

Enhanced Heterologous Production of Desosaminyl Macrolides and Their Hydroxylated Derivatives by Overexpression of the *pikD* Regulatory Gene in *Streptomyces venezuelae*[∇]

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To elevate the production level of heterologous polyketide in *Streptomyces venezuelae*, an additional copy of the positive regulatory gene *pikD* was introduced into the pikromycin (Pik) polyketide synthase (PKS) deletion mutant of *S. venezuelae* ATCC 15439 expressing tylosin PKS genes. The resulting mutant strain showed enhanced production of both ty lactone (TL) and desosaminyl ty lactone (DesTL) of 2.7- and 17.1-fold, respectively. The notable increase in DesTL production strongly suggested that PikD upregulates the expression of the desosamine (*des*) biosynthetic gene cluster. In addition, two hydroxylated forms of DesTL were newly detected from the extract of this mutant. These hydroxylated forms presumably resulted from a PikD-dependent increase in expression of the *pikC* gene that encodes P450 hydroxylase. Gene expression analysis by reverse transcriptase PCR and bioconversion experiments of 10-deoxymethynolide, narbonolide, and TL into the corresponding desosaminyl macrolides indicated that PikD is a positive regulator of the *des* and *pikC* genes, as well as the Pik PKS genes. These results demonstrate the role of PikD as a pathway-specific positive regulator of the entire Pik biosynthetic pathway and its usefulness in the development of a host-vector system for efficient heterologous production of desosaminyl macrolides and novel hydroxylated compounds.

Polyketides are a group of secondary metabolites that exhibit remarkable diversity in their structures and possess a wealth of pharmacologically important activities (14). As the number of cloned and sequenced genes involved in the biosynthesis of useful polyketide natural products has increased, the production of heterologous polyketide by genetically and physiologically well-characterized hosts has received considerable attention due to the difficulties in genetic manipulation of many original producers (21). The transfer of biosynthetic genes to a more amenable and robust heterologous host has, therefore, emerged as a viable alternative to producing high levels of desired compounds or to providing a basis for subsequent combinatorial biosynthetic approaches. Among several well-known actinomycete heterologous hosts, a pikromycin (Pik)/methymycin-producing strain, *Streptomyces venezuelae* ATCC 15439, has recently been developed as a promising host in that it is amenable to genetic manipulation and requires a short culture period (3 to 4 days) for metabolite production compared to other *Streptomyces* species (7, 8, 19, 23, 24). In addition, the presence of two post-polyketide synthase (PKS) tailoring enzymes that have unusual substrate flexibility, DesVII glycosyltransferase (2, 3, 6, 25) and PikC P450 monooxygenase (11, 13, 18), offers it another advantage as a heterologous host for combinatorial biosynthesis of novel compounds. However, one of the key challenges in developing *S.*

venezuelae as a competent heterologous host is the relatively low production of heterologous polyketides (8).

The PikD regulatory factor that originates from *S. venezuelae* was the sole transcriptional regulator of the Pik biosynthetic cluster (Fig. 1A) and required for the macrolide antibiotic biosynthesis (22). This factor belongs to one of the newly characterized regulatory groups, the large ATP-binding regulators of the LuxR family (LAL), which contains nucleotide triphosphate-binding motifs and a C-terminally located helix-turn-helix motif (22). The other LAL family regulators, such as RapH from the rapamycin biosynthetic pathway of *Streptomyces hygroscopicus*, have also been reported as positive regulatory elements of their biosynthetic cluster (10). The positive roles of LAL family regulators can be applied to improve the native antibiotic production.

In the present study, we exploited the PikD positive regulator for enhancing heterologously produced ty lactone (TL) and desosaminyl ty lactone (DesTL) in *S. venezuelae* (Fig. 1). The promotion of TL and DesTL production, as well as the generation of novel hydroxylated DesTL compound, seemed to result from the activation of the entire Pik biosynthetic pathway by PikD, which was verified by gene expression analysis and bioconversion experiments.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and genetic manipulation. *S. venezuelae* DHS2001, in which the *pikAI-VI* genes were deleted (8), was propagated on SPA medium (1 g of yeast extract, 1 g of beef extract, 2 g of tryptose, 10 g of glucose, trace amount of ferrous sulfate, and 15 g of agar/liter). Transformants of *S. venezuelae* were selected on R2YE agar plates (9). Genetic manipulations were

