Direct Involvement of the Master Nitrogen Metabolism Regulator GlnR in Antibiotic Biosynthesis in Streptomyces

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GlnR, an OmpR-like orphan two-component system response regulator, is a master regulator of nitrogen metabolism in the genus Streptomyces. In this work, evidence that GlnR is also directly involved in the regulation of antibiotic biosynthesis is provided. In the model strain Streptomyces coelicolor M145, an in-frame deletion of glnR resulted in markedly increased actinorhodin (ACT) production but reduced undecylenicodigiosin (RED) biosynthesis when exposed to R2YE culture medium. Transcriptional analysis coupled with DNA binding studies revealed that GlnR represses ACT but activates RED production directly via the pathway-specific activator genes actII-ORF4 and redZ, respectively. The precise GlnR-binding sites upstream of these two target genes were defined. In addition, the direct involvement of GlnR in antibiotic biosynthesis was further identified in Streptomyces avermitilis, which produces the important anthelmintic agent avermectin. We found that S. avermitilis GlnR (GlnRsav) could stimulate avermectin but repress oligomycin production directly through the respective pathway-specific activator genes, aveR and olmR/RII. To the best of our knowledge, this report describes the first experimental evidence demonstrating that GlnR regulates antibiotic biosynthesis directly through pathway-specific regulators in Streptomyces. Our results suggest that GlnR-mediated regulation of antibiotic biosynthesis is likely to be universal in streptomycetes. These findings also indicate that GlnR is not only a master nitrogen regulator but also an important controller of secondary metabolism, which may help to balance nitrogen metabolism and antibiotic biosynthesis in streptomycetes.

Actinobacteria, such as the soil-dwelling genus Streptomyces, are characterized by their robust capacity to produce a vast array of valuable secondary metabolites, such as antibiotics, anticancer drugs, immunosuppressants and anthelmintic agents. The biosynthesis of secondary metabolites in actinomycetes is governed by complex regulatory networks involving both global/pleiotropic and pathway-specific regulators (1–4). A better understanding of these regulatory networks is critically important for strain improvement by metabolic engineering or synthetic biology approaches (1, 5).

GlnR, an OmpR-type orphan two-component response regulator, is a well characterized master regulator of nitrogen metabolism in actinomycetes (6, 7). GlnR-mediated nitrogen regulation has been most intensively studied in the model strain Streptomyces coelicolor. The GlnR regulon has been determined and includes at least 15 genes that encode proteins (mainly enzymes) involved in nitrogen source uptake and signal transduction (the actII-glnK-glnD operon), alternative nitrogen source utilization (nirB, nasA, and ureA) and glutamine/glutamate synthesis (glnA, glnII, and gdhA) (6). The role of GlnR has also been investigated in other actinomycetes, including Streptomyces venezuelae (8), Amycolatopsis mediterranei (9), and Saccharopolyspora erythraea (10), among others. For instance, in S. venezuelae, a genome-wide analysis by chromatin immunoprecipitation (ChIP) assay coupled with microarray analysis (ChIP-chip) was performed and revealed the presence of 36 GlnR-binding sites, most located upstream of genes involved in nitrogen metabolism (8).

Increasing evidence has revealed that GlnR also plays an important role in carbon metabolism and mediates the interplay between nitrogen and carbon metabolism in actinomycetes. In Streptomyces coelicolor, GlnR was identified as a transcriptional regulator for the expression of the agl3EFG operon, which encodes a putative carbohydrate transporter (11). Recent reports have demonstrated that GlnR directly regulates the uptake and utilization of non-phosphotransferase system carbon sources in S. erythraea. The expression of most (13 of 20) carbohydrate ATP-binding cassette (ABC) transporters was activated by GlnR in response to nitrogen availability (12). Therefore, GlnR-mediated control of carbohydrate transport may be highly conserved among actinomycetes.

In addition to its global regulation in primary metabolism (i.e. nitrogen and carbon metabolism), GlnR may also be closely
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associated with antibiotic biosynthesis in actinomycetes. For instance, in the rifamycin-producing strain *A. mediterranei*, GlnR is involved in the suppression of rifamycin biosynthesis in the absence of nitrate (9). In *S. coelicolor*, the *glnR* mutation led to defective production of two colored antibiotics, blue-colored actinorhodin (ACT) and red-pigmented undecylprodigiosin (RED) (7). Previously, it was generally believed that the role of GlnR in antibiotic biosynthesis was an indirect consequence of its activity in nitrogen metabolism. However, increasing evidence has suggested the direct involvement of GlnR in antibiotic biosynthesis in streptomycetes. In *S. venezuelae*, ChiP-chip analysis indicated that GlnR is likely to directly activate the expression of *jadR*, a pathway-specific activator of jadomycin biosynthesis (8). A differential role for GlnR was described in *S. coelicolor* (13). Nevertheless, whether GlnR-mediated regulation of antibiotic biosynthesis is a universal strategy adopted by *Streptomyces* strains is still unknown.

Recently, cross-regulation in nitrogen metabolism between GlnR and the two-component system (TCS)\(^5\) AfsQ1-Q2, which functions as an activator of antibiotic biosynthesis, has been identified in *S. coelicolor* (14). The response regulator AfsQ1 was found to specifically bind to the promoter regions of GlnR-regulated target genes, such as *glnA* and *nirB*, thereby regulating nitrogen metabolism. Moreover, the minimal binding sequences recognized by AfsQ1 upstream of the pathway-specific regulatory genes *redZ* and *cdaR* are very similar to two copies of the “a site” (GTnAC) from the previously identified *S. coelicolor* GlnR box (6). These data suggest a reciprocal cross-regulation between GlnR and AfsQ1-Q2 and that GlnR might exert its function on antibiotic biosynthesis by directly binding to the promoter regions of the pathway-specific regulatory genes.

Here, the mechanism underlying the function of GlnR in antibiotic biosynthesis in *S. coelicolor* was studied. We demonstrate that GlnR regulates the biosynthesis of ACT and RED via direct interactions with the promoter regions of the pathway-specific activator genes, *actII-ORF4* and *redZ*, respectively. Furthermore, the regulatory mechanism underlying the function of GlnRsav (encoded by *SAV_4042*, a GlnR homologue from *Streptomyces avermitilis*) in avermectin and oligomycin production was also determined. Our results suggest that GlnR-mediated regulation of antibiotic biosynthesis may be universal among streptomycetes.

