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Functional Analysis of SGR4635-Induced Enhancement of Pigmented Antibiotic Production in *Streptomyces lividans*

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The Gram-positive mycelium-producing bacterium *Streptomyces* undergoes complex morphological differentiation after autolytic degradation of the vegetative mycelium. Cell-wall breakdown during growth stimulates cell development and secondary metabolite production by *Streptomyces*. *N*-acetylglucosamine (GlcNAc) produced by cell-wall lysis acts as a signal molecule, triggering the production of secondary metabolites in *S. coelicolor* A3(2). Here, we report that introduction of multiple copies of the GlcNAc-internalizing gene (*sgr4635*, encoding *nagE2*) of *S. griseus* activates actinorhodin and undecylprodigiosin production during the late growth of *S. lividans* in the absence of GlcNAc. Furthermore, the repressor-type transcriptional regulator DasR binds to two operator sites upstream of *sgr4635*. Our findings indicate that *sgr4635* induces DasR-mediated antibiotic production by internalizing the GlcNAc accumulated from cell-wall lysis.

Keywords: *Streptomyces*, antibiotic production, *N*-acetylglucosamine, transcriptional regulator, DasR

Members of *Streptomyces* are widely used as industrial producers of various secondary metabolites, such as antibiotics, anticancer agents, fungicides, and enzymes of industrial importance, (e.g. glucose isomerase and transglutaminase) (Horinouchi, 2002). When *Streptomyces* grows on solid media, the spores germinate to grow into filamentous, multinucleated hyphae (vegetative mycelia) that contain relatively sparse, thin, single layered vegetative septa. Subsequently, vegetative mycelia develop into aerial mycelia, whereupon the hyphae branch out, forming chains of unigenomic compartments (spores) separated by double-layered sporulation septa. Morphological differentiation and secondary metabolism of the *Streptomyces* are simultaneously controlled by environmental nutrients, such as carbon, nitrogen, phosphorus, and trace elements. A pleiotropic autoregulatory factor (A-factor, 2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) triggers both morphological and physiological differentiation of *Streptomyces* (Horinouchi *et al*., 1984; Hong *et al*., 1993; Horinouchi and Beppu, 1994; Chater and Horinouchi, 2003; Kato *et al*., 2005; Horinouchi, 2007; Chi *et al*., 2011).

Rigali *et al*. (2008) recently showed that the pleiotropic transcriptional repressor DasR triggers antibiotic production through the response of *Streptomyces* to environmental nutrient stress. In *S. coelicolor* A3(2), *N*-acetylglucosamine (GlcNAc) acts as a signal for antibiotic production. High extracellular concentrations of *N*-acetylglucosamine prevent *S. coelicolor* from progressing beyond the vegetative state. The signal is transmitted via the global regulator DasR which represses the *N*-acetylglucosamine regulon, including the *pts* genes *ptsH*, *ptsI* and *crr* needed for uptake of *N*-acetylglucosamine. When the extracellular concentration of GlcNAc is high, GlcNAc is transported and metabolized to GlcN-6-P, resulting in inhibition of DasR DNA-binding ability, which leads to induction of the GlcNAc regulon. Accordingly, the PTS components transfer the phosphate group from PEP to GlcNAc and thus prevent the PTS phosphotransfer-mediated control of development-specific proteins (Rigali *et al*., 2006). GlcNAc is transported into the cell and phosphorylated by the phosphotransferase system (PTS). The resulting GlcNAc-6-P is deacetylated to glucosamine-6-phosphate (GlcN-6-P), which induces the transcription of *actII*-ORF4 [encoding an activator of actinorhodin (Act) production] and *redZ* [an activator of undecylprodigiosin (Red) production] by functioning as an allosteric effector of DasR. GlcNAc addition induced antibiotic production in *S. coelicolor* A3(2), *S. venezuelae*, *S. clavuligerus*, *S. hygroscopicus*, *S. collinus*, and *S. griseus* grown on agar (Bentley *et al*., 2002; Bibb, 2005; Colson *et al*., 2006; Rigali *et al*., 2008). In *S. griseus*, DasR represses an adjacent gene cluster encoding a putative sugar ABC transporter (*dasABC*) that is responsible for the ectopic sporulation (ESP) phenotype, as well as its own transcription in an autoregulatory manner (Seo *et al*., 2002). In *S. coelicolor* A3(2), the GlcNAc-specific enzyme IIC (*nagE2*) is a member of the DasR regulon and it mediates nutrient signals to control the development of cells and antibiotic production (Nothaft *et al*., 2010).

