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AllR Controls the Expression of *Streptomyces coelicolor* Allantoin Pathway Genes

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Streptomyces **species are native inhabitants of soil, a natural environment where nutrients can be scarce and competition fierce. They have evolved ways to metabolize unusual nutrients, such as purines and its derivatives, which are highly abundant in soil. Catabolism of these uncommon carbon and nitrogen sources needs to be tightly regulated in response to nutrient availability and environmental stimulus. Recently, the allantoin degradation pathway was characterized in *Streptomyces coelicolor*. However, there are questions that remained unanswered, particularly regarding pathway regulation. Here, using a combination of proteomics and genetic approaches, we identified the negative regulator of the allantoin pathway, AllR. *In vitro* studies confirmed that AllR binds to the promoter regions of allantoin catabolic genes and determined the AllR DNA binding motif. In addition, effector studies showed that allantoic acid, and glyoxylate, to a lesser extent, inhibit the binding of AllR to the DNA. Inactivation of AllR repressor leads to the constitutive expression of the AllR regulated genes and intriguingly impairs actinorhodin and undecylprodigiosin production. Genetics and proteomics analysis revealed that among all genes from the allantoin pathway that are upregulated in the *allR* mutant, the *hyi* gene encoding a hydroxypyruvate isomerase (Hyi) is responsible of the impairment of antibiotic production.**

Streptomyces species are Gram-positive filamentous bacteria of high importance in industrial and medicinal applications. *Streptomyces* spp. represent one of our main sources of natural antibiotics and other secondary metabolites with important biological activities like eukaryotic cell differentiation inducers, apoptosis inhibitors, protein C kinase inhibitors, and compounds with antitumor activity (1). About two-thirds of the industrially manufactured antibiotics are produced by members of the *Streptomyceae* family (2). As native inhabitants of soil, *Streptomyces* spp. generally live in nutrient-limited and highly competitive conditions (3). As such, they have evolved sophisticated mechanisms to rapidly adjust to new nutritional and environmental conditions, through a plethora of catabolic and anabolic pathways (4), tightly regulated by uncharacterized regulatory mechanisms.

Purines and their derivatives (uric acid and allantoin) are abundant in soil, offering a rich source of nitrogen to its inhabitants (5, 6). Nutrient composition of soil has severe consequences on the microbial community; thus, increased levels of purines typically result in larger microbial diversity, as well as in living microbial biomass (6). Some bacteria are capable of metabolizing purine derivatives as both carbon and nitrogen sources, whereas others can only use them as a nitrogen source (7). Purine degradation processes also differ upon oxygen availability; for example, *Escherichia coli* and other members of the *Enterobacteriaceae* family can grow on allantoin as nitrogen source only under anaerobic conditions (8).

Despite the importance of purine catabolism, only a few reports have looked at its regulation in bacteria (9–11). In *E. coli*, for example, the genes encoding the enzymes involved in allantoin catabolism are organized in three transcriptional units that constitute the allantoin regulon. These transcriptional units are coordinately regulated by the repressor protein AllR and an activator protein AllS (9). In contrast, in *Bacillus subtilis*, a unique transcriptional regulator, PucR, controls the expression of genes for allantoin metabolism and other purine catabolic enzymes (10, 11).

In *Streptomyces coelicolor*, we have recently identified seven new functional enzymes of the allantoin pathway namely allantoinase (AllB), allantoicase (Alc), malate synthase (AceB1), glyoxylate carboligase (Gcl), tartronate semialdehyde reductase (GlxR), hydroxypyruvate isomerase (Hyi), and ureidoglycolate lyase (Ugl) (12). Most of these genes are encoded in two distantly located gene clusters. One of them contains the genes *allB*, *alc*, and *aceB1*; the second one, located in a different region of the genome, comprises *gcl*, *glxR*, and *hyi* (Fig. 1A). In the present study, the transcriptional regulator AllR, which controls expression of the allantoin catabolic genes, was characterized. The relevance of the transcriptional regulator was evident by the fact that construction of an *allR* mutant had remarkable consequences in actinorhodin and undecylprodigiosin production.

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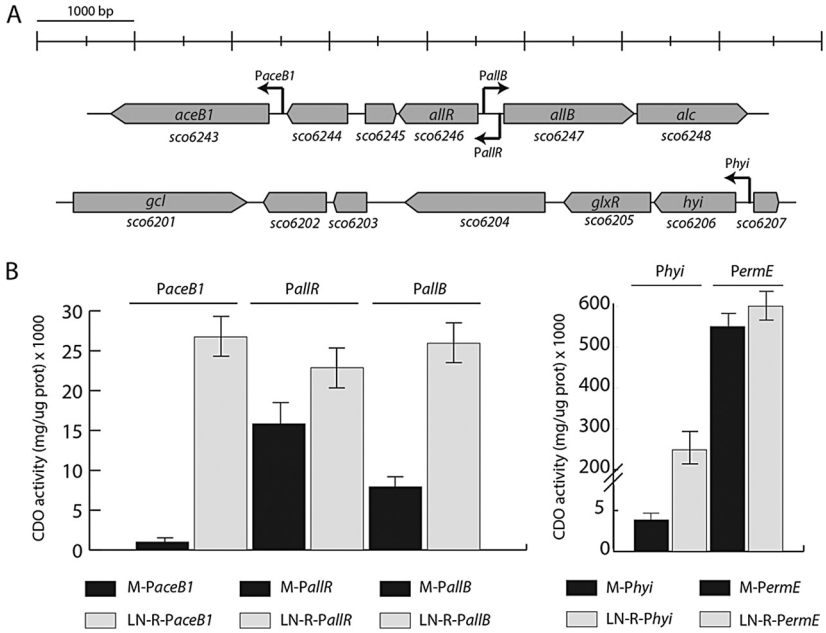

FIG 1 (A) Allantoin gene cluster. (B) Effect of *allR* mutation on the expression of allantoin pathway genes. *S. coelicolor* strains M145 and LN-R harboring a transcriptional fusion of the P*aceB1*, P*allB*, P*allR*, P*hyi*, or P*ermE* promoters to the *xylTE* cassette were grown in SMM for 24 h. The CDO specific activity was assayed in cell extracts of the individual cultures. The results represent the averages of three independent experiments ± the standard deviations (SD).

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* strains were grown either on solid or liquid Luria-Bertani medium at 37°C and supplemented with 100 mg/liter ampicillin (Ap), 50 mg/liter kanamycin (Km), 20 mg/liter chloramphenicol (Cm), or 50 mg/liter apramycin (Am) when needed. *Streptomyces* strains were grown at 30°C on soy-mannitol agar or supplemented liquid minimal medium (SMM) using glucose as a carbon source with Am, hygromycin (Hyg), or Km at final concentrations of 50 mg/liter when needed (13). The strains and plasmids used in the present study are shown in Table 1.