Results

GlnR Plays a Differential Role during Antibiotic Biosynthesis in *S. coelicolor* M145—To investigate the role of the master nitrogen metabolism regulator GlnR in the antibiotic biosynthesis of *S. coelicolor* M145, the production of two colored antibiotics, ACT and RED, was compared between the ΔglnR mutant and the wild-type strain M145 under MS and R2YE culture conditions, respectively. The ΔglnR mutant was generated previously using the CRISPR/Cas9-mediated genome editing system (15). In comparison with M145, the ΔglnR mutant exhibited impaired bacterial growth on both culture conditions, particularly during the early growth stage (supplemental Figs. S1 and S2). The defective growth of the mutant was possibly due to its lack of nitrogen assimilation and sugar transportation after glnR removal. Moreover, we observed that under the tested culture conditions, glnR inactivation resulted in altered antibiotic production. Quantitative analysis of ACT and RED production showed that on R2YE agar plates, compared with M145, the ΔglnR mutant exhibited reduced ACT biosynthesis at the early stages (before 72 h) but enhanced ACT biosynthesis after 72 h. The reduced ACT production of the mutant during the early stage is likely due to the greatly impaired bacterial growth. Decreased RED production was observed throughout the experiment course in the ΔglnR mutant (supplemental Fig. S1). On MS agar, only enhanced ACT levels were detected in the mutant when compared with M145; no RED production was detected in either strain (supplemental Fig. S2). Herein, we aimed to perform an in-depth study into the role of GlnR in antibiotic biosynthesis under the R2YE culture condition, which is a rich medium often used for the analysis of antibiotic production in *S. coelicolor*.

To verify that the phenotypic changes in the ΔglnR mutant were indeed ascribed to glnR removal, a complementation assay was performed. Three strains, namely, M145 with the empty *pB139* vector (*M145/pB139*), ΔglnR with *pB139* (ΔglnR/*pB139*), and the complemented strain (ΔglnR/*pB139-glnR*) were constructed, and the phenotypes were analyzed on R2YE agar plates. The phenotypic alterations in bacterial growth and ACT and RED production could be restored upon introduction of the wild-type *glnR* gene under control of the strong and constitutive promoter *ermE*\(^*p*\) (Fig. 1). The data presented here clearly suggested that the master nitrogen regulator GlnR plays a differential role in antibiotic biosynthesis under R2YE culture conditions.

GlnR Possibly Regulates ACT and RED Biosynthesis Directly via the Respective Pathway-specific Activator Genes *actII-ORF4* and *redZ*—To assess whether GlnR exerts its effects on ACT and RED biosynthesis directly via the respective pathway-specific activator genes, *actII-ORF4* and *redZ/redD* (for RED production), we performed electrophoretic mobility shift assay (EMSA) analysis using purified His\(_{6}\)GlnR protein. Three probes containing the respective promoter regions of the three pathway-specific activator genes were amplified from the genomic DNA of M145. The glnR promoter region, which has no interaction with GlnR (16), was used as a negative control. Purified His\(_{6}\)-GlnR specifically bound to the promoter regions of *actII-ORF4* and *redZ* but not to the *redD* and *glnR* probes (Fig. 2A).

Subsequently, we compared the transcript levels of *actII-ORF4* and *redZ* between the ΔglnR mutant and M145 by real-time RT-PCR (qRT-PCR). RNA samples were isolated from these two strains grown on R2YE agar medium for 48 and 72 h, respectively. As shown in Fig. 2B, *actII-ORF4* expression was

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\(^5\) The abbreviations used are: TCS, two-component system; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated proteins; FAM, fluorescent 6-carboxyfluorescein; MS, mannitol soya; sgRNA, single guide RNA; TSS, transcriptional start site; nt, nucleotide(s); qRT-PCR, real-time reverse transcription-PCR.
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FIGURE 1. Effects of glnR deletion on antibiotic biosynthesis and bacterial growth of S. coelicolor. A, phenotypic analysis of the ΔglnR mutant grown on R2YE agar plates. Three S. coelicolor strains, including M145/pIB139, the ΔglnR mutant with pIB139 (ΔglnR/pIB139), and the complemented strain ΔglnR/pIB-glR, were incubated on R2YE agar for three different time intervals (48, 96, and 120 h) before imaging. B, growth curves and quantitative analysis of ACT and RED production. Three S. coelicolor strains were grown on R2YE plates covered with sterile cellophane. The cultures were collected at six time points (48, 60, 72, 96, 120, and 144 h).

FIGURE 2. GlnR regulates ACT and RED biosynthesis through binding to the respective promoter regions of actII-ORF4 and redZ. A, EMSA analysis. The amounts of His6-tagged GlnR protein (GlnR, μM) used are as indicated. In competition assays, 200-fold excess amounts of specific (unlabeled actII-ORF4 or redZ probe, S) or nonspecific probe DNA (sperm DNA, NS) were added. Free Cy5-labeled probes and GlnR-DNA complexes are shown by arrows or an open brace. B, transcriptional analysis of actII-ORF4 and redZ upon glnR deletion. RNA samples were isolated from the cultures of M145 and the ΔglnR mutant grown on R2YE plates covered with sterile cellophane for 48 and 72 h. The values were determined after normalization to the internal control, hrdB. The relative values for two tested genes in M145 tested at 48 h were arbitrarily assigned as 1. The relative transcription levels presented are averages of three independent biological replicates, and the error bars indicate the standard deviations.

Increased by over 2-fold upon glnR deletion at 72 h. However, its expression was drastically down-regulated in the ΔglnR mutant compared with that in M145 at 48 h. Expression of redZ was decreased by more than 2-fold in the mutant when compared with that of M145 at 48 h. Because the ΔglnR mutant exhibited markedly impaired growth during the early stage (especially before 48 h), we could not exclude the possibility that the down-regulations in actII-ORF4 and redZ expression in the ΔglnR mutant were due to the greatly impaired growth of the mutant.