In this study, we showed that antibiotic production was affected by the heterologous expression of *S. griseus nagE2* (*sgr4635*) in *S. lividans* TK21 in the absence of DasR control.

Materials and Methods

General recombinant DNA studies

S. griseus IFO13350 was obtained from Professor Sueharu Horinouchi, The University of Tokyo, Japan. *S. lividans* TK21 was used as the host for *sgr4635* overexpression. Strains of *Streptomyces* were grown

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on R2YE agar (2% agar) (Hopwood *et al*., 1985). R2YE medium was also used for the regeneration of protoplasts. *Escherichia coli* JM109 and the vector pBluescript II KS(+) was used for DNA manipulation. *E. coli* ET12567 was used as a host to prepare non-methylated DNA of *Streptomyces* for transformation*. E. coli* was maintained on M9 agar and routinely cultured in Luria-Bertani medium at 37°C with periodic agitation (Sambrook and Russel *et al*., 2001). *E. coli-Streptomyces* shuttle vector, pWHM3 was used for *sgr4635* expression in *S. lividans* TK21. Restriction enzymes, T4 DNA ligase, and Taq DNA polymerase were purchased from TaKaRa Bio Inc. (Japan). DNA manipulation techniques were performed according to standard methods described by Maniatis *et al*. (1982).

Gel mobility shift assay

Purification of DasR-H from *E. coli* BL21(DE3) pLysS and the gel mobility shift assay were performed according to the methods described previously (Kato *et al*., 2005). Briefly, the *dasR* coding region was amplified from *S. griseus* chromosomal DNA by polymerase chain reaction (PCR) with the primer pair: dasR-F (5′-GCG*GGATCC*CATATGGGCGCCGAAGGGGGCAGTACG-3′; italicized letters: *Bam*HI site, underlined letters: *Nde*I site, and ATG in the *Nde*I site: start codon of *dasR*) and dasR-R (5′-GCT*AAGCTT*CTCGAG*GTC*CGTGGGCCGCTTCAGGCGGG-3′; italicized letters: *Hin*dIII site, and underlined letters: *Xho*I site). The DNA fragment was digested with *Bam*HI and *Hin*dIII and inserted into pUC19. The *Nde*I-*Xho*I fragment containing *dasR* was inserted into pET26b(+), generating pET26b-dasR. *E. coli* BL21(DE3) pLysS harboring pET26b-dasR was cultured at 37°C for 3 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). DasR with a His-tag at the C-terminus was purified from the soluble fraction by using Ni^{2+}-NTA Spin Column (QIAGEN, Germany), and the purified DasR-H was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). For the gel mobility shift assay, the DNA fragment acting as probe was amplified by PCR with the primer pair: NAGE2-F (5′-CGGATCTGCTACTGCGAATAAGCA-3′) and NAGE2-R (5′-ACACGGTGCCGCCCAGGTCGTTGA-3′), and labeled with ^{32}P using [γ-^{32}P] ATP and T4 polynucleotide kinase. The DasR-DNA complex was analyzed on a 6% polyacrylamide gel.