Construction of mutant strains of *S. coelicolor*. To disrupt the *allR* gene, the 2H4.2.D01 cosmid from the transposon insertion cosmid library of *S. coelicolor* was used (14). The Am resistance (Am^r) marker of Tn*5062* in cosmid 2H4.2.D01 was replaced by the Hyg^r marker from Tn*5066* using the plasmid pQM5066 through Red-mediated recombination in *E. coli* (15). The resultant 2H4.2.D01 Hyg^r cosmid was introduced into *S. coelicolor* M145 by conjugation using *E. coli* ET12567/pUZ8002 as a donor (16). Two independent Hyg^r Km^s exconjugants were isolated and checked by PCR using specific primers for the *allR* gene, 5′-CATATGTCCGAAGCTGAACTG-3′ (upper) and 5′-ACTAGTTCAGGCGGCCGGGTTGCC-3′ (lower), and for the transposon, 5′-ATGCGCTCCATCAAGAAGAG-3′ (EZR1) and 5′-TCCAGCTCGACCAGGATG-3′ (EZL2), verifying that allelic replacement had occurred. To isolate LN-RA, LN-RG, and LN-RH mutant strains, cosmids 2H4.2.A01, SC2G5.2.F07, and 2G5.2.G11, respectively, were individually introduced into *S. coelicolor* LN-R (Hyg^r) by conjugation using *E. coli* ET12567/pUZ8002 as a donor. For each mutant, two independent Am^r Hyg^r Km^s exconjugants were isolated and checked by PCR using specific primers for the *aceB1*, *gcl*, *hyi* genes, 5′-GCGGACGACTCCTGGAAG-3′ (upper) and 5′-TGCCGAGGTCCAGCAGAC-3′ (lower), 5′-CATATGGCTCGTATGACCGCTGC-3′ (upper) and 5′-ACTAGTCAGACCTTCAGCGTCCT-3′ (lower), and 5′-CATATGGGATTCGCAGACCAGCGCTTC-3′ (upper) and 5′-ACTAGTCAGCGGGCCGCGCGGGCCCC-3′ (lower), respectively, and for the transposon (primers EZL1 and EZL2). For construction of LN-RBC mutant strain a transferable suicide plasmid (pLN04) was introduced into *S. coelicolor* LN-R (Hyg^r) by conjugation using *E. coli* ET12567/pUZ8002 as a donor. Plasmid pLN04 was constructed by cloning *allR* and *alc* genes into a XbaI-SphI fragment of pSET152 vector (16). Selected Am-sensitive (Am^s) Hyg^s candidates were checked by Southern blot using an adequate ^{32}P-labeled probe, verifying that correct allelic replacement had occurred. The lack of expression of *allR* and *alc* genes was verified by semiquantitative reverse transcription-PCR (sqRT-PCR) using the primers 5′-ACGGGGACGAGATCGTGT-3′ (upper) and 5′-GCGTGGTGATGGTCTTGTC-3′ (lower) and the primers 5′-ACGCCTCCAACCTCTTCTAC-3′ (upper) and 5′-ATCCAGTCGTTGCCCTTG-3′ (lower), respectively.

Assay of catechol 2,3-dioxygenase in cell extracts. DNA fragments corresponding to positions 255 bp upstream of *allB* (P*allB*), 255 bp upstream of *allR* (P*allR*), 184 bp upstream of *aceB1* (P*aceB1*), or 156 bp upstream of *hyi* (P*hyi*) translation start sites, were amplified by PCR using the primer pairs 5′-GCGGCCGCACGTTCCTTTCGGGG-3′ (upper) and 5′-CATATGCCGGATCCCCTTCTCTGC-3′ (lower), 5′-TTTCGGACACCCGGATCCCCTTCTCTG-3′ (upper) and 5′-TTTCATATGACGTTCCTTTCGGGGCTG-3′ (lower), 5′-GGATCCTTGAGTGAGCGAGGTGGC-3′(upper)and5′-CATATGCTGTCACTTCCTTCAGC-3′(lower), and 5′-GCGGCCGCTCACCGCTCGCCCTCTCCC-3′ (upper) and 5′-CATATGCCACGGCGCGAGGGAAGGCG-3′ (lower), respectively, using *S. coelicolor* M145 genomic DNA as the template. Each fragment was cloned upstream of the reporter genes *xylTE* encoding catechol 2,3-dioxygenase using plasmid pB130 (17) to give pJM27 (P*allR*), pJM28 (P*aceB1*), pJM31 (P*allB*), and pLN39 (P*hyi*). Plasmids containing amplified promoters were verified by DNA sequencing. Each plasmid was introduced into M145 and LN-R strains and Km^r exconjugants were isolated (Table 1). Strains containing an ectopic integration of *xylTE* genes under the control of the promoter *PaceB1* (M-P*aceB1* and LN-R-P*aceB1*), P*allB* (M-P*allB* and LN-R-P*allB*), *PallR* (M-P*allR* and LN-R-P*allR*), or *Phyi* (M-P*hyi* and LN-R-P*hyi*) were used to assay catechol 2, 3-dioxygenase activity at 24 h of growth in SMM. Catechol 2, 3-dioxygenase activity was measured as indicated in Kieser et al. (13). The protein concentration was determined with Bradford reagent (18). Catechol 2,3-dioxygenase activities were standardized to protein concentration.

Cloning of *allR*, *glxR*, and *hyi* genes. *allR* (*sco6246*), *glxR* (*sco6205*), and *hyi* (*sco6206*) genes were PCR amplified from *S. coelicolor* M145

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
S. coelicolor		
M145	Parental strain, SCP1$^-$ SCP2$^-$	13
LN-R	M145, *sco6246*::Tn*5066* (Hyg^r)	This study
LN-RA	LN-R (Hyg^r), *sco6243*::Tn5062(Am^r)	This study
LN-RG	LN-R (Hyg^r), *sco6201*::Tn5062(Am^r)	This study
LN-RH	LN-R (Hyg^r), *sco6206*::Tn5062(Am^r)	This study
LN-RBC	LN-R (Hyg^r), Δ*PallB-sco6247*	This study
LN-Rc	LN-R (Hyg^r), *attB*$_\Phi$BT1::pLN01(Km^r)	This study
LN-RHc	LN-R (Hyg^r), *attB*$_\Phi$BT1::pLN35(Km^r)	This study
M-P*aceB1*	M145, *attB*ΦBT1::pJM28(Km^r)	This study
LN-R-P*aceB1*	LN-R (Hyg^r), *attB*ΦBT1::pJM28(Km^r)	This study
M-P*allB*	M145, *attB*ΦBT1:: pJM31(Km^r)	This study
LN-R-P*allB*	LN-R (Hyg^r), *attB*ΦBT1::pJM31(Km^r)	This study
M-P*allR*	M145, *attB*ΦBT1::pJM27(Km^r)	This study
LN-R-P*allR*	LN-R (Hyg^r), *attB*ΦBT1::pJM27(Km^r)	This study
M-P*hyi*	M145, *attB*ΦBT1::pLN39(Km^r)	This study
LN-R-P*hyi*	LN-R (Hyg^r), *attB*ΦBT1::pLN39(Km^r)	This study
E. coli		
DH5α	*E. coli* K-12 F$^-$ *lacU169*(ϕ80*lacZ*ΔM15) *endA1 recA1 hsdR17 deoR supE44 thi-1 l2 gyrA96 relA1*	Life Technologies
ET12567	*supE44 hsdS20 ara14 proA2 lacY galK2 rpsL20 xyl 5 mtl 1 dam dcm-hsdM* (Cm^r)	16
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdSB*(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7* gene 1 ind1 sam7 nin5])	Stratagene
Plasmids		
pB130	Phagemid plasmid carrying *xylTE* genes; Ap^r	17
pET28a	Phagemid vector for expression of recombinant proteins under the control of strong T7 transcription and translation signals	Novagen
pJM27	pXE2 derivative carrying *xylTE* genes under the P*allR* promoter	This study
pJM28	pXE2 derivative carrying *xylTE* genes under the P*aceB1* promoter	This study
pJM31	pXE2 derivative carrying *xylTE* genes under the P*allB* promoter	This study
pLN01	pRT802 derivative carrying *allR* gene under the P*allR* promoter	This study
pLN02	pET28a derivative with an insert carrying a *sco6246* His tag fusion gene under the control of strong T7 transcription and translation signals	This study
pLN04	Mobilizable suicide vector derivative of pSET152 carrying genes the *allR* and *alc*	This study
pLN16	pET28a derivative with an insert carrying a *sco6206* His tag fusion gene under the control of strong T7 transcription and translation signals	This study
pLN17	pRT802 derivative carrying a *hyi* gene under the P*ermE** promoter	This study
pLN24	pET28a derivative with an insert carrying a *sco6205* His tag fusion gene under the control of strong T7 transcription and translation signals	This study
pLN35	pRT802 derivative carrying *hyi* gene under the P*hyi* promoter	This study
pLN39	pXE2 derivative carrying a *xylTE* genes under the P*hyi* promoter	This study
pRT802	Integrative vector based on ΦBT1 integrase; Km^r	19
pSET152	Integrative vector based on ΦC31 integrase; Am^r	16
pUZ8002	RK2 derivative with defective *oriT* (Km^r)	16