Defining the Precise GlnR-binding Sites Upstream of actII-ORF4 —To determine the precise GlnR-binding sequences for regulation of actII-ORF4 expression, DNase I footprinting assays were conducted using a FAM-labeled actII-ORF4 probe, as reported previously (17). A 32-nt GlnR-protected area was present in the actII-ORF4 promoter region, spanning from nucleotide position −77 to −46 relative to the transcriptional start site (TSS) (Fig. 3).

In the GlnR-protected area upstream of actII-ORF4, only one 5-nt GlnR box (5′-GGAGATCCGCTTGTGAC-3′) similar to the previously described sequence (6) was found. Base mutations of the 16-nt sequence (5′-GGAGATCGCTTGTGAC-3′) had no effect on the binding affinity of GlnR, indicating that this sequence is not required for GlnR binding. Further mutations of the remaining 16-nt DNA sequence led to a complete loss of GlnR binding affinity, indicating that the 16-nt sequence 5′-TAATTTTTGTACATAA-3′ is required for GlnR-mediated regulation of actII-ORF4 expression (Fig. 3). However, no typical GlnR boxes as identified previously in Actinobacteria were detected (6).

To further verify the role of GlnR in ACT biosynthesis, base substitutions of the putative GlnR-binding sequence 5′-CGTACGACTTGGACCT-3′ upstream of actII-ORF4 in the M145 genome were created (Fig. 4A). Unfortunately, we failed to generate the mutant with targeted base mutations in a single step using the CRISPR/Cas9-mediated technology. Therefore, we tried a two-step approach, including deletion of the actII-ORF4 promoter region and then in situ complementation of the mutated promoter region, as described under “Experimental Procedures,” and finally obtained the actIIpmu mutant. Subsequently, the phenotypic changes in ACT production and actII-ORF4 transcription were compared between the actIIpmu mutant and M145. As seen in Fig. 4, we observed that mutations in the GlnR-binding site in the actII-ORF4 promoter region resulted in markedly enhanced ACT production throughout the time course assessed. The actIIpmu mutant produced 3.3,
3.1, 3.3, 4.5, and 5.1 times more ACT levels than did the parental strain M145 at 60, 72, 96, 120, and 140 h, respectively. In addition, we observed that the actIIpmu mutant achieved a higher level of ACT production in a shorter time than the ΔglnR mutant, possibly because bacterial growth in this strain was unaffected by mutating only the GlnR-binding site upstream of actII-ORF4. Similar to the phenotypic changes in ACT production, actII-ORF4 transcription was significantly up-regulated upon base substitutions in the GlnR-binding site. Therefore, it could be concluded that GlnR serves as a repressor of ACT
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Defining the Precise GlnR-binding Sites Upstream of redZ—

Similar to the actII-ORF4 studies, DNase I footprinting assays were performed using a FAM-labeled redZ probe to determine the GlnR-binding sequence upstream of redZ. As shown in Fig. 5A, the GlnR protection region upstream of redZ extended from the nucleotide position −103 to −57 with respect to the transcriptional start site (TSS).

The GlnR protection region upstream of redZ, a signature sequence, 5′-GAAAAC-n6-GTAC-3′, was found; this sequence is similar to the consensus GlnR-binding sequence 5′-gTnAAGC-Gn6-GAAc-3′ identified in a previous study (6) (Fig. 5, A and B). To confirm the importance of this signature sequence for GlnR binding, we introduced base mutations into the two 5-nt boxes, the intervening 6-nt spacer and the flanking sequences (Fig. 5C), and the binding affinities between GlnR and the mutated probes were assessed by EMSA. The binding activities of GlnR to the mutated probes (redZmutA, redZmutB, and redZmutC, with base substitutions in both or either of two 5-nt boxes) were completely abolished, whereas mutations in the intervening and the flanking sequences (redZmutD and redZmutE) had little effect on binding affinities (Fig. 5D). These results clearly demonstrated that the signature sequence (5′-GAAAAC-n6-GTAC-3′) is crucial for GlnR binding and is likely to serve as the operator of GlnR. In agreement with the positive role of GlnR in redZ transcription, the GlnR-binding site (5′-GAAAAC-n6-GTAC-3′) is located upstream of the redZ promoter and spans from the nucleotide position −80 to −65 relative to the redZ TSS.

To further assess the function of GlnR in RED biosynthesis and to exclude the possibility that the down-regulation of redZ expression is due to impaired bacterial growth upon glnR deletion, base substitutions in the GlnR-binding site, as illustrated in Fig. 6, were introduced into the redZ promoter region in the M145 genome using the CRISPR/Cas9-mediated genome editing method to generate the redZmu mutant. Then, the effects of base substitutions on RED production and redZ transcription were analyzed. Mutations of the GlnR-binding site led to reduced redZ expression (48 h) and accordingly slightly decreased RED biosynthesis (reduced to 67% of the level in M145), confirming the positive role of GlnR in RED production by binding to the redZ promoter region. Interestingly, we found that RED production in the ΔglnR mutant was much lower than that in the redZmu mutant (Figs. 1 and 6), possibly due to the markedly impaired bacterial growth upon glnR deletion.

GlnR-mediated Regulation of Antibiotic Biosynthesis May Be Highly Conserved in the Genus Streptomyces—GlnR homologues are widespread in Streptomyces strains, and GlnR-mediated regulation of nitrogen metabolism is highly conserved among different streptomycetes (6, 18). Here, to investigate whether GlnR-dependent regulation of antibiotic biosynthesis is also universal among Streptomyces, the function of GlnRsav (a homologue of GlnR encoded by SAV_4042) in S. avermitilis NRRL 8165 was studied, and a ΔglnRsav mutant with an in-frame deletion of the glnRsav gene was constructed. Compared with the wild-type strain NRRL 8165 grown on MS agar plates, ΔglnRsav grew more slowly and exhibited defective spore formation (Fig. 7, A and B). Further quantitative analysis of the production of two antibiotics, avermectin and oligomycin, demonstrated that glnRsav deletion up-regulated oligomycin but down-regulated avermectin biosynthesis. As seen in Fig. 7C, at two tested time points, no avermectin B1a (the main component of avermectin) was detected in the ΔglnRsav mutant (with the control vector pLB139). In contrast, oligomycin A (the main component of oligomycin) was produced.
FIGURE 6. Effects of base substitutions in the GlnR-binding site upstream of redZ on RED biosynthesis. A, illustration of base substitutions introduced into the redZ promoter region on the chromosome of S. coelicolor M145. The GlnR-binding site was mutated as indicated (from 5’-GAAAC-GTATC-3’ to 5’-TGGAG-GTATC-3’). The redZmu mutant was obtained. The numbers (−80, −65) indicate the nucleotide positions of the GlnR-binding motif with respect to the TSS of redZ. B, analysis of RED production in M145 and the redZmu mutant. Cultures grown on R2YE agar plates covered with sterile cellophane were collected from these two strains at the indicated time points. The error bars represent the standard deviations of three biological replicates. C, transcriptional analysis of redZ in the redZmu mutant with respect to M145. RNA samples were prepared from the cultures of two strains grown on R2YE plates covered with sterilized plastic cellophane for 48 and 72 h. The values were calculated after normalization to the internal control, hrdB. The relative value for redZ in M145 tested at 48 h was arbitrarily assigned as 1. The relative transcript levels presented are averages of three independent biological replicates, and the error bars indicate the standard deviations.

