Genenation of structural diversity in polyketides by combinatorial biosynthesis of polyketides:

Part I. Generation of multiple bioactive macrolides by hybrid modular polyketide synthases in *Streptomyces venezuelae*

Part II. Production of novel rifamycins by combinatorial biosynthesis

Yeo Joon Yoon

School of Chemical Engineering and Bioengineering,
University of Ulsan

Part I. Generation of multiple bioactive macrolides by hybrid modular polyketide synthases in ***Streptomyces venezuelae***

Abstract

The pikromycin biosynthetic system in *Streptomyces venezuelae* is unique for its ability to produce two groups of antibiotics that include the 12-membered ring macrolides methymycin and neomethymycin, and the 14-membered ring macrolides narbomycin and pikromycin. The metabolic pathway also contains two post polyketide-modification enzymes, a glycosyltransferase and P450 hydroxylase that have unusually broad substrate specificities. In order to explore further the substrate flexibility of these enzymes a series of hybrid polyketide synthases were constructed and their metabolic products characterized. The plasmid-based replacement of the multifunctional protein subunits of the pikromycin PKS in *S. venezuelae* by the corresponding subunits from heterologous modular PKSs resulted in recombinant strains that produce both 12- and 14-membered ring macrolactones with predicted structural alterations. In all cases, novel macrolactones were produced and further modified by the DesVII glycosyltransferase and PikC hydroxylase leading to biologically active macrolide structures. These results demonstrate that hybrid PKSs in *S. venezuelae* can produce a multiplicity of new macrolactones that are modified further by the highly flexible DesVII glycosyltransferase and PikC hydroxylase

tailoring enzymes. This work demonstrates the unique capacity of the *S. venezuelae* pikromycin pathway to expand the toolbox of combinatorial biosynthesis and to accelerate the creation of novel biologically active natural products.

Introduction and current progress in combinatorial biosynthesis using pik *PKS system*

Modular polyketide synthases (PKSs) are large multifunctional enzymes that are responsible for the biosynthesis of macrolides and other macrocyclic polyketides whose members have diverse structural and pharmacological properties[1]. These modular PKS assemblies are formed by giant multifunctional enzymes harboring one to many modules that catalyze serial condensations of activated coenzyme-A thioester monomers derived from small organic acids such as acetate, propionate, and butyrate [2-4]. Each module contains distinctive active site domains required for one cycle of polyketide elongation. Active sites required for condensation include an acyltransferase (AT), acyl carrier protein (ACP), and β-ketoacyl synthase (KS). Each condensation cycle results in a β-keto group that can undergo additional processing steps. Catalytic domains that perform these reactions include a keto reductase (KR), dehydratase (DH), and enoyl reductase (ER). The absence of any β-keto processing domain results in the presence of a ketone, a KR alone give rise to a hydroxyl, a KR and DH generate an alkene, while a KR, DH, and ER combination leads to complete

reduction to an alkane. After assembly of the polyketide chain, the molecule typically undergoes cyclization by a thioesterase (TE) domain at the C-terminus of the final module. The linearity between the catalytic domains present and the structure of its polyketide products makes modular PKSs attractive systems for combinatorial biosynthesis [5, 6]. A number of genetic engineering strategies have been used to generate hybrid PKSs including (1) inactivation, deletion, insertion and substitution of one or more catalytic domains, (2) deletion or exchange of complete modules, and (3) combining complete subunits from heterologous PKS clusters [1, 5, 7, 8].

The pikromycin (Pik) PKS of *S. venezuelae* has several remarkable features that make it a powerful system for combinatorial biosynthesis, including the ability to produce both 12- and 14-membered ring macrolides [9] (Figure 1A). Recent work has shown that alternative expression of the Pik PKS results in the generation of two macrolactone structures [10]. Expression of full length PikAIV (the last module required for heptaketide chain elongation) generates the 14-membered ring macrolactone narbonolide, while expression of an N-terminal truncated form of PikAIV (using the alternative translation start codon 600 amino acid downstream of the normal *pikAIV* start codon) results in skipping of the final condensation cycle to generate the 12-membered ring macrolactone 10-deoxymethynolide. The unusual nature of the *S.*

venezuelae system provides a potentially useful tool for combinatorial biosynthesis to generate multiple products from a single hybrid modular PKS [11, 12].

