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# Site-Directed Mutagenesis on Putative Macrolactone Ring Size Determinant in the Hybrid Pikromycin-Tylosin Polyketide Synthase

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**Abstract** *Streptomyces venezuelae* ATCC 15439 is notable in its ability to produce two distinct groups of macrolactones. It has been reported that the generation of two macrolactone structures results from alternative expression of pikromycin (Pik) polyketide synthase (PKS). It was previously reported that the hybrid pikromycin-tylosin PKS can also produce two different macrolactones but its mechanistic basis remains unclear. In order to address this question, a series of site-directed mutagenesis of tentative alternative ribosome binding site and translation start codons in *tylGV* were performed. The results suggest that macrolactone ring size is not determined by the alternative expression of TylGV but through other mechanism(s) involving direct interaction between the PikAIII and TE domain or skipping of the final chain elongation step. This provides new insight into the mechanism of macrolactone ring size determination in hybrid PKS as well as an opportunity to develop novel termination activities for combinatorial biosynthesis.

**Key words:** Polyketide, polyketide synthase, pikromycin, tylosin, domain skipping

The pikromycin (Pik) polyketide synthase (PKS) of *S. venezuelae* has several remarkable features that make it a powerful system for combinatorial biosynthesis (Fig. 1). Such features include the unique ability to produce 12- and 14-membered ring macrolactones, which has not been found in any other PKS systems [13, 14, 15]. The monomodular organization of the two final modules of Pik PKS is known to be the critical factor that determines ring size in *S. venezuelae* [13, 15]. The tylosin (Tyl) PKS from *S. fradiae* has a similar organization of the two final modules to the Pik PKS; however, it produces only one macrolactone structure, the 16-membered ring tylactone (also called protylactonolide) (Fig. 1).

It has been reported that the generation of two macrolactone structures results from alternative expression of the Pik PKS [13]. Expression of full-length PikAIV (the last module required for heptaketide chain elongation) generates the 14-membered ring macrolactone narbonolide, whereas expression of an N-terminally truncated form of PikAIV (with the alternative translation start codon 600 amino acids downstream of the normal *pikAV* start codon) leads to skipping of the final condensation cycle in the polyketide biosynthesis to generate the 12-membered ring macrolactone 10-deoxymethynolide.

There are some structural differences between PikAIV and TylGV multifunctional enzymes. First, the acyltransferase (AT) domain in Tyl module 7 is specific for malonyl-CoA extension as opposed to PikAIV, which specifies chain extension by methylmalonyl-CoA. Secondly, Tyl module 7 includes a keto-reductase (KR) domain that is absent from Pik module 6. To produce a diverse range of macrolactone structures, a *pikAIV*-deleted mutant (HK954) was previously generated and a plasmid expressing TylGV, pDHS3007, was constructed in order to complement PikAIV. Transformation of HK954 with pDHS3007 resulted in the production of novel 14-membered ring macrolactone structures with predicted structural alteration, 2-desmethyl-3-dihydro-narbonolide (9), 2-desmethyl-3-dihydro-pikromycin (10), and 2-desmethyl-3-dihydro-narbomycin (11) (Fig. 2). Surprisingly, the 12-membered ring macrolactones, 10-deoxymethynolides, were

