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Two Master Switch Regulators Trigger A40926 Biosynthesis in *Nonomuraea* sp. Strain ATCC 39727

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ABSTRACT

The actinomycete *Nonomuraea* sp. strain ATCC 39727 produces the glycopeptide A40926, the precursor of dalbavancin. Biosynthesis of A40926 is encoded by the *dbv* gene cluster, which contains 37 protein-coding sequences that participate in antibiotic biosynthesis, regulation, immunity, and export. In addition to the positive regulatory protein Dbv4, the A40926-biosynthetic gene cluster encodes two additional putative regulators, Dbv3 and Dbv6. Independent mutations in these genes, combined with bioassays and liquid chromatography-mass spectrometry (LC-MS) analyses, demonstrated that Dbv3 and Dbv4 are both required for antibiotic production, while inactivation of *dbv6* had no effect. In addition, overexpression of *dbv3* led to higher levels of A40926 production. Transcriptional and quantitative reverse transcription (RT)-PCR analyses showed that Dbv4 is essential for the transcription of two operons, *dbv14-dbv8* and *dbv30-dbv35*, while Dbv3 positively controls the expression of four monocistronic transcription units (*dbv4*, *dbv29*, *dbv36*, and *dbv37*) and of six operons (*dbv2-dbv1*, *dbv14-dbv8*, *dbv17-dbv15*, *dbv21-dbv20*, *dbv24-dbv28*, and *dbv30-dbv35*). We propose a complex and coordinated model of regulation in which Dbv3 directly or indirectly activates transcription of *dbv4* and controls biosynthesis of 4-hydroxyphenylglycine and the heptapeptide backbone, A40926 export, and some tailoring reactions (mannosylation and hexose oxidation), while Dbv4 directly regulates biosynthesis of 3,5-dihydroxyphenylglycine and other tailoring reactions, including the four cross-links, halogenation, glycosylation, and acylation.

IMPORTANCE

This report expands knowledge of the regulatory mechanisms used to control the biosynthesis of the glycopeptide antibiotic A40926 in the actinomycete *Nonomuraea* sp. strain ATCC 39727. A40926 is the precursor of dalbavancin, approved for treatment of skin infections by Gram-positive bacteria. Therefore, understanding the regulation of its biosynthesis is also of industrial importance. So far, the regulatory mechanisms used to control two other similar glycopeptides (balhimycin and teicoplanin) have been elucidated, and beyond a common step, different clusters seem to have devised different strategies to control glycopeptide production. Thus, our work provides one more example of the pitfalls of deducing regulatory roles from bioinformatic analyses only, even when analyzing gene clusters directing the synthesis of structurally related compounds.

The glycopeptide antibiotic A40926 (Fig. 1A) is the precursor for dalbavancin, a semisynthetic lipoglycopeptide approved for clinical use in 2014 by the U.S. Food and Drug Administration to treat acute bacterial skin and skin structure infections caused by bacteria, such as *Staphylococcus aureus* (methicillin-susceptible and methicillin-resistant strains) and *Streptococcus pyogenes*. Compared with the structurally related glycopeptide teicoplanin, dalbavancin possesses better potency and pharmacokinetic properties.

A40926 is produced by the actinomycete *Nonomuraea* sp. strain ATCC 39727 as a complex of related compounds. It consists of a heptapeptide containing the proteinogenic amino acid tyrosine and the nonproteinogenic amino acids 3,5-dihydroxyphenylglycine (DPG) and 4-hydroxyphenylglycine (HPG) (1–4). The heptapeptide is assembled by a nonribosomal peptide synthetase (NRPS) and then modified by oxidative cross-linking of the aromatic side chains to yield a rigid peptide scaffold. Further tailoring steps include halogenation, glycosylation, oxidation, methylation, acetylation, and acylation (5). The A40926 components differ by the type of *N*-acyl chain attached to the glucuronic acid moiety and by the presence or absence of an *O*-linked acetyl residue at position 6 of the mannose (6–8). The form with the acetylated mannose was proposed to be the final biosynthetic product, which in turn exerts an inhibitory effect on A40926 production by a yet unknown mechanism (8). Detailed studies of A40926 biosynthesis and its regulation will be crucial for the development of rational approaches towards overproduction of the dalbavancin precursor.

The *dbv* gene cluster for A40926 biosynthesis contains 37 protein-coding sequences (Fig. 1B) that participate in antibiotic biosynthesis, regulation, immunity, and export (5). Previous reverse transcription (RT)-PCR analysis of the intergenic regions (9) revealed that the cluster contains five monocistronic transcription

