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Identification of Three Positive Regulators in the Geldanamycin PKS Gene Cluster of *Streptomyces hygroscopicus* JCM4427

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In the *Streptomyces hygroscopicus* JCM4427 geldanamycin biosynthetic gene cluster, five putative regulatory genes were identified by protein homology searching. Among those genes, *gel14*, *gel17*, and *gel19* are located downstream of polyketide synthase genes. Gel14 and Gel17 are members of the LAL family of transcriptional regulators, including an ATP/GTP-binding domain at the N-terminus and a DNA-binding helix-turn-helix domain at the C-terminus. Gel19 is a member of the TetR family of transcriptional regulators, which generally act to repress transcription. To verify the biological significance of the putative regulators in geldanamycin production, they were individually characterized by gene disruption, genetic complementation, and transcriptional analyses. All three genes were confirmed as positive regulators of geldanamycin production. Specifically, Gel17 and Gel19 are required for *gel14* as well as *gelA* gene expression.

Keywords: Regulator, geldanamycin, biosynthesis, *Streptomyces*

Streptomycetes are filamentous soil bacteria known for their ability to produce a wide variety of secondary metabolites. The genes involved in the assembly of secondary metabolites from simple carbon precursors are clustered together with regulatory genes that coordinate their expression [28]. It has been well established that the regulation of secondary metabolite production in *Streptomyces* spp. involves a complex regulatory network that operates at several layers of control response to nutritional and environmental factors [2, 13, 22]. Analysis of these regulatory genes is crucial for understanding the mechanism of regulation, and to construct strains overproducing these compounds.

An entire family of regulatory genes has been identified based on sequence and motif homology analyses, as well as through complementation studies. Many of the pathway-specific regulatory proteins that control secondary metabolism in *Streptomyces* belong to the SARP (*Streptomyces* antibiotic regulatory proteins) family. These transcriptional activators contain a winged helix-turn-helix motif at their N-terminus that is related to the OmpR family of proteins [26]. TetR-like proteins also comprise a large family of prokaryotic transcriptional regulators, many of which function as repressors [23]. These proteins control genes involved in the biosynthesis of secondary metabolites, some of which act as signals to trigger cellular differentiation, whereas others can have an effect on antibiotic resistance. The regulatory mechanisms have been identified with the help of high-resolution crystal structures for the TetR family of proteins [10, 21]. These proteins are homodimers, with an N-terminal helix-turn-helix DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). Another important family of regulators has recently been found for several macrolide antibiotic pathways. They have an N-terminal ATP-binding domain represented by clear Walker A and B motifs [29], and a C-terminal LuxR-type DNA-binding domain [9]. Regulators belonging to this so-called LAL (large ATP-binding regulators of the LuxR) family have been identified [4] and characterized in several macrolide antibiotic pathways, including PikD for pikromycin from *Streptomyces venezuelae* [30], RapH for rapamycin from *Streptomyces hygroscopicus* [18], and TmcN for tautomycetin from *Streptomyces* sp. CK4412 [14]. Multiple LAL homologs also occur in the nystatin (three), amphotericin (three), and candicidin (three) gene clusters [17].

Geldanamycin is a member of the ansa-macrolide family of secondary metabolites, and this group of compounds is attracting increased interest owing to its anticancer activities *via* inhibition of the human heat-shock protein 90 (Hsp90) chaperone [20]. The geldanamycin biosynthetic gene cluster has been sequenced in *S. hygroscopicus* NRRL 3602 by Rascher *et al*. [24], in *S. hygroscopicus* 17997 by a Chinese group [8], and in *S. hygroscopicus* JCM4427 by

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our laboratory [11]. Each geldanamycin biosynthetic gene cluster encodes seven polyketide synthase (PKS) modules, comprising three multifunctional enzymes, three post-PKS modification enzymes, precursor producing enzymes, and regulatory proteins. Among the regulatory genes in *S. hygroscopicus* 17997, the functions of two LAL family regulatory genes, *gdmRI* and *gdmRII*, were recently reported [7].

