AEM
Journals.ASM.org

A MarR Family Transcriptional Regulator, DptR3, Activates Daptomycin Biosynthesis and Morphological Differentiation in *Streptomyces roseosporus*

Qinling Zhang, Qiong Chen, Shuai Zhuang, Zhi Chen, Ying Wen, Jilun Li

State Key Laboratory of Agro-Biotechnology and MOA Key Laboratory of Soil Microbiology, College of Biological Sciences, China Agricultural University, Beijing, People's Republic of China

Daptomycin produced by *Streptomyces roseosporus* is an important lipopeptide antibiotic used to treat human infections caused by Gram-positive pathogenic bacteria, including drug-resistant strains. The genetic basis for regulatory mechanisms of daptomycin production is poorly known. Here, we characterized the *dptR3* gene, which encodes a MarR family transcriptional regulator located adjacent to the known daptomycin biosynthetic (*dpt*) genes. Deletion of *dptR3* reduced daptomycin production significantly and delayed aerial mycelium formation and sporulation on solid media. Dissection of the mechanism underlying the function of DptR3 in daptomycin production revealed that it stimulates daptomycin production indirectly by altering the transcription of *dpt* structural genes. DptR3 directly activated the transcription of its own gene, *dptR3*, but repressed the transcription of the adjacent, divergent gene *orf16* (which encodes a putative ABC transporter ATP-binding protein). A 66-nucleotide DptR3-binding site in the intergenic region of *dptR3-orf16* was determined by DNase I footprinting, and the palindromic sequence TCATTGTTACCTATGCTCACAATGA (underlining indicates inverted repeats) in the protected region was found to be essential for DptR3 binding. *orf16*, the major target gene of DptR3, exerted a positive effect on daptomycin biosynthesis. Our findings indicate that DptR3 functions as a global regulator that positively controls daptomycin production and morphological development in *S. roseosporus*.

Streptomycetes are soil-dwelling filamentous bacteria characterized by complex morphological differentiation and the ability to produce a variety of antibiotics. The production of these antibiotics is a complex process that is usually accompanied by morphological differentiation and is controlled by multiple regulatory proteins that respond to nutritional status, population density, and a variety of environmental conditions (1–3). Generally, the lowest level of the regulatory network involves pathway-specific regulatory genes that are found within the respective antibiotic biosynthesis gene cluster and affect only a single antibiotic biosynthetic pathway.

Daptomycin is a cyclic lipopeptide antibiotic used clinically to treat complex skin infections caused by Gram-positive pathogens, including 15 genera and 35 species, notably, penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* spp., glycopeptide-insensitive *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (4). Daptomycin is a minor component of the A21978C complex produced by *Streptomyces roseosporus* via a nonribosomal peptide synthetase (NRPS) mechanism. Components of the complex have in common a 10-membered cyclic peptide nucleus and a three-amino-acid tail with various fatty acid moieties attached to the N-terminal tryptophan (Trp) (5). The fatty acid portion of daptomycin is straight-chain decanoic acid, and daptomycin production can be increased by adding the precursor decanoic acid or sodium decanoate to the fermentation broth (6, 7).

In view of the wide medical application of daptomycin, there have been many studies of its biosynthesis. The *dpt* gene cluster has been cloned and consists of at least 12 known genes (5, 8, 9). Among these, *dptA*, *dptBC*, and *dptD* encode the three subunits of an NRPS; *dptE* and *dptF* are responsible for the activation of fatty acids that are then catalyzed by the N-terminal C domain of DptA to form N-acylation of Trp; and *dptG*, *dptH*, *dptI*, *dptJ*, *dptM*, *dptN*, and *dptP* are involved in precursor supply, resistance, or transport. Many open reading frames (ORFs) flanking the *dpt* genes encode multicomponent transporters or hypothetical proteins. Three regulatory genes (*dptR1*, *dptR2*, *dptR3*) located adjacent to the known *dpt* genes encode LuxR, DeoR, and MarR family regulators, respectively. Because of their location, these three genes were presumed to encode the pathway-specific regulators of daptomycin production (5). However, *dptR2* was shown recently to be required for daptomycin production but not for expression of the *dpt* gene cluster, suggesting that the DptR2 protein does not function as a pathway-specific regulator (10). To our knowledge, *dptR2* is the only regulatory gene reported for the regulation of daptomycin production in *S. roseosporus*. The functions of *dptR1* and *dptR3* remain unknown. Identification and characterization of regulatory genes involved in daptomycin production are im-

Received 7 January 2015 **Accepted** 19 March 2015

Accepted manuscript posted online 27 March 2015

Citation Zhang Q, Chen Q, Zhuang S, Chen Z, Wen Y, Li J. 2015. A MarR family transcriptional regulator, DptR3, activates daptomycin biosynthesis and morphological differentiation in *Streptomyces roseosporus*. Appl Environ Microbiol 81:3753–3765. doi:10.1128/AEM.00057-15.

Editor: S.-J. Liu

Address correspondence to Ying Wen, wen@cau.edu.cn.

Q.Z. and Q.C. contributed equally to this work.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00057-15.

doi:10.1128/AEM.00057-15

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
S. roseosporus		
NRRL11379	WT strain	7
DR3	*dptR3* deletion mutant	This study
CR3	*dptR3*-complemented strain	This study
WT/pKC1139-ermpR3	*dptR3* overexpression strain	This study
WT/pKC1139	WT strain carrying empty vector pKC1139	This study
D16	*dpt16* deletion mutant	This study
E. coli		
JM109	General cloning host for plasmid manipulation	Laboratory stock
ET12567	Methylation-deficient strain	23
BL21(DE3)	Host for protein overexpression	Novagen
Plasmids		
pMD18-T	TA cloning vector	TaKaRa
pKC1139	Multiple-copy, temperature-sensitive *E. coli-Streptomyces* shuttle vector	36
pSET152	Integrative *E. coli-Streptomyces* shuttle vector	36
pET-28a(+)	Vector for protein overexpression in *E. coli*	Novagen
pJL117	pIJ2925 derivative carrying *Streptomyces* strong constitutive promoter *ermE*p*	37
pDR3	*dptR3* deletion vector based on pKC1139	This study
pD16	*orf16* deletion vector based on pKC1139	This study
pKC1139-ermpR3	*dptR3* overexpression vector based on pKC1139	This study
pSET152-ermpR3	*dptR3* complemented vector based on pSET152	This study
pET28-R3	*dptR3* overexpression vector based on pET-28a(+)	This study

portant for elucidation of the regulatory networks of daptomycin biosynthesis and for practical construction of strains with high daptomycin production levels.

The MarR (multiple antibiotic resistance regulator) family of transcriptional regulators is named for the almost completely characterized *Escherichia coli* MarR protein, a repressor of genes that regulate multiple antibiotic resistance operons (11, 12). MarR homologs are widely distributed in bacteria and archaea and control a wide range of cellular activities, including antibiotic resistance, stress responses, virulence, and catabolism of aromatic compounds (13–15). They are winged helix-turn-helix (HTH) DNA-binding proteins that exist as dimers and bind palindromic sequences within cognate promoters. They typically act as transcriptional repressors, although a few act as activators or dual repressors-activators. A common regulatory mechanism involves MarR homolog binding to the intergenic region between the *marR* gene and a divergently oriented gene (or operon), regulating the transcription of both genes positively or negatively. Such transcriptional regulation is blocked by conformational changes upon the binding of small-molecule ligands to MarR proteins. Over 12,000 MarR-like proteins have been annotated to date in bacterial and archaeal genomes (14), but only a few have been studied in *Streptomyces*. *Streptomyces coelicolor* OhrR acts as either a repressor or an activator in response to organic hydroperoxides (16). The orthologous proteins PenR and PntR serve as pathway-specific activators of the biosynthesis of the antibiotic phenalinolactone in *Streptomyces exfoliatus* and *Streptomyces arenae*, respectively (17). *S. coelicolor* TamR controls the expression of the gene encoding *trans*-aconitate methyltransferase (*tam*) and is important for metabolic flux through the citric acid cycle during oxidative stress (18). *S. coelicolor* PecS was proposed to function under oxidative stress conditions by responding to the ligand urate (19). *S. coelicolor* PcaV controls the transcription of genes encoding β-ketoadipate pathway enzymes (which are essential for aromatic catabolism) through its interaction with the pathway substrate protocatechuate (PCA) (20). The crystal structures of the PcaV-PCA complex (20) and *S. coelicolor* SCO5413 (21) have been determined.

The present study addressed the positive regulatory role of the MarR family regulator DptR3 in daptomycin production and morphological differentiation of *S. roseosporus*. We found that DptR3 regulates daptomycin biosynthesis indirectly at the transcriptional level.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *S. roseosporus* wild-type (WT) strain NRRL11379 is a producer of daptomycin. *S. roseosporus* strains were grown at 28°C on solid DA1 medium (0.4% glucose, 1% malt extract, 0.4% yeast extract, 0.2% $CaCO_3$, 1.5% agar) for sporulation or in liquid YEME medium (22) containing 25% sucrose for growth of mycelia. RM14 medium (23) was used for regeneration of protoplasts and for selection of transformants. R2YE and MM agar (22) were used for *S. roseosporus* phenotype observation. Seed medium and fermentation medium [1.1% yeast extract, 0.086% $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 1.07% glucose, 7.2% potato dextrin, 0.72% cane molasses, pH 7.0] were used for daptomycin production. The primary and secondary seed media contained 2.5% dextrin and 3% Trypticase soy broth. Fermentation medium was also used to cultivate mycelia for RNA isolation.

E. coli strains JM109 and BL21(DE3) (Novagen, Germany) were used as cloning and expression hosts, respectively. *E. coli* ET12567 (*dam dcm hsdS*) (23) was used to propagate nonmethylated DNA for transformation into *S. roseosporus*. The antibiotics used were described previously (24).

