Vol. 190, No. 1

A New TetR Family Transcriptional Regulator Required for Morphogenesis in *Streptomyces coelicolor*[▽]

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Both morphogenesis and antibiotic production in the streptomycetes are initiated in response to starvation, and these events are coupled. We previously described a transposon-generated mutant in *Streptomyces coelicolor*, SE293, that resulted in a *bld* strain that overproduced the antibiotic actinorhodin. The SCO1135 open reading frame identified by the insertion encodes a member of the TetR family of transcriptional regulators. Here we show that a constructed deletion of the SCO1135 open reading frame resulted in the same morphological and antibiotic production phenotype as the insertion mutant. The constructed deletion also resulted in constitutive expression of SCO1135 transcript, as well as that of the gene cluster immediately adjacent to it, SCO1134-1132, which encodes a putative molybdopterin binding complex. A His$_6$-tagged version of the SCO1135 protein product was shown to bind the intergenic region between SCO1135 and SCO1134, which contains the apparent transcription start sites for each gene mapped by primer extension analysis. Increased expression of the SCO1134-1132 transcript in the SCO1135 deletion mutant also resulted in increased expression of xanthine dehydrogenase activity, confirming the predictions about these open reading framed based on protein similarity. We have designated the SCO1134-1142 gene cluster *xdhABC* and the regulator encoded by SCO1135 *xdhR*. We speculate that the inappropriate expression of xanthine dehydrogenase affects purine salvaging pathways at the onset of development, creating artificially high concentrations of both GTP and ppGpp and perturbing the pathways these molecules participate in for the initiation of morphogenesis and antibiotic production.

The interpretation of environmental signals plays a key role in the ability of an organism to survive starvation, adapt to environmental changes, and initiate new programs of development. Bacteria must respond quickly and effectively to the constantly changing environments in which they live, and regulatory proteins that respond to small molecule signals or morphogens serve to activate or repress the transcription of genes that allow the organism to adapt. The regulator proteins control interconnected and often complex networks that involve the interaction of multiple signaling pathways. Signal transduction typically involves the binding of a small molecule to a transcriptional regulator to modulate its activity on gene expression. The TetR family is a common class of transcriptional regulator with more than 2,000 members found in a wide variety of bacteria, but only about 100 have been fully characterized (21). The first member of the group, TetR, was identified in *Escherichia coli* and controls expression of the genes encoding a tetracycline efflux pump responsible for drug resistance conferred by Tn*10*. In the absence of tetracycline, TetR binds to the *tet* promoter and represses transcription of the efflux pump genes. When tetracycline enters the cell it binds TetR and causes conformational changes within the protein that abolish protein binding, thus relieving repression (23). RsrA, another member of this class, has been shown to activate transcription of *rpoS*, a sigma factor responsible for transcription of stationary-phase genes in *Pseudomonas putida*, in response to cell density (3).

TetR-like regulators contain a conserved helix-turn-helix DNA-binding domain, form homodimers, and generally act as repressors of transcription. They function to regulate a wide range of cellular activities including drug efflux, antibiotic production, amino acid metabolism, and development (21). There are 151 predicted TetR-like transcriptional regulators in the *Streptomyces coelicolor* genome (2, 21). Two (ActII and CprB) are repressors of actinorhodin biosynthesis, two (Pip and PqrA) are repressors of drug resistance genes, and one (ScbR) is a repressor of γ-butyarlactone synthesis (1, 6–8, 27).

Both morphogenesis and antibiotic production in the streptomycetes are initiated in response to starvation. Upon sensing starvation, the substrate mycelia release small molecules that act as signals for the initiation of aerial hyphal growth, as well as for the production of antibiotics (29, 30). As the aerial hyphae grow, they coil and septate into uninucleoid cells that give rise to spores. Most of what is known about this process comes from the study of mutants that fail to produce aerial hyphae, called *bld* mutants, or those that initiate aerial hyphal growth but fail to produce mature spores, called *whi* mutants (13).

We previously described a transposon-generated mutant, SE293, that resulted in a *bld* strain that overproduced the antibiotic actinorhodin (25). The SE293 mutant also required arginine for growth on minimal medium (25). The SCO1135 open reading frame identified by the insertion encodes a member of the TetR family of transcriptional regulators. Here we show that a constructed deletion of the SCO1135 open reading frame resulted in the same phenotype as the insertion mutant but was prototrophic, suggesting that the auxotrophy of the original mutant resulted from a second, unrelated mutation. Deletion of the SCO1135 open reading frame resulted in con-

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TABLE 1. Primers used in this study

