J. Microbiol. Biotechnol. (2016), 26(1), 66–71
http://dx.doi.org/10.4014/jmb.1510.10085

jmb

Interspecies Complementation of the LuxR Family Pathway-Specific Regulator Involved in Macrolide Biosynthesis

SangJoon Mo[1]* and Yeo Joon Yoon[2]*

[1]*Clinical Trial Institute, Biosafety and Validation Center, Dankook University, Choenan 31116, Republic of Korea*
[2]*Department of Chemistry and Nano Science, Ewha Womans University, Seoul 03760, Republic of Korea*

Received: October 26, 2015
Revised: November 18, 2015
Accepted: November 25, 2015

First published online
November 26, 2015

**Corresponding authors*
Y.J.Y.
Phone: +82-2-322-4082;
Fax: +82-2-3277-3419;
E-mail: joonyoon@ewha.ac.kr
S.J.M.
Phone: +82-41-550-1819;
Fax: +82-41-559-7906;
E-mail: sjmo1107@dankook.ac.kr

pISSN 1017-7825, eISSN 1738-8872

PikD is a widely known pathway-specific regulator for controlling pikromycin production in *Streptomyces venezuelae* ATCC 15439, which is a representative of the large ATP-binding regulator of the LuxR family (LAL) in *Streptomyces* sp. RapH and FkbN also belong to the LAL family of transcriptional regulators, which show greatest homology with the ATP-binding motif and helix-turn-helix DNA-binding motif of PikD. Overexpression of *pikD* and heterologous expression of *rapH* and *fkbN* led to enhanced production of pikromycin by approximately 1.8-, 1.6-, and 1.6-fold in *S. venezuelae*, respectively. Cross-complementation of *rapH* and *fkbN* in the *pikD* deletion mutant (ΔpikD) restored pikromycin and derived macrolactone production. Overall, these results show that heterologous expression of *rapH* and *fkbN* leads to the overproduction of pikromycin and its congeners from the pikromycin biosynthetic pathway in *S. venezuelae*, and they have the same functionality as the pathway-specific transcriptional activator for the pikromycin biosynthetic pathway in the ΔpikD strain. These results also show extensive "cross-communication" between pathway-specific regulators of streptomycetes and suggest revision of the current paradigm for pathway-specific versus global regulation of secondary metabolism in *Streptomyces* species.

Keywords: LAL transcriptional regulator, pikromycin, polyketide, *Streptomyces venezuelae*, interspecies complementation

Streptomyces venezuelae ATCC 15439 produces methymycin and pikromycin, a series of six related polyketide macrolactones with an attached deoxysugar D-desosamine [7, 14]. The pikromycin polyketide synthase (Pik PKS) in *S. venezuelae* generates 12- and 14-membered ring aglycons, 10-deoxymethynolide, and narbonolide. These aglycons are then processed by glycosylation and hydroxylation reactions to produce methymycin and pikromycin through a unique gene regulation system controlled by the unusual pathway-specific regulator PikD [14]. PikD is the most representative of the large ATP-binding regulators of the LuxR family (LAL) in *Streptomyces* sp., which is composed of two major functional domains; namely, an N-terminal ATP-binding domain distinguishable from Walker A and B, and a C-terminal LuxR-like helix-turn-helix (HTH) motif for the DNA binding domain [11, 12]. Among many of the pathway-specific regulators that belong to the LAL-family regulators, PikD shows high homology to the known RapH from the rapamycin cluster and FkbN from the FK520 cluster.

Streptomycetes generally have pathway-specific and global regulators that control the onset of production of several secondary metabolites. The pathway-specific regulatory genes are situated on the biosynthetic gene clusters of secondary metabolites where they manipulate the production of secondary metabolites directly, and their roles are generally referred to as "low-level" regulatory genes. In contrast, global regulatory genes exert pleiotropic control over multiple aspects of secondary metabolites, such as antibiotic production and morphological differentiation [8]; they are located toward the exterior of the biosynthetic gene cluster and indirectly influence secondary metabolite production [5]. Their roles are generally referred to as "higher level" regulatory genes. As a general rule,

pathway-specific regulators that control the biosynthesis and overexpression of single antibiotics are associated with increased production of the corresponding antibiotic, whereas global regulators activate pathway-specific regulators situated within gene clusters encoding biosynthesis of their respective antibiotics. However, this notion has been complicated by a report of a pathway-specific regulator that can control other antibiotic biosynthetic pathways and, thus, acts as a global regulator [2].