Construction of *sgr4635* overexpression plasmid

A 1.3 kb DNA fragment containing *sgr4635* was amplified by PCR with the primers, SGR4635(EX)-F (5′-GCG*GGATCC*CATATGACCACGGCTGAGACCGCACCCGCG-3′; italicized letters: *Bam*HI site, underlined letters: *Nde*I site, and ATG in the *Nde*I site: start codon of *sgr4635*) and SGR4635(EX)-R (5′-GCG*GAATTC*TACTTCGCCTCGGCCTTCTGGAGCTCGG-3′, italicized letters: *Eco*RI site and underlined letters: stop codon of *sgr4635*), digested with *Bam*HI and *Eco*RI, then cloned into pBluescript II KS(+), and digested with the same restriction enzymes. The generated plasmid was named pUC19-sgr4635. The 350 bp promoter region of *hrdB* encoding a principal sigma factor of RNA polymerase was amplified by PCR with the primer pair: 5′-GCGAAGCTTGCGCCGCGCGAGCACTGACCGGGG-3′ (positions -351 to -327 with respect to the translational start codon of *hrd*; underlined letters: *Hin*dIII site) and 5′-GCG*GAATTC*CATATGAACCTCTCGGAACGATGGAAACG-3′ (underlined letters: *Nde*I site and the start codon, ATG of *hrdB*, and italicized letters: *Eco*RI site). The DNA fragment was digested with *Hin*dIII and *Eco*RI and inserted into pBluescript II KS(+) generating pUC19-phrdB. The *Hin*dIII-*Nde*I fragment containing the *hrdB* promoter region and the *Nde*I-*Eco*RI fragment containing *sgr4635* were inserted into pBluescript II KS(+), which was digested with *Hin*dIII and *Eco*RI, generating pBlue-phrdB- sgr4635. The cloned fragment was digested with *Hin*dIII and *Eco*RI and inserted into pWHM3 generating pWHM3-phrdB-sgr4635.

Assay for antibiotic production

Assays for the two pigmented antibiotics Act and Red produced by *S. lividans* were carried out as previously described (Kim *et al*., 2007). An agar plug (1×1 cm) of *S. lividans* lawn grown on R2YE agar was cut out and inoculated into 100 ml of R2YE broth in a 500 ml baffled flask. The strains were cultivated at 28°C for 13 days with vigorous shaking. Five milliliters of the culture broth were removed 24 h after inoculation, and centrifuged at 15,000 rpm for 30 min. The optical densities of Act and Red in the aqueous phase were

Fig. 1. Characterization of DasR in *S. griseus*. (A) Amino acid sequence alignment of *S. griseus* DasR and *S. coelicolor* A3(2) DasR. Asterisks indicate the identical amino acids in the two DasR sequences. Sgr, *S. griseus* DasR; Sco, *S. coelicolor* A3(2) DasR. (B) Alignment of the promoter sequences between *sgr4635* and its homologues in *Streptomyces* spp. Putative DasR-binding consensus sequences (GTNTANAC; N, any nucleotide) are indicated by boxes. Nucleotides conserved among the three sequences are indicated by asterisks. Sgr, *S. griseus* IFO13350; Sco, *S. coelicolor* A3(2); Sav, *S. avermitilis* MA4680.

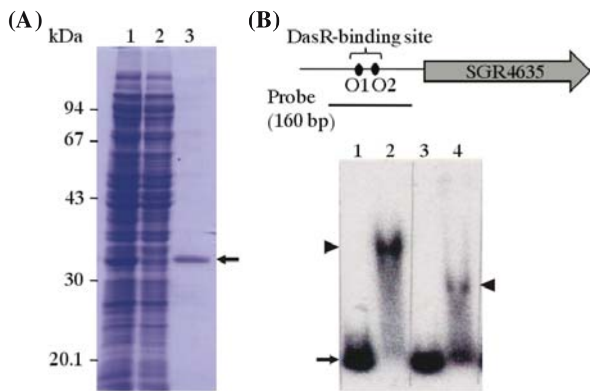

Fig. 2. Purification and DNA-binding assay of DasR. (A) Purification of DasR from *E. coli*. Purified DasR was analyzed by 12% SDS-PAGE. Molecular weight markers are indicated on the left side of the gel. Lanes: 1, soluble fraction of *E. coli* with pET26b-dasR; 2, soluble fraction of *E. coli* with pET26b(+); 3, DasR-H purified on a Ni^{2+}-NTA column. (B) Gel mobility shift assay. Probe for *sgr4635* contains putative DasR-binding sequences (operators 1 and 2). Lanes: 1, *sgr4635* probe without DasR-H; 2, *sgr4635* probe with 0.6 μg of DasR-H; 3, *dasA* probe (used as a control) without DasR- H; 4, *dasA* probe with DasR-H. The *dasA* probe used in this study contains an intact operator 1 (*O1*) and a mutated operator 2 (*O2*). The arrowheads indicate the shifted DasR-DNA complexes, whereas the arrow indicates the free probe.