Primer and purpose	Sequence
For construction of His$_6$-tagged XdhR protein	
His$_6$-XdhR Forward	AACCCGAAAGGAGGACATATGCCGCAGCCGAAGAAG
His$_6$-XdhR Reverse	GTGGAGACCGCCGAATTCGTCGAGCGCGCG
For RT-PCR	
XdhA RT Forward	CACCCTCGCCGACGTCCAGCGCC
XdhA RT Reverse	TGCCTTCGATGGTGGTGATCTCG
XdhR RT Forward	GCTCGGACGCCCAGCGCAACCGC
XdhR RT Reverse	CCATCCACTGGCGCAGGGCCAGG
WhiB Forward	GTCGACGACGCGGACGAGGAA
WhiB Reverse	AGATGCCGAAGCGCTCGTCGT
WhiG Forward	TGTGGCGGTCGTACAAGACGA
WhiG Reverse	ATCGCGTACGTCTCGAACTTG
WhiH Forward	AGCTGGGCCAGATGATCGTCT
WhiH Reverse	AAGGCACGCCATTCGATGATG
HrdB Forward	CGGCCGCAAGGTACGAGTTGATGA
HrdB Reverse	CCATGACAGAGACGGACTCGGCG
For construction of *xdhR* mutant	
XdhA Upstream	CGGGCAGGGCACGTTCTACCGCAACTTCCCGAACCGCGAATTCCGGGGATCCGTCGACC
XdhA Downstream	GACGCCCCGAACGCCGCTACCGCTTCCGGGGCGCGGTCATGTAGGCTGGAGCTGCTTC
P1	ATTCCGGGGATCCGTCGACCTGCA
P2	TGTAGGCTGGAGCTGCTTCGAAGT
For primer extension	
XdhA PE	AGACACCAAAGAAGGCTGATCAT
XdhR PE	AACCCGAAAGGAGGACGAGTGCC
PE seq Forward	GCGCTCGCGGTTGCGCTGGGCGT
PE seq Reverse	GTCAGCTGGTGCTTCTCGCCGTT

stitutive expression of the SCO1135 transcript, as well as that of a molybdopterin binding complex encoded by the gene cluster immediately adjacent to it, and increased the levels of xanthine dehydrogenase (XDH) activity, confirming the predictions about these open reading frames based on protein similarity. The SCO1135 gene product is a DNA-binding protein that interacts directly with the intergenic region between SCO1135 and SCO1134, which contains the apparent transcription start sites for both genes, to repress transcription.

MATERIALS AND METHODS

Strains and growth conditions. General techniques for bacterial growth were performed as described previously for *S. coelicolor* (16) and *E. coli* (24), respectively. *S. coelicolor* strains were grown on mannitol-soya flour (MS) agar medium with the addition of 10 mmol MgCl2 for mating experiments. Antibiotic selections were applied by overlay with soft nutrient agar (NSA). *Streptomyces* RNA was isolated from cells grown on cellophane discs placed on top of maltose-yeast extract-malt extract (MYM) agar. *E. coli* strains and growth conditions for the preparation of cosmids for marker replacement in *Streptomyces* were as described previously (9). The *S. coelicolor* strains used in the present study were M145 (SCP1$^-$ SCP2$^-$), SE293/*xdhR* (M145 SCO1135::Tn5::*apr*), and Δ*xdhR*::*aac(3)IV* [M145 SCO1135::*acc(3)IV*]. *E. coli* strains ET12576 [Δ(*dam dcm*)] containing the nontransmissible helper plasmid pUZ8002 and BL21(DE3) [F$^-$ *dcm ompT hsdS* (r$_B^-$ m$_B^-$) *gal*λ(DE3)] were used for mutant construction and protein expression, respectively. Primers used to amplify fragments for construction of the His tag fusion, construction of mutants, primer extension reactions, and reverse transcriptase PCRs are listed in Table 1.

Construction and confirmation of deletion mutants. A deletion of the SCO1135 open reading frame was constructed by using the PCR targeting method described by Gust et al. The Δ*xdhR*::*aac(3)IV* mutant was made by replacing the SCO1135 open reading frame with the apramycin resistance cassette [acc(3)IV]. The deletion extended from position +200 with respect to the translation start site to the end of the open reading frame, eliminating 130 of the 196 amino acids, allowing analysis of the 5′ end of the transcript of this gene in the deletion mutant itself. The presence and location of the apramycin resistance cassette was detected using the specific primers P1 and P2. The extent and location of the deletion was also confirmed by PCR. Marker replacement of the deletion with a wild-type copy of *xdhR* was accomplished by the same method using cosmid 2STG38.

Scanning electron microscopy. The procedure used in this analysis was as previously described (22). Samples from colonies grown for 5 days on MYM agar plates were mounted on a an aluminum stub with O.C.T. compound, submerged in liquid nitrogen slush at approximately −210°C and transferred to a Gatan Alto 2500 cryostage and cryoprep chamber (Gatan UK, Oxford, United Kingdom) attached to a LEO 982 field emission scanning electron microscope (LEO Electron Microscopy, Inc., Thornwood, NY). Samples were sublimated to remove surface frost at −95°C for 3 min, coated with platinum, placed on the cryostage in the main chamber of the microscope, at approximately −140°C, and viewed at 5.0 kV.