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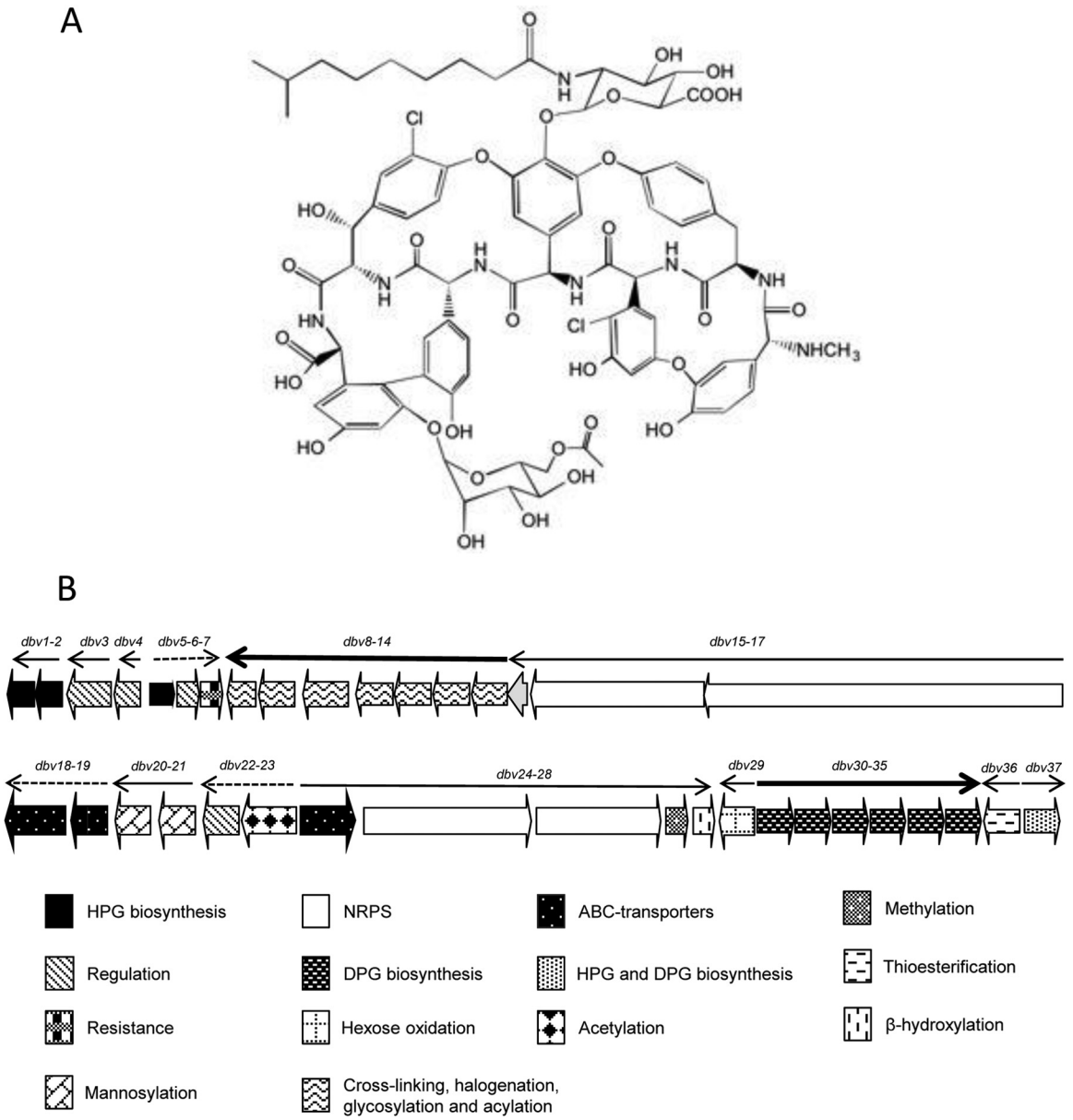

FIG 1 Structures of A40926 (A) and of the *dbv* gene cluster (B). The arrows indicate operons, as previously determined experimentally (9). The thin arrows represent the transcriptional units controlled by Dbv3, the thick arrows indicate the Dbv4-controlled operons, and the dashed arrows indicate operons whose control is unknown. *dbv* genes are grouped by category as indicated.

units (*dbv3*, *dbv4*, *dbv29*, *dbv36*, and *dbv37*) and nine operons (*dbv2-dbv1*, *dbv5-dbv7*, *dbv14-dbv8*, *dbv17-dbv15*, *dbv18-dbv19*, *dbv20-dbv21*, *dbv22-dbv23*, *dbv24-dbv28*, and *dbv30-dbv35*), indicated by arrows in Fig. 1B.

The cluster contains the positive regulatory gene *dbv4*, the putative regulatory gene *dbv3*, and the two-component system-encoding genes *dbv6-dbv22* (5). Most glycopeptide gene clusters encode a homolog of Dbv4, as well as conserved Dbv4-binding sites (10–12). Indeed, our previous analysis revealed that Dbv4 is a DNA-binding protein that acts as a positive regulator of A40926 biosynthesis by controlling expression of just two *dbv* operons: *dbv14-dbv8*, encoding the four cross-linking oxygenases, the halogenase, the *N*-acetylglucosamine transferase, and the *N*-acylase, and *dbv30-dbv35*, encoding the four enzymes involved in DPG biosynthesis, as well as the sodium-proton antiporter and a protein of unknown function (9). In previous work, we also showed that expression of *dbv4* is increased under phosphate-limiting conditions (9).

The putative regulator Dbv3 belongs to the LAL (large ATP-binding regulators of LuxR) family. Recently, several regulators of the LAL family have been identified in antibiotic and other secondary-metabolite gene clusters from actinomycetes (13–16). The last two positive regulators, *dbv6* and *dbv22*, encode a response regulator and a histidine sensory kinase, respectively, homologous to the *Streptomyces lividans* CutR/CutS pair that negatively regulates secondary metabolism (17).

TABLE 1 Bacterial strains and plasmids used in this work

Strain or plasmid	Description	Source or reference
Nonomuraea strains		
ATCC 39727	Wild type	ATCC
Δ*dbv3* mutant	Strain in which *dbv3* gene was replaced with an apramycin cassette	This work
Δ*dbv4* mutant	Strain in which *dbv4* gene was replaced with an apramycin cassette	This work
Δ*dbv6* mutant	Strain in which *dbv6* gene was replaced with an apramycin cassette	This work
Oe-*dbv3*	Strain carrying pIJ8600-*dbv3* integrated into the *attB* site in which an additional copy of the *dbv3* gene is under *tipA* promoter control	This work
Micrococcus luteus ATCC 9341	Used for bioassay of A40926	ATCC
E. coli strains		
DH10B	F$^-$ *endA1 recA1 galE15 galK16 nupG rpsL* Δ*lacX74* ϕ80*lacZ*ΔM15 *araD139* Δ(*ara-leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$; host for routine subcloning experiments	Invitrogen
ET12567(pUZ8002)	(*dam-13*::Tn*9 dcm-6*) pUZ8002$^+$ (Δ*oriT*); used for conjugative transfer of DNA and for demethylating plasmid DNA	39
BW25113/pIJ790	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-4*) *lacIp-4000*(*lacI*^{q}) λ$^-$ *rpoS369*(Am) *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*: used to generate recombinant cosmid 11A5	40
Plasmids		
11A5	Cosmid containing part of the *dbv* cluster from *dbv1* to *dbv16*	9
11A5 Δ*dbv3*	Derivative of 11A5 with the inactivated *dbv3* gene	This work
11A5 Δ*dbv4*	Derivative of 11A5 with the inactivated *dbv4* gene	This work
11A5 Δ*dbv6*	Derivative of 11A5 with the inactivated *dbv6* gene	This work
pIJ773	pUC19 containing the *aac*(*3*)*IV-oriT* cassette; source of the *aac*(*3*) gene	22
pIJ790	λ Red plasmid; contains the resistance marker *cat* and a temperature-sensitive origin of replication	40
pIJ8600	Actinomycete integrative vector with the *tipA* promoter	21
pIJ8600-*dbv3*	pIJ8600 derivative containing *dbv3* under *tipA* promoter control	This work