FIGURE 7. Phenotypic changes in the ΔglnRsav mutant in comparison with the wild-type strain NRRL 8165 grown on MS agar. A, phenotypic analysis of the ΔglnRsav strain on MS agar. Five S. avermitilis strains, including the wild-type strain NRRL 8165 (WT), the ΔglnRsav mutant (ΔglnRsav), WT with the empty plasmid pIB139 (WT/pIB139), the ΔglnRsav mutant with pIB139 (ΔglnRsav/pIB139), and the complemented strain (ΔglnRsav/pIB-glnRsav), were grown on MS agar for two different time intervals (3 and 9 days) before imaging. B, changes in bacterial growth upon glnRsav deletion. C, changes in avermectin production in the ΔglnRsav mutant compared with NRRL 8165 (WT). B1a is the main component of avermectin. D, changes in oligomycin production in the ΔglnRsav mutant compared with NRRL 8165 (WT). Oligomycin A (olmA) is the main component of oligomycin. For quantitative analysis of bacterial growth and antibiotic production, three strains as described above, including WT/pIB139, ΔglnRsav/pIB139, and ΔglnRsav/pIB-glnRsav, were grown on MS plates covered with sterilized cellophane, and the cultures were collected at 3 and 5 days. The experiments were performed with three independent biological replicates, and the error bars indicate the standard deviations.
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FIGURE 8. GlnRsav regulates avermectin and oligomycin biosynthesis through binding to the respective promoter regions of aveR and olmRII. A, EMSA analysis. The amounts of His6-tagged GlnRsav protein (μM) used are as indicated. In competition assays, 200-fold excess amounts of specific (unlabeled olmRII or aveR probe, S) and nonspecific probe DNA (sperm DNA, NS) were added. Free Cy5-labeled probes and GlnRsav-DNA complexes are shown by arrows. B, transcriptional analysis. Three pathway-specific regulatory genes, including aveR (for avermectin biosynthesis) and olmRII (for oligomycin production), were assessed for transcriptional analysis. RNA samples were prepared from cultures of S. avermitilis NRRL 8165 (WT) and the ΔglnRsav mutant grown on MS plates covered with sterile cellophane for 3 and 5 days. The values were determined after normalization to the internal control, rpoD. The relative values for each tested gene in NRRL 8165 (WT) tested at day 3 were arbitrarily assigned as 1. The relative transcription levels presented are averages of three independent biological replicates, and the error bars indicate the standard deviations.

~3-fold higher levels in the ΔglnRsav mutant (with pIB139) than in the wild-type strain (with pIB139) (Fig. 7D). Introduction of the wild-type glnRsav gene into the ΔglnRsav mutant under control of the strong and constitutive promoter ermE’p, readily restored antibiotic production, along with growth and development (Fig. 7, C and D). These results suggest that GlnRsav plays an important role in avermectin and oligomycin biosynthesis in S. avermitilis, inferring that GlnR-mediated antibiotic regulation might be universal in Streptomyces.

To determine whether GlnRsav regulates avermectin and oligomycin biosynthesis directly via the respective cluster-situated regulatory genes aveR (for avermectin biosynthesis) and olmRI/olmRII (for oligomycin biosynthesis), EMSA experiments were performed with purified His6-GlnRsav protein and three Cy5-labeled probes harboring the respective promoter regions of aveR, olmRI, and olmRII. The probe containing the promoter region of GlnRsav (glnRsavp) was used as a negative control. GlnR bound specifically to the promoter regions of all three tested probes but not to the control probe glnRsavp (Fig. 8A).

Subsequently, we assessed the effects of glnRsav deletion on antibiotic production at the transcriptional level. Transcription of these three regulatory genes was compared between the ΔglnRsav mutant and NRRL 8165 by qRT-PCR. RNA samples were isolated from the cultures grown on MS agar plates for 3 or 5 days. qRT-PCR analysis revealed that glnRsav deletion resulted in at least a 3-fold enhancement in olmRII/olmRII expression and significantly reduced aveR expression by over 2-fold (Fig. 8B), consistent with the phenotypic changes in antibiotic biosynthesis observed in the ΔglnRsav mutant. These results clearly suggest that GlnRsav plays a differential role in avermectin and oligomycin biosynthesis via directly regulating aveR and olmRII expression, respectively.

Discussion

In this study, we demonstrated that the master nitrogen metabolism regulator GlnR functions as an important player during antibiotic biosynthesis in two Streptomyces strains under the tested culture conditions, including the model strain S. coelicolor M145 and the avermectin-producing strain S. avermitilis NRRL 8165. We showed that GlnR-mediated regulation of the biosynthesis of four antibiotics in these two strains (ACT and RED from S. coelicolor and avermectin and oligomycin from S. avermitilis) is directly mediated by the pathway-specific regulators. To the best of our knowledge, this report describes the first experimental evidence indicating that GlnR regulates antibiotic biosynthesis directly through pathway-specific regulators in the genus Streptomyces. The results obtained here, when integrated with previous data (7–10, 13), clearly reveal that GlnR-dependent regulation of antibiotic biosynthesis appears to be conserved in Streptomyces, which further expanded our understanding of the function and the regulatory mechanisms of GlnR. However, here, we paid little attention to how GlnR coordinates its role in both primary (nitrogen and carbon metabolism) and secondary metabolism, an important issue that is the focus of our future research.