Fermentation and high-performance liquid chromatography (HPLC) analysis of daptomycin. Spores from various *S. roseosporus* strains cultured on DA1 plates for 10 days were added to 250-ml flasks containing 50 ml of primary seed medium and incubated at 28°C for 48 h on a rotary shaker (250 rpm). Cultures in primary seed medium were inoculated at

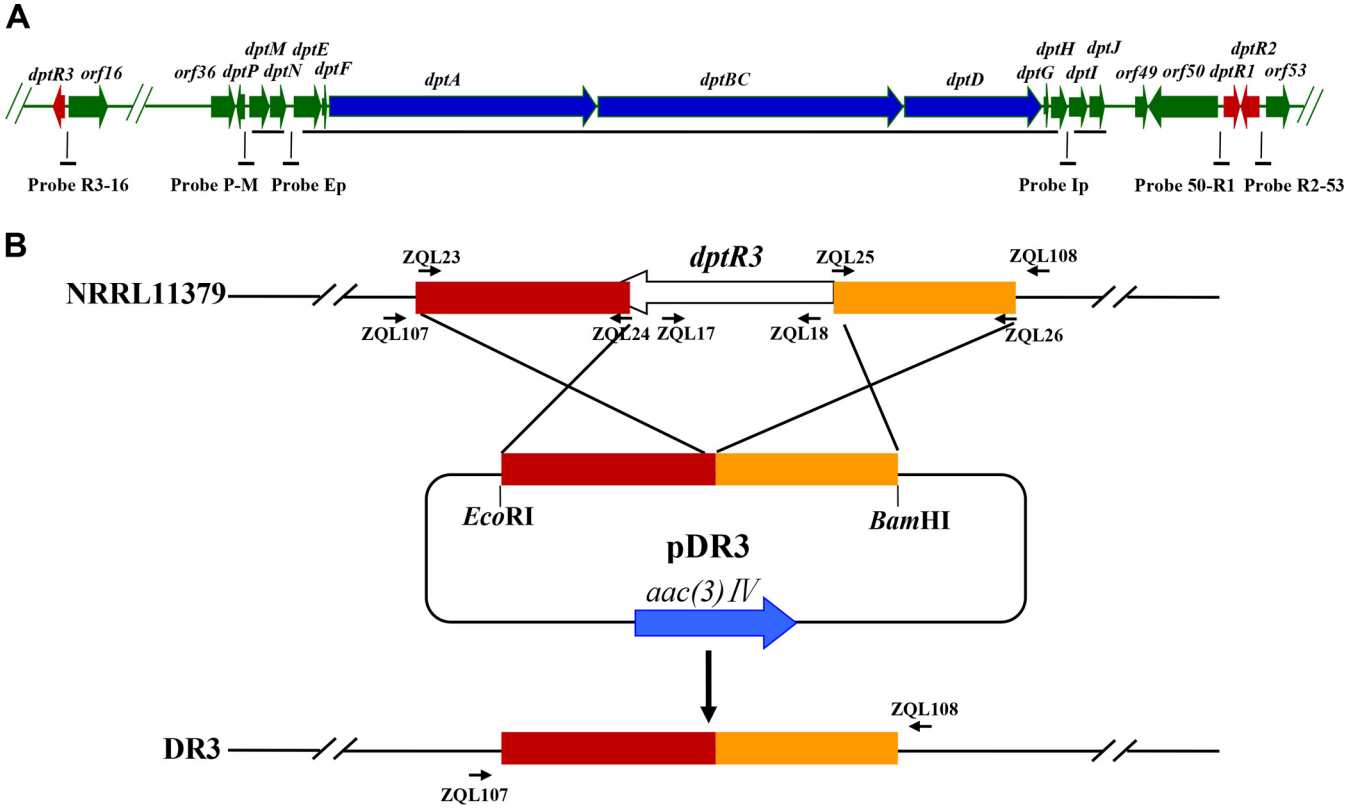

FIG 1 Genetic organization of 12 known *dpt* genes and the genes adjacent to them, from *dptR3* to *orf53*, in *S. roseosporus* WT strain NRRL11379 (A) and a schematic strategy for deletion of *dptR3* (B). (A) Long black lines, transcriptional units. Short lines at the bottom, probes of the promoter regions used for EMSAs. (B) Large arrows, genes and their directions. Short arrows, positions of primers used for cloning exchange regions and confirming gene deletion as described in Materials and Methods. Rectangles, homologous exchange regions used for deletion of *dptR3*.

6% (vol/vol) into 50 ml of secondary seed medium and incubated at 28°C with shaking for 30 h. Then a 6% (vol/vol) inoculation volume of cultures in secondary seed medium was transferred into 50 ml of fermentation medium and cultured further for 10 days. After 48 h of fermentation, sodium decanoate (final concentration, 0.02%, wt/vol) was added every 12 h until the end of the fermentation.

HPLC analysis of daptomycin production in fermentation culture was performed as described previously, with modification (7). In brief, the fermentation broth (1.0 ml) was centrifuged at 12,000 × *g* for 10 min, the supernatant was filtered (membrane pore size, 2.2 μm), and the filtrate was applied directly to an HPLC system (model 600; Waters, Milford, CT) with a C_{18} column (4.6 [inside diameter] by 250 mm). A mobile phase of 0.1% (vol/vol) trifluoroacetic acid in water and acetonitrile (55:45, vol/vol) was used at a flow rate of 1.0 ml · min^{-1}. Daptomycin was detected by measuring UV absorption at 218 nm and using authentic daptomycin samples (Shanghai Qiao Chemical Science, China) to draw calibrated standard curves.

Gene deletion, complementation, and overexpression. To construct a *dptR3* deletion mutant, two fragments flanking *dptR3* were prepared by PCR from genomic DNA of NRRL11379. A 481-bp 5′ flanking region (positions −413 to +68 relative to the *dptR3* start codon) was amplified with primers ZQL25 and ZQL26, and a 544-bp 3′ flanking region (positions −104 to +440 relative to the *dptR3* stop codon) was amplified with primers ZQL23 and ZQL24 (complementary to ZQL25). The two fragments were fused together by PCR with primers ZQL23 and ZQL26 and then ligated into EcoRI/BamHI-digested pKC1139 to generate *dptR3* deletion vector pDR3. Transformation of pDR3 into NRRL11379 and selection of double-crossover recombinant strains were performed as described previously (24). *S. roseosporus* does not sporulate on RM14 medium; transformants regenerated on RM14 plates were transferred to DA1 medium for sporulation. Spores of the transformants were collected and spread on DA1 plates containing apramycin. The plates were incubated initially at 28°C for 48 h and then at 39°C for 7 days. Because pKC1139 cannot replicate itself in *Streptomyces* when the temperature is higher than 34°C, only mutants in which pDR3 was integrated into the *S. roseosporus* chromosome by a single crossover can grow at 39°C on DA1 plates. Single-crossover mutants were transferred to DA1 plates under nonselection conditions for three rounds of sporulation, and double crossover took place only in colonies sensitive to apramycin. The putative *dptR3* deletion mutants were confirmed by PCR analysis with primers ZQL107, ZQL108, ZQL17, and ZQL18, followed by DNA sequencing. A 1.23-kb band was detected when primers ZQL107 and ZQL108, which flank the exchange regions, were used, whereas a 1.6-kb band was detected when genomic DNA of NRRL11379 was used as the template. When primers ZQL17 and ZQL18, located within the deletion region of *dptR3*, were used, only NRRL11379 produced a 405-bp PCR fragment, as predicted (data not shown). We thus obtained *dptR3* gene deletion mutant DR3, in which *dptR3* was mostly deleted by double-crossover recombination (Fig. 1B).

For complementation of DR3, a 590-bp DNA fragment carrying the *dptR3* ORF was amplified by PCR with primers ZQL21 and ZQL22. The PCR product was digested with HindIII/XbaI and ligated simultaneously with the EcoRI/HindIII *ermE*p* fragment from pJL117 and EcoRI/XbaI-digested pSET152 to generate the *dptR3*-complemented vector pSET152-ermpR3, in which the *dptR3* gene was controlled by the *ermE*p* promoter. pSET152-ermpR3 was introduced into DR3 to obtain complemented strain CR3. The same 590-bp *dptR3* ORF and *ermE*p* fragments were cloned into pKC1139 to produce *dptR3* overexpression vector pKC1139-

ermpR3, which was then introduced into NRRL11379 to obtain *dptR3* overexpression strain WT/pKC1139-ermpR3.

To construct an *orf16* deletion mutant, a 664-bp 5′ flanking region (positions −472 to +192 relative to the *orf16* start codon) and a 605-bp 3′ flanking region (positions −181 to +424 relative to the *orf16* stop codon) were amplified with primer pairs CQ37/CQ38 and CQ39/CQ40, respectively. The two fragments were fused together by PCR with primers CQ37 and CQ40 and then ligated into EcoRI/XbaI-digested pKC1139 to generate *orf16* deletion vector pD16, which was transformed into NRRL11379 protoplasts. The resulting *orf16* deletion mutant D16 was confirmed by PCR analysis with primers CQ41, CQ42, ZQL113, and ZQL114 (see Fig. 6A). A 1.54-kb band was detected when primers CQ41 and CQ42, which flank the exchange regions, were used, whereas a 3.0-kb band was detected from genomic DNA of NRRL11379. When primers ZQL113 and ZQL114, located within the deletion region of *orf16*, were used, only NRRL11379 produced a 151-bp PCR fragment, as predicted (data not shown). All of the primers used in this study are listed in Table 2.

Overexpression and purification of recombinant His_6-tagged DptR3 protein. A DNA fragment encoding the predicted 166 amino acids of the DptR3 protein was generated by PCR with primers ZQL35 and ZQL36. The PCR fragment was digested with NdeI/XhoI and inserted between the corresponding sites in expression vector pET-28a(+) to generate pET28-R3, which was confirmed by DNA sequencing and then introduced into *E. coli* BL21(DE3) for protein overexpression. Following induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), bacteria harboring pET28-R3 were incubated for 8 h at 16°C. Cells were collected, resuspended in lysis buffer (20 mM Tris-Cl [pH 8.0], 200 mM NaCl, 1 mM EDTA [pH 8.0], 0.5% NP-40), and disrupted by sonication. After centrifugation, the supernatant was recovered and the resulting recombinant His_6-tagged DptR3 protein was purified on a Ni^{2+}-nitrilotriacetic acid spin column (Qiagen, Germany) according to the manufacturer's instructions. The purified protein was used for electrophoretic mobility shift assays (EMSAs) and DNase I footprinting assays as described below.