We identified five putative regulatory genes through sequence analysis and three of these genes, *gel14*, *gel17*, and *gel19*, were located in the downstream region of the geldanamycin biosynthetic gene cluster in *S. hygroscopicus* JCM4427. In this study, the regulatory effects of these three genes on geldanamycin production were examined by gene knock-out and complementation experiments. The *gel14* and *gel17* genes, which encode LAL family regulatory proteins, are identical to previous reported regulators in *S. hygroscopicus* 17997. However, we also found that Gel19 is a TetR-type positive regulator of geldanamycin production. In addition, we analyzed in detail the transcriptional regulation of the geldanamycin biosynthetic gene cluster by performing reverse transcription PCR studies in strains mutated for each of these regulators.

Materials and Methods

Bacterial Strains, Media, and Plasmids

Wild-type geldanamycin-producing strain *S. hygroscopicus* subsp. *duamyceticus* JCM4427 was obtained from the Japanese Culture Collection of Microorganisms. These strains were grown in YEME to obtain mycelia for chromosomal DNA isolation and metabolite extraction [12]. *Escherichia coli* strain XL1-blue was used as the general cloning host, and *E. coli* ET12567(pUZ8002) was used as the donor strain for intergeneic conjugation [3]. *E. coli* strains were grown in Luria–Bertani (LB) medium or on LB agar supplemented with appropriate antibiotics. Apramycin (50 μg/ml), chloramphenicol (25 μg/ml), or kanamycin (50 μg/ml) was added to the growth medium as required. Gene disruption experiments were carried out using pKC1139 as a vector and by inserting the kanamycin resistance gene from pFDneo-S vector as a selective marker [5].

General Genetic Manipulation and Analysis of Metabolites

General molecular biological techniques were performed as described by Sambrook *et al*. [25]. Wild-type strain, gene inactivation mutants, and complemented strains were grown in 50 ml of liquid R2YE medium with appropriate antibiotics and incubated at 28°C for 7 days. The metabolites were extracted and analyzed as per our previously reported methods [27].

Gene Inactivation and Complementation Experiments

Gene inactivation was performed using plasmid pKC1139 to deliver the corresponding kanamycin resistance gene cassettes, as previously reported [27]. Genetic complementation experiments were carried out by introducing individual genes cloned from wild-type strains into the corresponding mutants by using replicating vectors derived from plasmid pWK-HisA, which includes the *ermE** promoter from the multiple cloning sequence of the pKC1139 vector [15]. Detailed primer information for each construction is summarized in Table 1. The GenBank accession number of the sequence reported in this paper is DQ249341.

Isolation of Total RNA and RT–PCR

S. hygroscopicus and mutant strains were grown for 24, 72, and 144 h in soluble R2YE medium. The cultures were harvested by centrifugation at 2,000 ×*g* for 20 min and washed three times with DEPC-treated water. Then the cells were immediately frozen by immersion in liquid nitrogen. Frozen mycelia were broken by grinding in a mortar. The RNeasy mini kit was used for RNA isolation according to the manufacturer's instructions (Qiagen). Total RNA preparations were treated with RNase free DNase I (Qiagen) to eliminate possible chromosomal DNA contamination. The absence of contaminating DNA was checked by PCR amplification, using primers corresponding to the 16S rRNA gene, the *hrdB* gene [15], and to geldanamycin biosynthetic genes (Table 1), indicating that the total RNA preparation was competent for subsequent reverse transcriptase PCR manipulations. Quantitation of isolated total RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. RT–PCR was done with 5 μg of RNA, 2 pmol of RT primer, SuperScript II reverse transcriptase (Invitrogen), and RNase H (Takara), according to the manufacturer's instructions. The PCR conditions were 97°C for 40 s, 50°C for 40 s, and 72°C for 1 min, in a total of 28 cycles. In every RT–PCR, no amplification from the genome DNA occurred without reverse transcriptase, indicating no contamination of DNA.