In this study, we characterize the functions of *dbv3*, *dbv4*, and *dbv6* in regulating A40926 biosynthesis in *Nonomuraea* sp. strain ATCC 39727 and demonstrate that most of the *dbv* genes are under the control of Dbv3.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria broth liquid medium at 37°C and were supplemented with 50 μg ml^{-1} apramycin when necessary to maintain plasmids. Frozen cell stocks of *Nonomuraea* strains were prepared by storing individual aliquots of the desired strain from midexponential cultures in R3 medium [10 g/liter glucose, 5 g/liter yeast extract, 0.1 g/liter Casamino Acids, 3 g/liter L-proline, 10 g/liter $MgCl_2 \cdot 6H_2O$, 4 g/liter $CaCl_2 \cdot 2H_2O$, 0.2 g/liter K_2SO_4, 0.05 g/liter KH_2PO_4, 5.6 g/liter *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 2 ml/liter trace elements, pH 7.2] at −80°C. Liquid cultures of *Nonomuraea* strains were prepared by inoculating 0.2 ml of the frozen cell stock into 20 ml of fresh R3 medium; after 120 h of growth, 1 ml of this culture was inoculated into 50 ml of fresh R3 medium in an orbital shaker (500 × *g*) in 250-ml baffled flasks at 30°C. The cultivation media were supplemented with 50 μg ml^{-1} apramycin to select for strains carrying integrated antibiotic resistance genes. For solid media, R3 was supplemented with 1.8% agar. To detect glycopeptide production, *Nonomuraea* strains were incubated in R3 liquid medium without apramycin, and aliquots of spent media or culture broths of the strains at different time points were withdrawn for analysis and stored at −80°C. To study the effect of *dbv3* expression in the *dbv3*-overexpressing (Oe-*dbv3*) strain, *Nonomuraea* sp. strain ATCC 39727 and the Oe-*dbv3* strain were grown in R3 liquid medium with 10^{-8} M thiostrepton. This concentration was found to have no effect on *Nonomuraea* sp. strain ATCC 39727 growth and *dbv3* transcription, and it did not inhibit *Micrococcus luteus* ATCC 9341 (18) and thus did not interfere with the assay of antibiotic activity due to A40926.

Analysis of antibiotic production. A paper disc diffusion method was used with *M. luteus* as the assay organism as described previously (19). *Nonomuraea* strains were grown in selected liquid media under the conditions specified above. After cultivation, 100 μl of spent medium was applied to Whatman 3 MM Chr paper discs (Whatman, Maidstone, United Kingdom). The wet discs were placed on the surface of LB soft agar inoculated with 100 μl of *M. luteus* (optical density at 600 nm [OD_{600}] = 1.2), and inhibition zones were measured after overnight incubation at 37°C. In parallel bioassays, the specific competitor D-Ala–D-Ala (20 μg/μl) was added to the spent medium of broth cultures to confirm A40926 antibacterial activity. For high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) analyses, 50 μl of broth cultures was collected at the time points indicated and mixed briefly with 2 volumes of methanol followed by 1 h of incubation at 40°C. After centrifugation to remove the cells, the supernatant was lyophilized and redissolved in 100 μl 10% dimethyl sulfoxide (DMSO). HPLC analyses were performed using an LC 2010A-HT (Shimadzu) equipped with a Merck LiChrosphere (5-μm; 4.6-mm by 100-mm) C_{18} column. Elution was performed at 1 ml min^{-1} at 50°C with a linear gradient from 10% to 90% phase B in 30 min. Phase A was 0.1% (vol/vol) trifluoroacetic acid (TFA) in H_2O, and phase B was CH_3CN. UV detection was set at 230 nm. LC-electrospray ionization (ESI)-MS data were recorded on an Ion Trap equipped with an LC Agilent 1100, using an Ascentis express Supelco RP18, 2.7-μm (50- by 4.6-mm) column kept at 40°C, eluting at 1 ml min^{-1} with a 7-min linear gradient from 95:5 phase A (0.05% [vol/vol] TFA in water)-phase B (0.05% [vol/vol] TFA in acetonitrile) to 100% phase B.

DNA manipulation. Standard genetic techniques with *E. coli* and *in vitro* DNA manipulations were performed as described previously (20).

Isolation of *Streptomyces* total DNA was performed by the salting-out procedure (21). Southern hybridization was carried out with probes labeled with digoxigenin by using the DIG DNA labeling kit (Roche Biochemicals). PstI- or PvuII (Invitrogen)-digested pIJ8600 was used as a probe. Chromosomal DNAs were digested with BamHI and PstI (Invitrogen).