RT-PCR. RNA was isolated from *S. coelicolor* M145 and the Δ*xdhR*::*aac(3)IV* mutant after 24, 48, and 72 h of growth on MYM agar medium overlaid with cellophane discs as for primer extension analysis. The One-Step PCR kit (Qiagen) was used with primers specific for each gene. The forward and reverse primers for *xdhA*, *xdhR*, *whiG*, *whiB*, *whiH*, and *hrdB* are listed in Table 1. Reaction mixtures contained 10 pmol of each primer and 100 ng of RNA in a total volume of 20 μl. Each primer was first tested using chromosomal DNA as a template and without a reverse transcription (RT) cycle to test for DNA contamination in the RNA. Reactions were run for up to 35 cycles with wild-type RNA, sampling every 5 cycles between 20 and 35 in order to determine the linear range of product formation. In all cases the 25th cycle was in the linear range and was chosen as the assay point. The experiments were done in triplicate, and the experiment shown in the figure is representative. HrdB was used as a control for RNA concentration. Products were displayed on a 1% agarose gel and visualized by staining with ethidium bromide.

Gel retardation assays. To construct a His-tagged version of the XdhR protein, the coding region was amplified by PCR from *S. coelicolor* genomic DNA using the primers His$_6$-XdhR Forward and His$_6$-XdhR Reverse (Table 1). The fragment was digested with NdeI (NEB) and EcoRI (NEB) and cloned into NdeI/EcoRI-digested pET28a. XbaI and HindIII digestion confirmed the correct orientation and fusion to the His tag. Expression and purification of the His$_6$-
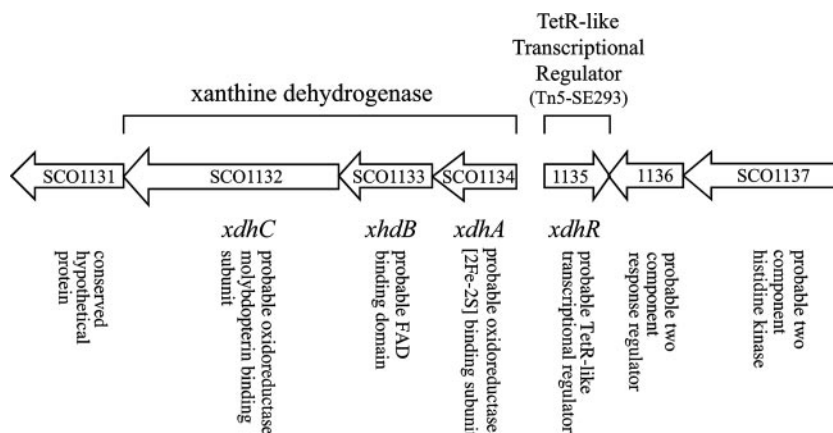
FIG. 1. Organization of the *S. coelicolor* genome showing the *xdhR* open reading frame identified by transposon insertion and adjacent genes. Gene notations are based on the Sanger Centre Sequencing Projects (http://www.sanger.ac.uk/Projects/S_coelicolor).

XdhR protein from *E. coli* was as previously described (12). Purified His$_6$-XdhR protein (5, 10, or 20 µg) or crude cell extract (20 µg of total protein) was mixed with the 305-bp PCR-generated DNA fragment containing the intergenic region between SCO1134 and SCO1135 used for primer extension experiments. The fragment was 5′ end labeled with [γ-^{32}P]ATP (MP Biomedicals) using T4 polynucleotide kinase (Promega) and purified on a 1% agarose gel. The labeled DNA fragment (1 ng; 6,000 cpm) was incubated with cell extracts or purified His-tagged XdhR protein for 20 min at 30°C in 20 µl (total volume) of binding buffer (20 mM Tris, 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [pH 8.0]) containing 1 µg of sonicated salmon sperm DNA and 3 µg of bovine serum albumin. For competitive inhibition of the binding reaction, 100 ng of unlabeled fragment or 100 µg of sonicated salmon sperm was added to 5 µg of the purified His$_6$-XdhR protein. Reactions were displayed on a nondenaturing 6% acrylamide Tris-borate-EDTA gel and visualized by autoradiography.

RNA isolation and primer extension analysis. Primer extension reactions were carried out as described previously (24). RNA was isolated from cells grown for 48 h on MYM agar plates overlaid with cellophane discs. Primers were labeled by using [γ-^{32}P]ATP 6,000 Ci/ml and OptiKinase (USB) according to the manufacturer's instructions. Then, 40 µg of RNA was hybridized to 1 pmol of either XdhA primer extension (PE) primer or XdhR PE-labeled primer by heating reactions to 65°C for 20 min and then allowing them to cool to room temperature for 10 min. The annealed primer and RNA mixture was added to a reaction mixture containing 40 mM sodium pyrophosphate and 1 U of avian myeloblastosis virus reverse transcriptase (Promega), followed by incubation at 42°C for 30 min. A 305-bp fragment, generated by PCR using the primers PE seq Forward and PE seq Reverse from chromosomal DNA, was used as a template. This fragment was also used to generate the DNA sequence ladder with the Fmol Cycle DNA Sequencing System (Promega) with 1 pmol of 5′ primer labeled with 6,000 Ci of γ-^{32}P/ml. Products from the DNA sequencing reactions were separated on a 6% denaturing polyacrylamide gel, and bands were visualized by autoradiography.

