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Novel Variable Modifications to Polyketide Synthase Pathway and Screen Process

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ABSTRACT

Polyketide synthase (PKS) pathway modifications offer a limitless diversity of products. Soil microbes such as the Acidobacteria phylum are physiologically diverse, however their fastidious nature does not allow them to be easily cultured *in vitro*. These bacteria have been found to be rich in polyketide production and are a desired focus for polyketide discovery, specifically antibiotics compounds. Environmental DNA (eDNA) offers a new route to examine these PKS pathways and assay their antibacterial activity, as well as their potential for antitumor and antiviral properties. eDNA transformed into *Escherichia coli* contain a sequence of genes to be tested for antibiotic activity. These samples are plated against the gram-positive *Bacillus subtilis*, in both liquid and solid media, to determine their antibiotic activity.

The efficacy of these natural products decreases when the target bacteria develop resistance to their effects. Chimera compounds, utilizing the deoxysugars, loading domains, and PKS pathways of other compound production mechanisms offer an untapped source of novel antibiotics, for which no bacterial resistance has yet to be seen. Erythromycin, a highly-effective antibiotic, has faced resistance from a multitude of prevalent bacteria today. Its synthesis consists of iterative addition of carbonyl groups to form the erythromycin precursor, 6-deoxyerythronolide B (6-dEB). This then undergoes post-translational modification with the addition of two-deoxysugar groups and methyl-hydroxylation. Through the genomic modification of the loading domain and diversifying which molecule the synthesis begins with as well as the sugar groups that are added onto the molecule, novel compounds are formed that can exhibit greater antibiotic activity than erythromycin.

INTRODUCTION

The goal of this study is dual-fold: to determine the protocol necessary to produce a successful titer of 6-dEB with structural modifications, and to develop a screening protocol for complex new natural product discovery. The primary specific aim of the study is the discovery of a novel class of antibiotics (A), while the secondary specific aim of the project is the discovery of a novel antibiotic from an existing class (B). In its current state, novel antibiotics are being discovered at a rate of approximately 2-3 per decade, while bacterial species are becoming resistant to existing antibiotics at an even faster rate. With the exponential increase in antibiotic resistant bacteria, it is crucial to develop a method of rapid novel drug discovery through high-throughput eDNA scanning, as well as through genetic modifications.

eDNA scanning results in the discovery of not only novel forms of antibiotics, but perhaps a novel class of antibiotics, of which none have been discovered since 1987 (ansamycins). Our supplier of eDNA, Lucigen, has spliced the collection of eDNA from soil microbes into bacterial artificial chromosomes (BACs) that were each identified as having a PKS pathway, into *E. coli*, resulting in 53 samples. *E. coli* cells transformed with the BACs were tested on their antibiotic activity against *Bacillus subtilis* in solid and liquid media, as well as extraction of cellular proteins onto a filter disk assay. *E. coli* is utilized due to its expansive manipulability as a heterologous host for these genes (as compared to the native fastidious host of the genes); as a gram-negative bacteria, it is generally more resistant to antibiotics, and is therefore a suitable host for their production, while *B. subtilis* is a gram-positive bacteria, and is more susceptible to infection and therefore more likely to react to an antibiotic produced by the *E. coli* sample. The filter disk assay is crucial if the *E. coli* are killed by the antibiotic they produce, thus resulting in a low efficacy against the *B. subtilis*, while maintaining a high native potency.

The eDNA screening protocol involves 53 samples in 4 different hosts, each with a slightly altered genetic makeup to facilitate the production of the different forms of PKS pathways. As the current study is proceeding, 6 out of the available 31 BACs show promise towards antibiotic activity.