Herein, we show that the GlnR-binding sites upstream of redZ and actII-ORF4 completely or partially overlap those recognized by AfsQ1, which acts as a transcriptional activator for ACT and RED biosynthesis in S. coelicolor (14). Therefore, it is possible that base mutations of the GlnR-binding sites on the chromosome would inevitably affect the binding of GlnR and...
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AfsQ1, thereby leading to functional inactivation of both regulators during the regulation of *actII-ORF4* and *redZ*. That is to say, the phenotypic changes in ACT and RED production in the mutants with mutations of the GlnR-binding sites upstream of *redZ* and *actII-ORF4* are likely to be the result of the combined influence of both regulators. In our previous study, we demonstrated that TCS AfsQ1/Q2 exerts its effects only under the conditions of minimal medium supplemented with a high concentration of glutamate as the sole nitrogen source, but not under the R2YE culture condition (14). Therefore, it could be concluded that under the tested culture condition (R2YE), the effects of mutations in the GlnR-binding sites on ACT and RED biosynthesis, in which RED synthesis was reduced but ACT synthesis was enhanced, were exerted by GlnR and not by AfsQ1.

In the respective promoter regions of *actII-ORF4* from *S. coelicolor* and those of *olmr1/R2* and *aveR* from *S. avermitilis*, no typical GlnR-box containing the conserved 16-nt consensus sequence 5’-gTnAc-n6-GaAAc-3’ was identified. This phenomenon has also been reported for a GlnR homologue from *Streptomyces hygroscopicus* 5008, which produces the antifungal agent validamycin A (13). These results clearly demonstrate the diversity of DNA sequences recognized by GlnR and its homologues. In the future, it will be interesting to precisely define the GlnR-binding site located in these promoter regions, which may help to identify more putative GlnR direct target genes and to shed more light on GlnR-mediated transcriptional regulation of gene expression in streptomycetes.

The regulation of *actII-ORF4* expression in *S. coelicolor* has been intensively studied during the past two decades (4). In addition to the aforementioned TCS AfsQ1/Q2, the involvement of a number of global or pleiotropic regulators has been described, including AdpA (a global regulator of both antibiotic biosynthesis and morphological differentiation) (19), AtrA (a TetR-family transcriptional activator) (20), DasR (a GntR-family regulator) (21), DraR and AbsA2 (the two-component response regulators) (22, 23), and AbsC (a MarR-family regulator) (24), among others. These data clearly demonstrated the complexity of transcriptional regulation of *actII-ORF4* expression. In this study, we characterized GlnR as a novel regulator involved in the regulation of *actII-ORF4* expression. Interestingly, we found that although GlnR acts as a repressor of *actII-ORF4* transcription, the sequence recognized by GlnR in the *actII-ORF4* promoter region is located upstream of the −35 nt region, in contrast with the previous model in which binding of transcriptional regulators to positions upstream of the −35 nt regions of gene promoters would activate transcription (25). Considering the complexity of the regulation of *actII-ORF4* transcription, it could be speculated that the negative role of GlnR is likely to reflect the combined roles of GlnR and other regulators.

A previous study reported that deletion of *glnR* based on *S. coelicolor* M145 (the same strain that we used in this study) led to defective ACT and RED production, but these authors did not show the detailed results in their publication (7). These phenotypic changes in ACT and RED biosynthesis upon *glnR* deletion are inconsistent with the results obtained in this study. There are two possible reasons for this disagreement. First, the difference might be due to the media used for phenotypic analysis. Here, we used the rich medium R2YE for phenotypic analysis; however, in the previous study, the phenotypic changes were assessed using nitrogen-rich or limited solid and liquid medium. Because ACT and RED biosynthesis is controlled by a wide variety of regulators, under these culture conditions, a different array of regulators could be activated, and the combined effects of these regulators could contribute to the difference in phenotypic changes in antibiotic production. Second, as we know, *glnR* deletion led to greatly impaired growth, particularly during the early stage (Figs. 1, and supplemental S1 and S2). If antibiotic production was assayed during the early stage, defective ACT and RED production would be observed. Actually, this phenomenon was also observed in our study, as ACT and RED biosynthesis in the Δ*glnR* mutant was not detected before 48 h, as described in Figs. 1, and supplemental S1 and S2.

The Δ*glnR* mutant produced a higher level of ACT than the wild-type strain M145 only during late growth stages (after the 96-h time point) (Fig. 5), possibly due to the very impaired bacterial growth after *glnR* deletion at the early stages. In contrast, the *actIIpmu* mutant with base mutations in the putative GlnR-binding site upstream of *actII-ORF4* achieved a higher level of ACT production at earlier stages (after 60 h) than the Δ*glnR* mutant. This observation is possibly ascribed to unaffected bacterial growth of the *actIIpmu* mutant upon decoupling of the global functions of GlnR by mutating the binding site upstream of *actII-ORF4* without affecting its role in other physiologic processes, including carbon and nitrogen metabolism. Recently, Qu et al. (13) has reported that GlnR acts simultaneously as an activator and a repressor for validamycin biosynthesis by binding different sites in the intergenic region of *valK-valA* in *S. hygroscopicus* 5008. Through mutating the GlnR-binding site, which is required for GlnR-mediated repression of validamycin production, they achieved a 2.5-fold improvement of antibiotic titer. Therefore, it could be suggested that this strategy (only removing the repression of GlnR on antibiotic biosynthesis) could also be applicable in the engineering of other *Streptomyces* strains in which GlnR functions as a repressor of antibiotic biosynthesis.