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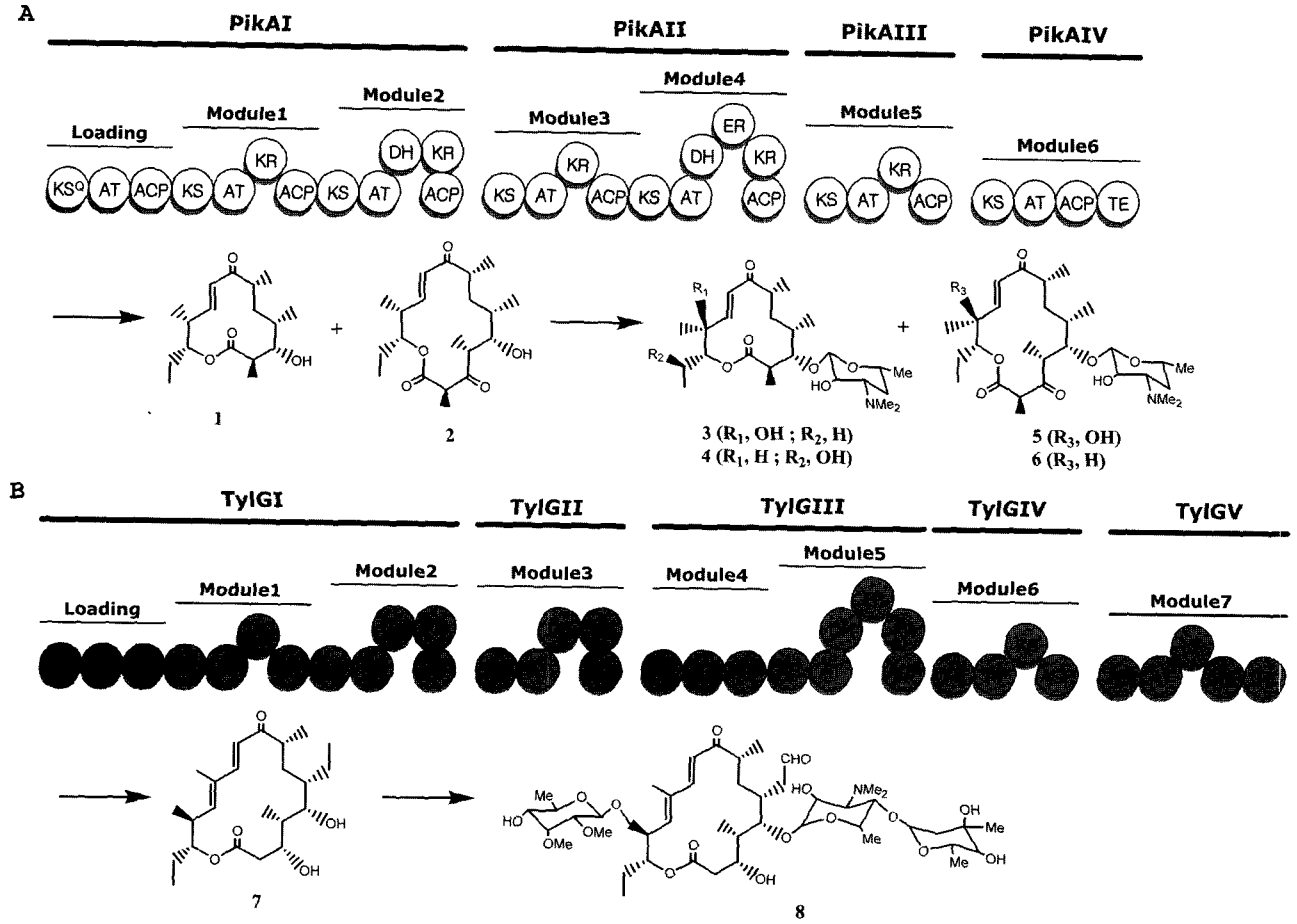



**Fig. 1.** Modular organization and the products of pikromycin PKS (A) and tylosin PKS (B).
Pik PKS produces 10-deoxymethynolide (1) and narbonolide (2). Final products are methymycin (3), neomethymycin (4), pikromycin (5), and narbomycin (6). Tyl PKS generates tylactone (7), which is modified further to tylosin (8).

also detected from the culture extract. However, it remains unclear whether the production of 10-deoxymethynolides resulted from the alternative expression of TylGV or whether other mechanism(s) enabled this hybrid PKS to generate 10-deoxymethynolide.

Wild-type *Streptomyces venezuelae* ATCC 15439 and the *pikAIV*-deleted mutant HK954 have been described previously [1, 16]. The manipulation and transformation of DNA in *E. coli* was performed in LITMUS28 (New England Biolabs, Hitchin, Hertfordshire, U.K.) by general molecular cloning procedures [11]. The polymerase chain reaction (PCR) was performed with Pfu Turbo polymerase (Stratagene, La Jolla, CA, U.S.A.) under the recommended conditions. *S. venezuelae* transformation was performed by the standard protoplast procedure [2, 4, 5, 7] and SGGP liquid medium [7] was used for propagation of *S. venezuelae*. The *S. venezuelae* wild-type strain and mutants were cultured on SPA solid medium [16] at 30°C for 3 days under appropriate antibiotic selection. The extract was analyzed on a reverse-phase $C_{18}$ column with 0–100% acetonitrile in water gradient by the combination of LC/MS [12] and MS/MS [16]. The hybrid polyketides were identified by mass spectral fragmentation pattern corresponding either to the predicted products or to known standards. Under the ionization conditions used, aglycones and their analogs generate signature dehydration patterns. The relative amount of each compound produced was compared by the peak intensity in the LC/MS.