EMSAs. EMSAs were performed with a DIG Gel Shift kit, 2nd generation (Roche) according to the manufacturer's instructions. The probes were amplified by PCR and labeled at the 3′ ends with nonradioactive digoxigenin (DIG). The reaction mixture (20 μl) contained the probes, proteins, and 1 μg of poly(dI-dC) (vial 9) in the binding buffer (vial 5). The mixture was incubated at 25°C for 30 min, and then 5 μl of loading buffer with bromophenol blue (vial 13) was added. Protein-bound and free DNAs were separated by electrophoresis on 5% native polyacrylamide gels with 0.5× Tris-borate-EDTA as the running buffer and then transferred to positively charged nylon membranes by electroblotting. The membranes were baked at 80°C for 10 min, and the DNA fragments were cross-linked by exposure to UV radiation for 10 min. Chemiluminescence detection was performed according to the manufacturer's instructions, and the membranes were exposed to X-ray film for 15 to 30 min.

DNase I footprinting assay. A nonradiochemical capillary electrophoresis method was used for DNase I footprinting (25). To characterize the DptR3 protein binding sites in the *dptR3-orf16* intergenic region, a 6-carboxyfluorescein (FAM) fluorescence-labeled DNA fragment was synthesized by PCR with primers FAM-ZQL103/ZQL104. The resulting 378-bp DNA fragments covered the entire intergenic region. Following purification with a Midi Purification kit (Tiangen, China), labeled DNA fragments (400 ng) and appropriate concentrations of His_6-tagged DptR3 protein were added to a final reaction volume of 50 μl and the mixture was incubated for 30 min at 25°C in binding buffer [20 mM HEPES (pH 7.6), 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 0.2% Tween 20, 30 mM KCl]. Digestion with DNase I (0.016 U) was performed for 40 s at 37°C and stopped by adding EDTA to a final concentration of 60 mM. The reaction mixture was heated to 80°C for 10 min to totally inactivate DNase I. The samples were subjected to phenol-chloroform extraction, ethanol precipitation, and capillary electrophoresis by loading into a DNA genetic analyzer (model 3730) together with the internal-lane size standard ROX-500 (Applied Biosystems). Electropherograms were analyzed with the GeneMarker program, v1.8 (Applied Biosystems).

Identification of TSPs by 5′ RACE. To determine the transcriptional start points (TSPs) of *dptR3* and *orf16*, total RNA was extracted from a 96-h culture of NRRL11379 grown in fermentation medium. Total RNA (2 μg) was used for reverse transcription (RT) with 20 pmol of gene-specific primer ZQL59 or ZQL64 with a 5′/3′ rapid amplification of cDNA ends (RACE) kit (2nd generation; Roche). The sample was purified with a PCR Product Purification kit (Beijing HT-Biotech Co. Ltd., China). An oligo(dA) tail was added to the 3′ end of the cDNA by using terminal deoxynucleotidyl transferase, followed by direct amplification of the tailed cDNA with an oligo(dT) anchor primer (GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV) and the second inner gene-specific primer ZQL60 or ZQL65. An additional round of PCR was performed with a 1,000-fold dilution of the original PCR product as the template, an anchor primer (GACCACGCGTATCGATGTCGAC), and nested primer ZQL62 or ZQL67 to obtain a single specific band. The final PCR products were cloned into the vector pMD18-T (TaKaRa, Japan) for sequencing. The first nucleotide following the oligo(dA) sequence was considered the TSP.

RNA preparation and real-time RT-PCR analysis. Mycelia of *S. roseosporus* grown in fermentation medium were collected, frozen in liquid nitrogen, and ground to a fine powder. RNA extractions were performed with TRIzol reagent (Tiangen) according to the manufacturer's instructions. To remove chromosomal DNA contamination, each RNA sample was treated with DNase I and tested by PCR to confirm the absence of chromosomal DNA. The treated RNA sample (2 μg) was reverse transcribed with Moloney murine leukemia virus (RNase H^-; TaKaRa), random hexamers (25 μM), and a deoxynucleoside triphosphate mixture (10 mM each). The cDNAs obtained were used as templates for real-time RT-PCR analysis with the primers listed in Table 2. The experiments were performed with FastStart Universal SYBR green master mix (ROX; Roche) and analysis with an ABI 7900HT sequence detection system with optical-grade 96-well plates. Template cDNA, 10 μl of FastStart Universal SYBR green master mix, and forward and reverse primers (each at 300 nM) were mixed in each reaction system (total volume, 20 μl). The PCR protocol was 95°C for 10 min and 40 cycles of 95°C for 10 s and 60°C for 30 s. The DNase I-treated RNA samples without RT were used as negative controls to rule out chromosomal DNA contamination. The *hrdB* gene (*SAV2444*), which encodes the major sigma factor in *Streptomyces*, was used as a positive internal control to normalize samples. Relative expression levels were calculated by the comparative cycle threshold method. Each experiment was performed in triplicate.

RESULTS

DptR3 is a positive regulator of morphological differentiation and daptomycin production. Miao et al. (5) reported that *S. roseosporus* WT strain NRRL11379 contains the *dpt* gene cluster involved in daptomycin biosynthesis. The *dptR3* gene contains 501 nucleotides (nt) and encodes a 166-amino-acid putative MarR family transcriptional regulator, including a conserved HTH DNA-binding motif homologous to MarR. There are 21 ORFs between *dptR3* and *dptP* at the left end of the *dpt* gene cluster (Fig. 1A). The divergently transcribed gene *orf16* is located upstream of *dptR3* and encodes a putative ABC transporter ATP-binding protein. The nucleotide sequences and deduced amino acid sequences of *orf16-dptR3* are highly homologous to those of *SCO1144-1145* in *S. coelicolor* and *SAV1564-1565* in *S. avermitilis* (DptR3, 60 and 65% identical to SCO1145 and SAV1565; ORF16, 81 and 83% identical to SCO1144 and SAV1564, respectively).

To elucidate the role of *dptR3* in *S. roseosporus*, we deleted the *dptR3* gene from the WT strain by homologous recombination (Fig. 1B). The deletion mutant DR3 showed strikingly delayed formation of aerial mycelia and spores on the solid media MM

TABLE 2 Primers used in this study

Application(s) and primer	DNA sequence (5′–3′)[a]	Use(s)
Gene disruption, complementation, and overexpression		
ZQL23	CCGGAATTCTCGTCCAGCACCTCCACG (EcoRI)	*dptR3* gene deletion
ZQL24	CTCTCGCGATTCCCGGGACCGCCGCAGAGGAGATCC	
ZQL25	GGATCTCCTCTGCGGCGGTCCCGGGAATCGCGAGAG	
ZQL26	CGGGATCCGATGAGGGTCTGGAGCACG (BamHI)	
ZQL21	CCCAAGCTTCTCCAGCAGCGGCATGTC (HindIII)	Complementation of DR3, overexpression of *dptR3* in *S. roseosporus*
ZQL22	GCTCTAGACGCACAGTGAGAGCCTAGC (XbaI)	
ZQL17	TCCGGCATTCCGTCGAC	Confirmation of *dptR3* deletion mutant DR3
ZQL18	GCTCGCCGAACAGCTGC	
ZQL107	CGGCAGCACGAAGGTGAG	
ZQL108	CAGGTCGTGCATGACGCG	
CQ37	CCGGAATTCCCCTCGTCGGTGATCTCG (EcoRI)	Deletion of *orf16* gene
CQ38	GCGATGGTGAGCGTGGTCGAGGATCTCGCGCAGCAG	
CQ39	CTGCTGCGCGAGATCCTCGACCACGCTCACCATC GC	
CQ40	TGCTCTAGACGCCTGGATCTTCACCACG (XbaI)	
CQ41	CAGCTCGCCGAACACCTC	Confirmation of *orf16* deletion mutant D16
CQ42	GGAACTCCGCGTCCCACT	
ZQL113	CTTCGGCGTGCTCCAGAC	
ZQL114	GTTGGCGATGCGGGACTG	
ZQL35	GGAATTCCATATGGACGCCCCGGATTCCCCC (NdeI)	Overexpression of His_6-tagged DptR3 protein in *E. coli*
ZQL36	CCGCTCGAGTCAGCAGTGGCGCTCCGG (XhoI)	
EMSA		
ZQL37	TCCCGGGAATCGCGAGAG	Probe R3-16
ZQL38	GAAGAGGCGGAGGATGCG	
ZQL39	GGCGTGGAACATACTGGCG	Probe Ep
ZQL40	GCACAGCGGCTCTCACTC	
ZQL47	GCGCGGTCAACAAGATTCTT	Probe Ip
ZQL48	CCTTGACGGTCACGTGGTAG	
ZQL49	TGACCTGGTCCGGCCGAT	Probe 50-R1
ZQL50	CCCCACCGGCGGAATTGT	
ZQL51	CCGTTCCGATGCGAGTGC	Probe R2-53
ZQL52	CGTGCAGGAAGGTGTTCGC	
ZQL57	CGGTCCGAACCGGCTCTTG	Probe P-M
ZQL58	AGGCGCTGCGGATCGATG	
GJ91	CCAAGGGCTACAAGTTCTCC	Probe *hrdB*
GJ92	TTGATGACCTCGACCATGTG	
5′ RACE		
ZQL59	GGATCTCCTCTGCGGCGG	Identification of TSP of *dptR3*
ZQL60	GGAACTGGGCCGGAGTGATGT	
ZQL62	CAGCTGGCGGCGCTGGAT	
ZQL64	GTTGGCGATGCGGGACTG	Identification of TSP of *orf16*
ZQL65	GAGATGAGGGTCTGGAGCACG	
ZQL67	GAAGAGGCGGAGGATGCGG	
Real-time RT-PCR		
ZQL75	TCCCGGGAATCGCGAGAG	*dptR3* ORF
ZQL76	CCCGGATTCCCCCGACTC	
ZQL79	GAACAGACCACCCTCCTCG	*dptBC* ORF
ZQL80	CTGTGGCCGATGGGGTAG	
ZQL81	CGGCGTACATCATCCAGACC	*dptA* ORF
ZQL82	GTCATGCTCAGTTCGGAGACG	
ZQL83	GAGTGAGAGCCGCTGTGC	*dptE* ORF
ZQL84	GGTGTCCCGTACGAGAACC	
ZQL85	CCAGATCCTCTCGACGGTG	*dptM* ORF
ZQL86	CCGATACAGGCGCGTACC	
ZQL87	TTCCGGTACGAGCGGCTG	*dptD* ORF
ZQL88	CGTCAGATCGAAGCGGCG	
ZQL89	TCCGCACCATCACGTTCAC	*dptH* ORF
ZQL90	GACTTCCTGGGCCACCTG	

