Fig. 6B with Fig. 4B), suggesting that *redD* expression is not autoregulated.

DISCUSSION

The lack of *redD*, but not of *act*II-ORF4, transcripts in a *bldA* mutant indicated the existence of a gene that is required for *redD* transcription, that depends on *bldA* for expression, and that might therefore contain a TTA codon. The data presented here, and those acquired by others (19, 23), suggest that this gene is *redZ*. Thus, as for Act production, *bldA* exerts its influence on Red biosynthesis through a pathway-specific activator gene, but via a different route. The *bldA* tRNA appears to be essential for the correct and efficient translation of the single UUA codon present in the *redZ* mRNA, and RedZ seems to be essential for transcription of *redD*, and consequently for the transcription of at least some of the *red* biosynthetic structural genes.

Transcription of *redZ*, at least in liquid-grown cultures, is regulated in a growth phase-dependent manner that might influence whether and when a critical level of RedZ is made for activation of *redD* transcription. Although transcription of *redZ* does not depend on *bldA*, translation of *redZ* mRNA does, and there is clearly scope for regulation of Red production via *bldA*. Moreover, since RedZ is unlikely to be activated by phosphorylation in a manner akin to other response regulator homologs (19), association with some ancillary factor may be necessary before RedZ can activate transcription from *redD*p and potentially from other *red* promoters. The apparent ability of RedZ to regulate negatively its own synthesis would also be consistent with a role for a low-molecular-weight effector. Consequently, the onset and extent of Red production may be determined by mechanisms acting at the transcriptional, translational, and posttranslational levels, each presumably responding to different environmental signals.

The presence of multiple copies (ca. 50 to 100) of *redD* in *S. coelicolor* M145(pIJ6014) resulted in high levels of *redD* transcription and Red production in exponentially growing cultures (47). This finding indicates that RedZ is not limiting for *redD* transcription under the conditions used. Since *redZ* is evidently subject to negative autoregulation, the likely titration of RedZ by its probable binding site on pIJ6014 presumably explains the increased levels of *redZ* transcription in M145(pIJ6014) (Fig. 6A). This elevation in *redZ* mRNA is likely to result in an increased level of RedZ, which is presumably responsible for the stimulation of *redD* transcription during exponential growth (compare Fig. 4B with Fig. 3B and 5B). This stimulatory effect on *redD* transcription may be sufficient to overcome any constraints on activation of *redD* expression that might normally be imposed by the putative translational and posttranslational mechanisms referred to above, permitting the observed precocious production of Red during exponential growth.

It is possible to suppress the Act$^-$ and Red$^-$ phenotypes of a *bldA* mutant by providing multiple copies of the TTA-containing pathway-specific regulatory gene (*act*II-ORF4 for Act [42] and *redZ* for Red). In both cases, suppression is observed on the rich and complex medium R2YE but not on the relatively simple and defined SMMS. Consistent with these observations, expression of the TTA-containing *aad* and *hyg*, encoding spectinomycin and hygromycin resistance, respectively, was dependent on *bldA* on minimal medium but not at all on R2YE (33). This conditional phenotype presumably reflects differences in translational accuracy under different nutritional conditions.

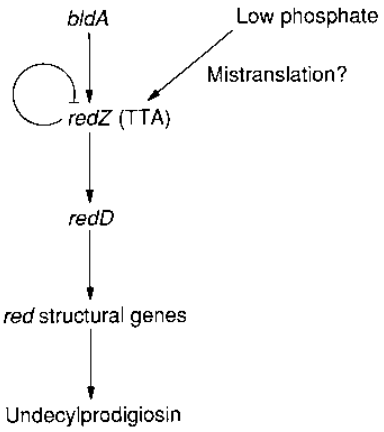

FIG. 7. Model for the regulation of undecylprodigiosin production in *S. coelicolor*.

At lower phosphate concentrations, Red synthesis is restored in *bldA* strains but remains absent in a *redZ* mutant. It thus seems likely that *redZ* is required for Red production by *bldA* mutants under these conditions, and yet *redZ* contains a TTA codon (the possibility that there is a *bldA*- and *redZ*-independent pathway that operates at low phosphate concentrations and that is activated in *bldA* mutants seems less attractive). We suggest that the suppression of *bldA* at low phosphate concentrations reflects mistranslation of the *redZ* TTA codon, potentially induced by reduced rates of translation. Since the expression of several other TTA-containing genes remains *bldA* dependent at low phosphate concentrations (31), the apparent specificity for the *redZ* TTA codon may simply reflect increased levels of *redZ* mRNA in *bldA* mutants, a prediction that stems from the negative autoregulation of *redZ* transcription (unfortunately, the growth characteristics of *bldA* mutants make confirmation of the latter difficult). This potential effect of transcript levels would be consistent with the ability to suppress the requirement for *bldA* for Act and Red production with multiple copies of *act*II-ORF4 and *redZ*, respectively. A model for the regulation of Red production is shown in Fig. 7.

The existence of two pathway-specific regulatory genes for Red production in *S. coelicolor* parallels the situation for daunorubicin synthesis in *S. peucetius*, in which *dnrI* and *dnrN* are homologs of *redD* and *redZ*, respectively (41, 46). In *S. peucetius*, transcription of *dnrI* depends on *dnrN*, and transcription of several biosynthetic structural genes depends on *dnrI*. However, in contrast to the situation in *S. coelicolor*, a mutation in *dnrN* created by the insertion of an antibiotic resistance gene could be suppressed by multiple copies of *dnrI*. Unless the disrupted *dnrN* retained some residual activity, this indicates either incomplete transcriptional dependence of *dnrI* on *dnrN* or transcriptional readthrough into *dnrI* from a promoter present on the vector used in the cloning experiment. Unlike *redZ*, *dnrN* does not contain a TTA codon, and there are no reported *bldA* mutants in this strain. The lack of transcription of *redD* and *dnrI* in *redZ* and *dnrN* mutants, respectively, suggests that RedZ and DnrN are transcriptional activators of *redD* and *dnrI*, respectively. Consequently, it is interesting that

the promoter regions of *redD* (47) and of *dnrI* (35, 46) possess the sequence CTTGC-A-GATGGA located one and four nucleotides upstream of their respective transcriptional start sites; this sequence is not found in the promoter region of *act*II-ORF4 (17), for which there is no corresponding *redZ*/*dnrN* homolog. While this motif might have represented the binding site for the homologous RedZ and DnrN proteins, recent studies indicate that DnrN binds at two locations further upstream of the *dnrI* transcriptional start site, between nucleotides −37 and −55 and between nucleotides −62 and −100 (20a). Strikingly similar sequences are not apparent upstream of the *redD* transcriptional start site. Perhaps the sequences shared by *redD* and *dnrI* identify the binding site for another regulatory protein involved in the regulation of antibiotic production.

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