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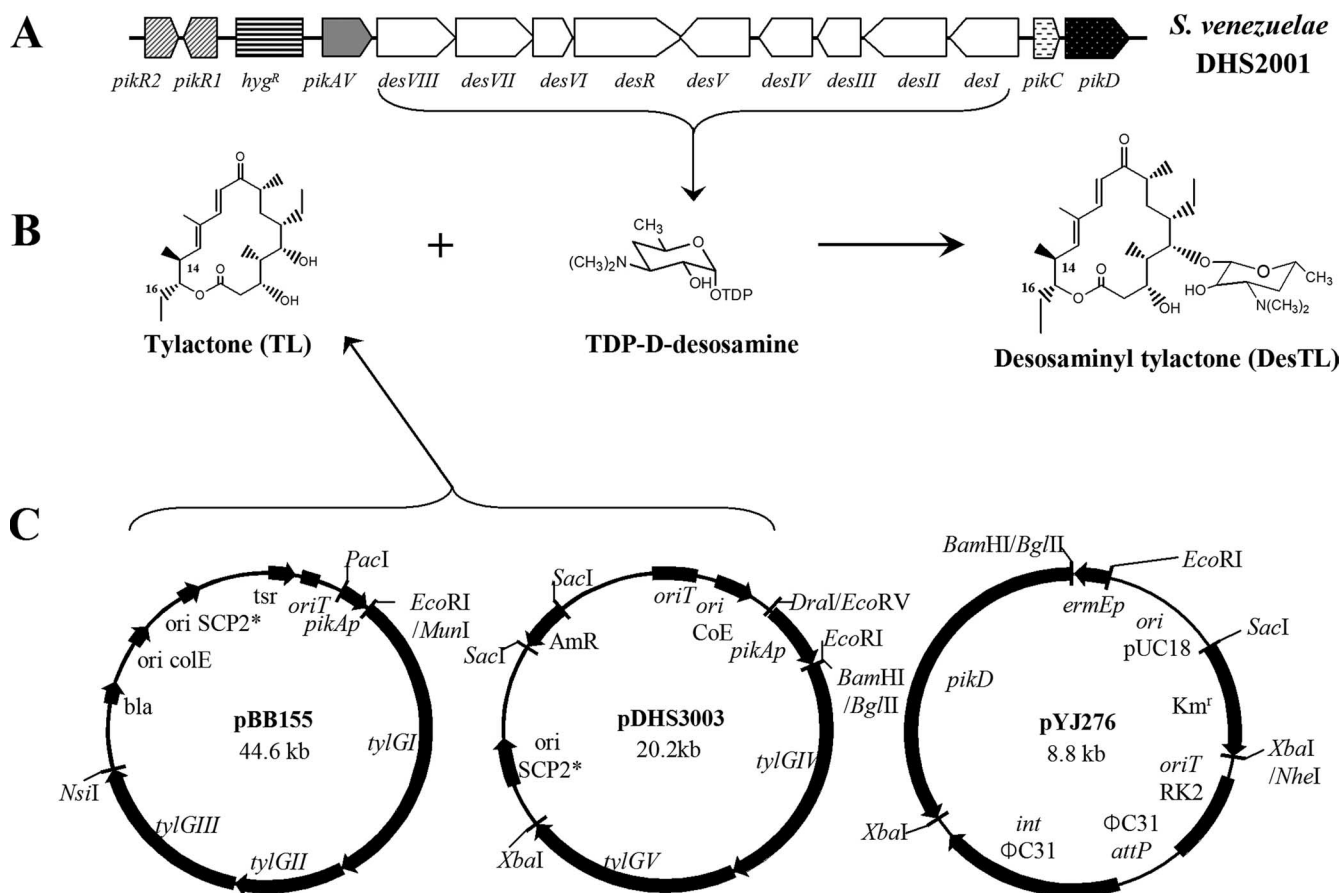


FIG. 1. Organization of the *Pik* gene cluster of *S. venezuelae* mutant DHS2001 in which the hygromycin resistance gene replaced *Pik* PKS (A), formation of DesTL by attaching TDP-D-desosamine to TL (B), and maps of three plasmids for expressing tylosin PKS (pBB155 and pDHS3003) and the *pikD* regulatory gene (pYJ276) (C).

carried out in *Escherichia coli* DH10B by using standard protocols (16). Transformation of DNA into protoplasts of *S. venezuelae* was performed according to standard protocols (9). Southern blot hybridization was performed according to the protocol recommended by a digoxigenin labeling and detection kit (Roche).

Construction of *pikD* expression vector pYJ276 and *S. venezuelae* mutant strains YJ112 and YJ113. A derivative of pSET152 (1), in which the apramycin resistance gene [*aac(3)IV*] was replaced by the kanamycin resistance gene (*aphIII*), was constructed. The *aphIII* gene (20) was amplified from plasmid pYJ100 (6) by PCR using the following primers: 5'-AAAGAGCTCCCCGAACCCCAGAGTCCC-3' and 5'-AAATCTAGACCTGATACCGCTCGCCG-3' (restriction sites are underlined). The PCR product of *aphIII* was cut by *SacI*/*XbaI* and ligated to *SacI*/*NheI*-digested pSET152, generating pYJ205. A 2.9-kb DNA fragment of *pikD* was amplified from the genomic DNA of *S. venezuelae* by PCR using a forward primer, 5'-ACGACTAGATCTCACGGAAGCCCCGATCG-3', and a reverse primer, CGGGGACACTCTAGATGGCTCAGGCCGTGA (the restriction sites are underlined). The *pikD* PCR product was cloned into pGEM-T Easy vector (Promega) and sequenced. This plasmid was digested with *BglIII*/*XbaI*, and the resulting *pikD*-containing fragment was placed into *BamHI*/*XbaI* sites of pYJ205, yielding pYJ276. Plasmid pYJ276 was integrated into the chromosomal DNA of *S. venezuelae* mutant DHS2001 (8), thus generating mutant strain YJ112. The two previously constructed replicating plasmids, pBB155 containing *tylGI-III* and pDHS3003 containing *tylGIV-V* (8), were subsequently transformed into the *pikD*-integrated mutant YJ112, generating YJ113.

Production and analysis of heterologous polyketides. All mutants were cultured and extracted as previously reported (8). The extracts were analyzed by the combination of liquid chromatography-electrospray ionization mass spectrometry (LC/ESI-MS) and electrospray ionization mass spectrometry-mass spectrometry (ESI-MS/MS) using a Waters/Micromass Quattro micro/MS apparatus. Separation was performed on a Nova-Pak C₁₈ (150 by 3.9 mm, 4.0 μ m, Waters)

reversed-phase column. The analytes were eluted at a flow rate of 180 μ l/min with a gradient of 5 mM (wt/vol) ammonium acetate–0.05% acetic acid (vol/vol) in water (solution A) and 80% (vol/vol) acetonitrile with the same additive concentrations (solution B) at 20 to 70% solution B for 25 min, to 90% solution B for 20 min, maintained at 90% solution B for 10 min, and then to 20% solution B for another 10 min for column re-equilibration. Authentic TL and 5-O-mycaminosyl tylosin standards (provided by Eric Cundliffe at University of Leicester) were used to generate calibration curves for the determination of TL and DesTL titer, respectively. The production levels of TL and DesTL were determined in triplicates.