XDH and AOR assays. Cells were grown for 36 h at 30°C in YEME medium, harvested by centrifugation at $3,500 \times g$ for 10 min, and resuspended in extraction buffer (0.1 M Tris-HCl [pH 7.6], 10 mM MgCl$_2$, 6 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride). Extracts were obtained by first homogenization by sonication, followed by centrifugation at $12,000 \times g$ for 15 min at 4° (16). XDH activity was measured spectroscopically as conversion of NAD$^+$ to NADH at 340 nm in 50 mM sodium pyrophosphate buffer (pH 8.5) containing 0.2 mM EDTA, 0.15 mM xanthine, and 0.5 mM NAD$^+$ (14). Then, 20 µg of cell extract was added, and measurements were taken at 5-, 10-, 15-, and 20-min intervals. The aldehyde oxidoreductase (AOR) activity was determined by reduction of the electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm in 50 mM Tris-HCl buffer (pH 7.6) containing 35 µM DCPIP and 50 µM acetylaldehyde (28). Next, 20 µg of cell extract was added, and measurements were taken at 5-, 10-, 15-, and 20-min intervals. Xanthene dehydrogenase (XDH) and AOR activities were normalized to glutamate dehydrogenase activity. The glutamate dehydrogenase activity was assayed by measuring NADH oxidation at 340 nm in 50 mmol of triethanolamine buffer containing 50 mmol of ammonium sulfate, 200 nmol of NADH, and 10 mmol of 2-oxoglutarate. Reactions were

measured every 6 min (20). Protein concentrations were determined by the Bradford assay (4).

RESULTS

A deletion of the *xdhR* open reading frame results in a *bld* mutant phenotype. A mutation in *xdhR* was first identified by a transposon insertion into the SCO1135 open reading frame (25). The location of SCO1135 and the organization of the genes on the chromosome near it are shown in Fig. 1. The *xdhR* open reading frame encodes a TetR family transcriptional regulator and is located directly adjacent to but in the opposite orientation of a gene cluster, *xdhA* (SCO1134), *xdhB* (SCO1133), and *xdhC* (SCO1132), predicted to encode a molybdopterin binding protein complex. The transposon mutant was completely defective in morphogenesis, failing to make the aerial hyphae associated with the initiation of development but overproduced the blue pigment associated with the polyketide antibiotic actinorhodin. While the original mutant had a single copy of the transposon, it also required arginine for growth on minimal medium. The arginine auxotrophy was presumed to be unrelated to the morphological phenotype (25). A deletion of the *xdhR* open reading frame was constructed by using the targeted marker replacement method developed by Gust et al. (9). The deletion mutant, Δ*xdhR::aac(3)IV*, had the same morphological phenotype as the insertion mutant but was able to grow on minimal medium, suggesting that the arginine auxotrophy was most likely a second and unrelated mutation.

Scanning electron microscopy of the Δ*xdhR::aac(3)IV* mutant (Fig. 2) grown on MYM sporulation medium revealed normal substrate mycelia but no evidence of aerial hyphae or spores. Unlike many *bld* mutants that are substantially delayed in morphogenesis but will eventually produce some spores, the Δ*xdhR::aac(3)IV* mutant never produces either aerial hyphae or spores even with prolonged (more than 10 days) growth on sporulation agar.

The morphogenic and antibiotic production phenotypes of the Δ*xdhR::aac(3)IV* mutant were not complemented with the wild-type allele of *xdhR* introduced on a pSET plasmid integrated at the φC31 attachment site. Similar experiments with the original