The alignment of nucleotide sequences of the 5' region of *pikAIV* with those of *tylGV* indicated that there was a high degree of homology between them, but a minor difference was identified in the predicted ribosome binding site (RBS) and in one of two possible start codons (Fig. 3). To understand the ring size determination mechanism in the hybrid PKS, a series of site-directed mutagenesis was performed, altering RBS and two start codons (ST1, ST2) in the *tylGV* of pDHS3007. Mutations of alternative RBS (GAAGGA) and both ST1 and ST2 (ATG and GTG) in *tylGV* were conducted using a Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.). The sequences of RBS, ST1, ST2, and both ST1 and ST2 in plasmid pDHS3007 were individually changed by site-

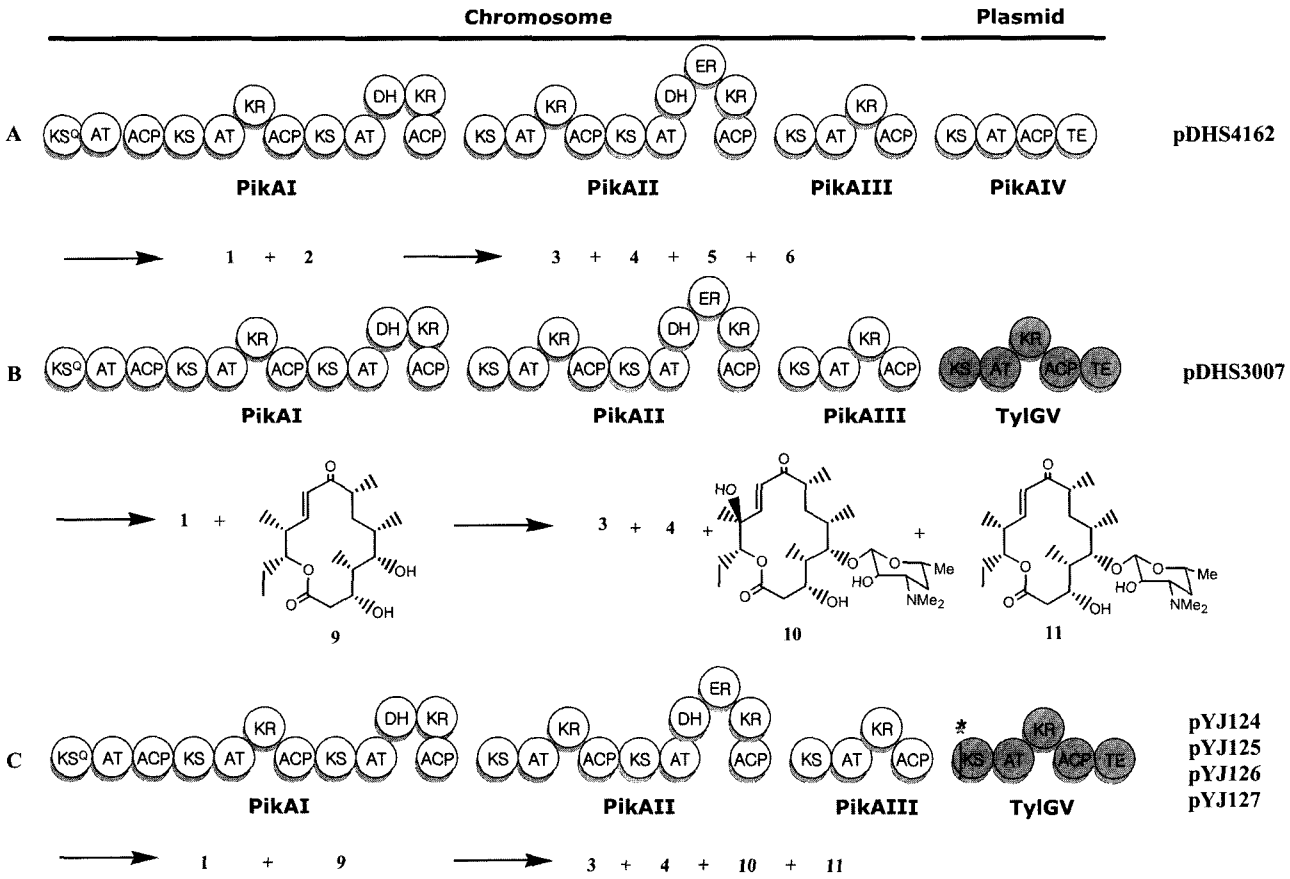
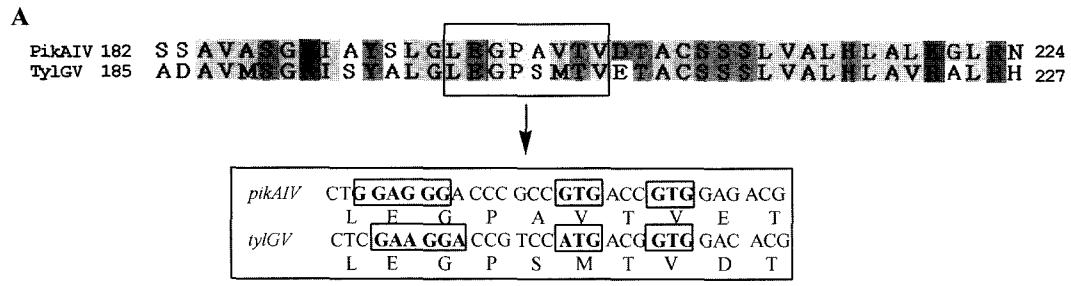