In this study, we showed that *rapH* and *fkbN* are heterologous regulators that have the same functionality as *pikD*, and they are able to successfully enhance and restore pikromycin and production of its derivatives, suggesting that *rapH* and *fkbN* may play positive regulatory roles in the *S. venezuelae* Pik PKS biosynthetic pathway. Furthermore, our results indicate that pathway-specific regulators functionally interact between different secondary metabolite biosynthetic pathways of *Streptomyces* species.

S. venezuelae ATCC 15439 and *Streptomyces hygroscopicus* ATCC 14891 strains were obtained from the American Type Culture Collection. The *Streptomyces hygroscopicus* NRRL 5492 strain was obtained from the Agricultural Research Service Culture Collection. The *Escherichia coli* strain, used as a host for the cloning experiments, was DH5α [9]. pGEM-T Easy vector (Promega, Madison, WI, USA) and Litmus28 (New England Biolabs, Ipswich, MA, USA) were used for subcloning, and the integrative *E. coli-Streptomyces* shuttle vector pSET152 [1] derivative containing the *ermE** promoter (P_{ermE*}) [10] was used for gene expression in *S. venezuelae* and the in-frame *pikD* deletion mutant (ΔpikD) strain. pKC1139 [1] was used for in-frame *pikD* deletion. *S. venezuelae* and its mutants were maintained and propagated at 30°C on SPA medium [3]. Liquid SGGP medium [6] was used for protoplast preparation. Protoplast transformation was accomplished according to a standard method [4]. The strains for pikromycin production were grown in liquid SCM medium [6]. Luria-Bertani, SOB, and SOC liquid media were used to grow *E. coli* [9]. Routine molecular biology experiments were carried out using standard methods [4, 9]. The integrative pSET152-based expression vector with a P_{ermE*} promoter system was used to overexpress genes, including *pikD* from *S. venezuelae* ATCC 15439 (GeneBank Accession No. AF079139.1), *rapH* from *S. hygroscopicus* NRRL 5491 (GeneBank Accession No. X86780.1), and *fkbN* from *S. hygroscopicus* ATCC 14891 (GeneBank Accession No. AF235504.1) in *S. venezuelae*. The EcoRI-XbaI fragment, including P_{ermE*} and *pikD* for *pikD* complementation and overexpression, was obtained from the pYJ276 plasmid [3] and then cloned into pSET152 to generate pPIKD. All primers for polymerase chain reaction (PCR) amplification of *rapH* and *fkbN* were designed along with their natural ribosomal binding site. *rapH* was obtained as BamHI-XhoI and XhoI-XbaI PCR fragments from *S. hygroscopicus* NRRL 5491 genomic DNA. The oligonucleotide primer set used to amplify the BamHI-XhoI PCR fragment was 5'-TCTGGATCCAGAAAACTG GAGGAGGTTGTG-3' (forward) and 5'-ATTATTCTCGAG GCCAGCGGGTTGCGG-3' (reverse) and contained the BamHI and XhoI sites (underlined), respectively. The oligonucleotide primer set used to amplify the XhoI-XbaI PCR fragment was 5'-ATTCTCGAGCCGGCACCTCGA CGA-3' (forward) and 5'-GGTCTAGAGGCTATTCCGCCT AGACGAGTTCGG-3' (reverse) and contained the XhoI and XbaI sites (underlined), respectively. The two amplified products were cloned into the integrative pSET152 vector derivative containing the *ermE** promoter and treated with BamHI and XbaI, via three-way ligation used to generate pRAPH. *fkbN* was obtained as BamHI-SacI and SacI-XbaI PCR fragments from *S. hygroscopicus* ATCC 14891 genomic DNA. The oligonucleotide primer set used to amplify the BamHI-SacI PCR fragment was 5'-TTTGGATCCTTCCACGAC GGTCGCGAG-3' (forward) and 5'- TAATATGAGCTCGCCGCG CAGGTGGTG-3' (reverse) and contained the BamHI and SacI sites (underlined), respectively. The oligonucleotide primer set used to amplify the SacI-XbaI PCR fragment was 5'-TAAGAGCTCGACGAGGCCGTCACCTAC-3' (forward) and 5'-TAATCTAGAGGCTCAGCCGGCGTCGGACAG-3' (reverse) and contained the SacI and XbaI sites (underlined), respectively. The two amplified products were cloned into the pSET152 derivative integrative vector containing the *ermE** promoter and treated with BamHI and XbaI via the three-way ligation used to generate pFKBN. For *pikD* disruption, the recombinant plasmid was constructed by PCR amplification of the left- and right-flanking fragments from genomic DNA derived from *S. venezuelae*. The HindIII-BamHI 1,070 bp PCR fragment, as a left-flanking fragment, was amplified by the primer pair 5'-CGG AAGCTTCGCTCAACCACAACATGCTG-3' (forward) and 5'-CAGGGATCCTAACCAGGTCTTGTTACGGCG-3' (reverse) and contained the HindIII and BamHI sites (underlined), respectively. A BglII-EcoRI 1,043 bp PCR fragment, as the right-flanking fragment, was amplified by the primer pair 5'-CGGAGATCTAACATGTGGCTCTCCGGG-3' (forward) and 5'-TAAGAATTCCGCTGGAAGACGCCGGCCTG-3' (reverse) and contained the BglII and EcoRI sites (underlined), respectively. Two PCR fragments were separately cloned in pGEM-T Easy Vector and then sequenced. The left and right fragments were cloned into pKC1139 that was treated