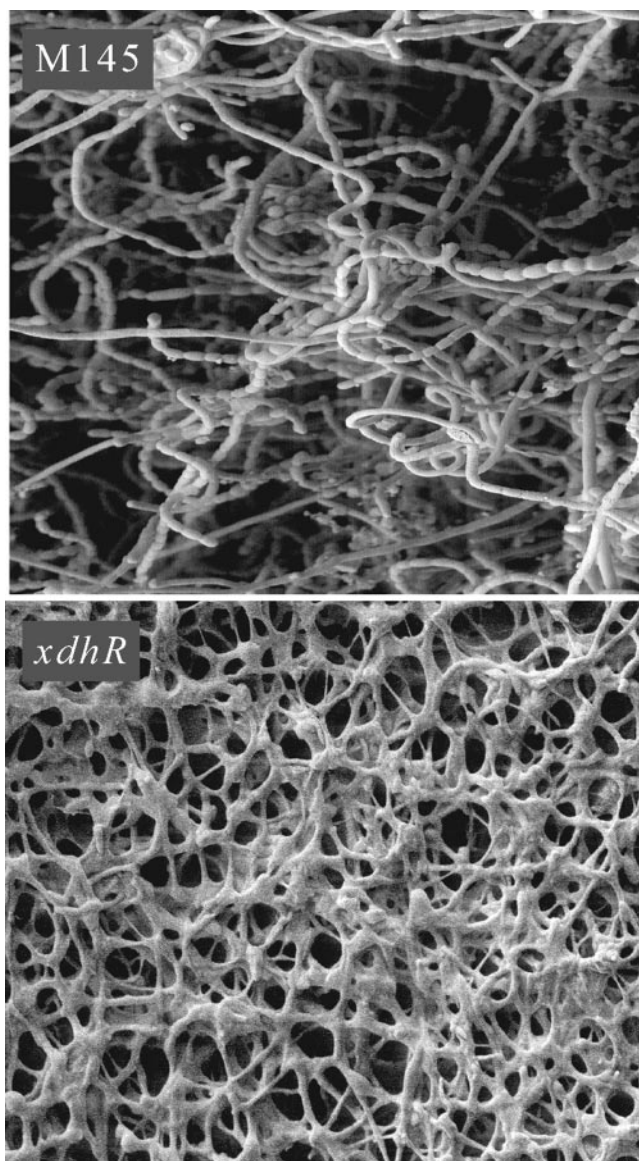FIG. 2. Scanning electron micrographs of wild-type *S. coelicolor* (M145) and the *xdhR* mutant [Δ*xdhR*::*aac(3)IV*] at ×2,000 magnification. Both strains were grown for 5 days at 30°C on MYM agar medium.

FIG. 3. RT-PCR analysis of transcripts from various morphological mutants in wild-type *S. coelicolor* (M145) and the *xdhR* deletion mutant [Δ*xdhR*::*aac(3)IV*]. *hrdB* was used as a control for RNA.

SE293 transposon insertion mutant also failed to show complementation by the wild-type allele (25). To test whether the observed phenotypes were due to the *xdhR* mutation, cosmid 2STG38 was introduced into the Δ*xdhR*::*aac(3)IV* mutant, and exconjugants containing a marker replacement of the mutation with the wild-type allele were obtained. The resulting strain was wild type. This suggests that the *xdhR* mutation, in fact, caused the defects in morphogenic and antibiotic production and while it is not clear why the mutation is not complemented by a wild-type copy of the gene, it raises the possibility that the *xdhR* gene product does not work in *trans*.

The *xdhR* gene product acts to repress its own transcription, as well as the transcription of an adjacent gene cluster that encodes a putative molybdoterin binding complex. To test
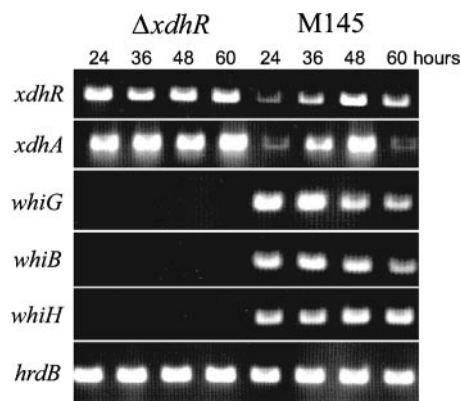
whether a mutation in *xdhR* affected regulation of itself or the adjacent gene cluster, RT-PCRs were performed with RNA isolated from wild-type *S. coelicolor* and the Δ*xdhR*::*aac(3)IV* mutant that had been grown for 24, 36, 48, or 60 h on MYM sporulation medium. This solid medium supports morphological development of the wild type, and the stages of development are clearly detectable. Cells harvested after 16 h were growing vegetatively (no aerial mycelia present). Aerial mycelia began to appear between 24 and 36 h and were abundant between 36 and 48 h, as was the blue pigment associated with actinorhodin production. The gray pigment associated with mature spores was evident at 60 h.

As shown in Fig. 3, transcript from *xdhA*, the first of a series of the genes adjacent to *xdhR*, was detected at low levels during growth phase and at higher levels as development proceeded with a peak in wild-type cells coincident with the aerial hyphae production. Transcription of *xdhA* was constitutive in the Δ*xdhR*::*aac(3)IV* mutant. RT-PCRs were also performed with RNA isolated from wild-type *S. coelicolor* and the Δ*xdhR*::*aac(3)IV* mutant to examine the effect of the mutant on its own expression. As shown in Fig. 3, in the wild-type strain, transcript from *xdhR* was similar to that of the adjacent cluster, present at low levels during growth phase and at higher levels as development proceeded with a peak in wild-type cells coincident with the aerial hyphae production. The transcription of *xdhR* was constitutive in the Δ*xdhR*::*aac(3)IV* mutant. The same RNA preparations used for this analysis were used for detection of the *hrdB* transcript, which served as a control for the level of RNA (Fig. 3). The PCR products shown are samples taken during the exponential phase of the PCR so that the amount of product is representative of quantitative differences in RNA level. These data suggest that the *xdhR* gene product acts to repress, either directly or indirectly, transcription of itself as well as the genes in the adjacent gene cluster.