Fig. 2. Polyketides produced by functional replacement and mutation in the *pikAIV*-deleted mutant.
Polyketides produced by complementation of *pikAIV*-deleted mutant by wild-type PikAIV (A), native (B), and mutated TylGV (C), respectively. The asterisk and line represent amino acids alteration through site-directed mutagenesis of *tylGV*.




B

| Mutation | Sequence | Plasmid |
|---|---|---|
| Wild Type | CTC GAA GGA CCG TCC ATG ACG GTG GAC ACG<br>L E G P S M T V D T | pDHS3007 |
| RBS | CTC GAA GGT CCG TCC ATG ACG GTG GAC ACG<br>L E G P S M T V D T | pYJ124 |
| ST1 | CTC GAA GGA CCG TCC GTC ACG GTG GAC ACG<br>L E G P S V T V D T | pYJ125 |
| ST2 | CTC GAA GGA CCG TCC ATG ACG GTC GAC ACG<br>L E G P S M T V D T | pYJ126 |
| ST1&ST2 | CTC GAA GGA CCG TCC GTC ACG GTC GAC ACG<br>L E G P S V T V D T | pYJ127 |

Fig. 3. (A) Partial sequence alignment of PikAIV and TylGV. (B) Changing RBS and start codon sequence by site-directed mutagenesis. Alternative ribosome binding site (RBS) and two possible start codons (ST1, ST2) are boxed. The sequences of RBS, ST1, ST2, and both ST1 and ST2 in plasmid pDHS3007 were individually changed by site-directed mutagenesis, yielding plasmids pYJ124, pYJ125, pYJ126, and pYJ127, respectively.