with HindIII and EcoRI via the three-way ligation used to generate pPIKD1, after being digested with the appropriate restriction enzymes. pPIKD1 was introduced into *S. venezuelae* by polyethylene glycol (PEG)-mediated protoplast transformation for in-frame *pikD* deletion [4] on R2YE medium. After a 30°C incubation for 12 h, the plates were overlaid with P buffer containing apramycin (50 μg/ml) and then incubated for a further 3 days. Resistant single colonies from plates were transferred to a SPA plate containing apramycin (50 μg/ml). The colonies that were apramycin-resistant at 37°C were identified as the integrating mutants, in which a single-crossover homologous recombination took place. These mutants were further cultured at 30°C for 3 days in liquid R2YE medium in the absence of apramycin for three rounds, to generate double-crossover mutants. The desired double-crossover mutants with an apramycin-sensitive phenotype were confirmed by Southern blot analysis [4]. pPIKD, pRAPH, and pFKBN were introduced into the ΔpikD strain and *S. venezuelae*, respectively, by PEG-mediated protoplast transformation through the procedure described above, and selected with apramycin (50 μg/ml) for complementation and overexpression. Fermentation of all strains was carried out in SCM medium, and pikromycin and its derivatives were extracted and analyzed according to a previous method [7, 13]. Pikromycin and its derivatives were analyzed by high performance liquid chromatography (HPLC) using a Waters Alliance system with a 5 μm Discovery HS C18 reversed-phased column (25 × 4.5 cm; Supelco Analytical, Bellefonte, PA, USA), a linear gradient system from solvent A (20% acetonitrile with 10 mM ammonium acetate) to solvent B (80% acetonitrile with 10 mM ammonium acetate) over 60 min at a flow rate of 1.0 ml/min, with detection at 220 nm. The production was the average of two series of duplicate separate cultures and extractions.

Wilson *et al*. [12] reported that PikD is a pathway-specific regulatory protein in the pikromycin biosynthetic system that belongs to LAL family transcriptional regulators. LAL-family transcriptional regulators have a common ATP-binding motif represented by Walker A and B motifs at the N-terminal region and a HTH DNA-binding motif at the C-terminal region (Fig. 1) [12]. BLASTp analysis of PikD showed high homology to the known regulatory protein RapH from *S. hygroscopicus* (34% identity) and FkbN from *S. hygroscopicus* ATCC 14891 (35% identity). According to a PikD characterization study [12], the ATP-binding motif is an integral part during pikromycin regulatory protein biosynthesis and has also been found in polyketide macrolide biosynthetic regulators, including RapH and FkbN (Fig. 1). In addition, the amino acid sequence analysis of these regulatory proteins showed the greatest homology to the ATP-binding motif, as they had a Walker A motif GXXXXGKT common with each other, which was functionally characterized as an ATP-requiring activator (Fig. 1).

pPIKD, pFKBN, and pRAPH were constructed under the control of the *ermE** promoter in a pSET152-based integrating plasmid (Fig. 2A), and then SVPIKD, SVRAPH,