Results and Discussion

Sequence Analysis of Putative Regulators in the Geldanamycin Biosynthetic Gene Cluster

In the *S. hygroscopicus* JCM4427 geldanamycin PKS gene cluster, *gel14*, *gel17*, *gel19*, *orf10*, and *orf11*, coding for putative regulatory genes, were analyzed by BLAST search and protein homology alignment. The divergently transcribed genes *orf10* and *orf11* are located upstream of the geldanamycin PKS genes. Orf10 and Orf11 are protein products with predicted polypeptides of 294 and 253 amino acids, respectively. A BlastP sequence homology search of Orf10 identified a putative Ara-C family domain, and a BlastP search of Orf11 identified a TetR family regulatory domain.

The amino acid sequences of Gel14 and Gel17 revealed a hypothetical N-terminal nucleotide triphosphate-binding domain, or Walker A motif GxxxGKT, as well as a possible C-terminus helix-turn-helix (HTH) DNA-binding domain belonging to the LuxR family (Fig. 1A). Several LAL regulatory proteins showing strong homology to Gel14 and Gel17 have been shown to be positive regulators, including PikD of the pikromycin-producing strain *S. venezuelae* [30], AveR of the avermectin-producing strain *S. avermitilis* [16], and TmcN of the tautomycetin-producing strain *Streptomyces* sp. CK4412 [14]. Specifically,

Table 1. Oligonucleotide primers used in this study.

Description	Name or target	Sequences (5'–3')	Restriction Enzyme site
gel14 gene disruption cassette	gel14-HS-s	G**AAGCTT**CCCGGCGATCTCCCGGTTGGTG	*Hin*dIII
	gel14-HS-a	CGTCCATCGCC**GTCGAC**TTACTGGAGC	*Sal*I
	gel14-SE-s	GCTCCAGTAA**GTCGAC**GGCGATGGACG	*Sal*I
	gel14-SE-a	CCCAGTC**GAATTC**CCGTCCGATGGGCG	*Eco*RI
gel17 gene disruption cassette	gel17-ES-s	**GAATTC**GGGGTAGTCGCGTATCTCGTCCA	*Eco*RI
	gel17-ES-a	**GTCGAC**TCGATGAGGCCGAGCGATATCTGC	*Sal*I
	gel17-SH-s	**GTC GAC** CAG CGC CAT GAG AAT GTC CATGT	*Sal*I
	gel17-SH-a	**AAG CTT** CGC GAC GTC CTC GAC GCG GCA G	*Hin*dIII
gel19 gene disruption cassette	gel19-HK-s	CCC **AAG CTT** CAC CGG TAT GGC CCG CGA GTGC	*Hin*dIII
	gel19-HK-a	CC**G GTA CC**A TAT GCG TCC GCA TCA GCC GCGG	*Kpn*I
	gel19-PE-s	AA**C TGC AG**T GCC CCG AGC AGC AGC GGC TGG	*Pst*I
	gel19-PE-a	CG**G AAT TC**C CGG ACG GCG GAA ACC CCG GCG	*Eco*RI
Gene disruption confirmation	neo L (*aphII)*	CAA TCC ATC TTG TTC AAT CAT GCG AAA	-
	neo R (*aphII*)	CGC ATC GCC TTC TAT CGC CTT CTT G	-
	g14- c (*gel14*)	GCC CTG CGC GAG GTG AAC AAG CTG CTCC	-
	g17- c (*gel17*)	GGA TCG TCC ATC CTC CCG AGG TTG AAC	-
	g19 –c (*gel19*)	CGA ATC GAG TCC GCC GAG TCC TGG CTC	-
Gene complementation	*gel14*	**CAT ATG** CCC TAC AGC TAT GCC ATG CCG TTG AAT	*Nde*I
		AAG CTT CAC GCC AAC TCC GGC CGG TCG ACG TACAG	*Hin*dIII
	gel17	**CAT ATG** ACT GCG GAG ATC AAC TCA TCG CTC AGA	*Nde*I
		AAG CTT CAG CAG GAG GAC GAT CCG GTG GCT CCGAT	*Hin*dIII
	gel19	GA**A GAT CT**A TGG TCC CCC GAA GCC CGT CGG TC	*Bgl*II
		CCC **AAG CTT** TCA CTC CGG CGT GCT CAT CCG GCT	*Hin*dIII
RT–PCR primer	*orf10*	GTG CAC CGC TGG AGC GAT ATC GACG	-
		ATG GTG CCA GTC GGT GAA GCG CTCT'	-
	orf11	CGC CGG TCT TGA ACC CTG CCA TCCG	-
		CGG GAA CGA AAG AAG CAG CGG ACCC	-
	gelA (polyketide gene)	CGC GAC GAC CTG GGC CTG GAC GAAA	-
		ACC TGA CCA GGT CCG CCG TGG CCAC	-
	gel8	GGT AGG TCT CCG GGT TGC CCC AGGG	-
		ACC GAA TAT CCG CGG GCA CCC CTGC	-
	gel14	CGG TCT CCC CCA GCT CCG CCA CGACCAC	-
		GGG GCT GCC GGA CAT CCT CCG CGCG	-
	gel16	CGG TGG CGA CCA CCT GCT GCT GGGT	-
		ACT TCA CCA TCC GGC GCA CCG AGGC	-
	gel19	CGG CCG TCC ACC AGC TGA TGC ACAT	-
		CTC CCA GTC CAG TCC GTA GCG GTGG	-
	hrdB	CGA GGA GGG TGT GAC GCT GAT GGTC	-
		GTG CGG CCT CCT CGA TGA CCT CGTC	-
	16s rRNA	ACG CTC CCG CAT GGG ATG CGT GTGG	-
		TGCAGGTACCGTCACTCTCGCTTCT	-