Extraction of total mRNA from *S. venezuelae* grown on an R2YE plate. At day 2 and day 3, total RNA was isolated from *S. venezuelae* mutants grown on R2YE plates. In order to prevent the degradation of mRNA, 2 volumes of RNA protect reagent (Qiagen) were mixed with 1 volume of phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄, and 800 ml of distilled H₂O; pH adjusted to 7.4 with HCl). The resulting solution was added to the plate, and the bacterial mycelia were obtained by using a sterile spreader. The cell pellets were harvested by centrifugation (5,000 \times g for 5 min at 4°C) and were then suspended in 1 ml of TRIzol reagent (Invitrogen), followed by incubation at room temperature for 5 min. The mixture was added to 200 μ l of chloroform, vigorously vortex mixed for 15 s, and centrifuged at 12,000 \times g for 15 min at 4°C. The supernatant was transferred into a new tube and mixed with 1 volume of 70% ethyl alcohol. The mixture was moved to an RNeasy Mini spin column (Qiagen) and centrifuged at 8,000 \times g for 15 s at room temperature. The spin column was washed two times with RPE washing buffer (Qiagen), and the resulting total RNAs were dissolved in 50 μ l of RNase-free water. Nucleic acid preparations were treated with DNase I (New England Biolabs) as recommended by the manufacturer.

Detection of mRNA transcripts by RT-PCR. Analysis of semiquantitative reverse transcriptase PCR (RT-PCR) was performed by using a One-Step RT-PCR

TABLE 1. Primers for RT-PCR

Primer	Sequence (5'-3')	Description
TylGIF	TTCGTCCTCTTCTCTCCGT	Forward primer for <i>tylGI</i>
TylGIR	TTCGGATGCCGACGACAAAA	Reverse primer for <i>tylGI</i>
TylGVF	ATCTGCTCTTCGACCGGGAC	Forward primer for <i>tylGV</i>
TylGVR	ACTCGTTCTCCCGGCAGAAA	Reverse primer for <i>tylGV</i>
DesVIF	TTCACCAAGGAGTTCGGCGA	Forward primer for <i>desVI</i>
DesVIR	AGATGGACGTCGGAGAAGTG	Reverse primer for <i>desVI</i>
DesIVF	TTGTTGCAGCAGCGGGTGAT	Forward primer for <i>desIV</i>
DesIVR	TGAGGTGATCGTCTCTGGACA	Reverse primer for <i>desIV</i>
PikCF	CGCTCAACCACAACATGCTG	Forward primer for <i>pikC</i>
PikCR	TGGCGATCAGATTGACCGTG	Reverse primer for <i>pikC</i>
PikDF	CGGGATGAATCTGGTGGAAC	Forward primer for <i>pikD</i>
PikDR	GCAGTACAGGAGGAACCTCA	Reverse primer for <i>pikD</i>

kit (Qiagen) as recommended by the manufacturer. Dimethyl sulfoxide (5% [vol/vol]) was added to all PCRs together with RNAsguard RNase inhibitor (Amersham Pharmacia). A total of 5 ng of DNase I-treated total RNA was used as a template for reverse transcription and PCR amplification under the following conditions: 50°C for 30 min, followed by 95°C for 15 min and then 32 to 35 cycles of 94°C for 1 min (*tylGI*, *tylGV*, *pikC*, and *pikD*, 32 cycles; *desVI* and *desIV*, 35 cycles), 55°C for 1 min, and 72°C for 45 s. Negative controls were carried out with each experimental reaction using *Taq* DNA polymerase (New England Biolabs) to confirm that amplified products were not derived from the chromosomal DNA.

Primer design for RT-PCR analysis. Primers (20-mers) were designed to possess a similar T_m value at 60°C and to generate PCR products of approxi-

mately 500 bp. Five primer pairs specific for Tyl PKS genes (*tylGI*, *tylGII*, *tylGIII*, *tylGIV*, and *tylGV*) and four primer pairs specific for *des* genes (*desVII*, *desVI*, *desIV*, and *desII*) were designed to detect the transcripts of Tyl PKS and the *des* cluster, respectively. Among these primer pairs, one pair was selected per expected mRNA transcript based on the visibility of the RT-PCR band. Consequently, two primer pairs for Tyl PKS genes (*tylGI* and *tylGIII*) and two primer pairs for the *des* cluster (*desVI* and *desIV*) were selected. The specific primer pairs for the *pikC* and *pikD* genes were also designed. The oligonucleotide primers and the target genes are summarized in Table 1.

Semiquantification of RT-PCR products. Image analysis of agarose gel electrophoresis was carried out to achieve the relative quantification of RT-PCR products by using Quantity One software (version 4.1.1). After RT-PCR amplification was complete, 20- μ l aliquots of the reaction product (total volume of 50 μ l) were electrophoresed onto a 1% agarose gel containing 0.5 μ g of ethidium bromide/ml and photographed under UV light by using Gel Doc 2000 (Bio-Rad). The relative amounts of amplified products were determined by calculating the average signal intensity of each DNA band in relation to that of a known quantity of the standard. All data were calculated from at least three replicates.