Construction of the Δ*dbv3*, Δ*dbv4*, Δ*dbv6*, and Oe-*dbv3* strains. *dbv3*, *dbv4*, and *dbv6* of *Nonomuraea* sp. strain ATCC 39727 were deleted by replacing the gene with a cassette containing an apramycin-selectable marker using PCR targeting (22). pIJ773 containing the apramycin resistance gene [*aac(3)IV*] and the *oriT* replication origin was used as a template. The PCR primers used to amplify the resistance cassette for deletion of the *dbv* genes are reported in Table S1 in the supplemental material. *E. coli* BW25113/pIJ790 bearing cosmid 11A5 (5) was electrotransformed with the deletion cassettes. The isolated mutant cosmids were first introduced into the nonmethylating *E. coli* strain ET12567 containing pUZ8002 and then transferred to *Nonomuraea* sp. strain ATCC 39727 by intergeneric conjugation. Briefly, *Nonomuraea* sp. strain ATCC 39727 was grown in 30 ml R3 medium in a 250-ml baffled flask and incubated at 30°C on a rotary shaker at 200 rpm for 40 h. Mycelium was collected, washed twice with ice-cold 10% (vol/vol) glycerol, and suspended in 10 ml of 10% (vol/vol) glycerol. Then, 0.5 ml of the mycelium was added to 0.5 ml *E. coli* donor cells prepared as described previously (21). The mixtures were plated out on R3 agar plates. The conjugation plates were incubated for 18 to 20 h at 30°C and then overlaid with 1 ml water containing 1 mg nalidixic acid and 1 mg apramycin. The plates were incubated at 30°C for 10 to 15 days. Exconjugants were picked off and analyzed by PCR (data not shown), using primers reported in Table S1 in the supplemental material. One clone (out of 3 analyzed) contained the interrupted *dbv3* gene, 3 clones (out of 12 analyzed) contained the interrupted *dbv4* gene, and 1 clone (out of 9) carried the interrupted *dbv6* gene. The fidelity of the PCR products was confirmed by sequencing (BMR Genomics).

To construct the Oe-*dbv3* strain, a PCR fragment prepared using the primers *dbv3* over for and *dbv3* over rev (see Table S1 in the supplemental material) was cloned into pGEM T-Easy (Promega), digested with BglII (Invitrogen) and NdeI (Invitrogen), and then inserted between the BamHI (Invitrogen) and NdeI (Invitrogen) sites of pIJ8600 (21) under the control of the thiostrepton-inducible *tipA* promoter. Cloning was confirmed by sequencing the insert. The plasmid was transferred into *Nonomuraea* sp. strain ATCC 39727 by conjugation from *E. coli* ET12567 (pUZ8002) as described above, generating a strain called Oe-*dbv3*. As a control, pIJ8600 was inserted into the *Nonomuraea* sp. strain ATCC 39727 chromosome. Southern hybridization using PstI-digested pIJ8600 as a probe was carried out (see Fig. S1 in the supplemental material). As expected for integration at the chromosomal *attB* site, hybridizing bands of about 4.3 and 3.9 kb were observed with BamHI-digested chromosomal DNA (see Fig. S1, lane 1, in the supplemental material) and four bands of about 6, 4.3, 2, and 0.8 kb (see Fig. S2, lane 2, in the supplemental material) were observed with PstI-digested chromosomal DNA. No hybridization signal was detected for the parental strain (see Fig. S1, lanes 3 and 4, in the supplemental material).

Total RNA isolation and qRT-PCR analysis. To perform transcriptional analysis of selected genes, mycelium was harvested from 6 ml of culture, resuspended in 1 ml P buffer (21) containing lysozyme (50 mg/ml), and then incubated for 20 min at 37°C. RNA was extracted by using the RNeasy midi kit (Qiagen) according to the manufacturer's instructions. DNase I (Roche) treatment was performed at 37°C for 1 h, and RNA was precipitated with 2 volumes of ethanol in the presence of 0.1 volume of 3 M sodium acetate. After a washing step with 70% (vol/vol) ethanol and drying in air, the RNA pellet was resuspended in water. As a control for RNA quality, an RT-PCR with 0.1 μg of total RNA and primer pairs internal to *hrdB*, encoding a vegetative sigma factor, was carried out using the Superscript One-Step RT-PCR kit (Invitrogen) under the conditions indicated by the supplier. PCRs using the same primers were performed on 0.5 μg of RNA samples using 40 cycles to exclude the presence of genomic DNA. For quantitative RT (qRT)-PCR, a two-step protocol was used. The High-Capacity cDNA Archive kit (Applied Biosystems) was used to retrotranscribe 2 μg of total extracted RNA in a total volume of 100 μl. Primer pairs for the genes analyzed by qRT-PCR were designed with Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3/) to fulfill the following criteria: length, 20 ± 2 bp; melting temperature (T_m), 60 ± 1°C; GC content, >50%; and amplicon lengths ranging from 200 to 500 bp (see Table S1 in the supplemental material). Primer specificity was controlled by BLAST analysis. The PCR efficiency was estimated by using a free Web-based service (http://srvgen.upct.es/efficiency.html) (23). Gene expression was analyzed quantitatively by using the Applied Biosystems 7300 real-time PCR system (Applied Biosystems) with SYBR green PCR master mix (Applied Biosystems) in 96-well plates. Two microliters of cDNA was added to 20 μl of PCR mixture. Amplification required activation of AmpErase UNG at 50°C for 2 min, followed by denaturation at 95°C for 10 min and then 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curves were performed from 60 to 98°C to validate the specificity of the PCR. Three independent measurements were performed for each gene. The threshold cycle (C_T) values were determined with the baseline set automatically. The results were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method, in which the amount of target RNA is adjusted to an internal reference (24). *hrdB*, encoding a vegetative sigma factor, and a 16S rRNA gene did not show significant expression variation, as revealed by absolute quantitative RT-PCR using RNA extracted after 24, 48, and 72 h from *Nonomuraea* sp. strain ATCC 39727 (see Fig. S2 in the supplemental material). Standard deviations were calculated from three independent experiments. The values were calculated using a standard curve obtained by measuring the fluorescence of known amounts (10, 100, 1,000, and 10,000 μg) of cDNA and were used as internal references to normalize the results. Two different sets of experiments were carried out using one of the endogenous genes; the relative transcription values were compared and reported as the average of these two sets of experiments. Expression ratios were expressed as $2\Delta\Delta C_T$. Each run included negative controls.