(Continued on following page)

TABLE 2 (Continued)

Application(s) and primer	DNA sequence (5′–3′)[a]	Use(s)
ZQL91	GAGATCGCCCTCCTGGAC	*dptR1* ORF
ZQL92	GTGGAAGCTCAGCCCCTC	
ZQL93	GGCCAGGATCGTGACGTC	*dptR2* ORF
ZQL94	CGGAACGGCAGGAGTTCATC	
ZQL113	CTTCGGCGTGCTCCAGAC	*orf16* ORF
ZQL114	GTTGGCGATGCGGGACTG	
ZQL115	GACAGATCGCCGAGTTCGT	*dptF* ORF
ZQL116	GACGCTCCACAGCAGCTC	
ZQL117	CTACCACGTGACCGTCAAGG	*dptI* ORF
ZQL118	GTCGTCGAACTCGTTTCCCTC	
ZQL119	CAACGACGGCAGCTACCTC	*dptG* ORF
ZQL120	GGCGCATATCGGTCCAGTTC	
ZQL121	CAATCCGCCGTACCAGGC	*dptP* ORF
ZQL122	GGACACGCCCGCTGTTGG	
DNase I footprinting assay		
FAM-ZQL103	GAAGAGGCGGAGGATGCG	*dptR3-orf16* intergenic region
ZQL104	GGGCCGGAGTGATGTCGAT	

[a] The restriction site in parentheses is underlined.

agar, R2YE, and DA1 (Fig. 2A). Deletion of *dptR3* also led to markedly delayed and reduced daptomycin production in comparison with that of the WT strain. Daptomycin production in DR3 was not detectable by HPLC until fermentation day 7, and the final yield after day 10 was only ~13% of that of the WT (Fig. 2B).

To demonstrate that these changes were due solely to the deletion of *dptR3*, we constructed *dptR3*-complemented strain CR3. The morphological phenotype and daptomycin production were restored in CR3 (Fig. 2A and B). To investigate whether the reduced daptomycin production of DR3 was due to altered cell growth, we analyzed the growth of the WT, DR3, and CR3 strains in fermentation medium. The results showed that deletion of *dptR3* had no notable effect on cell growth (Fig. 2C). These findings indicate that DptR3 plays a positive regulatory role in morphological differentiation and daptomycin biosynthesis.

In general, overexpression of a positive regulator can increase the production of the corresponding antibiotic. To examine this possibility, we introduced *dptR3* overexpression vector pKC1139-ermpR3 into the WT strain and evaluated its effects on daptomycin production. HPLC analysis of the fermentation products following 10 days of culture in fermentation medium showed that a transformant containing the empty vector pKC1139 produced nearly the same amount of daptomycin as the host strain, whereas the daptomycin yield was ~28 and 31% higher in *dptR3* overexpression strains WT/pKC1139-ermpR3-1 and -2, respectively (Fig. 2D). These findings further confirmed the role of *dptR3* as a daptomycin biosynthesis activator gene.

DptR3 activates *dpt* genes but represses the adjacent gene *orf16*. To further elucidate the role of DptR3 in daptomycin biosynthesis, we performed a real-time RT-PCR analysis with RNAs isolated from the WT and DR3 strains grown in fermentation medium for 2 (late exponential phase) or 4 (stationary phase) days. The transcription levels of structural genes *dptA*, *dptBC*, *dptD*, *dptE*, *dptF*, *dptG*, *dptH*, *dptI*, *dptM*, and *dptP* in the *dpt* gene cluster were reduced in DR3 at either both time points or one time point (Fig. 3), indicating that DptR3 affects daptomycin production by stimulating the transcription of *dpt* structural genes.

Given the common regulatory mechanism of MarR proteins (14, 15), we predicted that DptR3 regulates the expression of itself and of the adjacent, divergently transcribed gene *orf16*. The transcription levels of *dptR3* and *orf16* were thus tested with the same RNA preparations. The *dptR3* transcription level in DR3, measured with a 60-bp transcript amplified from the remainder ORF of *dptR3*, was lower than that in the WT, whereas *orf16* expression was strikingly upregulated in DR3 (Fig. 3). These results indicate that DptR3 functions as an autoactivator, as well as a repressor, of *orf16*.

The regulatory genes *dptR1* and *dptR2* adjacent to the *dpt* cluster were also subjected to transcription analysis. *dptR1* transcription was undetectable in the WT and DR3 strains by real-time RT-PCR with various primer pairs, suggesting either that the level was too low to be detected or that the *dptR1* gene was not expressed under our culture conditions. *dptR2* expression was downregulated in DR3 (Fig. 3), suggesting that DptR3 positively regulates *dptR2* transcription, either directly or indirectly.

DptR3 binds specifically to the bidirectional *dptR3-orf16* promoter region. To determine whether DptR3 directly regulates the genes mentioned in the preceding section, we performed EMSAs with a full-length recombinant, His_6-tagged DptR3 protein expressed in *E. coli*. In the *dpt* cluster, the core genes are oriented in the same direction (Fig. 1A). The contiguous genes from *dptE* to *dptH* were reported to be transcribed on a large polycistronic transcript (26). Given that *dptM* overlaps *dptN* and that *dptI* overlaps *dptJ*, it is possible that *dptM* and *dptN* are cotranscribed and that *dptI-dptJ* forms another transcriptional unit. The *dptP-dptM*, *orf50-dptR1*, *dptR2-orf53*, and *dptR3-orf16* intergenic regions lie between two divergently transcribed genes and presumably contain bidirectional promoters. The promoter regions of *dptE* and *dptI* were therefore designated probes Ep and Ip, respectively. Probes P-M, 50-R1, R2-53, and R3-16 were designed to cover the corresponding intergenic regions containing two divergent promoters (Fig. 1A). Results showed that purified His_6-DptR3 did not bind to probe Ep, Ip, P-M, 50-R1, or R2-53, even when the protein amount was large (200 ng) (only probe Ep is shown in Fig. 4A), indicating that the positive regulatory effect of DptR3 on daptomycin production and on *dptR2* expression is