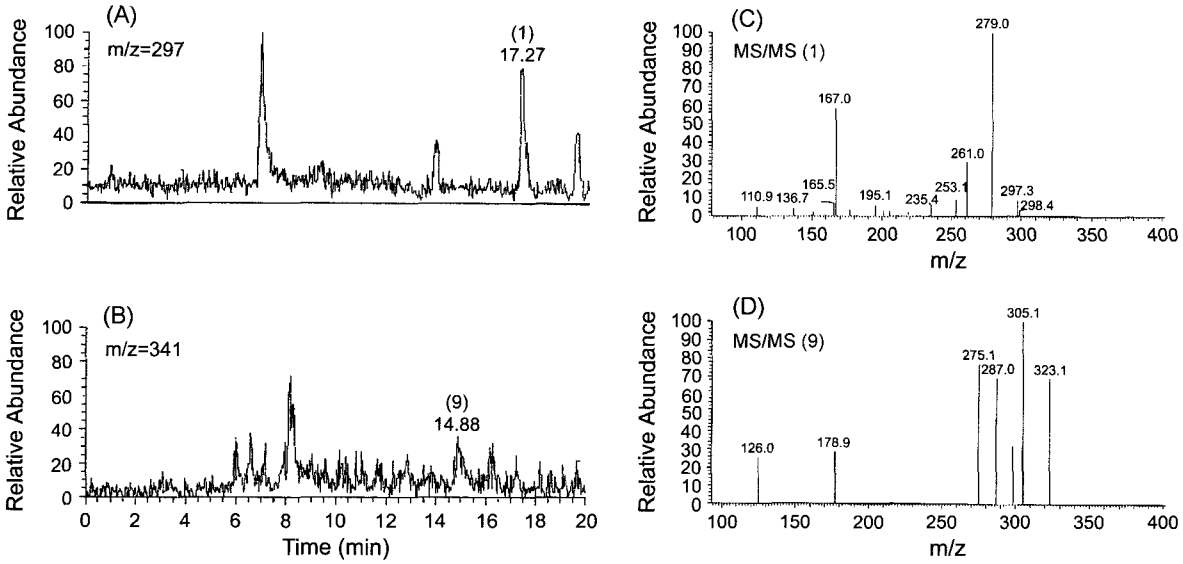



**Fig. 4.** LC/MS and MS/MS analyses of macrolactones produced from HK954/pYJ127 strain.
(A) LC/MS selected for amu=297, corresponding to compound **1**. (B) LC/MS selected for amu=341, corresponding to compound **9**. (C) MS/MS spectrum corresponding to compound **1**. (D) MS/MS spectrum corresponding to compound **9**.

directed mutagenesis [8], yielding plasmids pYJ124, pYJ125, pYJ126, and pYJ127, respectively (Fig. 3). The PCR primer pairs using mutation of RBS, ST1, ST2, and both ST1 and ST2 are 5'-TCG GTC TCG AAG GTC CGT CCA TGA CGG TGG AGA C-3' and 5'-GTC TCC ACC GTC ATG GAC GGA CCT TCG AGA CCG A-3', 5'-TCG GTC TCG AAG GAC CGT CCG TCA CGG TGG AGA C-3' and 5'-GTC TCC ACC GTG ACG GAC GGT CCT TCG AGA CCG A-3', 5'-TCG GTC TCG AAG GAC CGT CCA TGA CGG TCG AGA C-3' and 5'-GTC TCG ACC GTC ATG GAC GGT CCT TCG AGA CCG A-3', and 5'-TCG GTC TCG AAG GAC CGT CCG TCA CGG TCG AGA C-3' and 5'-GTC TCG ACC GTG ACG GAC GGT CCT TCG AGA CCG A-3', respectively (mutated RBS and two start codons are underlined). The mutations were confirmed by sequencing before and after introduction into *S. venezuelae*. Transformation of *S. venezuelae* HK954 (*pikAIV*-deleted mutant) with pYJ124 ($RBS^-$), pJY125 ($ST1^-$), pYJ126 ($ST2^-$), and pYJ127 ($ST1^-$ &$ST2^-$) was performed and, interestingly, all the transformed strains were observed to generate both 12-membered macrolactones and 14-membered novel hybrid compounds without any changes in production patterns to those of HK954/pDHS3007 (Fig. 4). The ability of these mutants to produce 12-membered ring macrolactone derivatives indicated that macrolactone ring size is not determined by the alternative expression of TylGV in this hybrid PKS. It is obvious that other mechanism(s) enable this hybrid PKS to produce macrolactones with two different ring sizes.

Recent work has shown that insertion of heterologous PKS module into erythromycin PKS leads to the production of novel compounds with the predicted structure, accompanied by substantial amounts of unextended normal macrolactone compounds [10]. This result demonstrates that heterologous module within a hybrid modular PKS can be bypassed during polyketide chain extension. Considering this theory, there is a possibility that TylGV could be recognized during polyketide biosynthesis as heterologous and could be skipped. The extending hexaketide chain intermediate may be transferred to the ACP domain in the TylGV without elongation and directly released *via* the adjacent TE domain to produce 12-membered ring macrolactones by domain skipping, which was previously reported in *S. venezuelae* [1]. Another proposed mechanism involves the direct association of PikAIII and TylGV containing TE domain into a head-to-tail assembly [6, 9], in which two equivalent catalytic centers are present at opposite ends of the PKS complex and each subunit contributes half of the KS and ACP domains to each catalytic center and KS and ACP from opposite subunits interact during the synthesis of a polyketide molecule. The TylGV TE domain in a head-to-tail assembly of PikAIII with TylGV could competitively reach out to the growing chains and attach to either the ACP domain of PikAIII in the opposite subunit of TylGV or the adjacent ACP domain of TylGV. Then, the TylGV TE domain would catalyze the release of hexaketide and heptaketide to produce two different macrolactone structures. These two mechanisms must be tested and confirmed by a series of complementation experiments. However, this study clearly demonstrates that the alternative translational start codons and putative RBS have no relation with the formation of two different macrolactone rings. Further studies on these mechanisms, therefore, would provide an opportunity to develop novel termination activities

as well as contribute to the potential for rational design of structural diversity for combinatorial biosynthesis.

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