measured at 615 nm (pH 12) and 468 nm (pH 2), respectively. To estimate the quantities of Act and Red secreted into the agar during solid culture, *S. lividans* transformants were cultivated on cellophane on R2YE agar. The strain was also cultivated at 28°C for 3 days in R2YE broth. Cells were collected by centrifugation and resuspended in the same volume of fresh R2YE medium, of which 300 μl was spread on cellophane placed on the surface of the agar. This set up was incubated under similar conditions for 13 days. To assay the extracellular antibiotics produced, the cell-free agar was homogenized and melted using heat before measuring the optical density under alkaline (pH 12) and acidic (pH 2) conditions. The removed cells were suspended in fresh R2YE liquid medium and collected by centrifugation at 8,000 rpm for 30 min. Twenty milligrams of dried cells were extracted with 5 ml of chloroform, to which an identical volume of 1 N NaOH was added; this mixture was centrifuged for 15 sec. To measure the intracellular concentration of Act and Red, the optical density of the aqueous and chloroform phases were measured at 615 nm under alkaline condition and at 540 nm under acidic condition.

Results and Discussion

sgr4635 is controlled by DasR

DasR is a repressor type transcriptional regulator that belongs to the HutC subfamily of transcriptional regulators in the GntR superfamily that contains two domains, namely the