Bioconversion experiments of polyketide aglycones using *S. venezuelae* mutant strains DHS2001 and YJ112. The native aglycones produced from *S. venezuelae*, 10-deoxymethynolide (10-DML) and narbonlide (NL), were obtained from the *des* deletion mutant YJ003 (6) by the previously described purification steps (12). These macrolactones (10-DML, 0.9 mg; NL, 1.5 mg; TL, 0.06 mg) suspended in ethanol were added to 1 ml of double-distilled water. The suitable feeding concentrations of the exogenous macrolactones for bioconversion experiments, which included 10-DML, NL, and TL, were determined on the basis of the production yields of wild-type *S. venezuelae* (7) and the yield of TL from the YJ005 mutant strain (8). After the mutant strains, DHS2001 and YJ112, were

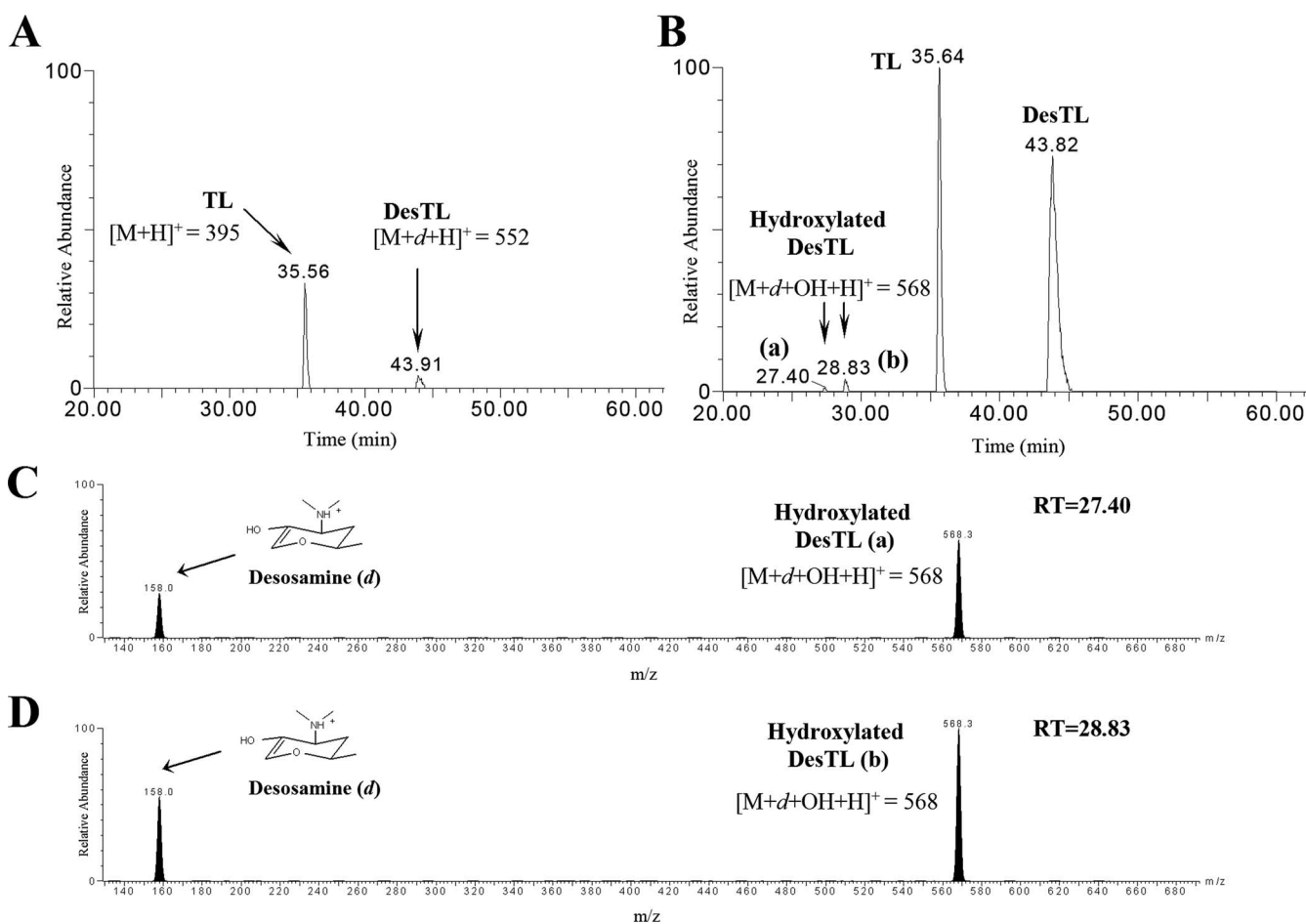


FIG. 2. LC/ESI-MS/MS chromatograms of TL and DesTL produced by YJ005 (A) and YJ113 (B). The ESI-MS/MS analysis of a hydroxylated DesTL (a) from the peak at 27.4 min (C) and the other hydroxylated DesTL (b) from the peak at 28.8 min (D).

cultured on R2YE solid medium at 30°C for 2 days, 1 ml of each solution was spread onto the solid culture medium, followed by incubation for four additional days. The resulting agar-grown culture was extracted as described above. The calibration curves using the purified 10-DML, NL, and TL were used for determining the conversion yields of each aglycone.