[a] Cm^r, chloramphenicol resistance; Am^r, apramycin resistance; Hyg^r, hygromycin resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

genomic DNA using the following oligonucleotides: 5′-**CATATG**TCCG AAGCTGAACTG-3′ (upper) and 5′-**GAATTCACTAGT**TCAGGCGGC CGGGTTGCC-3′ (lower), 5′-**CATATG**AGCACGCTCCCAAGGTCG-3′ (upper) and 5′-**GAATTCACTAGT**TCAGACCTGGTCGCCGGAG (lower), and 5′-**CATATG**GGATTCGCAGACCAGCGCTTC-3′ (upper) and 5′-**GAATTCACTAGT**CAGCGGGCCGCGCGGGCCCC-3′ (lower), respectively. The upper primers used were designed to have an NdeI site (indicated in boldface) overlapping the translational initiation codon, changing GTG start codon to ATG for *allR* gene. The lower primers contained EcoRI and SpeI sites (indicated in boldface) downstream of the stop codon. The resulting PCR products were cloned as NdeI-EcoRI fragments into a derivative the expression vector pET28a, which contains six His codons upstream of the NdeI site, obtaining pLN02, pLN24, and pLN16, respectively. Each NdeI-EcoRI insert in pLN02, pLN24, and pLN16 was verified by DNA sequencing. For complementation studies in *S. coelicolor*, each NdeI-SpeI fragment from pLN02 and pLN16 was cloned into the integrative vector pRT802 (19) containing the *allR* promoter or the *hyi* promoter to yield pLN01 (c_*allR*) and pLN35 (c_*hyi*), respectively. For the overexpression experiments, NdeI-SpeI fragment from pLN16 was cloned into the integrative vector pRT802 containing the *ermE** promoter to give pLN17. Plasmid pLN17 was introduced into *S. coelicolor* M145 by conjugation.

Expression and purification of AllR, Hyi, and GlxR. *E. coli* BL21(DE3) host strains (Stratagene) carrying each plasmid (pLN02, pLN16, or pLN24) were grown at 37°C in Luria-Bertani medium, induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and incubated for 20 h at 30°C. The cells were harvested by centrifugation at 4,000 × *g* for 20 min at 4°C, washed twice, and resuspended with buffer containing 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 10% glycerol, and 10 mM $MgCl_2$ (buffer A). Cell disruption was carried out in a French pressure cell at 1,000 MPa in the presence of 0.1% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich). The protein extract was cleared by

centrifugation at 15,000 × *g* for 30 min at 4°C, and the supernatant applied to a Ni_2-nitrilotriacetic acid-agarose affinity column (Qiagen) equilibrated with the same buffer supplemented with 20 mM imidazole. For Hyi and GlxR purification, the column was washed and the His-tagged protein eluted using buffer A containing 60 to 250 mM imidazole. Fractions were collected and analyzed by SDS-PAGE. For AllR purification, the column was treated with thrombin buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, 1 mM dithiothreitol, 100 mM NaCl, and 10 μg of thrombin/ml, followed by incubation at room temperature for 3 h to obtain AllR protein without His tag. Fractions containing purified proteins were dialyzed overnight using buffer A at 4°C. Pure proteins were stored at −80°C.

Electrophoretic mobility shift assays (EMSAs). Purified recombinant AllR was used to assess protein binding to *allR-allB* and *aceB1* promoter fragments (255 and 184 bp, respectively). The promoter DNA fragments for these assays were generated by PCR amplification from *S. coelicolor* genomic DNA with the primers described above for the assay of catechol 2,3-dioxygenase in cell extracts. These primers were end labeled with [γ^{32}P]ATP (3,000 Ci mmol^{-1}) using T4 polynucleotide kinase, and the PCR products were purified from agarose gels. Different concentrations of AllR were mixed with each γ-^{32}P-labeled probe (3,000 cpm) in a total volume of 20 μl of buffer containing 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5% (vol/vol) glycerol, 150 mM NaCl, 0.01% Triton X-100, and 1 mg of competitor DNA [poly(dI-dC)·poly(dI-dC)] for 20 min at 30°C. When indicated, allantoin (0 to 50 mM), allantoic acid (0 to 7.5 mM), glyoxylate (0 to 50 mM), urea (0 to 50 mM), or xanthine (0 to 1 mM) was added to the binding buffer, followed by incubation with the protein for 5 min at 30°C before the addition of the labeled probe. DNA-protein complexes were resolved by electrophoresis on a 6% (wt/vol) nondenaturing polyacrylamide gel in 1× TBE (Tris-borate-EDTA)–5% (vol/vol) glycerol at 150 V at 4°C and then visualized and digitalized with a Storm 840 scanner (Amersham). Unlabeled specific and nonspecific (nonrelated DNA) competitor DNA (50- or 100-fold molar excess) were incubated with AllR for 5 min at 30°C, followed by the addition of the labeled probe and incubation for 20 min at 30°C. The resulting DNA-protein complexes were then subjected to electrophoresis and developed and digitalized with the Storm 840 scanner.

DNase I footprinting. Radiolabeled P*aceB1* and P*allR-allB* promoter fragments (50,000 cpm) were incubated at 30°C for 20 min with different amounts of purified AllR protein in 200 μl of binding buffer. DNA was partially digested with DNase I (Promega) for 4 min at room temperature and digestion was stopped by adding 510 μl of stop solution (20 mM EDTA [pH 8.0], 200 mM NaCl, 100 μg of yeast RNA/ml) and 750 μl of phenol-chloroform (1:1). DNA precipitation was done with 1 ml of 100% ethanol, and the pellet was washed with 70% (vol/vol) ethanol, dissolved in 5 μl of formamide-dye mixture, heat denatured (94°C for 2 min), and immediately placed on ice. Digestion products were resolved on a 6% (wt/vol) denaturing polyacrylamide gel by electrophoresis. Appropriate sequencing reactions were loaded onto the gels, along with the footprinting samples, and used as a size ladder for identification of the sequences of protected sites. The results were developed and digitalized with a Storm 840 scanner.

RNA extraction and qRT-PCR assay. RNA was extracted from M145, LN-RBC, and LN-R strains grown for 24 or 46 h in SMM liquid medium using an SV total RNA isolation system (Promega). Second-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) with random primers and used in an sqRT-PCR or in a quantitative RT-PCR (qRT-PCR) with SYBR green as the indicator dye. The primer pairs RThrdBR (5′-GTTGATGACCTCGACCATGT-3′) and RThrdBL (5′-CAAGGGCTACAAGTTCTCCA-3′), RTactIIORF4R (5′-TACACGAGCACCTTCTCACC-3′) and RTactIIORF4L (5′-TGGAATCGTATCGGAATCTC-3′), and RTallBR (5′-AAGTCGTCGGTCTTGAGGTC-3′) and RTallBL (5′-GCACTACCTCACGCTCACC-3′), respectively, were used to analyze transcript levels of *hrdB*, *actII*-ORF4, and *allB*. The expression of each gene was quantified after normalization to *hrdB* mRNA levels. qRT-PCR was carried out in Eppendorf Realplex^2 PCR machine with the following cycling conditions: 95°C for 2 min, followed by 40 cycles at 94°C for 15 s, 58°C for 15 s, and 68°C for 20 s. qRT-PCR data are presented as the fold difference in expression in M145 cells relative to LN-R cells using the Pfaffl method (20), with *hrdB* used as a normalizing gene.