the homology between Gel14 and Gel17 showed 26.6% identity, using ClustalW alignment. The presence of multi–copies of this LAL regulator near to a biosynthetic gene cluster also occurs in the nystatin, amphotericin, and candicidin gene clusters.

The Gel19 protein is predicted to be a TetR N-family transcriptional regulator, with an N-terminal helix-turn-helix DNA-binding domain. Amino acid sequence analysis showed that Gel19 had homology to the ActII protein (a putative transcriptional regulator) from *Streptomyces coelicolor* [1], Sim16 from *Streptomyces antibioticus* [19], TcmR from *Streptomyces glaucescens* [6], and TetR (tetracycline repressor protein class D) from *E. coli* [21]. In the TetR crystal structure, this conserved stretch corresponded to the almost complete α-helix 1, the HTH domain formed by α-helices 2 and 3, and five residues of

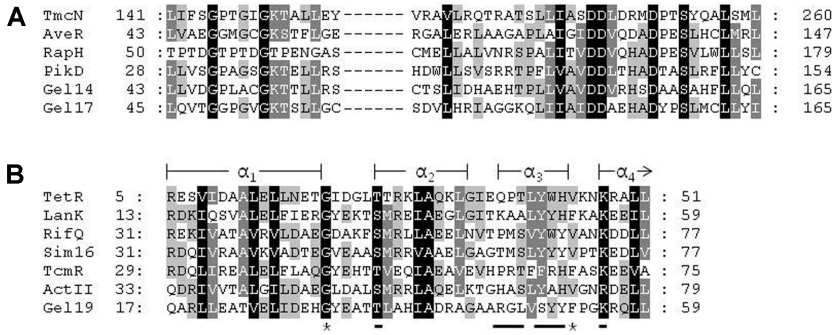

Fig. 1. Amino acid alignment of putative regulators.
A. LuxR family showed GxxxGKT at the N-terminal region and HTH motif at the C-terminal region. **B**. The bold underlines indicate α-helix residues involved in DNA contacts in the crystal structure of TetR. The asterisk indicates turns. The most conserved residues are shaded.

the α-helix that connects the DNA-interacting region with the core of the protein (Fig. 1B).