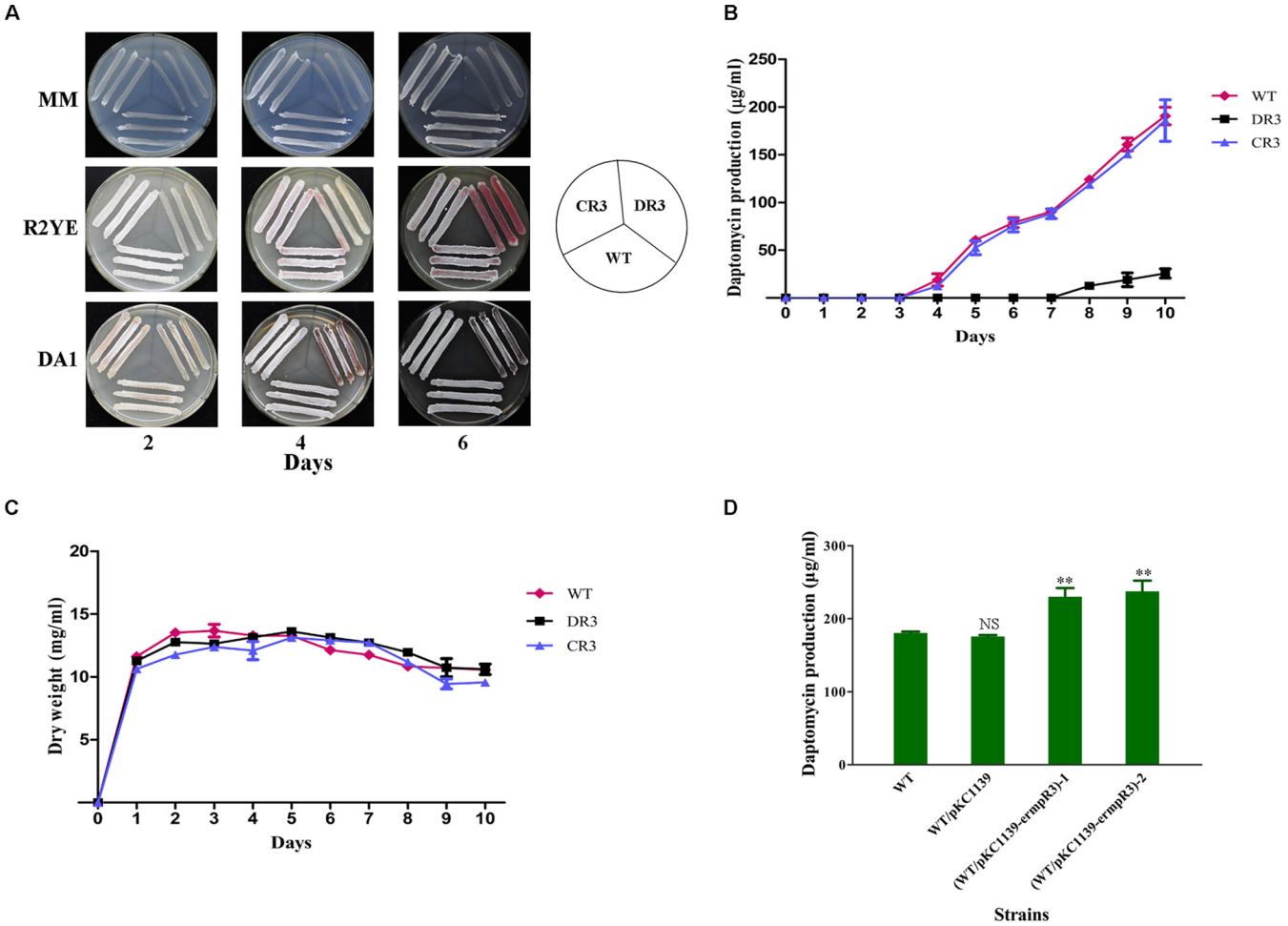

FIG 2 Daptomycin production and growth of *S. roseosporus* WT and *dptR3* mutant strains. (A) Effect of *dptR3* deletion on morphological development. The WT strain, the *dptR3* deletion mutant (DR3), and the complemented strain (CR3) were grown on MM agar, R2YE, and DA1 plates at 28°C, and the plates were photographed every 48 h. (B) Daptomycin yields of the WT (◆), DR3 (■), and CR3 (▲). (C) Growth curves of the WT, DR3, and CR3. Cell growth was measured in cell dry weight. (D) Comparison of daptomycin production in the WT, vector control strain WT/pKC1139, and *dptR3* overexpression strains WT/pKC1139-ermpR3-1 and -2 grown in fermentation medium for 10 days. *S. roseosporus* was cultivated in 250-ml shaking flasks containing 50 ml of seed medium or fermentation medium. Apramycin was added to primary and secondary seed medium of WT/pKC1139 and WT/pKC1139-ermpR3 for plasmid selection. Error bars show the standard deviation of three replicate flasks. Statistical significance was determined by comparing the mutant values to those of the WT strain. **, $P < 0.01$; NS, not significant.

indirect and that DptR3 does not function as a pathway-specific regulator of the *dpt* gene cluster.

His_6-DptR3 was observed to bind to the *dptR3-orf16* intergenic region (probe R3-16) and generated significantly shifted bands. The binding specificity was evaluated by the addition of excess unlabeled specific probe R3-16 (lane S), which competed strongly with labeled probe R3-16 for binding to DptR3, and by the addition of excess unlabeled nonspecific competitor DNA (lane N), which could not attenuate or abolish the retarded signal. The labeled nonspecific probe *hrdB* and bovine serum albumin (BSA) were used as negative controls (Fig. 4A). Our findings indicate that DptR3 directly regulates the transcription of its own gene, *dptR3*, and the adjacent gene *orf16* through interaction with their promoter regions.

The DNA-binding activity of MarR proteins is typically attenuated by the binding of small-molecule ligands (14, 15). Because DptR3 regulates daptomycin production, we examined the possibility that daptomycin functions as the ligand of DptR3. This possibility was ruled out by the finding that daptomycin did not induce dissociation of DptR3 from probe R3-16 even at a high concentration (20 mM) (Fig. 4B).

Identification of the DptR3-binding site in the *dptR3-orf16* intergenic region. To elucidate the mechanism whereby DptR3 regulates *dptR3* and *orf16*, we determined the promoter structures of the two genes and the specific binding site of DptR3 in their intergenic region. The TSPs of the genes were determined by 5′ RACE analysis. The *dptR3* TSP coincides with the first A of the putative ATG translational start codon of *dptR3* (Fig. 5A and C), which is used frequently in *Streptomyces*. This finding indicates that *dptR3* is transcribed as a leaderless mRNA, as are many other *Streptomyces* genes (27–29). The *orf16* TSP was mapped to G at a position 50 nt upstream of the translational start codon of *orf16*. Determination of these TSPs led to the proposal of the putative −10 and −35 promoter sequences indicated by boxes in Fig. 5C.

The precise binding sequence of DptR3 was determined by DNase I footprinting experiments with a 378-bp FAM-labeled

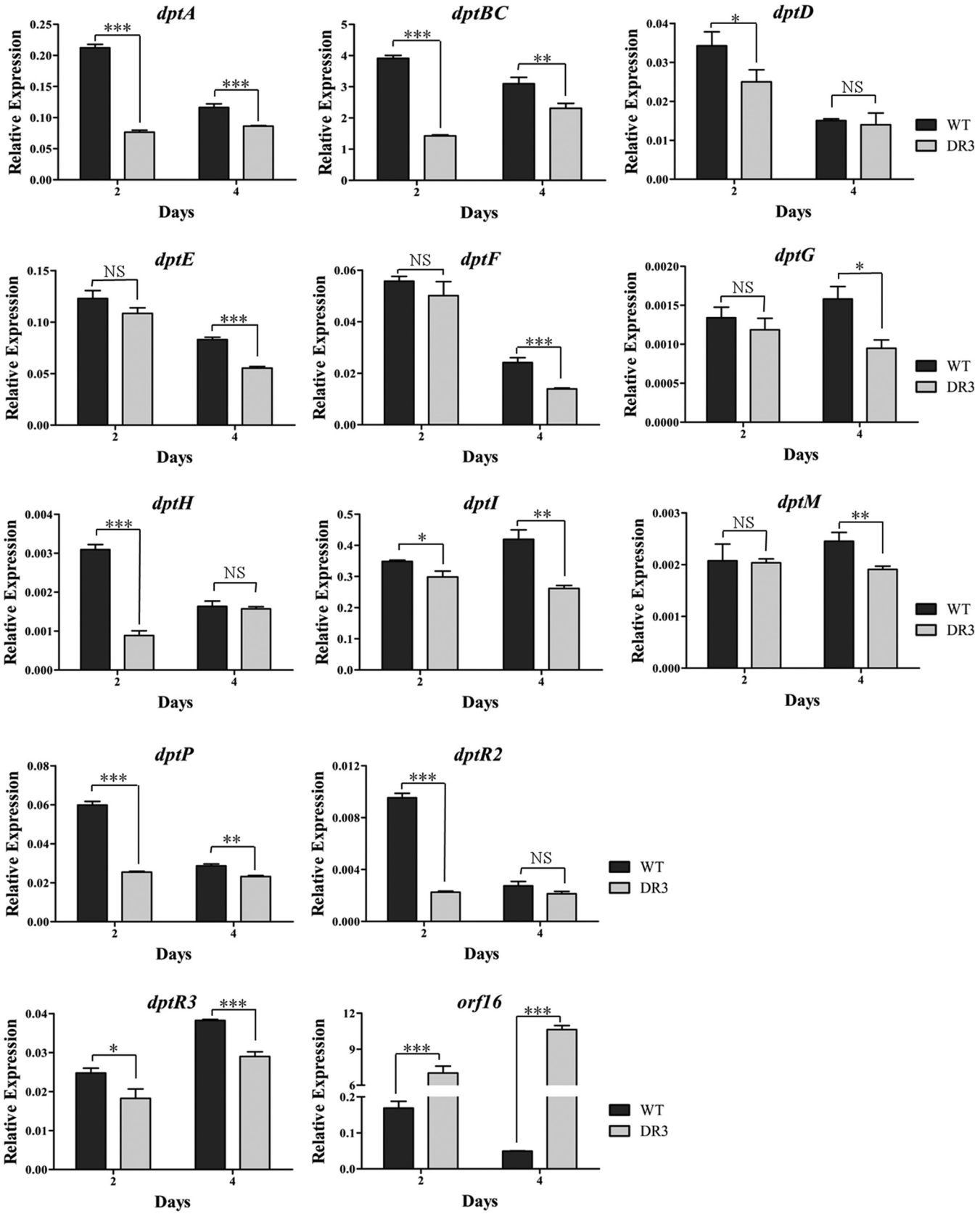

FIG 3 Real-time RT-PCR analysis of the *dpt* and *orf16* gene transcription levels in the WT and DR3 strains. RNA samples were isolated from 2- and 4-day fermentation cultures. The relative transcription levels of each gene were obtained after normalization against the internal reference *hrdB* at corresponding time points. *dptR3*, 60-bp transcript amplified from the remainder ORF of *dptR3* in DR3 with primers ZQL75 and ZQL76. Error bars show the standard deviation of three independent experiments. Statistical significance was determined by Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

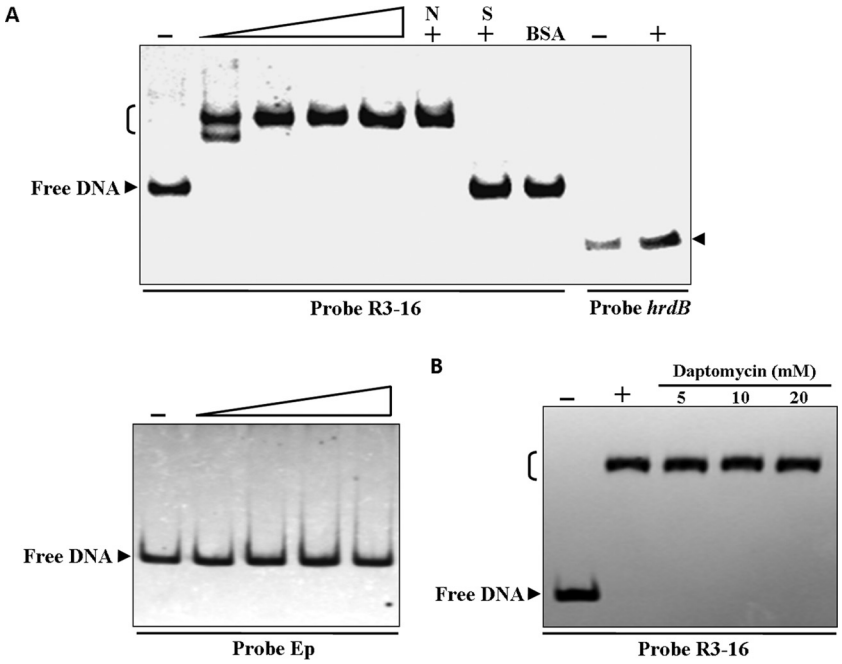

FIG 4 EMSAs of DptR3 binding to the *dptR3-orf16* intergenic region. (A) EMSAs of the interaction of promoter regions with purified His_6-DptR3 protein. Each lane contained 0.15 nM labeled probe. EMSAs with 50-fold unlabeled specific probe (S) or nonspecific (N) competitor DNA were performed to confirm the specificity of band shifts. Labeled probe *hrdB* was used to eliminate nonspecific binding of DptR3 protein. BSA was used as a negative control for DptR3 protein. The labeled probes were incubated in the absence (−) or presence of various amounts (50, 100, 150, 200 ng) of His_6-DptR3. Two hundred nanograms of His_6-DptR3 was used for control probe *hrdB* and competition experiments. (B) EMSAs of His_6-DptR3 (150 ng) interaction with daptomycin. Arrowhead, free probe; bracket, DptR3-DNA complex.