RESULTS AND DISCUSSION

Construction of the *S. venezuelae* mutant strain YJ113 expressing *pikD* and Tyl PKS genes. We recently reported that the expression of Tyl PKS using two SCP2*-type replicating plasmids, pBB155 and pDHS3003 (Fig. 1C), under the control of *pikAI* promoter in the *Pik* PKS deletion mutant DHS2001 led to the successful production of heterologous polyketides, TL and DesTL (8). To improve the production level of these compounds, we introduced an additional copy(ies) of *pikD* regulatory gene into the YJ005 mutant strain (expressing Tyl PKS in DHS2001), in which the native *pikD* gene remained intact. The Φ C31-based integrating plasmid pYJ276 (Fig. 1C) containing *pikD* regulatory gene under the control of a strong constitutive *ermEp1* Δ TGG (*ermE**) promoter (17), which carries a 3-bp deletion (TGG) in the -35 region of *ermEp1* promoter of *Saccharopolyspora erythraea*, was transformed into the DHS2001 mutant strain, generating YJ112. The integration of an extra copy(ies) of *pikD* into the chromosome of DHS2001 was confirmed by Southern hybridization. Chromosomal DNA of YJ112 was isolated and digested with EcoRI/XbaI. When the digested genomic DNA was probed with the digoxigenin-labeled 280-bp EcoRI-BamHI fragment of the *ermE** promoter region, a 3.1-kb DNA fragment was observed, whereas no DNA band was detected from the genomic DNA of DHS2001 (data not shown). The Φ C31 integrase of pYJ276 often yields cointegrates of several copies of the introduced gene, and these results showed that at least one extra copy of *pikD* was integrated into the chromosomal DNA of the YJ112 strain. A mutant strain YJ113 was constructed by introduction of the two plasmids pBB155 and pDHS3003 into the *pikD*-integrated mutant YJ112.

Production analysis of *S. venezuelae* YJ005 (expressing Tyl PKS) and YJ113 (expressing Tyl PKS and *PikD*). To compare the production of heterologous polyketides, TL and DesTL, between YJ005 and YJ113, both mutant strains were cultured on R2YE plates, extracted, and then analyzed by LC/ESI-MS and ESI-MS/MS. The peaks with the calculated molecular mass for TL ($m/z = 395$) and DesTL ($m/z = 552$) were observed at retention times of 35.6 and 43.9 min (Fig. 2A), respectively, from the LC/MS analysis of the YJ005 extract. MS/MS fragmentations of these compounds produced patterns identical to those of TL and DesTL, respectively, as reported previously (8). The titers of TL and DesTL from YJ005 were approximately 0.5 and 0.1 mg/liter, respectively. Approximately 1.4 mg of TL/liter and 1.8 mg of DesTL/liter were produced from the *pikD*-integrated mutant YJ113 (Fig. 2B), showing the 2.7- and 17.1-fold enhanced production of TL and DesTL, respectively, compared to those produced from YJ005. These results strongly indicated that the overproduction of *PikD* regulator resulted in upregulated expression of Tyl PKS and the *des* cluster by binding to *pikAI* promoters on two plasmids used to express Tyl PKS genes (pBB155 and pDHS3003), as well as promoters acting on the *des* cluster in the chromosomal DNA of *S. venezuelae*.

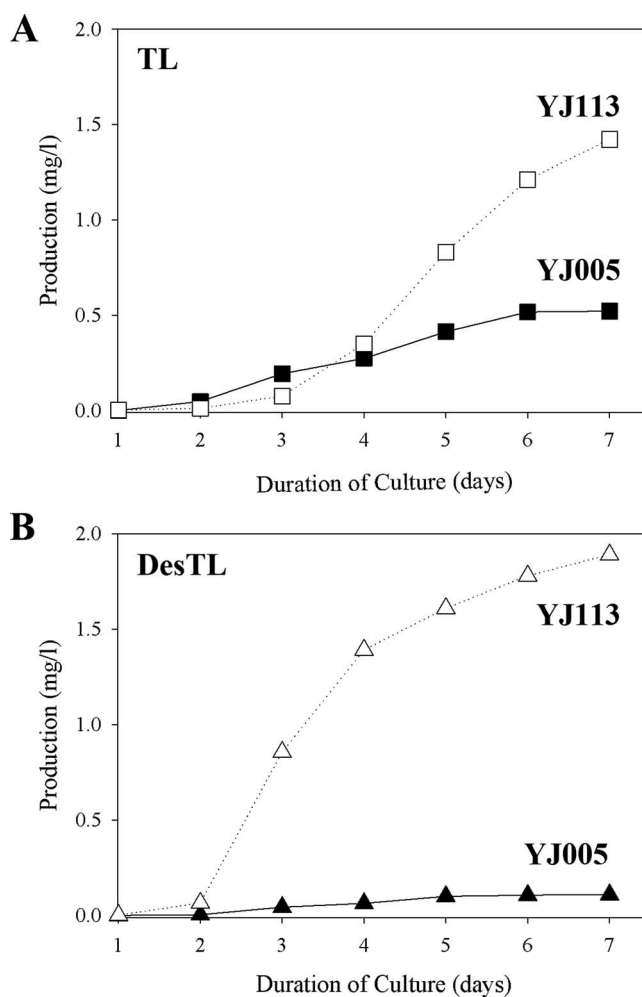


FIG. 3. Time courses of TL (A) and DesTL (B) production by the YJ005 and YJ113 strains. Incubation was carried out on R2YE solid medium for 7 days.