RESULTS

Transcript abundance of regulatory genes during growth. We evaluated the transcript levels of three regulatory genes present in the *dbv* cluster, *dbv3*, *dbv4*, and *dbv6*, during growth and in respect to A40926 production. *Nonomuraea* sp. strain ATCC 39727 growth and A40926 production were monitored in R3 liquid medium for 96 h. Microbiological bioassays indicated that A40926 biosynthesis was detectable after 60 h of growth (Fig. 2A). Total RNA was extracted before (at 24 and 48 h) and during (at 72 h) production and was analyzed for gene expression by qRT-PCR (Fig. 2B) using *hrdB*, encoding a vegetative sigma factor, as a control. The transcript levels for *dbv3*, *dbv4*, and *dbv6* were ~5-, ~20, and ~3-fold higher, respectively, at 48 and 72 h than at 24 h, indicating that transcription of all the regulatory genes is upregulated and largely precedes the detection of A40926 in the medium (Fig. 2). The largest increase was observed for *dbv4*.

Effects of inactivation of the *dbv* regulatory genes. Disruption of *dbv3*, *dbv4*, or *dbv6* did not significantly alter the growth kinetics or the morphology of the mutants on the different media used (data not shown), indicating that these genes are not critical for bacterial growth and differentiation under the conditions used. In contrast, bioassays showed that inactivation of *dbv3* and *dbv4* abolished A40926 biosynthesis, while the absence of *dbv6* did not have a measurable effect on production (Fig. 3A). HPLC analysis confirmed that the Δ*dbv4* and Δ*dbv3* mutants produced undetectable levels of precursors and/or final product. In contrast, the metabolite profile of the Δ*dbv6* mutant was indistinguishable from that of *Nonomuraea* sp. strain ATCC 39727 (data not shown). These results indicate that both Dbv3 and Dbv4 are nec-

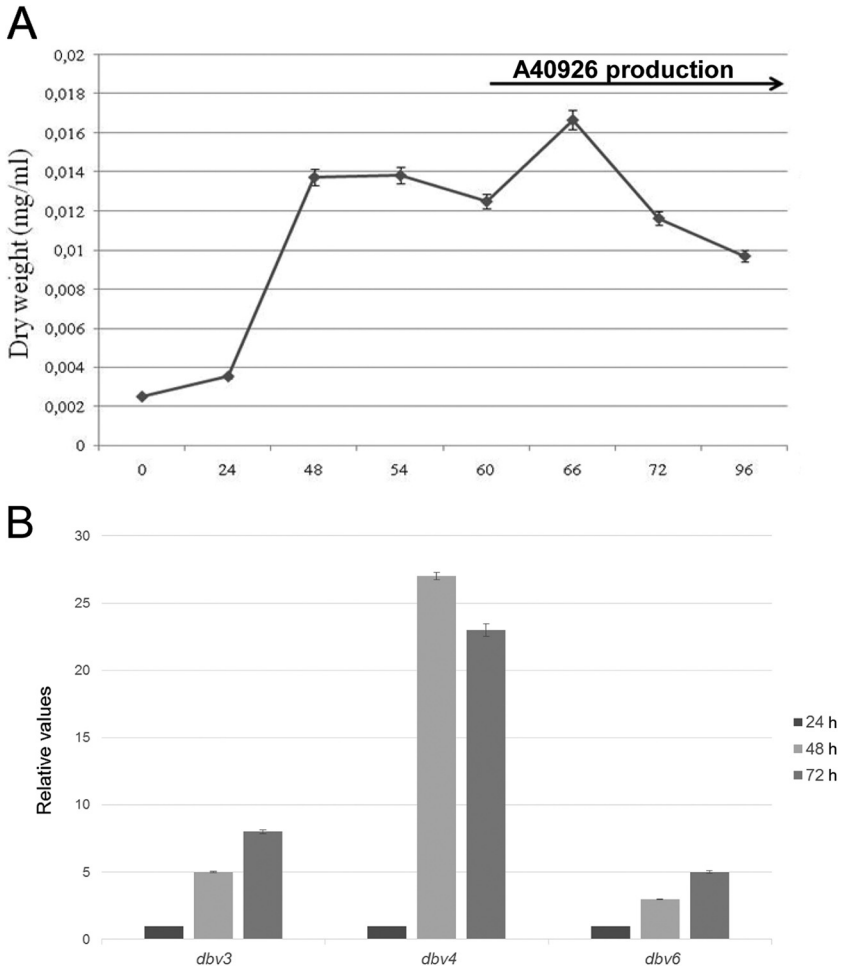

FIG 2 (A) Growth curve of *Nonomuraea* sp. strain ATCC 39727 grown in R3 medium. The arrow indicates A40926 detection in the supernatant. (B) qRT-PCR analysis of *dbv3*, *dbv4*, and *dbv6* after 24, 48, and 72 h of growth. mRNA levels are expressed relative to *hrdB* transcripts, with the ratio values for the 24-h sample arbitrarily set to 1. The standard deviations (indicated by error bars) were calculated from three independent determinations of mRNA abundance in each sample.

essary for antibiotic production, whereas Dbv6 is not. Since Dbv6 did not show a role in antibiotic biosynthesis, we further characterized only the Δ*dbv3* and Δ*dbv4* strains.

Interplay between the regulators. In order to establish any hierarchical control, qRT-PCR of the *dbv* regulatory genes was performed in the Δ*dbv3* and Δ*dbv4* mutant strains. In the Δ*dbv4* strain, *dbv3* and *dbv6* were transcribed as in the parental strain (Fig. 4), indicating that their expression is not strictly dependent

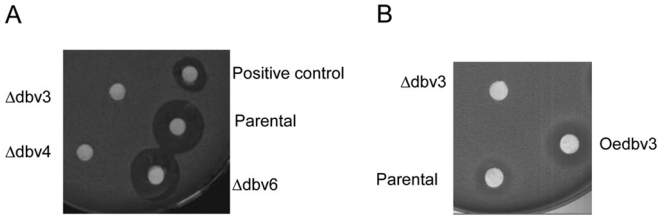

FIG 3 Bioassays of spent media from parental, Δ*dbv3*, Δ*dbv4*, and Δ*dbv6* strains (A) and from the Δ*dbv3* and Oe-*dbv3* strains (B) after 72 h of growth. The positive control was spent medium collected from the parental strain after 120 h.