DNA probe comprising the *dptR3-orf16* intergenic region, in the presence or absence of His_6-DptR3 protein. A 66-nt protected region was found on the coding strand of *dptR3* (Fig. 5B), extending from −87 to −22 nt relative to the *dptR3* TSP and from −42 to +24 nt relative to the *orf16* TSP (Fig. 5C). The 66-nt region overlaps the potential −10 and −35 regions of the *orf16* promoter, suggesting that DptR3 negatively regulates *orf16* transcription by blocking the access of RNA polymerase to its promoter region. The protection region is located upstream but overlaps the potential −35 region of the *dptR3* promoter. DptR3 may activate *dptR3* by stabilizing the RNA polymerase or by promoting the recruitment of RNA polymerase to the *dptR3* promoter.

MarR proteins typically form symmetric dimers and bind to palindromic sequences (14, 15). Analysis of the DptR3-binding region with the DNAMAN program revealed two perfect palindromic sequences termed a and b (Fig. 5B and C). To estimate the relative contributions of these two sequences to DptR3 binding, we performed EMSAs with a probe that contained either the intact palindromic sequence or a mutated sequence, as illustrated in Fig. 6A. DptR3 did not retard probe 1a containing palindromic sequence a (Fig. 6B), indicating that sequence a is not essential for DptR3 binding. The affinity of DptR3 for mutated probe bm1, which lacked inverted repeats, was abolished completely, whereas strong shifted signals were observed between DptR3 and corresponding WT probe b containing palindromic sequence b (Fig. 6B). Seven base pairs were deleted from the spacer region between the inverted repeats in probe bm2. For probe bm3, base changes were introduced into the spacer sequence. The affinity of DptR3 for mutated probes bm2 and bm3 was still present but very weak. These findings indicate that the inverted repeats in sequence b are essential for DptR3 binding and that the spacer sequence and length are also important for effective interaction with DptR3.

Effect of *orf16* deletion on daptomycin production. Because *orf16* was identified as the target gene of DptR3, we constructed *orf16* deletion mutant D16 (Fig. 7A) and further investigated the role of this gene in daptomycin production and morphological development. D16 displayed normal growth on the solid media DA1, R2YE, and MM agar (data not shown). In comparison with that of the WT strain, daptomycin production was reduced to ~56 and 55% in deletion mutants D16-1 and D16-2, respectively (Fig. 7B). Deletion of *orf16* did not affect biomass accumulation in fermentation medium (Fig. 7C) but attenuated expression of daptomycin biosynthetic genes (see Fig. S1 in the supplemental material). The transcription levels of *dpt* structural genes in the WT and D16 were consistent with the daptomycin yield data for these strains. These results indicate that *orf16* has a positive effect on daptomycin biosynthesis. The expression level of *orf16* in the WT strain was low. Deletion of *dptR3* led to a striking increase in the *orf16* transcription level (Fig. 3) and reduced daptomycin production (Fig. 2B). These findings suggest that the alteration of daptomycin production in the DR3 mutant did not result from varying expression of *orf16* and that other DptR3 targets may affect daptomycin biosynthesis.

DISCUSSION

Little is known regarding the regulation of daptomycin production at the transcriptional level, although the biosynthetic pathway (5, 9), combinational biosynthesis (30), metabolic engineering (31, 32), and fermentation optimization (6, 7) of this lipopeptide antibiotic have been extensively studied. We have shown here that DptR3 functions as a global activator of dapto-

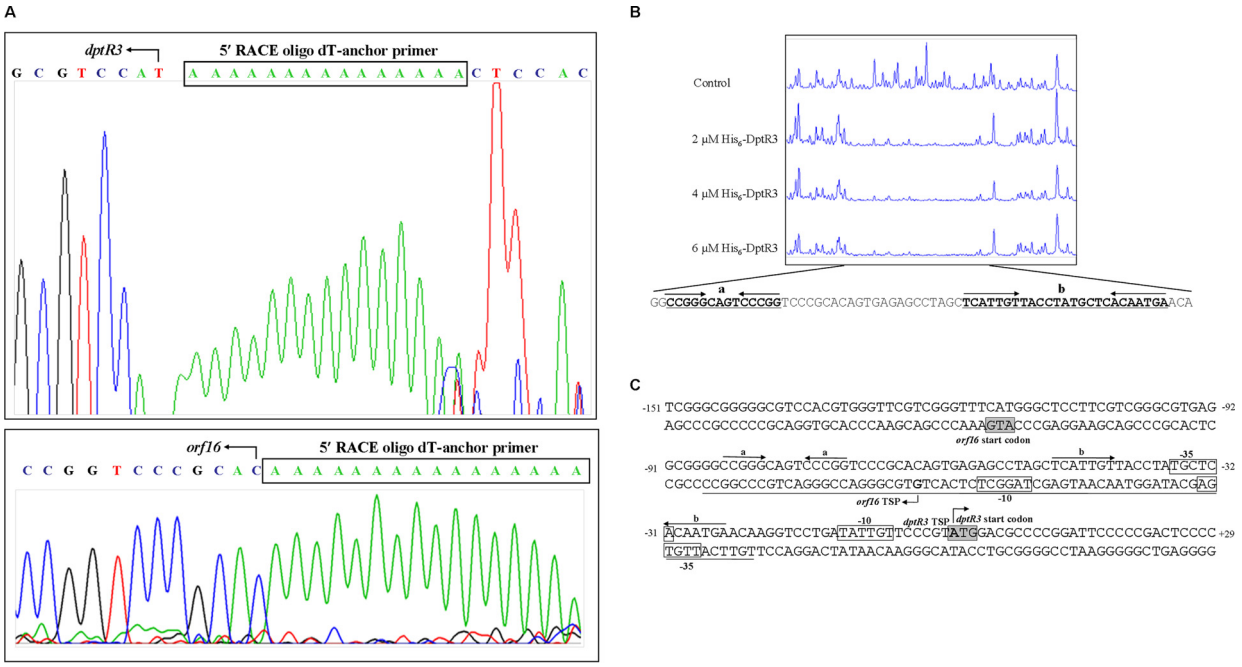

FIG 5 *dptR3* and *orf16* promoter structures and DptR3-binding site in the *dptR3-orf16* intergenic region. (A) Determination of TSPs of *dptR3* and *orf16* by 5′ RACE PCR. Boxed areas, complementary sequences of 5′ RACE oligo(dT) anchor primers. Bent arrows, complementary bases of TSPs. (B) DNase I footprinting assay of DptR3 in the *dptR3-orf16* intergenic region. Fluorograms correspond to control DNA (10 μM BSA) and to protection reactions with increasing concentrations of His_6-DptR3 protein. (C) Nucleotide sequences of the *dptR3-orf16* promoter region and the DptR3-binding site. The numbers are distances (in nucleotides) from the TSP of *dptR3*. Solid line, DptR3-binding site. Straight arrows, inverted repeats. Bent arrows, TSP and transcription orientation. Boxed areas, putative −10 and −35 regions. Shaded areas, translational start codon.

mycin production and morphological differentiation in *S. roseosporus*. Our findings extend the limited knowledge of the complex regulation of daptomycin biosynthesis.

Transcription and binding experiments have shown that DptR3 stimulates daptomycin production at the transcriptional level by altering the expression of structural genes in the *dpt* gene cluster; however, this stimulatory effect is indirect, indicating that DptR3 does not function as a pathway-specific regulator of daptomycin biosynthesis. Another group demonstrated that, of the three regulatory genes (*dptR1*, *dptR2*, *dptR3*) adjacent to the

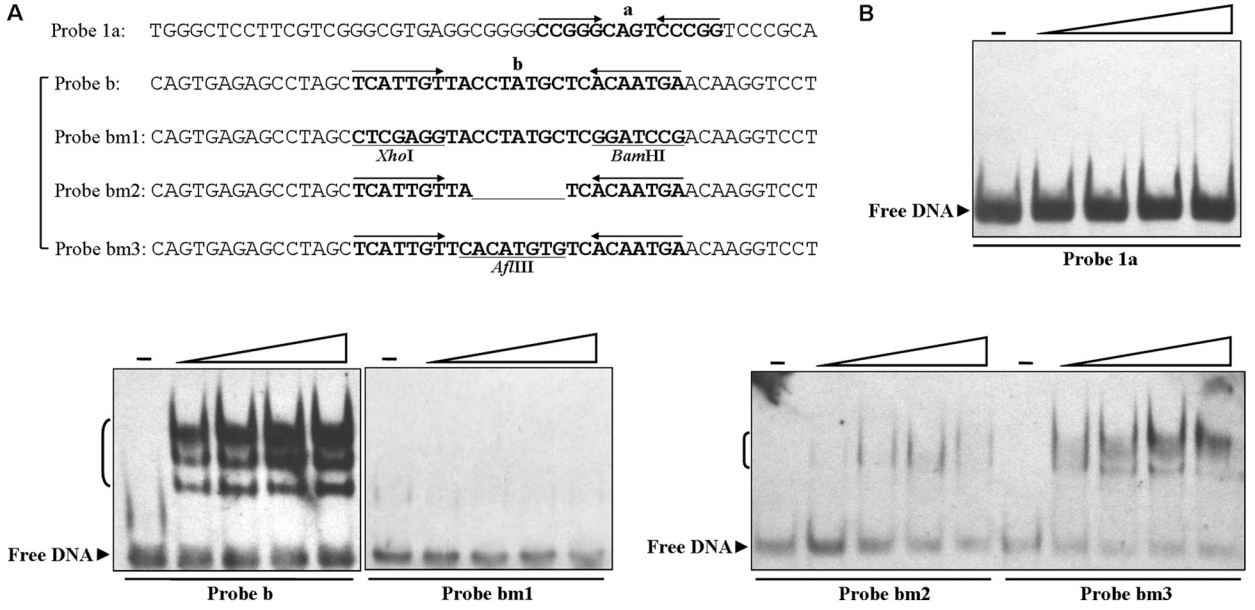

FIG 6 Mutational analysis of the DptR3-binding site. (A) DNA probes containing an intact palindromic sequence in the DptR3-binding site or mutated sequences. Probes 1a and b, 50-bp WT DNA containing intact palindromic sequences a and b, respectively. Mutations were introduced into probe b to produce mutated probes bm1, bm2, and bm3, respectively. Altered nucleotides are underlined. (B) EMSAs with various DNA probes. Each probe was incubated with 50, 100, 150, and 200 ng of His_6-DptR3 (concentrations increase from left to right in each series of four lanes, as indicated by the triangles above the blots).