Two small peaks with $m/z = 568$ were also detected from the extract of YJ113 at retention times of 27.4 and 28.8 min (Fig. 2B), which might correspond to the hydroxylated forms of DesTL. The structures of those two compounds were predicted by ESI-MS/MS analysis. The two compounds with identical $m/z = 568$ values were fragmented into peaks at $m/z = 158$ and 568, which corresponded to the oxonium ion fragment from desosamine (4) and the hydrogen adduct of hydroxylated DesTL, respectively (Fig. 2C and D). Two newly detected hydroxylated forms of DesTL were presumably synthesized by the action of *PikC* P450 hydroxylase. Therefore, the generation of hydroxylated DesTL in the *pikD* overexpression mutant YJ113 suggested that the overproduced *PikD* also activated the *pikC* gene expression. The two novel hydroxylated compounds seem to be synthesized by the introduction of a hydroxyl group into position C-14 or C-16 of DesTL, respectively (Fig. 1B), according to the intrinsic catalytic specificity of *PikC*. However, the exact hydroxylation positions could not be characterized due to their low concentrations. Further scale-up separation and structural analyses, such as nuclear magnetic resonance spec-

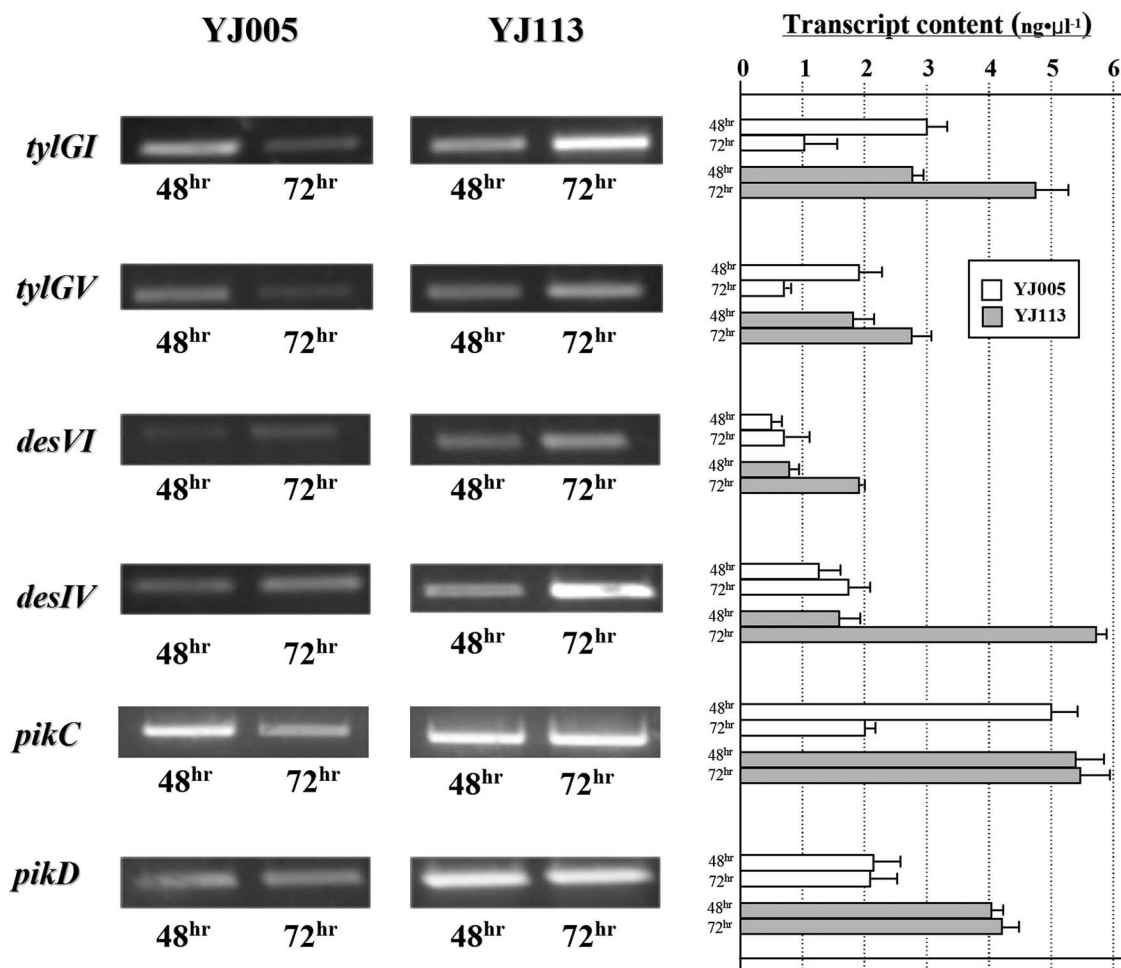


FIG. 4. Gene expression analyses by RT-PCR applied to the messenger RNAs isolated from *S. venezuelae* YJ005 and YJ113 strains at two different time intervals. The expression levels of those genes were analyzed by semiquantitative RT-PCR experiments.

troscopy, are needed to confirm the hydroxylated site of DesTL.

Monitoring the production of TL and DesTL by YJ005 or YJ113 strains. The production of TL and DesTL in the YJ005 or YJ113 strain was monitored throughout a 7-day incubation period (Fig. 3). The TL production level of the mutant YJ113 showed a notable increase between 3 and 4 days compared to that of YJ005 (Fig. 3A). In the case of DesTL, the YJ005 strain produced only a small amount of DesTL (0.1 mg/liter) until day 7, while a significantly enhanced production of DesTL was observed in the mutant YJ113 between 2 and 3 days and was maintained up through day 7 (Fig. 3B). The total sum of the 16-membered ring heterologous polyketides (TL plus DesTL) produced by the YJ113 strain was greatly increased between day 2 and day 3, and a wider gap was exhibited between YJ005 and YJ113 after 3 days (Fig. 3).