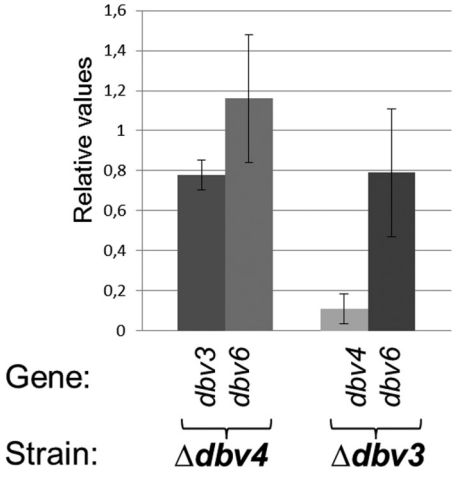

FIG 4 qRT-PCR analysis of *dbv3* and *dbv6* in the Δ*dbv4* mutant and of *dbv4* and *dbv6* in the Δ*dbv3* mutant after 60 h of growth. The transcription levels in the mutants were compared to those of the parental strain, taken as 1.0. The standard deviations (indicated by error bars) were calculated from three independent determinations of mRNA abundance in each sample. *hrdB* was used as an endogenous control.

on Dbv4. In contrast, qRT-PCR analysis of the Δ*dbv3* strain revealed that *dbv4* transcription was 10-fold reduced with respect to the parental strain, while *dbv6* transcription was not changed significantly (Fig. 4). These results suggest that Dbv3 controls *dbv4* transcription but does not have a major role, if any, in regulating *dbv6* transcription.

Dbv4 was previously shown to bind to the upstream regions of *dbv14* and *dbv30* (9). Consistently, transcription of these two genes was strongly reduced in the Δ*dbv4* mutant (data not shown), indicating that Dbv4 is essential for transcription of *dbv14* and *dbv30* and, presumably, of the *dbv14-dbv8* and *dbv30-dbv35* operons (Fig. 1B).

Identification of Dbv3 target genes. To identify Dbv3 target genes, total RNA was extracted from *Nonomuraea* sp. strain ATCC 39727 and the Δ*dbv3* strain, grown in R3 medium for 60 h, when antibiotic becomes detectable in the parental strain, and expression of *dbv* genes was analyzed by qRT-PCR. We concentrated on a subset of *dbv* genes, selecting them on the basis of transcriptional organization (Fig. 1B): the two monocistronic genes *dbv4* and *dbv29* and *dbv1*, *dbv6*, *dbv14*, *dbv15*, *dbv20*, and *dbv32* as representatives of the *dbv1-dbv2*, *dbv5-dbv7*, *dbv14-dbv8*, *dbv17-dbv15*, *dbv20-dbv21*, and *dbv30-dbv35* operons, respectively (Fig. 1B). The results clearly demonstrated that *dbv1*, *dbv4*, *dbv14*, *dbv15*, *dbv20*, *dbv29*, and *dbv32* either are not transcribed or are 10-fold less transcribed in the Δ*dbv3* strain than in the parental strain (Fig. 5). In contrast, *dbv6* transcript levels in the Δ*dbv3* strain were similar to those in the parental strain, indicating again that Dbv3 does not control *dbv6* transcription.

Increasing A40926 production by overexpression of *dbv3*. A *dbv3*-overexpressing strain (Oe-*dbv3*) was constructed by placing *dbv3* under the control of the thiostrepton-inducible *tipA* promoter and integrating the construct at the chromosomal *attB* site (see Fig. S1 in the supplemental material). In agreement with the results obtained with the Δ*dbv3* mutant, no differences were observed between growth of the parental and Oe-*dbv3* strains in liquid R3 medium (data not shown). To evaluate the effect of *dbv3* overexpression on A40926 production, bioassays were performed using spent culture medium collected at different time points. The Oe-*dbv3* strain produced more A40926 than the parental strain (Fig. 3B). HPLC and LC-MS analyses confirmed that the Oe-*dbv3* strain produced 1.7- to 2.2-fold more A40926 than the parental strain (Fig. 6A). (The control strain, containing a copy of the empty vector, showed production profiles similar to those of the parental strain, thus excluding an effect of pIJ8600 integration on antibiotic production [data not shown].)

Transcriptional control of A40926 production by Dbv3. To study the influence of Dbv3 on transcription of a subset of 20 *dbv* genes, RNA was extracted from *Nonomuraea* sp. strain ATCC 39727 and from the Oe-*dbv3* mutant grown in the presence of 10^{-8} M thiostrepton for 60 h and analyzed by qRT-PCR. With the exception of *dbv19*; the three cotranscribed genes *dbv5*, *dbv6*, and *dbv7*; and perhaps *dbv23*, the expression of all of the analyzed genes was significantly enhanced in the Oe-*dbv3* strain compared with the parental strain (Fig. 6B), with enhancement levels ranging from 2-fold (e.g., *dbv1*, *dbv25*, and *dbv28*) to 12-fold (*dbv15* and *dbv17*). Thus, from the established operon structure of the *dbv* gene cluster (Fig. 1B), we infer that Dbv3 may act as a positive regulator of 29 out of 37 *dbv* genes: those belonging to the six operons (*dbv2-dbv1*, *dbv14-dbv8*, *dbv17-dbv15*, *dbv21-dbv20*, *dbv24-dbv28*, and *dbv30-dbv35*) and the four monocistronic transcription units *dbv4*, *dbv29*, *dbv36*, and *dbv37*.