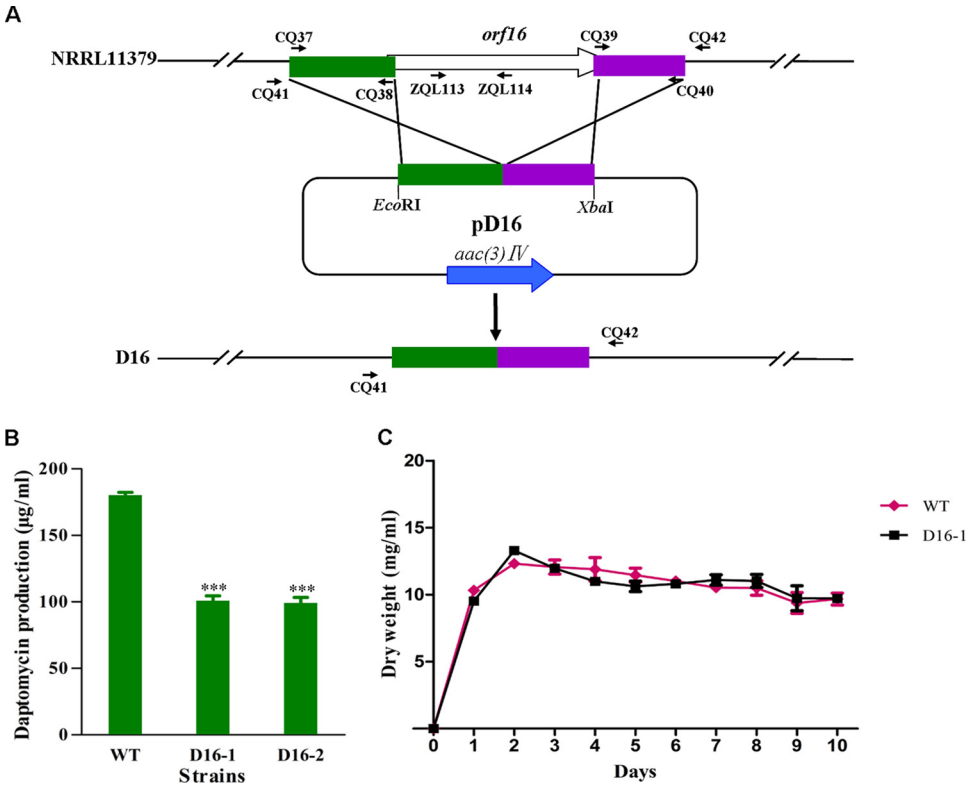

FIG 7 Effect of *orf16* deletion on daptomycin production and cell growth. (A) Strategy used for deletion of *orf16*. (B) Comparison of daptomycin production in WT and *orf16* deletion mutants D16-1 and -2 grown in fermentation medium for 10 days. ***, $P < 0.001$. (C) WT and D16-1 growth curves.

known *dpt* structural genes, *dptR2* is not a pathway-specific regulatory gene (10). We found that *dptR1* transcription under fermentation conditions is not detectable by real-time RT-PCR and that deletion of *dptR1* has no notable effect on daptomycin production (unpublished data), suggesting that *dptR1* is also not a pathway-specific regulatory gene. It therefore appears that the *dpt* cluster, similar to the erythromycin biosynthetic (*ery*) cluster in *Saccharopolyspora erythraea* (33), lacks a pathway-specific regulatory gene. BldD, a key developmental regulator whose gene resides apart from the *ery* cluster on the *S. erythraea* chromosome, was shown to directly control the expression of the *ery* cluster (34). The altered and coordinated expression of nearly all of the *dpt* genes suggests the existence of a common direct regulator of the *dpt* cluster. BldD homologs are present throughout the sporulating actinomycetes, including *S. roseosporus*. The consensus target site of BldD in *S. coelicolor* is 5′-NTNCNC(A/T)GNGTNAN-3′ (3). Similar binding motifs are present in some promoter regions of the *dpt* cluster, i.e., promoter regions of *dptE* and *dptI*, and the bidirectional promoter region of *dptP-dptM*, suggesting that the *dpt* genes may be direct targets of a BldD homolog in *S. roseosporus*. Further studies are necessary to identify the direct regulator(s) of the *dpt* cluster in order to clarify the daptomycin biosynthesis regulatory network.

Previous studies showed that most MarR homolog genes are oriented divergently from their neighboring genes and that the transcription of both genes is tightly controlled by binding of MarR proteins to the intergenic region (13, 14). The case of *dptR3* and *orf16* is similar. We found that DptR3 directly regulates the transcription of its own gene and *orf16* by binding to the 25-bp palindromic sequence TCATTGTTACCTATGCTCACAATGA (the underlining indicates inverted repeats) in the *dptR3-orf16* intergenic region. We used this palindromic sequence to scan the *S. roseosporus* genome in a search for new putative target genes of DptR3. No such sequence was found in other promoter regions. However, other DptR3 target genes may be present in *S. roseosporus*. The opposite effects of *dptR3* deletion on the *orf16* transcription level and of *orf16* deletion on the daptomycin production level and the positive regulation of morphological development by DptR3 suggest the existence of other DptR3 targets besides *dptR3* and *orf16*. Future studies using chromatin immunoprecipitation-DNA sequencing and bacterial one-hybrid systems will lead to the identification of other DptR3 target genes, and the roles of the new target genes in daptomycin production and morphological development can then be investigated.

We found that *dptR3* is positively autoregulated. Overexpression of *dptR3* in the WT strain promoted daptomycin production, indicating that endogenous DptR3 is not present in saturating amounts. Thus, enhancement of the *dptR3* expression level is a practical strategy for increasing daptomycin production. Although DptR3 regulates daptomycin biosynthesis, daptomycin is not the ligand of DptR3, and this may be due to the indirect control of daptomycin production by DptR3. The physiological roles of ~100 MarR proteins have been characterized in detail to date. Because the natural ligands of ligand-responsive MarR family proteins are unknown in most cases (13, 14, 21), our knowledge of the molecular regulatory mechanisms of these proteins remains limited. *orf16* encodes a putative ABC transporter ATP-binding protein, which is one of the components of the ABC transport system

and is repressed directly by DptR3. In the present study, deletion of *orf16* resulted in reduced daptomycin production, suggesting that this gene plays an important role in the uptake of compounds necessary for daptomycin biosynthesis. *SCO1144*, the *orf16* homolog gene in *S. coelicolor*, encodes a protein belonging to a lipid exporter family in the ABC-type export system. The substrates of this exporter family include lipoproteins, lipopolysaccharides, lipid A, multiple drugs, and peptides (35). Another possibility is that the *orf16* product is involved in the expulsion of harmful or toxic by-products generated from the biosynthesis of daptomycin or that it has an effect on daptomycin resistance through the expulsion of daptomycin. *orf16* is not a regulatory gene and may not influence gene expression directly. Therefore, the effect of *orf16* deletion, i.e., reducing the expression of daptomycin biosynthetic genes, is indirect. The as-yet-unknown substrate of the Orf16 protein may be the ligand of a regulatory gene(s) involved in daptomycin biosynthesis. More experiments are needed to test these possibilities.

The mechanism whereby DptR3 regulates morphological differentiation is unclear. AdpA and BldD are global regulators of secondary metabolism and morphological differentiation in *S. coelicolor* (3). We found that DptR3 could bind to promoter regions of *adpA* and *bldD* homologous genes in *S. roseosporus* (see Fig. S2 in the supplemental material), raising the possibility that DptR3 regulates development by controlling the expression of *adpA* and *bldD*, and this requires further detailed investigation.

This is the first report that a transcriptional regulator in *S. roseosporus* controls both daptomycin production and morphological development. Improved knowledge of DptR3 target genes and their functions and the specific ligand of DptR3 will lead to more effective strategies for increasing daptomycin production.

ACKNOWLEDGMENTS

This study was supported by the Chinese Universities Scientific Fund (grant 2014QT018).

We are grateful to Yinghua Lu (Xiamen University, Xiamen, China) for providing *S. roseosporus* NRRL11379 and to S. Anderson for editing the English of the manuscript.