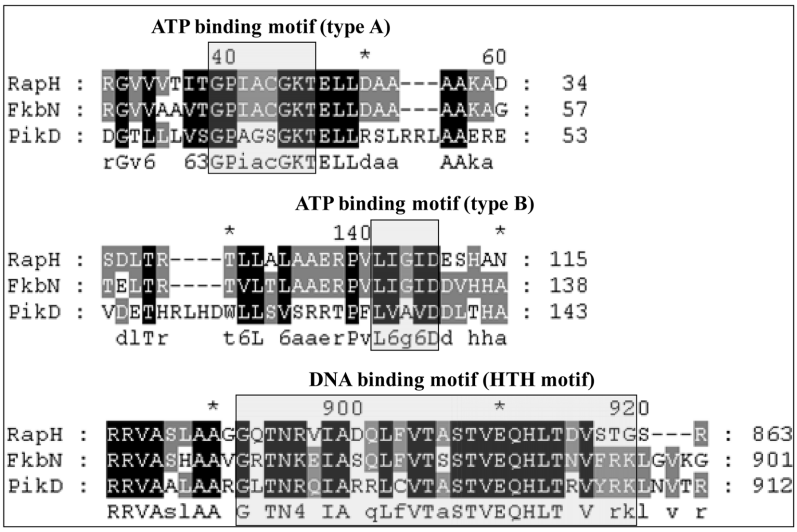

Fig. 1. Domain structure and amino acid alignment of the LAL-family proteins RapH, FkbN, and PikD.
Sequence alignment of these proteins characterized the LAL-family proteins, which consist of an N-terminal ATP-binding domain and a C-terminal LuxR-type HTH DNA-binding domain. The shaded box signifies ATP- and DNA-binding motifs.

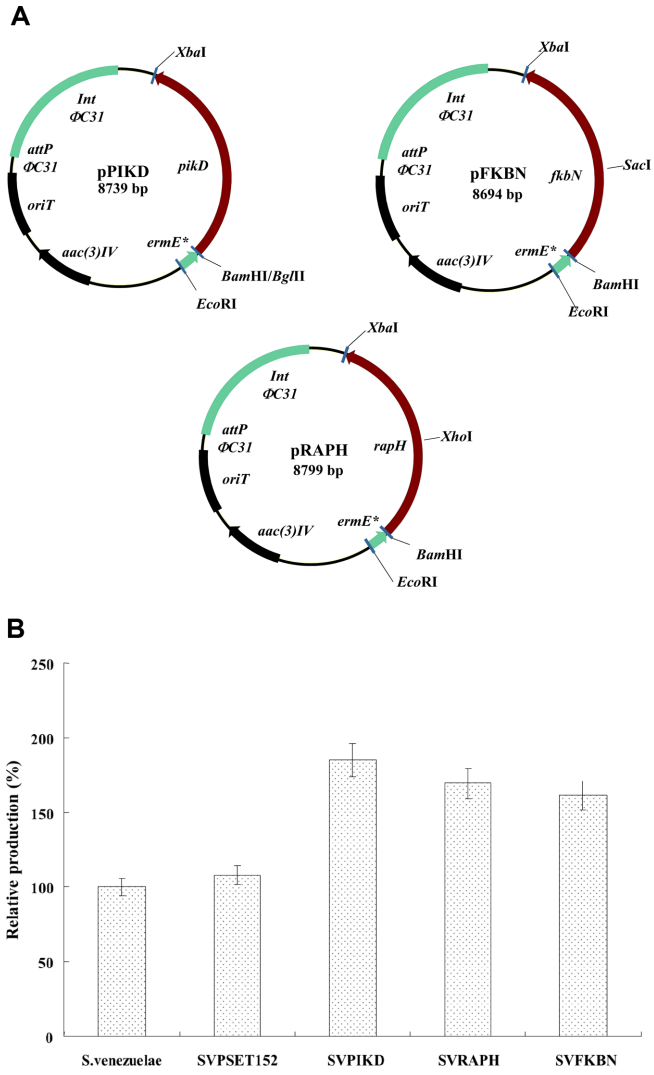

Fig. 2. Maps of the three plasmids for expressing LAL-family transcriptional regulators (pRAPH, pFKBN, and pPIKD) and pikromycin production by these plasmids.