Functional Analysis of Three Regulatory Genes, *gel14*, *gel17*, and *gel19*

To functionally characterize these putative transcriptional regulators, *gel14*, *gel17*, and *gel19* were chromosomally deleted using a kanamycin resistance gene (*aphII*) replacement cassette. Knock-out strains carrying the disrupted genes were confirmed by PCR and sequencing analysis using total genomic DNA as template (Fig. 2). The mutant strains, Δgel14 and Δgel17, had growth and morphological characteristics identical to those of wild-type *S. hygroscopicus* JCM4427 when grown on solid media. These results indicate that the *gel14* and *gel17* genes play no role in bacterial growth or differentiation. However, mutant Δgel19 showed no spore production under these culture conditions. Whereas wild-type cells produced geldanamycin under these conditions, no trace of geldanamycin or pathway intermediates were observed in all the Δgel14, Δgel17, or

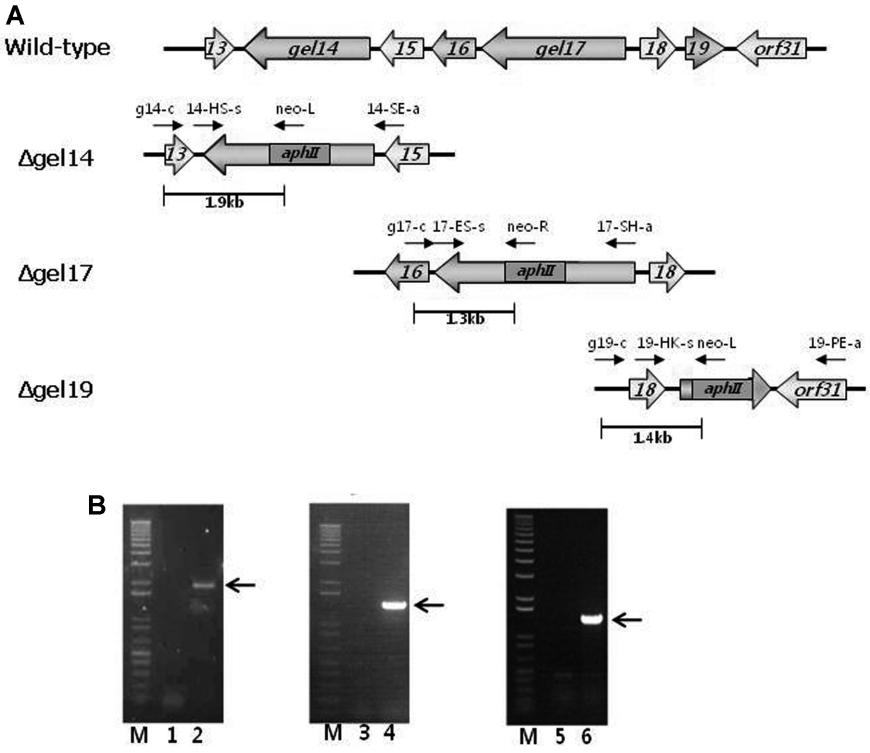

Fig. 2. Gene inactivation experiments of the regulatory genes.
A. Genetic organization of the regulatory genes in the geldanamycin biosynthetic gene cluster and construction of the gene knock-out mutants, Δgel14, Δgel17, and Δgel19. **B**. Confirmation of constructed mutant strains by PCR. Lane M, 1 kb ladder (SolGent Co.); 1, 3, and 5, PCR was performed with confirmation primers on *S. hygroscopicus* JCM4427 genomic DNA; 2, with 14-c and neo-L primers on Δgel14 genomic DNA; 4, with 17-c and neo-R primers on Δgel17; 6, with 19-c and neo-L primers on Δgel19.