The *xdhR* gene product binds the intergenic region between *xdhR* and *xdhA* in vitro. To test whether the XdhR protein made direct contact with the *xdhR* and/or *xdhA* promoter regions, an His6-XdhR protein was constructed and expressed in *E. coli* and used in a gel mobility shift assay with a DNA fragment containing the intergenic region between *xdhR* and *xdhA*. As shown in Fig. 4, crude extracts from *E. coli* cells
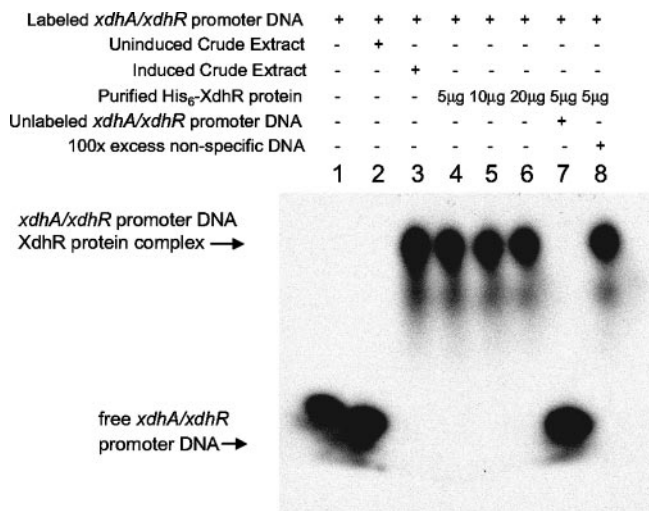
FIG. 4. Gel mobility shift assays using a DNA fragment containing the intergenic region between *xdhR* and *xdhA* and the His$_6$-XdhR protein. Lane 1, labeled fragment; lane 2, crude extract from uninduced cells containing the His$_6$-XdhR construction; lane 3, crude extract from induced cells containing the His$_6$-XdhR construction; lanes 4 to 6, labeled fragment with 5, 10, or 20 μg of purified His$_6$-XdhR protein; lane 7, labeled fragment with 100 ng of unlabeled promoter-containing fragment and 5 μg of purified His$_6$-XdhR protein; lane 9, labeled fragment with 100 μg of sonicated salmon sperm DNA and 5 μg of purified His$_6$-XdhR protein.
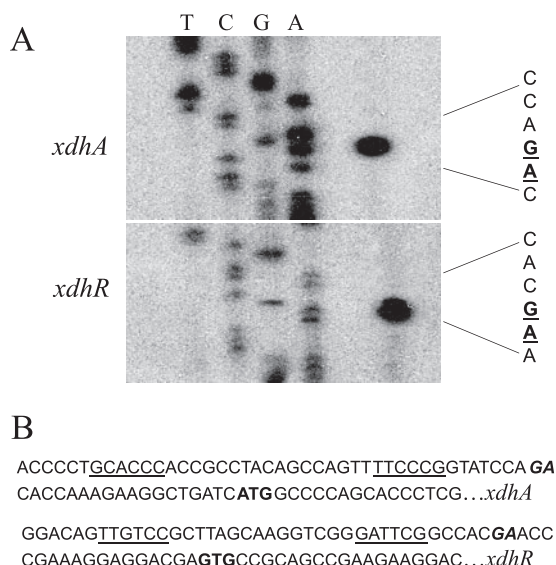

FIG. 5. (A) Primer extension analysis of transcripts originating upstream of *xdhR* and *xdhA*. (B) DNA sequence upstream of the apparent transcription start sites (indicated in boldface italics) of *xdhR* and *xdhA*. Potential RNA polymerase recognition sequences are underlined. The annotated translation starts are indicated by boldface with no italics.

expressing the His$_6$-XdhR protein retarded the promoter-containing fragment, whereas extracts from uninduced cells did not. Furthermore, the amount of probe shifted in the retardation assay was reduced in the presence of unlabeled probe but not nonspecific DNA, suggesting that binding of XdhR to the promoter-containing fragment was sequence or motif specific.