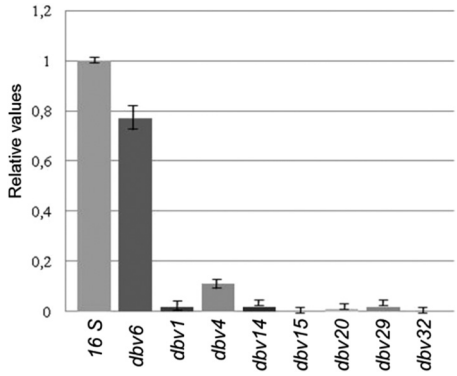

FIG 5 qRT-PCR of selected *dbv* genes in the Δ*dbv3* mutant after 60 h of growth. The transcription levels in the mutant were compared to those of the parental strain, taken as 1.0. The standard deviations (indicated by error bars) were calculated from three independent qRT-PCR experiments. The values were normalized using an endogenous 16S rRNA gene.

DISCUSSION

The present study was designed to elucidate the regulatory network for A40926 biosynthesis in the actinomycete *Nonomuraea* sp. strain ATCC 39727. Our experiments demonstrated that the transcriptional activators Dbv3 and Dbv4 are both required for A40926 biosynthesis, while Dbv6 does not influence A40926 biosynthesis (Fig. 3).

Disruption of any of these regulators did not significantly alter growth in *Nonomuraea* sp. strain ATCC 39727. This is in accordance with recent work showing that inactivation of *tei*15* (*dbv4*-like) or *tei*16* (*dbv3*-like) in *Actinoplanes teichomyceticus* does not influence growth but leads to a complete block of teicoplanin production (12).

qRT-PCR analysis showed that, in the absence of *dbv4*, transcription of *dbv3* and *dbv6* is not affected (Fig. 4), whereas transcription of the *dbv14-dbv8* and *dbv30-dbv35* operons is (data not shown), confirming that Dbv4 acts as a positive pathway-specific regulator of A40926 biosynthesis. Moreover, *dbv4*, *dbv14-dbv8*, and *dbv30-dbv35* transcription was previously demonstrated to be activated by phosphate limitation, and mobility shift assays demonstrated that a recombinant His-Dbv4 binds to a consensus sequence present upstream of *dbv14* and *dbv30* (9). Similarly to Tei15* (12) from the teicoplanin gene cluster and differently from Bbr (11) and StrR (25) from the balhimycin- and streptomycin-biosynthetic gene clusters, respectively, Dbv4 was reported previously not to control its own expression (9).

Expanding on previous results, we demonstrate that *dbv4* transcription is controlled by Dbv3, a regulator belonging to the LAL protein family. We found that Dbv3 plays a positive role in A40926 production, since the Δ*dbv3* strain completely lost the ability to produce the antibiotic (Fig. 3) and transcription of *dbv4* and of many other *dbv* genes was drastically inhibited (Fig. 5). Several members of the LAL family are pathway-specific transcriptional activators of secondary-metabolite gene clusters in a range of actinomycetes (13, 14, 26–32). Recently, two LAL proteins were identified as pleiotropic regulators affecting various cellular processes in *Streptomyces coelicolor* (15).