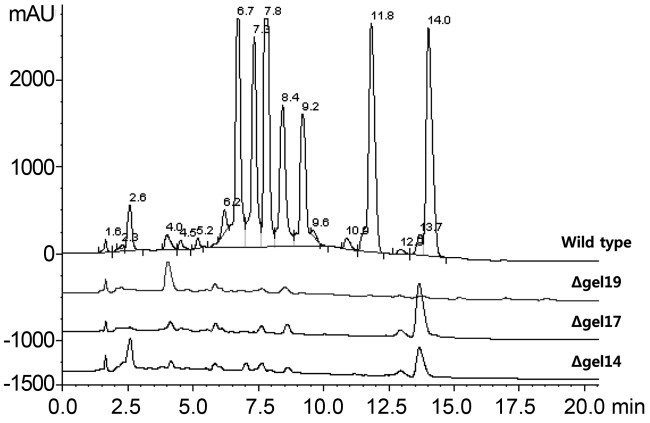

Fig. 3. HPLC analyses from the ethylacetate extracts of culture broth obtained from the geldanamycin regulation gene mutants in comparison with that of culture broth from *S. hygroscopicus* JCM4427.
Peak on t_R 14.0 min, geldanamycin; Peak on t_R 13.7 min, unknown compounds.

Δgel19 strains (Fig. 3). These results indicate that all three regulators are required for geldanamycin production, and act as positive regulators. The absence of geldanamycin and other intermediates demonstrates that these regulators exhibit control of the early stages of polyketide biosynthesis. We hypothesize that they affect the initiation of macrolide production by positively regulating expression of the *gelA*-encoded polyketide synthase gene.

To further demonstrate that the inactivation of these three regulators was indeed responsible for eliminating geldanamycin production, we performed genetic complementation experiments in each mutant strain by expressing each gene under the control of the *ermE** promoter. We did not use their own promoters, to exclude the effect of feedback between these genes. We used the conjugative vector pWK-HisA [15], which includes the *ermE** promoter from the multiple cloning sequence of the pKC1139 vector. Three complementation plasmids, pWK-g14c, pWK-g17c, and pKC-g19c, were constructed. Each of the plasmids was introduced into their respective mutant strains, and selected by apramycin resistance. HPLC analysis confirmed that geldanamycin productivity was restored in each strain to significant levels comparable to the wild-type strain (data not shown, [15]). From these results, we conclude that Gel14, Gel17, and Gel19 work as pathway-specific activators of geldanamycin production.