Actinorhodin and undecylprodigiosin determination. For actinorhodin determination, 1 ml of whole broth was added to a KOH solution, giving a final concentration of 1 M; the solution was mixed vigorously and centrifuged at 4,000 × *g* for 5 min. The supernatant absorbance at 640 nm was determined, and the actinorhodin concentration was calculated using a molar absorption coefficient at 640 nm of 25,320 (21). For undecylprodigiosin determination, 1 ml of broth was centrifuged at 5,000 × *g* for 10 min, and the cells were resuspended in 1 ml of methanol. The pH was adjusted to 1.5 with 1 N HCl, and the solution was mixed vigorously and centrifuged at 4,000 × *g* for 5 min. The supernatant absorbance at 530 nm was determined, and the undecylprodigiosin concentration was calculated using a molar absorption coefficient at 530 nm of 100,500 (22).

Hydroxypyruvate isomerase and tartronate semialdehyde reductase enzymatic assay. Hydroxypyruvate isomerase activity was assayed by the measurement of tartronate semialdehyde formed from hydroxypyruvate (Sigma) using *S. coelicolor* purified tartronate semialdehyde reductase enzyme (23–25). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7), 0.4 mM NADH, 10 U of tartronate semialdehyde reductase, 100 mM hydroxypyruvate, and the enzyme itself in a final volume of 0.5 ml. The reaction was carried out at 30°C and started with the addition of enzyme and monitored by measuring the initial change in absorbance at 340 nm after NADH oxidation.

For determination of hydroxypyruvate isomerase kinetic parameters hydroxypyruvate concentration varied from 2.5 to 150 mM. One unit of enzyme activity is defined as the amount of enzyme resulting in the production of 1 μmol of tartronate semialdehyde from hydroxypyruvate per min. For tartronate semialdehyde enzyme, one unit of enzyme was defined as the amount that catalyzed the formation of 1 μmol of D-glycerate from tartronate semialdehyde per min.

Sample preparation for iTRAQ. M145 and LN-R cells from the exponential and stationary phases grown in 50 ml of SMM medium were harvested in duplicates by filtration with 0.45-μm-pore-size nitrocellulose membranes, washed with phosphate-buffered saline solution (pH 7.5; 8 g of NaCl, 0.2 g of Cl, 1.44 g of Na_2PO_4, and 0.24 g of KH_2PO_4 per liter), and resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], Thermo protease inhibitor). Cells were disrupted by sonication, and cellular debris was removed by centrifugation (10,000 × 10 min at 4°C). DNase I (Fermentas) and RNase A (Fermentas) were added to the lysate to further remove impurities. The crude protein extract was dialyzed against MilliQ water and concentrated by lyophilization. Lyophilized proteins were trypsin digested and labeled with iTRAQ reagents as described by the manufacturer's protocol. Briefly, 50 μg of protein from each condition and two biological replicates were reduced with 50 mM Tris (2-carboxyethyl)-phosphine (TCEP) at 60°C for 1 h and cysteine residues alkylated with 200 mM methyl methane-thiosulfonate (MMTS) at room temperature for 15 min. Enzymatic digestion was performed with trypsin (Promega Gold trypsin; 1:20 [wt/wt]) at 37°C for 16 h. Labeling was performed for 2 h with one isobaric amine-reactive tag per sample, and labeled samples were pooled, evaporated to dryness in a vacuum centrifuge, resuspended in SCX buffer, and cleaned to remove the reducing reagent, SDS, calcium chloride, and excess iTRAQ using a cation-exchange cartridge system from AB Sciex.

Liquid chromatography-mass spectrometry (LC-MS) analysis. Samples were analyzed using a nano-LC hybrid Triple-TOF 5600 (AB-Sciex) equipped with a Shimadzu Prominence nano-LC system. The LC was equipped with a C_{18} column (Vydac, MS C18, 300 Å, 150 by 0.3 mm; Grace Davison, Discovery Sciences, USA) operated at 30°C with a gradient running from 0 to 80% acetonitrile (in 0.1% formic acid) for 220 min at a flow rate of 3 μl/min. Peptides were sprayed with a Nano-ESI III source.

TABLE 2 Allantoin pathway proteins showing increased levels in the AllR$^-$ mutant strain

Allantoin metabolism	Protein function	Ratio[a]	
		20 h	46 h
GlxR, SCO6205	Tartronate semialdeyde reductase	32.51	42.07
Hyi, SCO6206	Hydroxypyruvate isomerase	41.69	20.89
Gcl, SCO6201	Glyoxylate carboligase	20.90	12.36
AceB1, SCO6243	Malate synthase	11.69	7.80
AlC, SCO6248	Allantoicase	2.88	1.49*
Ugl, SCO2850	Ureidoglycolate lyase	3.02	ND

[a] That is, the AllR$^-$/M145 protein level ratio detected at 20 or 46 h. *, $P < 0.1$. ND, not determined.

Gas and voltage settings were adjusted as required. Proteins were identified by information-dependent acquisition of the fragmentation spectra of one to four charged peptides with a precursor selection window of 100 to 1,800 *m/z* using enhanced pulsed extraction of fragments for 0.5 s, followed by data-dependent acquisition of 20 peptides with intensity above 100 counts across *m/z* 40 to 1,800 (0.05 s per spectrum) with rolling collision energy. Peptides were identified using the paragon algorithm using Fasta-formatted protein sequences for the finished *S. coelicolor* genome obtained from NCBI (Protein Pilot software 4.0; Applied Biosystems) (26). Search parameters included trypsin as the enzyme, MMTS as the Cys modification, and a "thorough" search setting. Only proteins with a ProteinPilot confidence score of ≥95% (i.e., an estimated global FDR of ≤5%) were accepted. Ratios for iTRAQ and *P* values were determined using the Paragon method, described elsewhere (26).

RESULTS

***sco6246* encodes AllR, the repressor of the allantoin pathway in *S. coelicolor*.** In previous work we used a proteomic and genetic approach to identify and characterize the enzymes involved in allantoin metabolism in *S. coelicolor* (12). Upstream of *allB* and *alc* genes, which encode the first two enzymes of the pathway, allantoinase and allantoicase, respectively, we found the gene *sco6246*, which had previously been predicted to be the transcriptional regulator of the glyoxylate shunt (IcIR) (27) (Fig. 1A). Interestingly, the amino acid sequence analysis of the *sco6246* open reading frame (ORF) showed a 33% amino acid sequence similarity compared to AllR the negative regulator of the allantoin pathway of *E. coli*, suggesting that SCO6246 could potentially be the regulator of the allantoin catabolic genes in *S. coelicolor*.

To investigate whether *sco6246* encodes the allantoin regulator protein in *S. coelicolor*, we constructed a *sco6246* mutant strain and named it LN-R. The mutant was further used to perform quantitative proteomic studies. Total protein extracts of *S. coelicolor* M145 and the isogenic mutant LN-R were prepared from cultures grown in minimal medium at two different time points (20 and 46 h), followed by iTRAQ labeling quantification. Labeled proteins were analyzed by LC-MS. About 450 proteins were identified with at least two peptides with 99% confidence. Statistical analysis showed that totals of 44 and 46 proteins, for each time point, were differentially expressed ($P < 0.05$) between the parental strain and the LN-R mutant (see Table S1 in the supplemental material). Proteins in the allantoin pathway showed >10-fold-increased levels in the *allR*-defective strain compared to the parental strain (Table 2). The only exception found was Alc, which only increased 3-fold in the mutant. The protein AllB was not found in proteomics; thus, we performed qRT-PCR and found a (2.60 ± 0.64)-fold increase in its transcription (see Fig. S1 in the supplemental material).