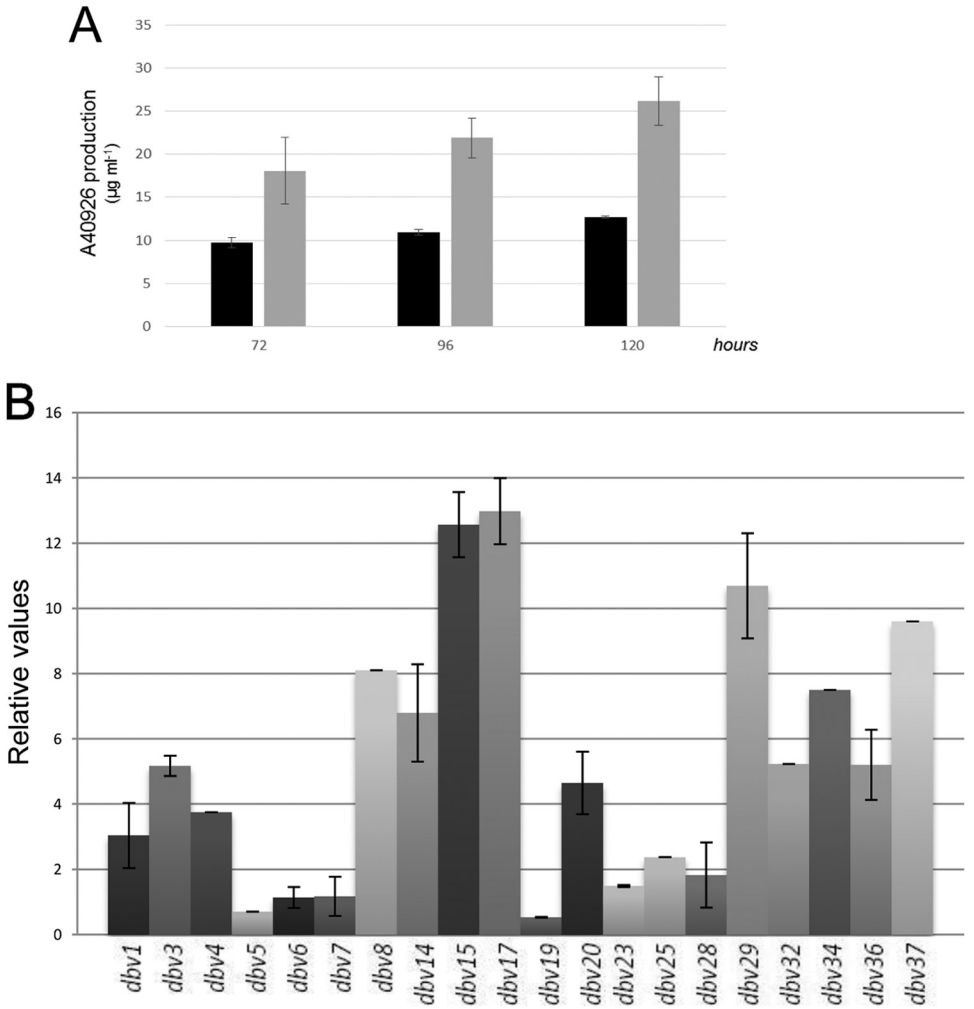

FIG 6 A40926 production and qRT-PCR of selected *dbv* genes in the Oe-*dbv3* strain. (A) A40926 production (micrograms per milliliter) by the parental (black bars) and Oe-*dbv3* (gray bars) strains after 72, 96, and 120 h in R3 liquid medium. (B) qRT-PCR of selected *dbv* genes in the Oe-*dbv3* strain after 60 h in R3 liquid medium. The transcript levels in the Oe-*dbv3* strain were compared to those of the parental strain, taken as 1.0. The relative values were determined by comparison to the transcript levels of endogenous *hrdB* and 16S rRNA genes. Error bars indicate standard deviations.

Improvement of A40926 production by *Nonomuraea* sp. strain ATCC 39727 has been successfully obtained using different growth conditions (33–36). One example of successful genetic manipulation has also been reported, since deletion of *dbv23*, responsible for adding the acetyl group to the mannose moiety, led to a 2-fold increase in A40926 yield (8). In our studies, overexpression of *dbv3* also resulted in higher antibiotic production, providing a potentially useful example of knowledge-based strain improvement.

Taken together, our results demonstrate that both Dbv3 and Dbv4 act as pathway-specific activators of A40926 biosynthesis, with Dbv3 hierarchically controlling *dbv4* transcription. Our data suggest a cascade-like regulatory mechanism (Fig. 7) in which Dbv3 triggers transcription of *dbv4* and, as a consequence, positively controls DPG biosynthesis and some key reactions that are the hallmark of glycopeptides (the four cross-links, halogenation, glycosylation, and acylation); in addition, Dbv3 controls HPG biosynthesis and heptapeptide backbone biosynthesis, as well as other tailoring reactions (mannosylation and hexose oxidation) and A40926 export. To our knowledge, the transcriptional activa-

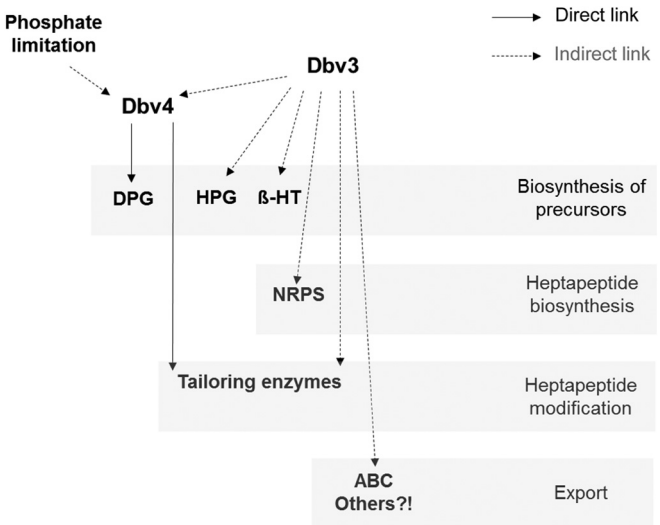

FIG 7 Model proposed for regulation of A40926 biosynthesis by Dbv3 and Dbv4. The solid and dashed arrows indicate direct and likely indirect links, respectively. β-HT, β-hydroxytyrosine.

tion of the NRPS genes by Dbv3 (Fig. 6B) represents the first example of a LAL regulator positively controlling the expression of NRPS genes, while a few examples of LAL regulators acting on polyketide synthase (PKS) gene expression have been reported (14).

Dbv4 and Dbv3 appear to be controlled by different stimuli, i.e., *dbv4* transcription and the two Dbv4-controlled biosynthetic steps were demonstrated to be negatively regulated by phosphate, while the Dbv3-controlled genes were not (9). Surprisingly, while the intergenic regions preceding Dbv4-controlled genes are highly conserved (9, 10), we could not identify any conserved sequences that could be the target of Dbv3, nor has a binding site for Tei16* been reported (12). Thus, it remains to be determined whether Dbv3 exerts its influence by direct binding to regulatory regions or if other mechanisms are involved. The absence of conserved DNA sequences in all of the *dbv* genes whose transcript levels are higher after *dbv3* overexpression suggests that Dbv3 control of A40926 biosynthesis might be indirect.

The *dbv* cluster was reported to have a mosaic structure (10) in which the various subclusters, responsible for different functional portions of the pathway, originated from different sources. Remarkably, the highly conserved Dbv4 regulator controls the expression of two operons devoted to important checkpoints along the A40926-biosynthetic pathway: one for synthesis of the specialized precursor DPG and the other for cross-linking, halogenation, and *N*-acetylglucosamine addition and acylation. The latter set of reactions contribute to the characteristic features of glycopeptides (multiple cross-links of the aromatic residues) and of the teicoplanin family in particular (acylated glucosamine attached to amino acid 4). It should also be noted that the P450 mono-oxygenases act during heptapeptide synthesis (37), and thus, lack of expression of the *dbv14-dbv8* operon, in which the mono-oxygenase genes reside, is likely to result in truncated peptides devoid of antibacterial activity, as seen with balhimycin (38).

When comparing transcriptional regulation in different glycopeptide gene clusters, it is striking that the StrR-like regulators Dbv4, Bbr, and Tei15* all control one common biosynthetic step, DPG biosynthesis. However, beyond this common step, different clusters seem to have devised different regulatory strategies to control glycopeptide production: a single StrR-like regulator for balhimycin production, a StrR-like regulator controlling a LAL regulator in teicoplanin biosynthesis, and a LAL regulator controlling a StrR-like regulator for A40926 production. Our results not only enlarge our knowledge of the regulatory mechanisms used to control glycopeptide biosynthesis but also provide a basis for rational approaches for the generation of overproducers of the dalbavancin precursor.

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We declare that we have no competing interests.

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