Experimental Procedures

**Bacterial Strains, Primers, Plasmids, and Growth Conditions**—All of the bacterial strains and plasmids used or constructed in this study are listed in Table 1. The primers are listed in supplemental Table S1. *S. coelicolor* M145, *S. avermitilis* NRRL 8165, and their derivatives were grown on mannitol soya flour medium (MS) at 30 °C for spore suspension preparation (26). When used for the preparation of spore suspensions of Δ*glnR*, Δ*glnR/pIB139, Δ*glnRsav*, and Δ*glnRsav/pIB139, 40 mM glutamine (Glu) was added. MS agar plates were also used for intergeneric conjugation between *Escherichia coli* ET12567/pUZ8002 and *Streptomyces* strains (26). For analysis of *S. coelicolor* growth and antibiotic production, R2YE plates covered with sterilized cellophane were used (26). For quantitative determination of *S. avermitilis* growth and avermectin and oligomycin production, MS plates covered with sterilized cellophane were used (27). *E. coli* DH5α and BL21(DE3) were used as hosts for routine cloning and protein overexpression, respec-
### TABLE 1
The strains and plasmids used in this study

| Strains | Characteristics | Source |
|---------|----------------|--------|
| **S. coelicolor** | | |
| M145 | Parental strain; SCP1 | 26 |
| ΔglnR | M145 with an in-frame deletion of the glnR gene | 15 |
| M145/pIB139 | M145 containing the control vector pIB139 | This work |
| ΔglnR/pIB-glR | ΔglnR with the control vector pIB-glR | This work |
| actIIpumu | Mutant with base mutations in the GlnR-binding site upstream of actII-ORF4 in the M145 genome | This work |
| redZpumu | Mutant with base mutations in the GlnR-binding site upstream of redZ in the M145 genome | This work |
| **S. avermitilis** strains | | |
| NRRL8165 | Parental strain, also named MA-4680 (ATCC31267), the model avermectin-producing strain | 34 |
| glnRsav NRRL8165 | with an in-frame deletion of the glnRsav gene | This work |
| WT/pIB139 | The model strain NRRL8165, containing the control vector pIB139 | This work |
| ΔglnRsav/pIB139 | ΔglnRsav with the control vector pIB139 | This work |
| ΔglnRsav/pIB-glR | ΔglnRsav with the complemented vector pIB-glRav | This work |
| **E. coli** strains | | |
| DH5α | F- * lacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (Rk- Mk-) supE44 thi-1 gyrA96 relA1 | Gibco-BRL |
| BW25113/pIJ790 | BW25113 containing the temperature-sensitive plasmid pIJ790, encoding the ARED recombination system | 29 |
| ET12567/pUZ8002 | Non-methylating ET12567 containing the non-transmissible RP4 derivative plasmid pUZ8002 | 29 |
| BL21(DE3) | F- ompT hsdS gal dcm (DE3) | Novagen |
| **Plasmids** | | |
| pHAQ31 | Cosmid vector, *amp for melC* | Xia et al. (unpublished data) |
| SK-39 | pHAQ31 carrying the sequence from 4943037 to 4982218 bp of the S. avermitilis NRRL8165 genome, containing the glrR gene | Xia et al. (unpublished data) |
| pMD18-T simple | TA-cloning vector | TaKaRa |
| pMD-actII-ORF4p | Recombinant plasmid with the sequence of the actII-ORF4 promoter region (actII-ORF4p for EMSA analysis) cloned in pMD18-T | This work |
| pMD-redZp | Recombinant plasmid with the sequence of the redZ promoter region (redZp for EMSA analysis) cloned in pMD18-T | This work |
| pUN1/CRE | Replicative vector for actinomycetes, containing the synthetic cre(a) gene, *Ts* Ampr | This work |
| pIB139 | Integrative vector with the strong constitutive promoter ermE* p | 35 |
| plB-glR | pIB139 with the glrR gene cloned between the Ndel and EcoRI sites in which glrR is under control of the constitutive promoter ermE* p | This work |
| plB-glnRsav | pIB139 with the glnRsav gene cloned between the Ndel and EcoRI sites in which glnRsav is under control of the constitutive promoter ermE* p | This work |
| pET28a | Expression vector, *kan* | Novagen |
| pET-glR | pET28a carrying the glrR gene cloned between Ndel and EcoRI | This work |
| pET-glnRsav | pET28a carrying the glnRsav gene cloned between Ndel and EcoRI | This work |
| pKCa9actII-ORF4 | A CRISPR/Cas9 editing plasmid, containing the actII-ORF4 gene in S. coelicolor containing the actII-ORF4-specific sgRNA and two homologous arms | This work |
| pKCa9actII-ORF4pdel | A CRISPR/Cas9 editing plasmid, containing the actII-ORF4 downstream region (294 bp with respect to the translation start codon) in the genome, containing the cassette for the transcription of actIIpdel-specific sgRNA and two homologous arms | This work |
| pKCa9actII-ORF4pcom | A CRISPR/Cas9 editing plasmid, containing the complementation of the mutated actII-ORF4 promoter region of the genome containing the cassette for the transcription of actIIpcom-specific sgRNA and two homologous arms | This work |
| pKCa9redZpumu | A CRISPR/Cas9 editing plasmid, containing the base substitutions of the GlnR-binding site upstream of redZ in the genome, containing the cassette for the transcription of redZ-specific sgRNA and two homologous arms with the mutated sequences | This work |
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tively. E. coli strains were grown in Luria-Bertani (LB) broth or on LB agar plates (28). When necessary, antibiotics were added at the following concentrations (μg ml⁻¹): ampicillin, 100; kanamycin, 50; and apramycin, 50.

Construction of the ΔglnRsav Mutant—The S. avermitilis ΔglnRsav mutant with an in-frame deletion of the entire glnRsav (SAV_4042) coding region was constructed using the PCR-targeting system (29). Briefly, the mutant cosmids with the deletion of glnRsav was generated by electro-transformation of E. coli BW25113/pJ790 containing the cosmid 3–59 (harboring the glnRsav gene) with a disruption cassette, which was obtained by PCR using the primer pair DglnRsav-fw/rv and plasmid pIJ773 as the template. The correct deletion of glnRsav in the cosmid was verified by PCR using the primer pair JglnRsav-fw/rv. The resulting cosmid (3–59ΔglnRsavvac3JIV) was transferred into S. avermitilis NRRL 8165 by conjugal transfer between the nonmethylating E. coli ETI2567/pUZ8002 and NRRL 8165. The apramycin-resistant and kanamycin-sensitive exconjugants were selected, and the glnRsav disruption mutant (ΔglnRsavvac3JIV) was verified by colony PCR with primers JglnRsav-fw/rv and the genomic DNA of M145 as the template. The integrative vector pIB139 was used for the construction of two complemented S. avermitilis strains by conjugal transfer, which resulted in two complemented vectors, in which the product was digested with NdeI and EcoRI and then cloned in the primers glnRex-fw/rv and glnRsavex-fw/rv. Each PCR coding regions were amplified using the respective primer pairs glnRex-fw/rv and glnRsavex-fw/rv. Finally, the apramycin-resistant exconjugants were selected, and the glnRsav mutant was obtained by PCR using the primer pair DglnRsav-fw/rv and the genomic DNA of M145 as the template. The correct deletion of glnRsav was confirmed by PCR with primers DglnRsav-fw/rv containing the synthetic cre(a) gene (30), which resulted in the ΔglnRsav mutant. The UWLCRE was removed by three continuous passages on MS agar medium without antibiotics.