ERYTHROMYCIN SYNTHESIS

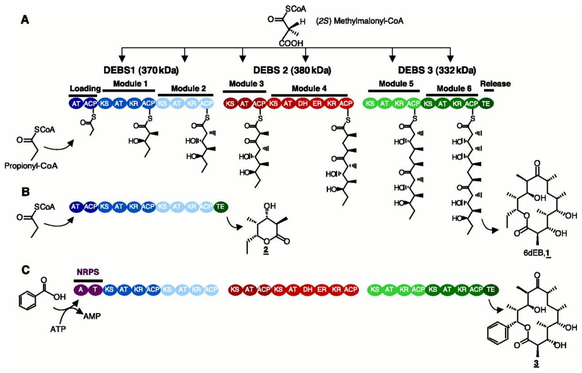


Figure 1. (*A*) The 6-deoxyerythronolide B synthase. Catalytic domains: KS, ketosynthase; AT, acyl transferase; ACP, acyl carrier protein; KR, ketoreductase; ER, enoyl reductase; DH, dehydratase, TE, thioesterase. DEBS utilizes 1 mole of propionyl-CoA and 6 moles of (2*S*)-methylmalonyl-CoA to synthesize 1 mole of 6-deoxyerythronolide B (6-dEB). (*B*) Truncated DEBS1+TE produces the triketide lactone. (*C*) The rifamycin synthetase is a polyketide synthase that is naturally primed by a nonribosomal PKS loading module, comprised of two domains: an ATP-dependent adenylation domain (A) and a thiolation domain (T). Substitution of this A-T didomain in place of the loading didomain of DEBS yields an engineered "hybrid" synthase that utilizes exogenous acids such as benzoic acid to synthesize substituted macrocycles such as in an engineered strain of *E. coli* (Pfeifer et al. 2001).

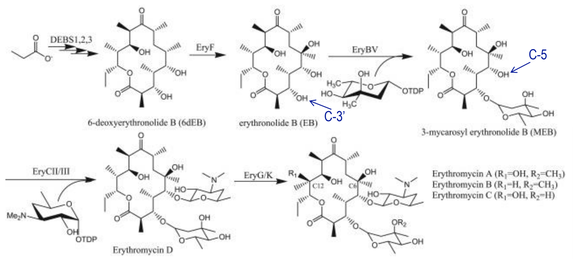


Figure 2. Post-PKS Tailoring Steps for Erythromycin synthesis from 6-dEB (Jiang and Pfeifer, 2013).

PIKROMYCIN SYNTHESIS

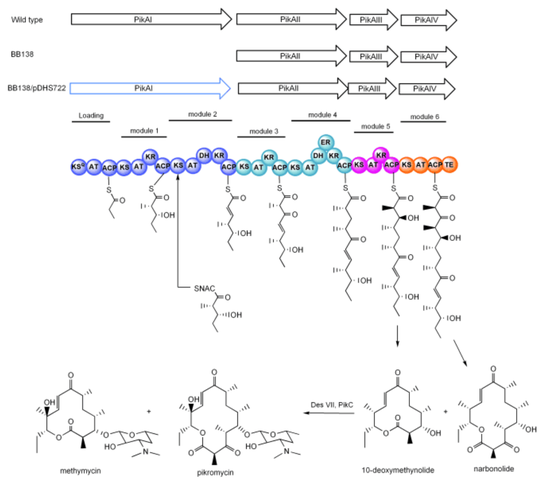
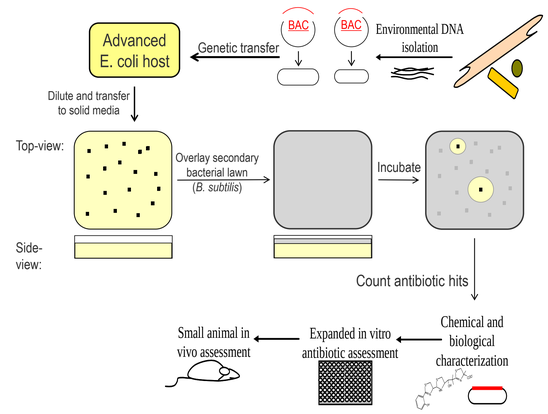


Figure 3. Pikromycin synthesis via PKS pathway to form mixed precursors narbonolide and 10-deoxymethynolide. Post-PKS tailoring via pikC produces mixed products pikromycin and methymycin (Yan et al. 2009).