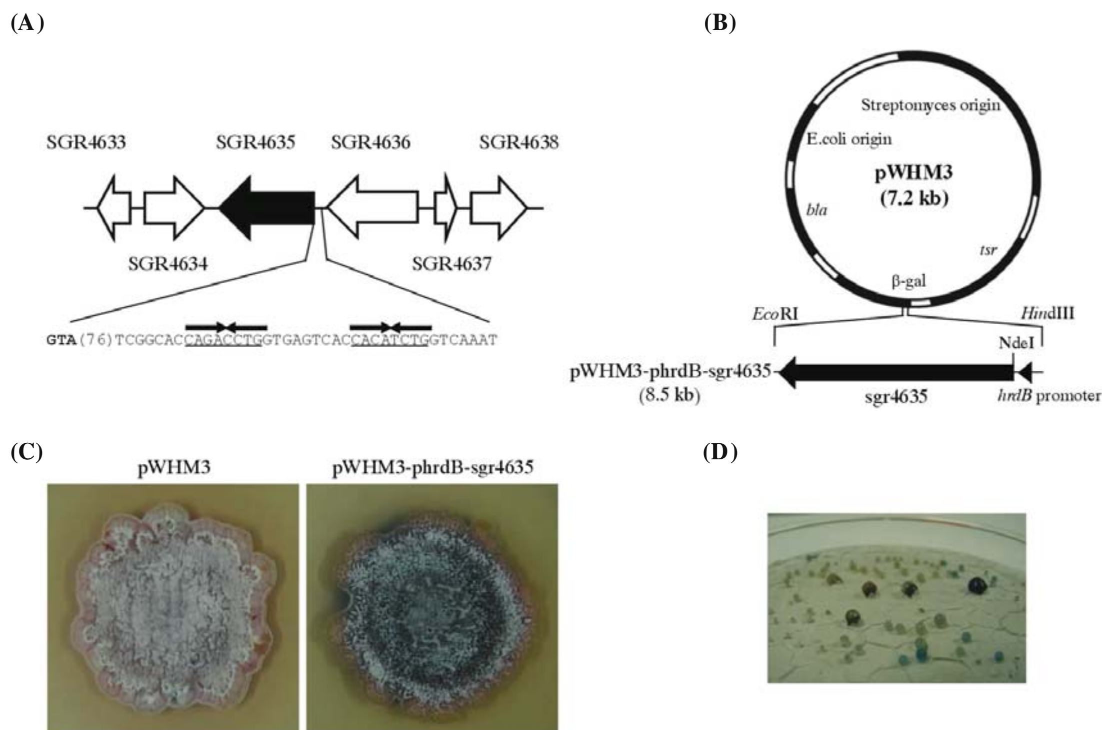

Fig. 3. Constitutive expression of *sgr4635* in *S. lividans* TK21. (A) Gene organization of *sgr4635* on *S. griseus* chromosome. The size and orientation of the genes indicated by arrows were deduced from the nucleotide sequence. *sgr4635* is indicated by a filled arrow, and two DasR-binding sequences are located at the promoter region of *sgr4635*. (B) Construction of expression vector for *sgr4635* in *Streptomyces* spp. The *sgr4635* promoter was substituted with the *hrdB* promoter encoding a sigma factor and constitutively expressed during growth (see 'Materials and Methods' section). The filled triangle indicates the *hrdB* promoter. (C) Photographs *of S. lividans* transformants. The transformants were cultivated on R2YE agar at 28°C for 8 days. (D) Antibiotic droplet production by *S. lividans* harboring pWHM3-phrdB-sgr4635 cultivated on a cellophane surface overlaying R2YE. Antibiotic droplets were observed on the surface of the spores.

helix-turn-helix (HTH) DNA binding domain in the amino-terminal region and the oligomerization and ligand-binding domain in the carboxyl-terminal region (Rigali *et al*., 2006).

We suggested that *S. griseus* DasR and *S. coelicolor* DasR are functionally similar because the amino acids of *S. griseus* DasR displayed high identity (89%) with those of *S. coelicolor* DasR (Fig. 1A), and homologous proteins of *S. coelicolor* DasR regulon were regulated by *S. griseus* DasR in *S. griseus* (our unpublished data). Furthemore, DasR-target genes in *S. griseus*, as well as the *S. coelicolor* DasR regulon (Rigali *et al*., 2006), also contain highly conserved DasR-binding consensus sequences in their promoter region (our unpublished data). The promoter region of *S. griseus nagE2* (*sgr4635*) contains two DasR-binding consensus sequences, namely 5′-GTCTACAC-3′ and 5′-GTCCAGAC-3′ (Fig. 1B), that were recognized by DasR (Fig. 2B).

S. griseus DasR was overexpressed in *E. coli* BL21(DE3) pLysS and purified to homogeneity (Fig. 2A). The gel mobility shift assay was performed to determine whether DasR-H would bind to a promoter region containing 2 operators. A DasR-DNA complex was observed at a different position than that in the control DNA with an operator (Fig. 2B), indicating that multimerized DasR recognized the 2 operators. Our findings are in agreement with those from previous studies indicating that the GntR-family transcriptional regulator forms a dimer that binds to the target site, and these dimers form a multimer that binds to the multi-binding sites of a target gene (Rigali *et al*., 2006).

Functional expression of *sgr4635* enhances antibiotic production in *Streptomyces*

S. lividans TK21 is very closely related to *S. coelicolor* A3(2), which is a genetically well-characterized strain of *Streptomyces.* Though the gene clusters for Act and Red production are present in both *S. lividans* and *S. coelicolor* A3(2), *S. lividans* normally produces very little of the two pigmented antibiotics compared to *S. coelicolor*. However, *S. lividans* produced a significant amount of antibiotics through alteration of the carbon source used for culture (Kim *et al*., 2001). Recent studies on *S. coelicolor* A3(2) PTS showed that GlcNAc derived from cell-wall hydrolysis is a substrate for NagE2, and its derivatives (GlcN-6-P) act as inducers for antibiotic production (Nothaft *et al*., 2010).