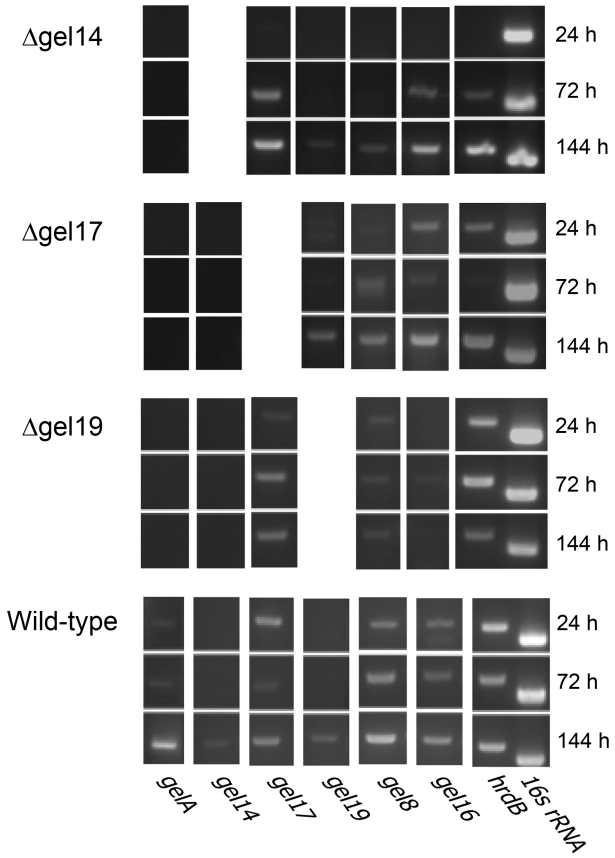

Fig. 4. Transcriptional analysis of the geldanamycin biosynthetic genes.
Transcripts from *S. hygroscopicus* JCM4427 wild-type, Δgel14, Δgel17, and Δgel19 strains were analyzed by RT–PCR. Total RNA was extracted from all four strains after 24, 72, and 144 h fermentation. Routinely, 28 cycles of PCR were used; whenever this generated no apparent product, the analysis was repeated at 32 cycles in attempts to detect transcript. Transcriptions of the 16S rRNA and *hrdB* gene were examined as an internal control.

Transcriptional Control of Geldanamycin Biosynthetic Genes

In order to determine the transcription levels of these regulatory genes and geldanamycin biosynthetic genes in

Table 2. Summary of the relationships between geldanamycin production and transcription of each regulatory gene in mutant strains.

	Geldanamycin production	Gene expression					
		gelA	*gel8*	*gel16*	*gel14*	*gel17*	*gel19*
Wild-type	○	○	○	○	○	○	○
Δgel14	×	×	○	○	-	○	○
Δgel17	×	×	○	○	×	-	○
Δgel19	×	×	○	△	×	○	-

○ and × represent strong and no expression, respectively. △ represents weak and delayed expression.

the wild-type strain as well as in these three mutants, RT–PCR was performed with total RNA isolated after 24, 48, and 72 h of growth in liquid R2YE media. Primers for RT–PCR were specific to sequences within the geldanamycin biosynthetic cluster genes (Table 1), and were designed to produce cDNAs of approximately 300 bp. The transcriptional activity of the *gelA* gene, which is the first of the three PKS genes responsible for geldanamycin production, was abolished in the Δgel14, Δgel17, and Δgel19 strains (Fig. 4). We also examined the transcriptional activity of post-PKS modification genes, *gel8* (carbamoyl transferase gene) and *gel16* (p450 hydroxylase gene), and found they were not significantly affected by disruption of the *gel14*, *gel17*, or *gel19* genes. Interestingly, expression of the *gel8* and *gel16* genes was greatly reduced at early culture time points in the Δgel14 mutant. Moreover, expression of the *gel16* gene, which is involved in the last post-PKS modification step, was delayed in the Δgel19 mutants. However, overall, these results demonstrated that the geldanamycin production deficiency in Δgel14, Δgel17, and Δgel19 was primarily caused by a lack of *gelA* gene expression.

Transcription of *gel17* and *gel19* was normal in the Δgel14 strain, whereas transcription of the *gel14* gene was abolished in the Δgel17 and Δgel19 strains. This result suggests that the *gel14* gene is regulated downstream of *gel17* and *gel19*. These results contrast with the results reported previously by He *et al*. [7]. The *gdmRI* and *gdmRII* genes in *S. hygroscopicus* 17997, which are related to *gel14* and *gel17*, do not regulate each other's transcription. In addition, the *gdmRI* and *gdmRII* mutants do not express the cytochrome P450 gene, identified in *S. hygroscopicus* JCM4427 as *gel16*. We suggest that Gel17 and Gel19 are required for *gel14* as well as *gelA* gene expression. However, we could not identify the genetic relationship between the *gel17* and *gel19* genes.

Our data demonstrate that *gel14* and *gel17* encode LAL-family pathway-specific activators of the geldanamycin biosynthetic pathway in *S. hygroscopicus* JCM4427. The discovery of Gel19 as a positive regulator of geldanamycin production has significant implications. Relatively few activators have been identified or analyzed in detail for polyketide pathways, and little is known about their precise mechanism of genetic control. These results suggest that the Gel14, Gel17, and Gel19 regulators might act in a cascade to regulate the geldanamycin biosynthetic pathway at multiple levels.

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