Another strength of the *S. venezuelae* system is the presence of two tailoring enzymes that have unusual substrate flexibility (recognizing both 12- and 14-membered ring macrolactones). Specifically, there is one DesVII glycosyltransferase that can accept both 12- and 14-membered ring aglycones [13]. PikC (a P450 hydroxylase) also accepts both macrolide substrates and is active at two positions on the macrolactone system [14]. Since structural modifications are often critical for biological activity, a current challenge for combinatorial biosynthesis is to develop approaches that lead not only to novel macrolactones, but ones that provide fully elaborated structures.

Here we report novel combinatorial biosynthetic approaches in *S. venezuelae* using a set of hybrid modular systems based on the Pik, tylosin (Tyl) and erythromycin (DEBS) PKSs (Figure 1A, B and C). The three systems were chosen based on biochemical architecture (e.g. the presence of bimodular PikAI, DEBS1 and TylGI; two final mono-modular PKSs in PikAIII/PikAIV and TylGIV/TylGV) as well as the structural similarity of their product profiles. These attributes provided a framework from which to address new questions about molecular recognition between heterologous mono-

modular PKSs, as well as to probe the flexibility of the DesVII glycosyltransferase and PikC hydroxylase toward novel substrates.

Part II. Production of novel rifamycins by combinatorial biosynthesis

1. Inactivation of the β-ketoacyl:acyl carrier protein reductase (KR)domain in module 8 of rifamycin polyketide synthase by site-specific mutagenesis of NADPH binding site

Abstract

The polyketide backbone of rifamycin B is assembled through successive condensation and β-carbonyl processing of the extender units by the modular rifamycin PKS. The eighth module, in the RifD protein, contains nonfunctional DH domain and functional KR domain, which specify the reduction of the β-carbonyl group resulting in the C-21 hydroxyl of rifamycin B. A four amino acid substitution and one amino acid deletion were introduced in the putative NADPH binding motif in the proposed KR domain encoded by *rifD*. This strategy of mutation was based on the amino acid sequences of the corresponding motif of the KR domain of module 3 in the RifA protein, which is believed dysfunctional, so as to introduce a minimum alteration and retain the reading frame intact, yet ensure loss of function. The resulting strain produces linear polyketides, from tetraketide to octaketide, which are also produced by a *rifD* disrupted mutant as a consequence of premature termination of polyketide assembly.

2. Replacement of the dehydratase (DH) domain in module 7 of the rifamycin polyketide synthase (PKS) with DH domain in module 7 of the rapamycin PKS

Abstract

Much of the structural diversity within the polyketide superfamily of natural products is due to the ability of PKSs to vary the reduction level of every other alternate carbon atom in the backbone. Thus, the ability to introduce heterologous reductive segments such as ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) into modules that naturally lack these activities would increase the power of the combinatorial biosynthetic toolbox. The dehydratase domain of module 7 of the rifamycin PKS, which is predicted to be nonfunctional in view of the sequence of the apparent active site, was replaced with its functional homolog from module 7 of rapamycin-producing polyketide synthase. The resulting mutant strain behaved like a *rifC* disrupted mutant, i.e., it accumulated the heptaketide intermediate and its precursors. This result points out a major difficulty we have encountered with all the *Amycolatopsis mediterranei* strain containing hybrid polyketide synthases: all the engineered strains prepared so far accumulate a plethora of products derived from the polyketide chain assembly intermediates as major products instead of just analogs of rifamycin B or its ansamycin precursors.

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