These results suggest that the gene *allR* encodes a transcriptional regulator that represses the expression of the allantoin catabolic genes in *S. coelicolor*. To confirm this hypothesis, we performed a transcriptional analysis of the allantoin pathway genes, namely, *aceB1*, *allB*, *allR*, and *hyi* (Fig. 1A), by constructing transcriptional fusions of the corresponding promoter regions to the catechol dioxygenase (CDO) reporter cassette. Using Softberry BPROM, a putative promoter within 100 bp from the start codon of each gene was found. The region 150 to 250 bp upstream of the start codon of each gene was cloned upstream of the catechol dioxygenase reporter gene in plasmid pXE2 (17). The plasmids containing the different transcriptional fusions were integrated into *S. coelicolor* M145 and LN-R strains. CDO activity assays were performed in cell extracts for both LN-R and M145 derivative strains. As shown in Fig. 1B, the CDO activity indicated an increased expression of all promoters tested in the absence of the AllR protein. As a control, we also constructed LN-R and M145 derivative strains carrying the CDO gene under a constitutive promoter (P*ermE*) and detected no variation for their CDO activities (Fig. 1B). These results confirmed that AllR regulates not only the expression of *aceB1*, *allB*, and *hyi* genes but also its own transcription.

Characterization of the AllR regulon. In order to determine whether AllR directly regulates the expression of the allantoin pathway genes, we carried out EMSAs using purified AllR protein from *E. coli* and PCR-amplified fragments derived from *aceB1*, *allR-allB*, and *hyi* promoter regions. The results shown in Fig. 2A reveal that AllR bind these DNA fragments in a concentration-dependent manner, forming one or two different protein-DNA complexes. The specificity of the gel shifts was tested by performing competition assays for each of the promoter regions using a 50- or 100-fold excess of unlabeled specific or unspecific DNA (Fig. 2B). The results showed that unspecific DNA had no effect on the formation of the AllR-DNA promoter complex, while the competition with the specific sequences disrupted the complex. These results confirmed that the AllR protein directly binds to the promoter regions of the *aceB1*, *hyi*, *allB*, and *allR* genes in order to regulate their expression.

The AllR DNA binding site was identified through DNase I footprinting assays using the coding and noncoding strands of the *aceB1* and *allR-allB* promoter regions as DNA probes. We found protected DNA regions upstream of *aceB1* and *allB* genes, between positions bp −91 to −126 and positions bp −135 to −175 bp from the start codon, respectively (Fig. 3A). *In silico* analysis of these sequences revealed a putative inverted repeat (TTCCXCXX XGXGGAA) highly conserved in both promoter regions and also present in the upstream region of orthologous genes of other *Streptomyces* species (Fig. 3B). Motif-based sequence analysis using MEME (28) revealed a 15-bp pseudopalindromic sequence located about 100 to 200 bp upstream of the translational start site (Fig. 3C). *In silico* analysis of the *S. coelicolor* chromosome also showed the presence of a highly similar pseudopalindromic sequence of AllR in the promoter regions of *hyi* and *gcl* genes. This result suggests that *hyi* and *gcl* may be also regulated by AllR, as previously observed in proteomic and transcription analyses (Fig. 3B).

Allantoic acid is the inducer of the allantoin pathway transcription in *S. coelicolor*. In order to identify the ligand recog-

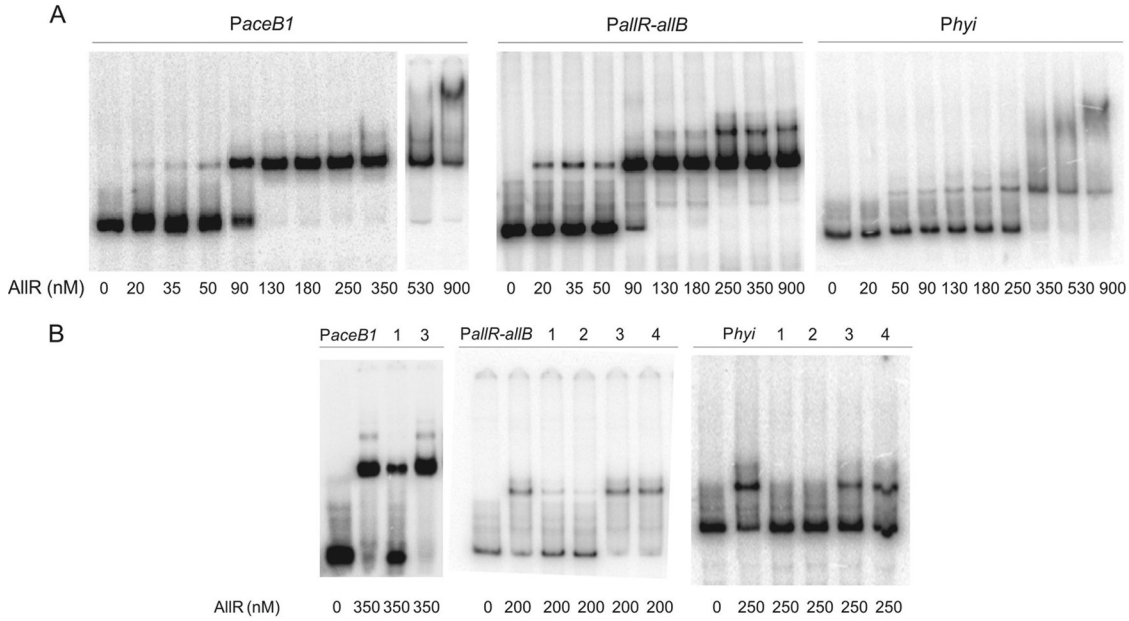

FIG 2 AllR binds to P*aceB1*, P*allR-allB*, and P*hyi* promoters *in vitro* (A) Gel-shift assay experiments were performed with a 184-bp P*aceB1*, a 255-bp P*allR-allB*, or a 156-bp P*hyi* fragment. The probes were labeled with [γ-^{32}P]ATP. (B) Competition assays. A fixed concentration of AllR (350, 200, or 250 nM) was incubated in the presence of P*aceB1*, P*allR-allB*, and P*hyi* fragments with a 50-fold excess of unlabeled P*aceB1*, P*allR-allB*, or P*hyi* fragment (lane 1), a 100-fold excess of unlabeled P*allR-allB* or P*hyi* fragment (lane 2), a 50-fold excess of unlabeled nonspecific 300-bp DNA fragment (lane 3), or a 100-fold excess of unlabeled nonspecific 300-bp DNA fragment (lane 4).

nized by AllR, we tested the effect of several metabolites from the allantoin pathway (allantoin, allantoic acid, glyoxylate, urea, and xanthine) on the binding of the AllR protein to its target sequences. Among all the metabolites tested, allantoic acid was able to alter the mobility shift during the EMSAs for *allR-allB* and *hyi* promoter regions, suggesting that this metabolite modulates the binding of the regulatory protein AllR *in vivo* (Fig. 4). We also found that glyoxylate altered the interaction of AllR and P*allR-allB* (Fig. 4); however, no effect was observed for the interaction of AllR with the *hyi* promoter region. Further experiments are needed to elucidate the effect of glyoxylate on the regulation of the allantoin pathway.

Mutation in AllR showed a severe defect in actinorhodin and undecylprodigiosin production. Considering that antibiotic production is tightly linked to the availability of C and N, we tested the effect of the *allR* mutation in growth rate and antibiotics production. To this end, we assayed the production of both actinorhodin and undecylprodigiosin in the wild-type strain, the LN-R mutant, and the LN-Rc complemented strains growing in liquid SMM. As shown in Fig. 5, despite the fact that growth was unaffected, the production of actinorhodin and undecylprodigiosin were impaired. To confirm this result, a new copy of the *allR* gene was expressed under its own promoter in the mutant strain and restored antibiotic production (Fig. 5).