We overexpressed *sgr4635* in the absence of a DasR control in *S. lividans* TK21, and in the absence of GlcNAc, with the *hrdB* promoter that encodes a housekeeping gene for constitutive expression instead of the original *sgr4635* promoter containing 2 operators (Fig. 3B). The promoter and ribosomal binding site of *hrdB* were able to facilitate efficient transcription and translation of *sgr4635*. *S. lividans* TK21 harboring

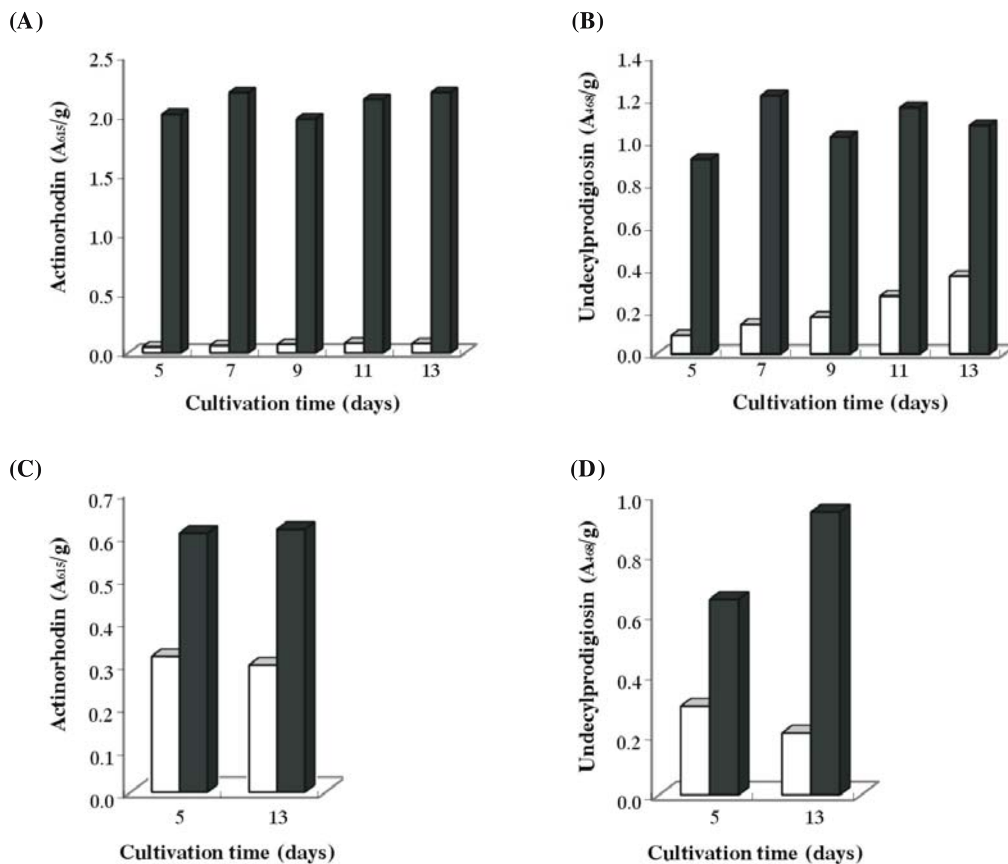

Fig. 4. Effects of *sgr4635* expression on extracellular antibiotic production (A and B) and on intracellular antibiotic production (C and D) on R2YE agar. The white and black bars indicate *S. lividans* strain harboring pWHM3 and pWHM3-phrdB-sgr4635, respectively.