Gene expression analysis in *S. venezuelae* YJ005 and YJ113 strains. A previous genetic analysis of PikD using the *xylE* reporter system showed that the PikD regulator activates the *pikAI* promoter, whereas it directly or indirectly represses *desI* promoter, and does not participate in *pikC* regulation in wild-type *S. venezuelae* (23). Our observations that the overexpres-

sion of the *pikD* regulatory gene triggered a remarkable enhancement of DesTL production (17.1-fold), as well as the generation of novel hydroxylated DesTL compounds, were inconsistent with the previous report. To determine the effect of PikD on the expressions of Tyl PKS genes, which are under the control of the *pikAI* promoter, and especially on the *des* cluster and *pikC*, transcriptional analysis was performed by using semiquantitative RT-PCR. The total RNA was isolated from both YJ005 and YJ113 mutant strains after 48 or 72 h growth (when the production of DesTL notably increased) to compare the expression levels of the Tyl PKS genes, the *des* gene cluster, and *pikC* between the two strains. After an agarose gel electrophoresis, the band intensities of the PCR products of YJ005 were compared to those of YJ113 (Fig. 4). In RT-PCR analysis of the expressions of the Tyl PKS genes, there was an observable decrease in the expression levels of Tyl PKS genes (*tylGI*, 0.3-fold; *tylGV*, 0.4-fold) in the YJ005 strain at 72 h compared to 48 h, whereas the expression levels of those genes in the YJ113 were increased (*tylGI*, 1.7-fold; *tylGV*, 1.5-fold) during the same culture period. These results showed the positive role of PikD on the *pikAI* promoter of the two Tyl PKS expression vectors (pBB155 and

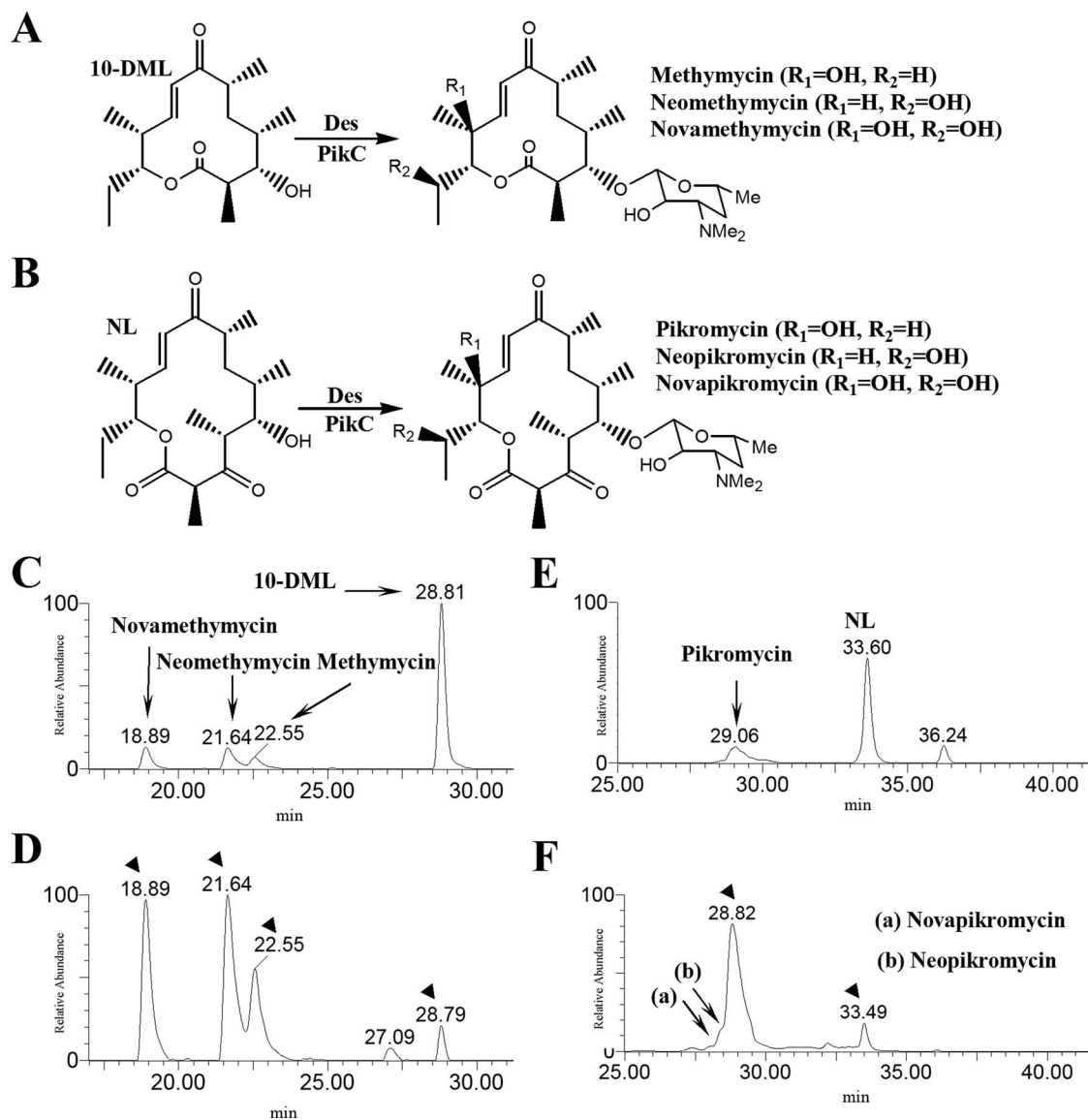


FIG. 5. The conversion schemes of 10-DML to 12-membered ring series (methymycin, neomethymycin, and novamethymycin) (A) and NL to 14-membered ring series (Pik, neopikromycin, and novapikromycin) (B). LC/ESI-MS chromatograms of 12-membered ring macrolides (C) and 14-membered ring macrolides (D) generated from DHS2001 fed with 10-DML and NL, respectively, and 12-membered ring macrolides (E) and 14-membered ring macrolides (F) generated from YJ113 fed with 10-DML and NL, respectively.