To determine whether the reduced actinorhodin production was directly caused by decreased expression level of its specific gene cluster activator, ActII-ORF4, RNA was extracted from M145 and LN-R cultures and quantified by qRT-PCR analysis. A (30 $\pm$ 0.09)-fold decrease in the levels of *actII-ORF4* RNA was detected in LN-R compared to the wild-type strain M145 (see Fig. S1 in the supplemental material).

The double-mutant LN-RH strain restores actinorhodin and undecylprodigiosin production. Since AllR is the repressor of the allantoin catabolic pathway, increased proteins levels from this metabolic route may be responsible for the alteration in antibiotic production in LN-R strain. To test this hypothesis, double mutant strains of *S. coelicolor* (LN-RA, LN-RG, and LN-RH) and a triple mutant (LN-RBC) were constructed and tested for undecylprodigiosin and actinorhodin production at 60 and 72 h of culture in SMM, respectively. Only the inactivation of *hyi* gene in LN-R strain was capable of restoring actinorhodin and undecylprodigiosin production (LN-RH strain) (Fig. 6). This result indicates that the overexpression of the Hyi protein might affect antibiotic production in the LN-R strain. To confirm this result, LN-RH strain was complemented with a wild-type copy of the *hyi* gene under its own promoter, and impairment of actinorhodin and undecylprodigiosin was observed as expected (Fig. 6).

Biochemical characterization of the enzyme hydroxypyruvate isomerase. To confirm that Hyi is a hydroxypyruvate isomerase enzyme, we examined the reverse direction reaction using hydroxypyruvate as the substrate, which required a coupled enzymatic assay using tartronate semialdehyde reductase as previously described (24). Thus, the first enzyme catalyzes the conversion of hydroxypyruvate into tartronate semialdehyde, and the second reaction catalyzes the NADH-dependent reduction of tartronate semialdehyde to glycerate. To this end, we used Hyi and GlxR proteins from *S. coelicolor* expressed as His tag recombinant proteins in *E. coli* BL21 and purified by affinity chromatography. Biochemical assays using excess of GlxR confirmed that Hyi catalyzes the conversion of hydroxypyruvate to tartronate semialdehyde with a V_{max} of 26.8 $\pm$ 1.8 U mg^{-1} and a K_m for hydroxypyruvate of 55 $\pm$ 7.5 mM. The kinetic parameters obtained are comparable to the values reported for other bacterial orthologues (23–25). On the other hand, biochemical assays using an excess of

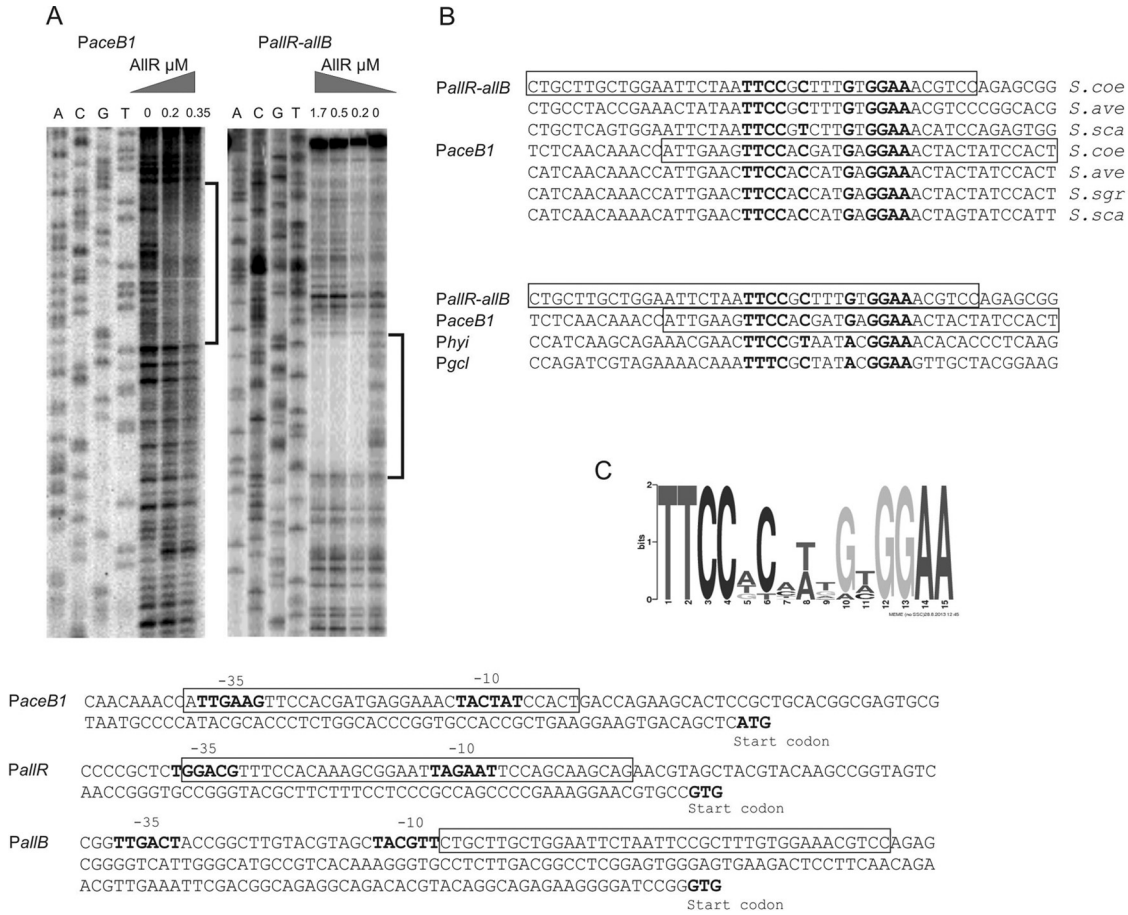

FIG 3 Identification of AllR binding sites in the P*aceB1* and P*allR-allB* promoter regions. (A) P*aceB1* and P*allR-allB* promoter sequences were labeled with [γ^{32}P]ATP and protected from DNase I nuclease activity with three different concentrations of AllR. The protected regions are indicated with black brackets. Lanes A to T, DNA sequence of the probe. Partial sequences of P*aceB1*, P*allR*, and P*allB* are shown at the bottom. The boxes indicate the protected region by AllR for each promoter. Putative −35 and −10 regions and translational start codons are indicated in boldface. (B) Promoter sequences from *S. coelicolor* (*S. coe*), *Streptomyces avermitilis* (*S. ave*), *Streptomyces scabies* (*S. sca*), and *Streptomyces griseus* (*S. sgr*) were aligned using CLUSTAL W (http://www.ebi.ac.uk/clustalw/). Sequences from other actinomycetes were taken from upstream regions of the appropriate orthologues genes. Boldface letters indicate the conserved inverted repeat sequence. An alignment of P*aceB1* and P*allR-allB* protected sequences with P*hyi* and P*gcl* promoter regions from *S. coelicolor* is shown. The presence of a highly similar inverted repeat sequence in P*hyi* and P*gcl* is indicated in boldface letters. The boxes indicate the protected region by AllR for each promoter. (C) Sequence analysis of the putative AllR binding regions of several species of streptomyces with the motif-based sequence analysis tool MEME led to the identification of a motif highly conserved in *Streptomyces*.

Hyi confirmed that GlxR protein is a tartronate semialdehyde reductase with a specific activity for the conversion of tartronate semialdehyde into glycerate V_{max} of 4.2 ± 0.5 U mg^{-1}.