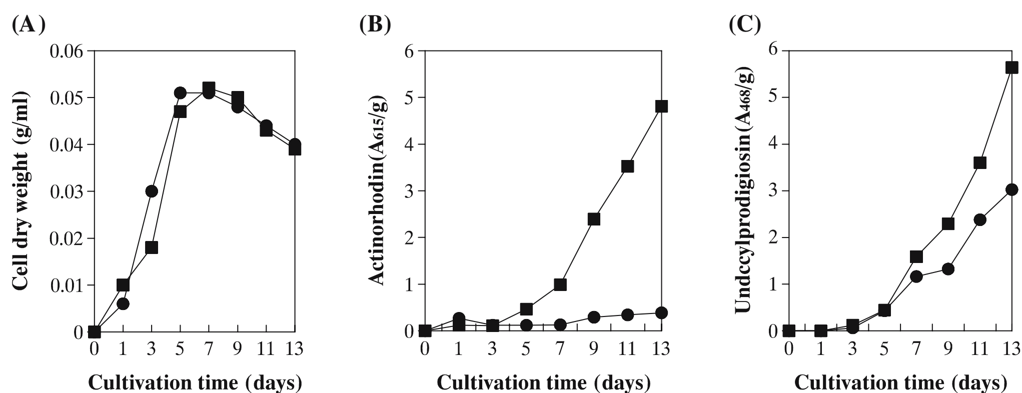

Fig. 5. Growth curve of *S. lividans* transformants, (A) and the effect of *sgr4635* expression on extracellular Act (B) and Red (C) production in R2YE broth. The filled circles and filled squares indicate *S. lividans* strains harboring pWHM3 and pWHM3-phrdB-sgr4635, respectively.

pWHM3-phrdB-sgr4635 produced antibiotics on R2YE rich medium (Figs. 3C and 4) and secreted pigmented antibiotic droplets on the hydrophobic surface of aerial mycelia and spores on the cellophane on R2YE agar plates (Fig. 3D). The antibiotic droplets are often observed in *S. coelicolor* A3(2) cells grown in a rich medium; however, this phenomenon has never been observed in *S. lividans*. *S. lividans* strains demonstrated formation of aerial mycelia (white) 2 days and spore formation (gray) 3 days after cultivation on the cellophane surface.

The transformants were cultivated on a cellophane surface on R2YE agar at 28°C for 13 days to determine whether *sgr4635* overexpression induces antibiotic production. The cells were harvested after 5 days and sonicated to measure the intracellular concentration of antibiotics and the agar was homogenized and melted to measure the concentration of secreted antibiotics. Both extracellular and intracellular antibiotic productions were strongly enhanced by *sgr4635* overexpression through the growth of the transformant (Fig. 4). In particular, the extracellular production of Act and Red by the *sgr4635*-overexpressing strain increased by approximately 20 to 40 and 3 to 10 times, respectively, compared to that by the control strain harboring empty vectors (Figs. 4A and B). The *sgr4635*-overexpressing strain also produced slightly increased levels of intracellular Act and Red, about 2.1 and 4.7 times, respectively, at maximum level.

The transformants were cultivated in R2YE broth at 28°C for 13 days to measure antibiotic production in liquid culture induced by *sgr4635* overexpression. The growth of the *sgr4635*-overexpressing strain was similar to that of the control strain (Fig. 5A), indicating that *sgr4635* would not influence the growth of *Streptomyces* under the conditions in this study. The *sgr4635*-overexpressing strain produced extremely high levels of Act (12.6 times) and Red (1.9 times) after approximately 7 days of cultivation compared to the control strain (Figs. 5B and C).

Overexpression of *sgr4635* had no effect on antibiotic production during early stages of growth (log and exponential phases) in nutrient-rich liquid medium; however, antibiotic production significantly increased during the late stages of growth (stationary and death phases) after undergoing cell-wall hydrolysis. This suggests that initiation of the cell lysis process triggers accumulation of GlcNAc in the medium, and overexpressed NagE2 (SGR4635) transports GlcNAc into the cell, where it is converted to GlcN-6-P, inhibiting the DNA binding activity of DasR, which then induces transcription of activators for antibiotic production. Our findings are consistent with the experimental observations in which GlcNAc is accumulated through cell-wall hydrolysis and internalized by GlcNAc-specific PTS permeases (NagE2 and MalX2) and metabolized to GlcN-6-P, which induces development of cells and antibiotic production by inhibiting the DasR DNA-binding ability in *S. coelicolor* A3(2) (Hong *et al*., 1993; Fernandez and Sanchez, 2002; Manteca *et al*., 2008; Rigali *et al*., 2008).

In conclusion, we determined that *sgr4635* transcription is controlled by DasR, which can be bound to 2 operator sites found upstream of *sgr4635*. In addition, we demonstrated that SGR4635 overproduction in *S. lividans* TK21 led to the intracellular accumulation of the GlcNAc during cell-wall hydrolysis, which subsequently induced antibiotic production by inactivating DasR, a transcriptional repressor of activators for antibiotic production.

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