(**A**) pPIKD, *pikD* from *S. venezuelae*, pFKBN, *fkbN* from *S. hygroscopicus* ATCC 14891, pRAPH, and *rapH* from *S. hygroscopicus* NRRL 5491 were constructed into the pSET152 derivative containing the *ermE** promoter. The plasmids have a ΦC31 attachment site for genomic interaction, *oriT* for transformation, and *aac(3)IV* for selecting transformants in *E. coli* and *Streptomyces* sp. (**B**) Comparison of pikromycin production from *S. venezuelae*, SVPSET152, SVPIKD, SVRAPH, and SVFKBN. Pikromycin was extracted twice with an equal volume of ethyl acetate and then the level of pikromycin production was determined by HPLC. Values are averages of two series of duplicate experiments.

and SVFKBN were developed to investigate the effect of the above-mentioned three regulatory proteins on production of pikromycin and its derivatives. Pikromycin was overproduced by expressing all integrating plasmids, and the level of pikromycin production in the SVPIKD strain increased about 1.8-fold compared with that in the wild-type strain. Furthermore, heterologous expression of the *rapH* and *fkbN* genes in *S. venezuelae* resulted in an approximate 1.6-fold increase in pikromycin production compared with that in the wild-type strain (Fig. 2B). In addition, all pikromycin series of antibiotics were enhanced with a similar increase in rate (data not shown). These results demonstrate that the integrated plasmid pSET152 derivative containing P_{ermE*} serves as a heterologous expression plasmid for secondary metabolites in *S. venezuelae* [3]. Moreover, we suspect that heterologous expression of *rapH* and *fkbN* in *S. venezuelae* might control pikromycin and its series of produced antibiotics.

The *pikD* inactivation mutant, ΔpikD, was constructed by excising the 573 bp internal fragment from the genomic DNA. The genomic DNA was treated with BamHI and XhoI and subjected to Southern blot analysis using the left-flanking fragment of pPIKD1 as a probe. As expected, the *S. venezuelae* wild-type strain presented one band corresponding to a 2.7 kb fragment, whereas the single-crossover mutant revealed two bands of 2.7 and 5.1 kb. The double-crossover mutant ΔpikD showed a 2.0 kb band (Fig. 3A, lanes 1–3). The ΔpikD strain did not produce pikromycin or its derivatives, as shown in the analytical HPLC trace (Fig. 3B). This result demonstrates that the *pikD* ATP-binding motif is required for pikromycin biosynthesis and shows that the loss of productivity of pikromycin and its derivatives in the ΔpikD strain was due to the absence of the specific *pikD* ATP-binding motif.

The above results show that the ATP-binding motif acts as an essential domain of the PikD regulator during pikromycin biosynthesis. This result gave rise to the question of whether the two regulators, *rapH* and *fkbN*, play equivalent or opposites role to the *pikD* mechanism. We carried out complementation of the *rapH* and *fkbN* expression construct in the ΔpikD strain to investigate cross-regulation between the heterologous regulators, *rapH* and *fkbN*, and the pikromycin biosynthetic mechanism. Complementation experiments in the ΔpikD strain using pRAPH and pFKBN (Fig. 2A) led to a dramatic restoration of pikromycin production, although they produced a low level of pikromycin compared with that of pPIKD (Fig. 3B). Complementation of the ΔpikD strain with the pRAPH plasmid showed that the pikromycin and methymycin titers were restored about 27% and 24% compared with that of the wild-type strain, respectively. Complementation of the ΔpikD strain with the pFKBN plasmid showed that