pDHS3003). In the case of the *des* cluster, slightly enhanced amounts of *desVI* (1.4-fold) and *desIV* (1.3-fold) transcripts were observed at 72 h compared to 48 h in the YJ005 strain, whereas the expression levels of those genes in YJ113 were remarkably increased (*desVI*, 2.7-fold; *desIV*, 3.6-fold) between 48 and 72 h. These results showed that the *desIV* gene selected as a representative of the *desI-desV* gene set was more highly expressed than the *desVI* gene chosen among the oppositely directed *des* gene set (*desVIII-desR*) in both the YJ005 strain and the YJ113 strain (Fig. 1, Fig. 4). This result also indicated that two apparent operons of the *des* gene cluster that are transcribed in the convergent direction are separately controlled by a *PikD* regulator. In addition, the remarkably enhanced transcriptions of both *desVI* and *desIV* at 72 h were observed compared to those detected at

48 h in the YJ113 strain (Fig. 4), which showed that both parts of the *des* cluster (*desI-desV* and *desVIII-desR*) were positively affected by the overexpression of *pikD*.

The transcriptional level of the *pikC* P450 hydroxylase gene in the YJ005 strain was decreased at 72 h compared to 48 h (0.4-fold), whereas the amount of *pikC* transcript in YJ113 at 48 h was similar to that in YJ005 at 48 h and was maintained for up to 72 h. Therefore, after 72 h of cultivation, the *pikC* gene of the YJ113 strain was expressed 2.7-fold more than that of YJ005, suggesting the overexpression of *pikD* stimulates *pikC* expression. The *pikD* regulatory gene was constantly expressed in both the YJ005 strain and the YJ113 strain during the culture time from 48 to 72 h. However, the amount of *pikD* gene transcript in YJ113 was 2.0-fold higher than that in YJ005.

Bioconversion of polyketide aglycones using *S. venezuelae* DHS2001 (*pikA*-deleted mutant) and YJ112 (*pikD*-overexpressed DHS2001). In order to investigate the desosaminylation and hydroxylation activity in the *pikD* overexpression mutant, aglycone bioconversion experiments were conducted using DHS2001 and YJ112. At first, the purified TL (0.06 mg) was fed onto 30 ml of solid medium containing DHS2001 and YJ112. Approximately 0.1 and 1.7 mg of DesTL/liter was biosynthesized by the DHS2001 and YJ112 strains, respectively (data not shown), and their amounts were similar to those of YJ005 (0.1 mg/liter) and YJ113 (1.8 mg/liter), respectively (Fig. 3). In the extract of YJ112 fed with TL, two novel hydroxylated forms of DesTL were also found, as was the case for the YJ113 strain. When the larger amount of TL (>0.06 mg) was used for the bioconversion experiment, an extra amount of TL remained unconverted (data not shown). In addition, purified 10-DML (0.9 mg) and NL (1.5 mg) were supplemented onto the 30 ml of growing medium containing DHS2001 and YJ112 (Fig. 5A and B). Both DHS2001 and YJ112 converted 10-DML to 12-membered ring macrolides including methymycin, neomethymycin, and novamethymycin (26). The conversion yield of 10-DML to the total 12-membered ring macrolides represented ca. 91% in YJ112, showing a 5.1-fold improvement (17%) over that in DHS2001 (Fig. 5C and D). Approximately 14% of the NL was successfully bioconverted to the Pik, together with a small amount of neopikromycin and novapikromycin (12) in DHS2001 fed with NL (Fig. 5E), whereas a 91% conversion of NL to the 14-membered ring macrolides, including Pik, neopikromycin, and novapikromycin, was observed in YJ112 (Fig. 5F). Overexpression of *pikD* in the DHS2001 strain resulted in 6.3-fold-enhanced bioconversion of NL to 14-membered ring macrolides. These results also supported our hypothesis that PikD activates the expression of the *des* cluster and *pikC* gene.

As mentioned above, these results are not consistent with the previous analysis using the *xylE* reporter system, which determined that the PikD regulatory element downregulates the *des* cluster and does not take part in regulation of the *pikC* expression (22). However, in the same report, the results obtained using the intermediate feeding experiment to wild-type *S. venezuelae* and *pikD* deletion mutant showed that the desosaminylation activity of 10-DML and NL disappeared in the *pikD* deletion mutant, which supports our hypothesis. In addition, although the *xylE* assay used in the previous report has been widely used as a reporter system, it has been suggested to use caution in interpreting data derived from *xylE* reporter system under aerobic conditions (5). The exact reason for these discrepancies has yet to be further revealed; however, our results using RT-PCR and bioconversion experiment would provide more direct evidence for the positive role of PikD regulator on the *des* cluster and *pikC* hydroxylase gene.

The present study shows that improved production of heterologous polyketides can be achieved by the overexpression of a *pikD* regulatory gene in *S. venezuelae*. The positive role of PikD toward the *des* cluster, as well as *pikC*, can be also harnessed for the combinatorial biosynthesis of a variety of desosaminyl macrolides and their hydroxylated forms. Our efforts are currently being focused on enhancing the production levels of heterologous polyketides by genetic engineering, as well as a metabolic strategy including direct supplementation

with limiting coenzyme A ester (15), which will facilitate the development of *S. venezuelae* as an efficient heterologous host and combinatorial biosynthetic system.

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