Genetic Complementation Assays—The integrative vector plB139 was used for the construction of two complemented vectors, including plB-glnR and pIB-glnRsav, which contained the glnR gene from S. coelicolor M145 and the glnRsav gene from S. avermitilis NRRL8165, respectively. The glnR and glnRsav coding regions were amplified using the respective primer pairs glnRex-fw/rv and glnRsavex-fw/rv. Each PCR product was digested with NdeI and EcoRI and then cloned in plB139 to yield two complemented vectors, in which glnR and glnRsav were under control of the strong and constitutive promoter ermE’p. Subsequently, plB-glnR and pIB-glnRsav were introduced into the ΔglnR and ΔglnRsav mutants, respectively, by conjugal transfer, which resulted in two complemented strains ΔglnR/pIB-glnRandΔglnR/pIB-glnR. Four strains, namely, M145/plB139, WT/plB139, ΔglnR/plB139, and ΔglnRsav/plB139, with introduction of the empty vector plB139 into M145, NRRL 8165, and the ΔglnR and ΔglnRsav mutants, were constructed, respectively, and used as controls.

Determination of Cell Growth and Antibiotic Production—For the quantitative determination of bacterial growth and antibiotic (ACT and RED) production, S. coelicolor strains with equivalent spore amounts (spore suspension OD₄₅₀ = 1.0) were grown on R2YE plates (8-cm diameter) covered with sterilized plastic cellophane, and the cultures were collected at 48, 60, 72, 96, 120, and 144 h. For bacterial growth, mycelia from the whole agar plates were collected, dried, and weighed. ACT and RED production levels were assayed using a previously described method (31). As ACT was produced both extracellularly and intracellularly (26), the cultures on the plastic cellophane and the agar were collected for determinations of ACT production. Quantitative determinations of bacterial growth and avermectin B1a and oligomycin A production from the S. avermitilis strains were performed as described previously (27). S. avermitilis and its derivatives with equivalent spore amounts (spore suspension OD₄₅₀ = 1.0) were grown on MS plates (8-cm diameter) covered with sterilized plastic cellophane and collected at 3 and 5 days. For bacterial growth measurements, mycelia from the whole agar plates were collected and weighed. Avermectin B1a and oligomycin A (olmA) production was analyzed by HPLC. These experiments were performed in triplicate and repeated twice.

RNA Preparation and Real-time RT-PCR Analysis—For RNA isolation, S. coelicolor and S. avermitilis strains were spread on R2YE and MS agar plates covered with sterilized plastic cellophane, respectively. The cultures were collected at different time points, and the samples were immediately frozen in liquid nitrogen and ground into powder. Total RNA was prepared with TRIzol™ reagent (Invitrogen) following the procedures recommended by the manufacturer. Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Takara, Dalian, China). Reverse transcription of total RNA was performed with a reverse transcriptase kit (Invitrogen) and 6-mer random primers (Takara, Dalian, China). qRT-PCR analysis was performed as described previously (32). The values were determined after normalization to the internal control hrdB (in S. coelicolor) or rpoD (in S. avermitilis). The relative values for each gene in S. coelicolor M145 or S. avermitilis NRRL 8165 assayed at the first time point were arbitrarily assigned as 1. The relative transcription levels presented are averages of three independent biological replicates, and the error bars indicate the standard deviations.

Protein Overexpression and Purification—The S. coelicolor glnR gene sequence was obtained by PCR amplification using the primers glnRex-fw/rv and the genomic DNA of M145 as the template. The purified PCR product was cloned into the expression vector pET28a between the NdeI and EcoRI sites, which led to pET-glnR that was further verified by DNA sequencing. Similarly, the glnRsav gene was amplified using the primer pair glnRsavex-fw/rv and then cloned into pET28a between the NdeI and EcoRI sites to yield the recombinant plasmid pET-glnRsav. Protein overexpression and purification were performed as described previously (22). The purities of His₆-GlnR and His₆-GlnRsav were analyzed by SDS-PAGE. The recombinant protein concentrations were determined by the Bradford method using a commercial kit (Sangon, Shanghai, China).

Electrophoretic Mobility Shift Assays (EMSAs)—EMSAs were performed according to a previously described method with some modifications (6). In brief, EMSA probes harboring the respective promoter regions of the tested genes were obtained by PCR amplification using the genomic DNA of S. coelicolor M145 or S. avermitilis NRRL 8165 as the template followed by labeling with a Cy5-labeled primer (5’-AGCCAGTGGCGATAAG-3’) via a second round of PCR. The conditions for EMSA analysis and detection were as previously described (22). To verify the specificity of GlnR (or GlnRsav)-probe interactions, 200-fold excess amounts of each specific unlabeled probe or sperm DNA (nonspecific DNA) were preincubated with purified His₆-GlnR or His₆-GlnRsav protein for 20 min at 25 °C, followed by addition of the corresponding labeled probes and
an additional 20 min of incubation. The samples were run in 0.5 X Tris acetate EDTA (TAE) buffer on non-denaturing 5% polyacrylamide gels. Finally, the gels were scanned directly with a FLA-9000 phosphorimagier (FujiFilm, Japan).