Primer extension analysis (Fig. 5A) was used to identify apparent transcription start sites for *xdhR* and *xdhA*. The start site of *xdhR* maps to an adenine or guanine nucleotide located 19 or 20 nucleotides upstream of the annotated translational start codon. The sequences centered at −10 and −35 with respect to the apparent start site show little homology to known consensus sequences for RNA polymerase binding. The start site for *xdhA* maps to guanine or adenine located 18 or 19 nucleotides upstream of the annotated translational start site and like *xdhR* the −10 and −35 regions show little homology to known RNA polymerase binding sites. The distance between the apparent transcription start sites of *xdhA* and *xdhR* is 150 bp. While the leader region of these predicted transcripts is relatively short, each contains a potential ribosome-binding site, in fact, the putative ribosome-binding site for *xdhR* was previously annotated in the genome sequence.

The *xdhR* mutant is defective in the expression of other developmental genes. The Δ*xdhR*::*aac(3)IV* mutation results in a severe *bld* phenotype. Many of the *bld* mutants affect the expression, either directly or indirectly, of *whi* genes. To test the effect of the Δ*xdhR*::*aac(3)IV* mutation on the expression of other developmental genes, RT-PCR was performed on RNA isolated from wild-type *S. coelicolor* M145 and the Δ*xdhR*::*aac(3)IV* mutant. Primers specific for *whiG*, *whiB*, *whiH*, *xdhA*, *xdhR*, and *hrdB*, a constitutively expressed gene as a control for RNA, were used. Although transcripts from

whiG, *whiB*, and *whiH* were readily detected from the wild-type strain, no expression of these genes was detected in the Δ*xdhR*::*aac(3)IV* mutant. The *whiG*, *whiB*, and *whiH* genes play early roles in the cascade of *whi* gene expression, and their expression is dependent on several *bld* genes. *xdhR* is clearly one of them.

The SCO1132-34 gene cluster likely encodes a XDH/AOR enzyme complex. The predicted protein products of the *xdhA* (SCO1134), *xdhB* (SCO1133), *xdhC* (SCO1132) gene cluster show significant similarity to a molybdopterin binding protein in the AOR/XDH family (19). XdhC shows 63% similarity (50% identity) to the molybdodenum binding subunit of *S. erythraea* XDH; *xdhB* shows 73% similarity (61% identity) to the flavin adenine dinucleotide (FAD)-binding subunit and *xdhA* shows 73% similarity (60% identity) to the [2Fe-2S] binding subunit (http://www.ncbi.nlm.nih.gov/BLAST/). Molybdenum is often bound to a pterin cofactor, and the synthesis of the molybdopterin cofactor has been shown to require more than 12 proteins that are highly conserved in all organisms (11). Molybdenum-containing enzymes perform a variety of functions, but all involve oxidation or reduction reactions. These enzymes take advantage of the ability of molybdenum to exist in a variety of oxidation states under physiological conditions. This allows the enzyme to catalyze redox reactions that require the movement of one or two electrons (11). Molybdenum enzymes are grouped in three families: the AOR/XDH family, the sulfite oxidase family, and the dimethyl sulfoxide reductase family. XDH enzymes are sometimes made up of multienzyme complexes or a single protein with multiple enzymatic functions or domains. Like many XDH and AOR proteins, the putative heterotrimeric protein encoded by the *xdhABC* gene cluster (Fig. 6) is composed a [2Fe-2S] iron-sulfur binding subunit, a FAD binding subunit, and a molyb-

A

xdhA	xdhB	xdhC
[2Fe-2S]	FAD	MOCO/Dimerization

XDH [2Fe-2S] | FAD Binding | MOCO/Dimerization

Hypoxanthine ⟶ Xanthine ⟶ Uric Acid

AOR [2Fe-2S] | FAD Binding | MOCO/Dimerization
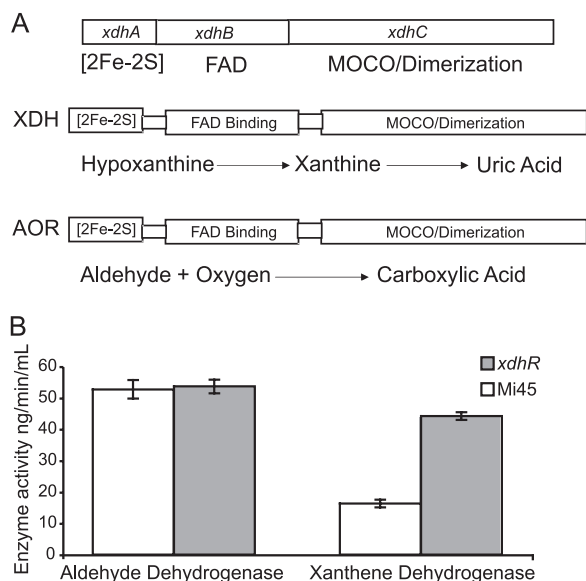
Aldehyde + Oxygen ⟶ Carboxylic Acid

B

FIG. 6. (A) Comparison of the predicted products of *xdhABC* with XDH and AOR. (B) Enzyme assays using cell extracts from either the wild type (M145) or the *xdhR* deletion mutant [$\Delta xdhR::aac(3)IV$]. The AOR activity was determined by the reduction of DCPIP. The XDH activity was measured spectroscopically as the conversion of NAD$^+$ to NADH at 340 nm.

dopterin binding/dimerization subunit (17). The enzyme catalyzes the reactions that convert hypoxanthine into xanthine and then xanthine to uric acid.