DISCUSSION

S. coelicolor has a distinct regulation of the allantoin pathway from those previously described (Fig. 7). We found that AllR acts as a repressor protein, inhibiting production of most of the enzymes in the allantoin pathway (i.e., AllB, Alc, AceB1, Gcl, and Hyi). In *E. coli*, the allantoin pathway is regulated by two proteins, AllR and AllS, acting as repressor and activator proteins, respectively (9). In *B. subtilis* PucR controls the expression of the allantoin pathway as both repressor and activator protein (11). AllR from *S. coelicolor* belongs to the large IclR family of transcriptional regulators, sharing 40% sequence identity with the *E. coli* IclR regulator (29), the founding member of this family. Members of IclR family have conserved domain architecture with a characteristic N terminus helix-turn-helix DNA binding motif (30). Effector molecule binding is proposed to occur at the C-terminal region of these regulators (31); however, as proposed by Walker et al. (30), the low conservation of amino acid residues participating in small molecule binding among IclR regulators reflects a potential chemical diversity of effector molecules. AllR is widely distributed in *Streptomyces* spp. It has a high percentage of amino acid sequence identity, ranging from 85 to 100%, compared to orthologous proteins from *S. avermitilis*, *S. griseus*, *S. scabies*, *S. venezuelae*, and *S. clavuligerus*. In addition, a cluster configuration of *allR*, *allB*, and *alc* putative genes is conserved in these *Streptomyces* spp.

S. coelicolor AllR was found to bind to its target sequences by recognizing specific pseudopalindromic inverted repeats TTCCX CXXXGXGGAA. The presence of this inverted repeats suggests symmetry of binding of AllR to its target DNA sequences. In *E. coli*, AllR binds as a tetramer to the promoter regions it controls, requiring four HTH domains for an effective binding interaction (30); this may also be the case of *S. coelicolor* AllR. In fact, the appearance of a second protein-DNA complex in the EMSA may

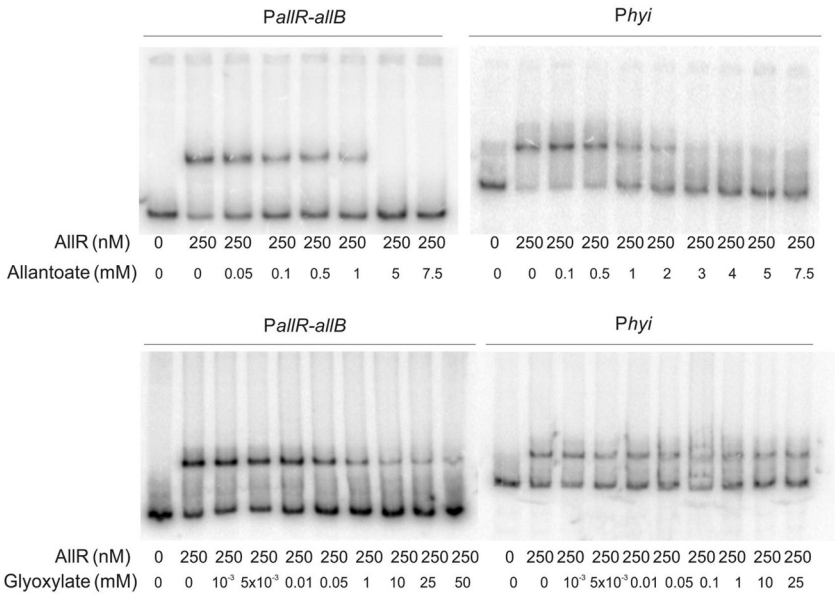

FIG 4 Binding of AllR to the P*allR-allB* and P*hyi* promoter regions is inhibited by allantoate (P*allR-allB* and P*hyi*) and glyoxylate (P*allR-allB*). Gel shift assays were performed by incubating the γ-^{32}P-labeled P*allR-allB* and P*hyi* probes with 250 nM AllR in the presence of allantoate or glyoxylate at different concentrations.

correspond to AllR binding to the promoter region as a dimer at low concentration and as a tetramer at higher concentrations; further studies will be necessary to confirm this hypothesis. The AllR specific binding site is also conserved in the promoter regions of the *allR*, *allB*, *aceB1*, and *gcl* genes from *S. avermitilis*, *S. venezuelae*, *S. griseus*, and *S. scabies* and in the promoter regions of the *allR*, *allB*, and *aceB1* putative genes from *S. clavuligerus*. In *S. coelicolor*, allantoic acid was identified as an effector of AllR. The presence of this compound appears to activate the expression of the allantoin catabolic genes by interacting with AllR and decreasing its binding affinity for the operator regions. Similarly, glyoxylate also seems to decrease the AllR binding to its cognate sequence; however, this effect was only observed for the *allR-allB* promoter region. Taken together, these results differ from studies performed in *E. coli*, where allantoin and glyoxylate have been proposed as effector molecules of AllR protein but act as a corepressor and a coactivator of the allantoin pathway, respectively (32).

On the other hand, the absence of the AllR protein in *S. coelicolor* resulted in a marked decrease in the production of actinorhodin and undecylprodigiosin. According to transcriptional analysis, this effect could be correlated, at least for actinorhodin, with the lower levels of ActII-ORF4, the activator of the *act* cluster (33). However, bioinformatic analysis showed that AllR binding site is not present in either of the promoter regions of the two pathways specific regulators, *actII-ORF4* or *redD* (the activator of the red cluster). This observation suggests that the lower production of these two antibiotics is an indirect metabolic effect caused by the absence of AllR. Quantitative LC-MS proteomics revealed increased levels of the allantoin catabolic enzymes in LN-R strain. Interestingly, after inactivating several genes from the AllR regulon in LN-R background, we found that inactivation of *hyi* gene encoding a hydroxypyruvate isomerase, the enzyme that catalyzes the conversion of tartronate semialdehyde into hydroxypyruvate, restores actinorhodin and undecylprodigiosin production. To test whether the increased level of Hyi was the only cause for the decrease production of antibiotics in LN-R, the *hyi* gene was expressed in *S. coelicolor* M145 strain using plasmid pLN17, and the levels of actinorhodin and undecylprodigiosin were determined (data not shown). Overexpression of the *hyi* gene in M145 parent

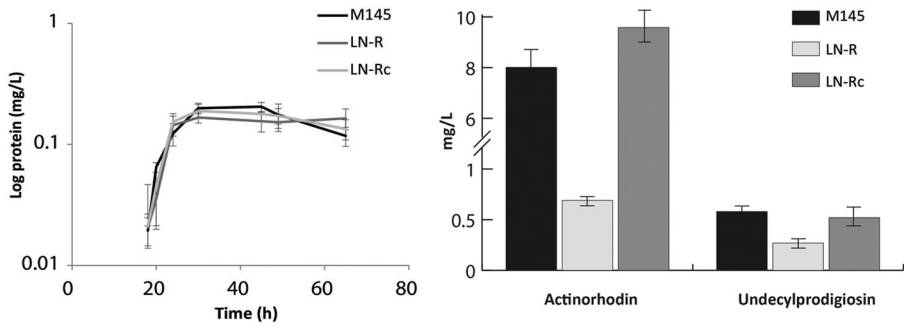

FIG 5 Growth curve and antibiotic production of M145, LN-R and LN-Rc strains in SMM. Actinorhodin and undecylprodigiosin production was assayed at 60 and 72 h, respectively. The results represent the averages of three independent experiments ± the SD.