REFERENCES

1. **Bibb MJ.** 2005. Regulation of secondary metabolism in streptomycetes. Curr Opin Microbiol **8:**208–215. http://dx.doi.org/10.1016/j.mib.2005.02.016.
2. **van Wezel GP, McDowall KJ.** 2011. The regulation of the secondary metabolism of *Streptomyces*: new links and experimental advances. Nat Prod Rep **28:**1311–1333. http://dx.doi.org/10.1039/c1np00003a.
3. **Liu G, Chater KF, Chandra G, Niu G, Tan H.** 2013. Molecular regulation of antibiotic biosynthesis in *Streptomyces*. Microbiol Mol Biol Rev **77:**112–143. http://dx.doi.org/10.1128/MMBR.00054-12.
4. **Baltz RH.** 2009. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. Curr Opin Chem Biol **13:**144–151. http://dx.doi.org/10.1016/j.cbpa.2009.02.031.
5. **Miao V, Coëffet-Legal MF, Brian P, Brost R, Penn J, Whiting A, Martin S, Ford R, Parr I, Bouchard M, Silva CJ, Wrigley SK, Baltz RH.** 2005. Daptomycin biosynthesis in *Streptomyces roseosporus*: cloning and analysis of the gene cluster and revision of peptide stereochemistry. Microbiology **151:**1507–1523. http://dx.doi.org/10.1099/mic.0.27757-0.
6. **Huber FM, Pieper RL, Tietz AJ.** 1988. The formation of daptomycin by supplying decanoic acid to *Streptomyces roseosporus* cultures producing the antibiotic complex A21978C. J Biotechnol **7:**283–292. http://dx.doi.org/10.1016/0168-1656(88)90040-5.
7. **Ng IS, Ye C, Zhang Z, Lu Y, Jing K.** 2014. Daptomycin antibiotic production processes in fed-batch fermentation by *Streptomyces roseosporus* NRRL11379 with precursor and medium optimization. Bioprocess Biosyst Eng **37:**415–423. http://dx.doi.org/10.1007/s00449-013-1007-2.
8. **Liao G, Shi T, Xie J.** 2012. Regulation mechanisms underlying the biosynthesis of daptomycin and related lipopeptides. J Cell Biochem **113:**735–741. http://dx.doi.org/10.1002/jcb.23414.
9. **Wittmann M, Linne U, Pohlmann V, Marahiel MA.** 2008. Role of DptE and DptF in the lipidation reaction of daptomycin. FEBS J **275:**5343–5354. http://dx.doi.org/10.1111/j.1742-4658.2008.06664.x.
10. **Wang F, Ren NN, Luo S, Chen XX, Mao XM, Li YQ.** 2014. DptR2, a DeoR-type auto-regulator, is required for daptomycin production in *Streptomyces roseosporus*. Gene **544:**208–215. http://dx.doi.org/10.1016/j.gene.2014.04.044.
11. **Martin RG, Rosner JL.** 1995. Binding of purified multiple antibiotic-resistance repressor protein (MarR) to *mar* operator sequences. Proc Natl Acad Sci U S A **92:**5456–5460. http://dx.doi.org/10.1073/pnas.92.12.5456.
12. **Martin RG, Nyantakyi PS, Rosner JL.** 1995. Regulation of the multiple antibiotic resistance (*mar*) regulon by *marORA* sequences in *Escherichia coli*. J Bacteriol **177:**4176–4178.
13. **Wilkinson SP, Grove A.** 2006. Ligand-responsive transcriptional regulation by members of the MarR family of winged helix proteins. Curr Issues Mol Biol **8:**51–62.
14. **Perera IC, Grove A.** 2010. Molecular mechanisms of ligand-mediated attenuation of DNA binding by MarR family transcriptional regulators. J Mol Cell Biol **2:**243–254. http://dx.doi.org/10.1093/jmcb/mjq021.
15. **Grove A.** 2013. MarR family transcription factors. Curr Biol **23:**R142–R143. http://dx.doi.org/10.1016/j.cub.2013.01.013.
16. **Oh SY, Shin JH, Roe JH.** 2007. Dual role of OhrR as a repressor and an activator in response to organic hydroperoxides in *Streptomyces coelicolor*. J Bacteriol **189:**6284–6292. http://dx.doi.org/10.1128/JB.00632-07.
17. **Zhu DQ, Wang YP, Zhang MM, Ikeda H, Deng ZX, Gene DE.** 2013. Product-mediated regulation of pentalenolactone biosynthesis in *Streptomyces* species by the MarR/SlyA family activators PenR and PntR. J Bacteriol **195:**1255–1266. http://dx.doi.org/10.1128/JB.02079-12.
18. **Huang H, Grove A.** 2013. The transcriptional regulator TamR from *Streptomyces coelicolor* controls a key step in central metabolism during oxidative stress. Mol Microbiol **87:**1151–1166. http://dx.doi.org/10.1111/mmi.12156.
19. **Huang H, Mackel BJ, Grove A.** 2013. *Streptomyces coelicolor* encodes a urate-responsive transcriptional regulator with homology to PecS from plant pathogens. J Bacteriol **195:**4954–4965. http://dx.doi.org/10.1128/JB.00854-13.
20. **Davis JR, Brown BL, Page R, Sello JK.** 2013. Study of PcaV from *Streptomyces coelicolor* yields new insights into ligand-responsive MarR family transcription factors. Nucleic Acids Res **41:**3888–3900. http://dx.doi.org/10.1093/nar/gkt009.
21. **Holley TA, Stevenson CE, Bibb MJ, Lawson DM.** 2013. High resolution crystal structure of Sco5413, a widespread actinomycete MarR family transcriptional regulator of unknown function. Proteins **81:**176–182. http://dx.doi.org/10.1002/prot.24197.
22. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA.** 2000. Practical *Streptomyces* genetics: a laboratory manual. John Innes Foundation, Norwich, United Kingdom.
23. **MacNeil DJ, Klapko LM.** 1987. Transformation of *Streptomyces avermitilis* by plasmid DNA. J Ind Microbiol **2:**209–218. http://dx.doi.org/10.1007/BF01569542.
24. **Zhao J, Wen Y, Chen Z, Song Y, Li JL.** 2007. An *adpA* homologue in *Streptomyces avermitilis* is involved in regulation of morphogenesis and melanogenesis. Chin Sci Bull **52:**623–630. http://dx.doi.org/10.1007/s11434-007-0105-4.
25. **Zianni M, Tessanne K, Merighi M, Laguna R, Tabita F.** 2006. Identification of the DNA bases of a DNase I footprint by the use of dye primer sequencing on an automated capillary DNA analysis instrument. J Biomol Tech **17:**103–113.
26. **Coëffet-Legal MF, Thurston L, Rich P, Miao V, Baltz RH.** 2006. Complementation of daptomycin *dptA* and *dptD* deletion mutations in *trans* and production of hybrid lipopeptide antibiotics. Microbiology **152:**2993–3001. http://dx.doi.org/10.1099/mic.0.29022-0.
27. **Hoskisson PA, Rigali S, Fowler K, Findlay KC, Buttner MJ.** 2006. DevA, a GntR-like transcriptional regulator required for development in *Streptomyces coelicolor*. J Bacteriol **188:**5014–5023. http://dx.doi.org/10.1128/JB.00307-06.
28. **Xu D, Seghezzi N, Esnault C, Virolle MJ.** 2010. Repression of antibiotic production and sporulation in *Streptomyces coelicolor* by overexpression

of a TetR family transcriptional regulator. Appl Environ Microbiol **76:** 7741–7753. http://dx.doi.org/10.1128/AEM.00819-10.

29. **Romero DA, Hasan AH, Lin YF, Kime L, Ruiz-Larrabeiti O, Urem M, Bucca G, Mamanova L, Laing EE, Wezel GP, Smith CP, Kaberdin VR, McDowall KJ.** 30 September 2014. A comparison of key aspects of gene regulation in *Streptomyces coelicolor* and *Escherichia coli* using nucleotide-resolution transcription maps produced in parallel by global and differential RNA sequencing. Mol Microbiol http://dx.doi.org/10.1111/mmi.12810.
30. **Nguyen KT, Ritz D, Gu JQ, Alexander D, Chu M, Miao V, Brian P, Baltz RH.** 2006. Combinatorial biosynthesis of novel antibiotics related to daptomycin. Proc Natl Acad Sci U S A **103:**17462–17467. http://dx.doi.org/10.1073/pnas.0608589103.
31. **Liao G, Wang L, Liu Q, Guan F, Huang Y, Hu C.** 2013. Manipulation of kynurenine pathway for enhanced daptomycin production in *Streptomyces roseosporus*. Biotechnol Prog **29:**847–852. http://dx.doi.org/10.1002/btpr.1740.
32. **Huang D, Wen J, Wang G, Yu G, Jia X, Chen Y.** 2012. In silico aided metabolic engineering of *Streptomyces roseosporus* for daptomycin yield improvement. Appl Microbiol Biotechnol **94:**637–649. http://dx.doi.org/10.1007/s00253-011-3773-6.
33. **Oliynyk M, Samborskyy M, Lester JB, Mironenko T, Scott N, Dickens S, Haydock SF, Leadlay PF.** 2007. Complete genome sequence of the erythromycin-producing bacterium *Saccharopolyspora erythraea* NRRL23338. Nat Biotechnol **25:**447–453. http://dx.doi.org/10.1038/nbt1297.
34. **Chng C, Lum AM, Vroom JA, Kao CM.** 2008. A key developmental regulator controls the synthesis of the antibiotic erythromycin in *Saccharopolyspora erythraea*. Proc Natl Acad Sci U S A **105:**11346–11351. http://dx.doi.org/10.1073/pnas.0803622105.
35. **Getsin I, Nalbandian GH, Yee DC, Vastermark A, Paparoditis PC, Reddy VS, Saier MH, Jr.** 2013. Comparative genomics of transport proteins in developmental bacteria: *Myxococcus xanthus* and *Streptomyces coelicolor*. BMC Microbiol **13:**279. http://dx.doi.org/10.1186/1471-2180-13-279.
36. **Bierman M, Logan R, Obrien K, Seno ET, Rao RN, Schoner BE.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene **116:**43–49. http://dx.doi.org/10.1016/0378-1119(92)90627-2.
37. **Li LL, Guo J, Wen Y, Chen Z, Song Y, Li JL.** 2010. Overexpression of ribosome recycling factor causes increased production of avermectin in *Streptomyces avermitilis* strains. J Ind Microbiol Biotechnol **37:**673–679. http://dx.doi.org/10.1007/s10295-010-0710-0.