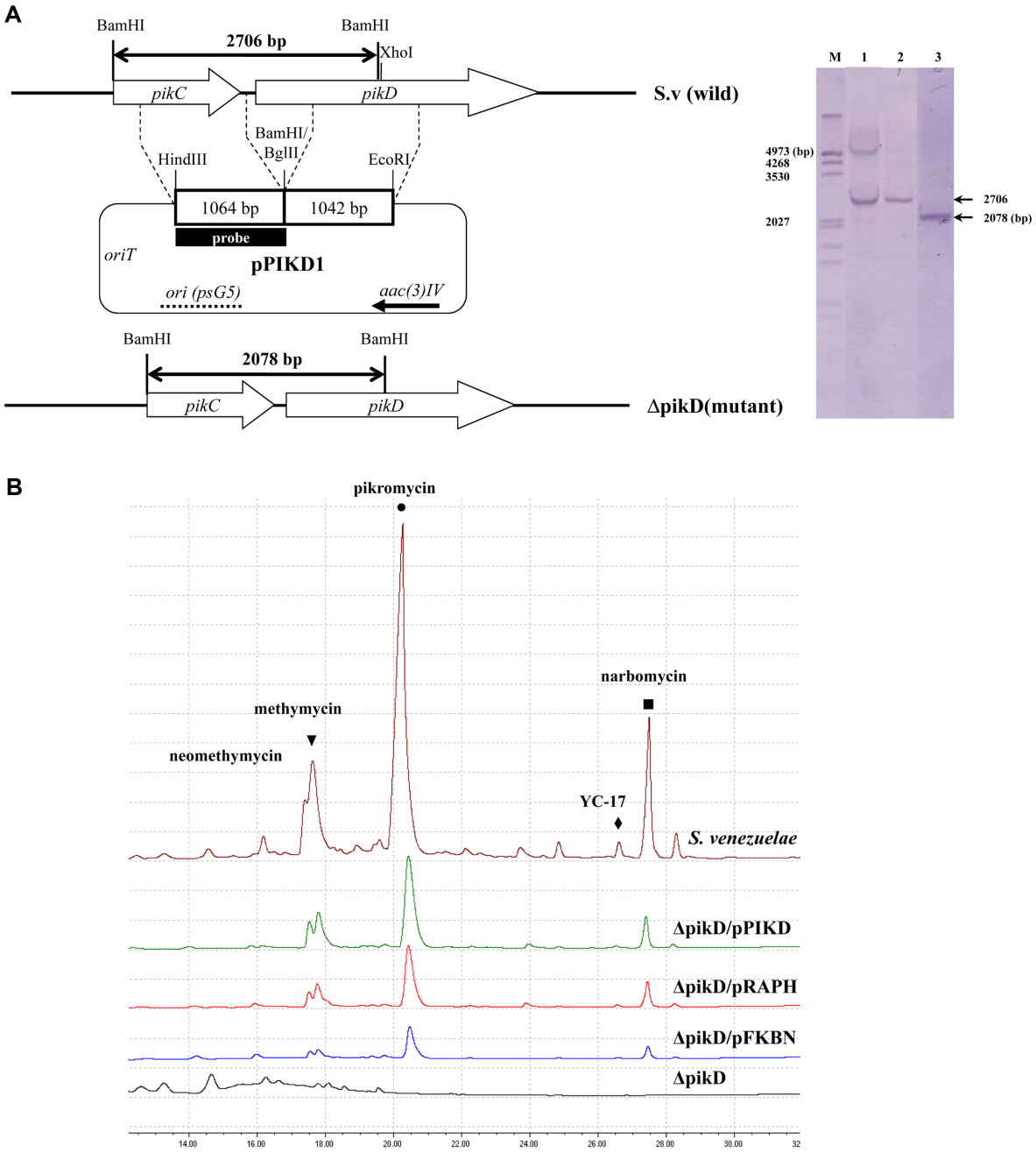

Fig. 3. In-frame deletion of *pikD* and HPLC analysis of fermentation products from the *S. venezuelae* ΔpikD strain and the complementation strains.

(**A**) Schematic representation of homologous recombination of construct pPKID1 into the genome of *S. venezuelae* (left). Verification of the in-frame deletion of *pikD* by Southern blot analysis (right). Lane: M, DIG-labeled DNA molecular weight marker III; 1, genomic DNA of single-crossover *pikD* mutant; 2, genomic DNA of *S. venezuelae*; 3, genomic DNA of double-crossover *pikD* mutant. (**B**) Pikromycin production was restored by expressing the heterologous LAL-family genes. The pikromycin, methymycin, YC-17, neomethymycin, and narbomycin peaks are indicated by a black circle, black inverted triangle, black lozenge, arrow, and black square, respectively.

the pikromycin and methymycin titers were restored about 19% and 11% compared with that of the wild-type strain. Moreover, YC-17 and narbomycin were restored about 21% by complementation of the ΔpikD strain with the pRAPH

plasmid, which were restored about 19% and 18% by complementation of the ΔpikD strain with the pFKBN plasmid, respectively, compared with the titers present in the wild-type strain (Fig. 3B). There were no differences in growth or morphology between the mutant strains and the wild-type strain when grown in R2YE medium (data not shown). This observation that pikromycin and its derivatives in the ΔpikD strain can be recovered by *rapH* and *fkbN* overexpression demonstrates that these regulatory genes act on a parallel biosynthetic mechanism. The results indicate the existence of functional interactions among associates in the *S. venezuelae* pikromycin production program; the ability of the regulator located at the interior of the biosynthetic gene clusters to control other antibiotic biosynthetic gene clusters as well as its own, and, thus, have global actions.

Acknowledgments

This work was partially supported by the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (MISP) (2013R1A2A1A01014230) and High Value-added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

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