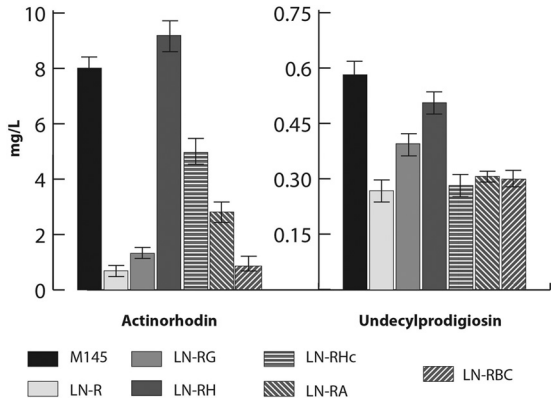

FIG 6 Antibiotic production in single-, double-, and triple-mutant strains. Actinorhodin and undecylprodigiosin production was assayed at 60 and 72 h of growth in SMM, respectively. The results represent the averages of three independent experiments ± the SD.

strain did not reproduce the LN-R mutant strain phenotype in any of the media tested, suggesting that metabolic changes beyond the Hyi activity might affect antibiotic production in the LN-R strain.

Since the autoxidation of hydroxypyruvate to oxalate and H_2O_2 has previously been demonstrated as a source of reactive oxygen species (ROS), Hyi might be directly linked to oxidative stress homeostasis (34). In addition, hydroxypyruvate has been proposed to increase the levels of intracellular glycolaldehyde, which is further oxidized to glyoxal; this process is accompanied by a large amount of ROS formation (35). The presence of ROS in the cell not only triggers an adaptive response to scavenge these toxic molecules and repair molecular damage but also plays a role as secondary messenger in cell signaling (36, 37). The restoration of antibiotic production to the wild-type levels in the double mutant strain LN-RH supports this hypothesis.

In addition to allantoin catabolic enzymes, proteins SCO6204 and SCO2396 showed increased levels in LN-R strain; these proteins may also play a role in oxidative stress responses. Protein SCO6204 has a conserved catalase domain and a high degree of similarity to KatE from *E. coli*, and its coding gene is located upstream of *glxR* gene. Protein SCO2396 has been described as a putative organic hydroxyperoxide resistance protein, likely involved in the prevention of lipid peroxidation (38). In addition, LN-R strain showed significant lower levels of proteins related to oxidative stress response mechanisms such as superoxide dismutase SodF2 (SCO0999), catalase (SCO0379), 50S ribosomal protein RlpA (SCO4649), 30S ribosomal protein RspD (SCO1505), putative Clp-family ATP-binding protease SCO3373, 50S ribosomal protein RplE (SCO4717), and 30S ribosomal protein RpsC (SCO4708) (see Table S1 in the supplemental material). Even though the molecular mechanisms are not completely understood, the consequences of the adaptive response to oxidative stress extend beyond the primary effect of defense into alterations

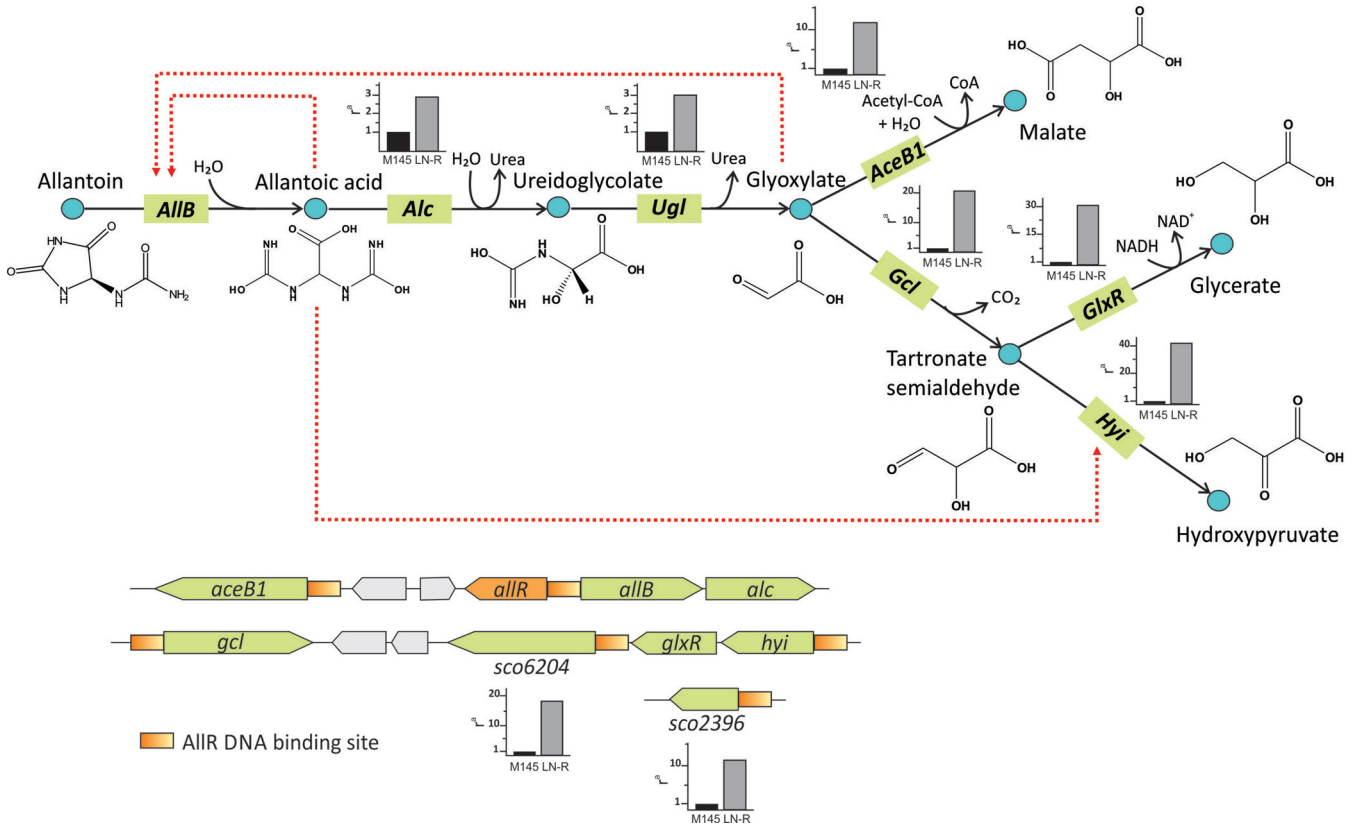

FIG 7 Regulation of allantoin pathway by AllR. Column plots indicate expression of each protein from the allantoin pathway in LN-R strain compared to the wild-type strain ("r[a]" is the ratio of protein levels detected in LN-R/M145). Dashed red lines indicate the activation of the pathway by degradation intermediates interacting with AllR and inhibiting binding to the promoter sequences. AllB, Alc, Ugl, AceB1, Gcl, GlxR, and Hyi correspond to allantoinase, allantoicase, ureidoglycolate lyase, malate synthase, glyoxylate carboligase, tartronate semialdehyde reductase, and hydroxypyruvate isomerase activities, respectively.

in the secondary metabolism profile (37). Cross talk between ROS homeostasis and secondary metabolic programs has been demonstrated in *S. natalensis* ATCC 27448 (37) and *S. clavuligerus* (39). As proposed by Hahn and coworkers (40–42), the production of oxidative stress resistance proteins in *S. coelicolor* seems to require specific regulators for individual enzymes. This brings up the question of whether AllR is also involved in the specific regulation of antioxidant enzymes not previously characterized, such as SCO6204 and SCO2396. Interestingly, sequence analysis showed a putative AllR binding site in the promoters of the genes encoding these proteins. Altogether, these results suggest that the reduced production of actinorhodin and undecylprodigiosin in LN-R mutant may be related to a deregulation of the oxidative homeostasis in *S. coelicolor*; however, further studies are required to validate this hypothesis.

We have demonstrated here an interesting crossing point between the regulation of purine catabolism and antibiotic production, and we have highlighted the complex and intriguing connection between primary and secondary metabolism in this fascinating group of bacteria.

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