To test whether this gene cluster in fact encoded an XDH complex, enzyme assays were preformed on extracts from wild type and $\Delta xdhR::aac(3)IV$ mutant cells. XDH activity was determined spectrophotometrically by assaying conversion of NAD$^+$ to NADH at 340 nm in the presence of xanthine. In the presence of xanthine, the rate of NAD$^+$ to NADH increased 2.7-fold in the $\Delta xdhR::aac(3)IV$ mutant strain. AOR activity was assayed by reduction of DCPIP as measured by increased absorbance at 600 nm, and there was no significant difference between the wild type and the $\Delta xdhR::aac(3)IV$ mutant for this activity. Bradford assays were used to determine the protein concentration of cell extracts. All enzyme activities were normalized to glutamate dehydrogenase activity.

DISCUSSION

Scanning electron microscopy of the $\Delta xdhR::aac(3)IV$ mutant showed that the mutation resulted in the complete loss of morphological development (no aerial mycelium even after prolonged incubation on sporulation medium) while apparently increasing actinorhodin antibiotic production. RT-PCR analysis of transcripts from *xdhR* and *xdhABC*, the first gene in an adjacent gene cluster, suggested that *xdhR* acts to repress its own transcription, as well as the transcription of *xdhABC*. Gel mobility shift experiments using His$_6$-XdhR protein and the *xdhR-xdhA* intergenic region showed that the XdhR protein binds this region directly in vitro and primer extension analysis identified apparent transcription start sites for *xdhR* and *xdhABC* within this region. XDH assays showed that the level of enzyme activity was significantly increased in the *xdhR* mu-

tant supporting the prediction from BLAST analysis that the *xdhABC* gene cluster encodes a XDH complex. RT-PCR analysis of the transcription of other genes required for morphological development in *S. coelicolor* showed that *xdhR* is required for the transcription of *whiG*, *whiB*, and *whiH*.

XdhR is a member of the TetR family of transcriptional regulators and, like other members of this group, is a DNA-binding protein that acts to repress transcription. XDH is a molybdo-flavoenzyme that participates in purine catabolism and catalyzes the conversion of hypoxanthine to xanthine and of xanthine to uric acid. XDH is a heterodimeric protein composed of two [2Fe-2S] cluster-binding domains, an FAD binding domain, and a domain for dimerization and binding of the Moco molybdenum cofactor (5). Electrons from the substrate are passed from the Moco center to FAD by the two [2Fe-2S] clusters. Once electrons reach the FAD site, they are transferred either to molecular oxygen or to NAD$^+$ to form NADH. In *E. coli* the conversion of hypoxanthine to xanthine by XDH plays a role in the purine salvage pathway. Deletion mutants of *xdhA* were sensitive to exogenous adenine, a phenotype previously shown to be due to inefficient conversion of adenine to guanine due to decreased xanthine availability (18, 31).

Why should the overexpression of XDH result in loss of morphogenesis while increasing antibiotic production? The purine tetraphosphate, ppGpp, is produced under conditions of amino acid limitation by the activity of RelA (10), which phosphorylates GTP to ppGpp and pppGpp. These molecules have been implicated in the sensing of nutritional shifts in both *E coli* and *S. coelicolor*. Interestingly, *S. coelicolor relA* mutants are defective in antibiotic production and delayed in morphogenesis, but the delay in morphogenesis occurs only under conditions of nitrogen limitation (26). Antibiotic production is restored by overexpression of *relA*, suggesting that increased levels of ppGpp restore antibiotic production. *Streptomyces clavuligerus relA* mutants are defective in antibiotic production and are *bld* under all conditions (15). In *S. clavuligerus*, ppGpp synthesis is accompanied by depletion of the cellular GTP pool (15). If, as in *E. coli*, the XDH encoded by the *xdhABC* cluster is involved in purine salvage, under starvation conditions such as those that signal the initiation of morphogenesis and antibiotic production in *Streptomyces species*, induction of purine salvage might be a signal for the initiation of antibiotic production and morphological development. Inappropriate expression of this XDH might interfere with this signaling pathway and lead to a defect in the ability of the organism to appropriately sense or interpret starvation signals. Overexpression of a cluster could result in an abnormally high level of xanthine in the cells and an elevated level of GTP during the time when ppGpp synthesis is strongest. High levels of ppGpp synthesis that result in depletion of the intracellular GTP pool is normally enough to trigger morphological differentiation in *Streptomyces* species (15). A large GTP pool could cause an arrest in morphological development, while at the same time increased ppGpp production could cause overproduction of actinorhodin.

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