DNase I Footprinting Assays—DNase I footprinting assays were performed using purified His<sub>6</sub>-GlnR protein as described by Wang et al. (17). The probes for actII-ORF4p and redZp containing the respective promoter regions were obtained by PCR amplification using the primer pairs, actII-ORF4p-FAM-fw/rv and redZp-FAM-fw/rv, respectively, which were then cloned into the T-vector pUC18B-T (TOLO BIOTECHNOLOGY, Shanghai, China) and verified by DNA sequencing. Two FAM-labeled probes were obtained by PCR amplification using the primer pairs, actII-ORF4p and pMD-redZp. Subsequently, PCR amplification of the respective promoter regions were obtained by PCR using the primer pairs actIIpdel-fw/rv and actIIpcom-fw/rv. The PCR products were treated with the restriction enzyme DpnI to remove methylated PCR templates. The resulting PCR products were transferred into E. coli DH5α competent cells, and the transformants were grown on LB agar overnight at 37°C. The obtained strain was named redZpmu, with base mutations in the GlnR-binding site from 5’-TAA-TTTTGTGATCAATA-3’ to 5’-CGTACGCATTGACCGT-3’.

Mutations of the GlnR-binding Sites in the Probes of actII-ORF4p and redZp—Mutations of the GlnR-binding sites in the EMSA probes of actII-ORF4p and redZp were performed according to a previously described method (33). Briefly, PCR products of the wild-type probe sequences (actII-ORF4p and redZp) were cloned into the pMD-18T simple vector (Takara, Dalian, China), resulting in the recombinant plasmids pMD-actII-ORF4p and pMD-redZp. Subsequently, PCR amplification was performed using two partially complementary primer pairs (containing the mutated DNA sequences) listed in supplemental Table S1, and pMD-actII-ORF4p and pMD-redZp were used as the PCR templates, respectively. The PCR products were treated with the restriction enzyme DpnI to remove methylated PCR templates. The resulting PCR products were transferred into E. coli DH5α competent cells, and the transformants were grown on LB agar overnight at 37°C. The correct mutated plasmids were verified by DNA sequencing and were then used as the templates to generate the Cy5-labeled actII-ORF4p and redZp mutated probes via PCR with the same primer pairs as those used for the amplification of the wild-type EMSA probes.

Base Substitutions of GlnR-binding sites in the Respective Promoter Regions of actII-ORF4 and redZ in the M145 Genome—Base substitutions of the GlnR-binding site upstream of actII-ORF4 were made in two steps. The first step is to delete the upstream region of actII-ORF4 (294 bp with respect to the translation start codon), and the second step involved in situ complementation of the deleted region using the mutated actII-ORF4 upstream region. Both steps were performed using the CRISPR/Cas9-mediated method (15). For deletion of the upstream region of actII-ORF4, a cassette for the transcription of a specific single guide RNA (sgRNA; in this case, actIIpdel-sgRNA) was amplified from the plasmid pCB003 using the primer pair actIIpdel-grNA-fw/rv. The corresponding upstream and downstream homologous arms were obtained by PCR with two respective primer pairs, including actIIpdel-upf-wfv and actIIpdel-down-fw/rv, using the genomic DNA of S. coelicolor M145 as the template. Then, three DNA fragments, including the sgRNA transcription cassette and the downstream and upstream homologous arms, were ligated into one fragment by overlapping PCR. The resulting PCR product was cloned into pKCCas9dO (between SpeI and HindIII) to yield pKCCas9actIIpdel, which was transformed into E. coli ET12567/pUZ8002 and then conjugated into M145 (26). Correct deletion of the actII-ORF4 upstream region was verified by PCR using the primer pair actIIpdel-fw/rv followed by sequencing. The editing plasmid pKCCas9actIIpdel was removed by growing on MS plates without antibiotics for two rounds at 37°C. The obtained strain was named M145ΔactII-ORF4p.

For in situ complementation of the mutated actII-ORF4 upstream region, a cassette for the transcription of a specific sgRNA (actIIpcom-sgRNA) was amplified using primers actIIpcom-grNA-fw/rv. The designed guide RNA (20 nt) of actIIpcom-sgRNA could only cleave the genome of M145ΔactII-ORF4p but not the complemented strains. Two homologous arms were obtained by PCR using the primer pairs actIIpcom-upf-wfv and actIIpcom-down-fw/rv. Similarly, these three fragments were inserted into pKCCas9dO (between SpeI and HindIII) to yield pKCCas9actIIpcom, which was transferred into M145 by conjugation. The correct in situ complementation of the mutated actII-ORF4 upstream region was checked by PCR using the primers actIIpcom-fw/rv followed by PCR product sequencing. The obtained strain was named actIIpmu, with base mutations in the GlnR-binding site from 5’-TAAAA-TTTTGTGATCAATA-3’ to 5’-CGTACGCATTGACCGT-3’.

The mutant with base mutations in the GlnR-binding site upstream of redZ was generated in one step using CRISPR/Cas9-mediated genome editing technology (15). The primer pair redZpmu-grNA-fw/rv was used for amplification of the specific sgRNA (redZpmu-sgRNA) transcription cassette. Two homologous arms were obtained by PCR using the primer pairs redZpmu-upf-wfv and redZpmu-down-fw/rv. The resulting editing plasmid was named pKCCasredZpmu. The correct mutations of the redZ upstream region were verified by PCR using the primer pair JredZpmu-fw/rv followed by DNA sequencing. The generated strain was named redZpmu, with targeted mutations in the GlnR-binding site from 5’-GAAAAC-CAGTTGTATAC-3’ to 5’-TGGGACATTCTGGAG-3’.

Author Contributions—J. M. H. and Y. H. L. designed the study and wrote the paper. G. Q. Z. and W. H. J. coordinated the study and wrote the paper with J. M. H. and Y. H. L. J. M. H. designed, performed, and analyzed the experiments shown in Figs. 1, 7, and 8. H. Z. performed and analyzed the experiments of Figs. 2 and 6. G. S. Z. and P. P. L. designed, performed, and analyzed the experiments of Figs. 3–5. J. W. and G. P. Z. provided technical assistance and contributed to the preparation of Figs. 3 and 5. All authors approved the final version of the manuscript.

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Direct Involvement of the Master Nitrogen Metabolism Regulator GlnR in Antibiotic Biosynthesis in *Streptomyces*
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