SCREENING PROCESS



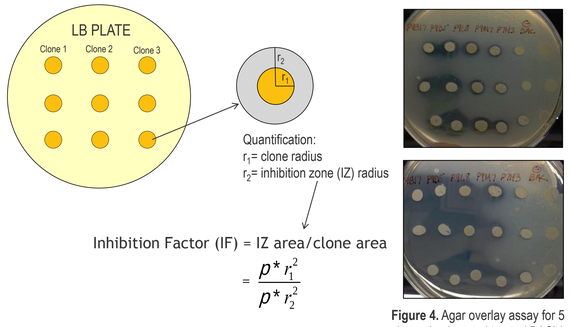
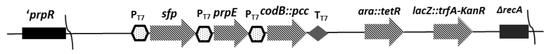


Figure 4. Agar overlay assay for 5 clones (and control 'empty' BAC) in 30° C (top) and 22° C (bottom).

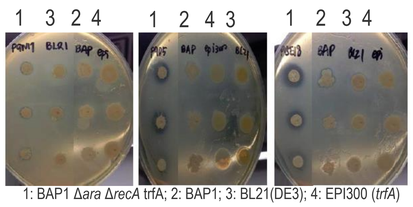
E. coli HOST SYSTEM

Advance *E. coli* strain for heterologous expression of natural products

E. Coli BTRAP



Gene	Approach	Functionality
trfA	knock-in	amplify fosmid copy number
ara	knock-out	eliminate arabinose catabolism
sfp	knock-in	post-translationally modify PKS/NRPS
prpE	enhance expression	accumulate propionyl-CoA
recA	knock-out	stabilize foreign DNA
pccAB	knock-in	accumulate methylmalonyl-CoA



1: BAP1 Δ*ara* Δ*recA* trfA; 2: BAP1; 3: BL21(DE3); 4: EPI300 (*trfA*)

DEOXYSUGAR VARIANCE

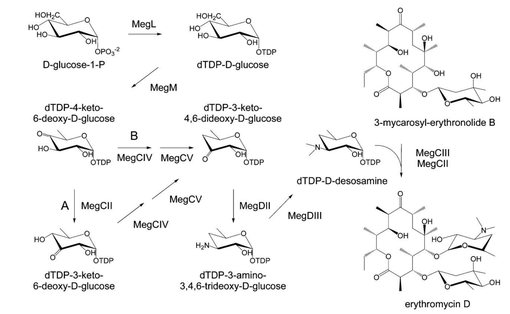


Figure 5. The dTDP-D-desosamine biosynthetic pathway towards erythromycin production from MEB (Rodriguez et al. 2006).

Deoxysugars To Be Tested:

- *-TDP-D-mycaminose*
- *-TDP-L-noviose*
- *-TDP-6-deoxy-D-allose*
- *-TDP-L-eremosamine*
- *-TDP-D-forosamine*
- *-TDP-L-olivose*
- *-TDP-D-oliose*
- *-TDP-L-oleandrose*
- *-TDP-L-digitoxose*
- *-TDP-D-vicenisamine*
- *-TDP-D-digitoxose*
- *-TDP-L-ravidosamine*
- *-TDP-3-N-acetamido-3,6-dideoxy-D-galactose*
- *-TDP-L-megosamine*

CONTINUED RESEARCH

- Express sugar pathways in E. coli BL21(DE3), detect all TDP-sugar production using LC-MS
- Test TDP-sugars that can be transferred to C-3' position hydroxyl group, feeding 6dEB
  - -TDP-L-mycarose (positive control) and extend to all sugars
- Test TDP-sugars that can be replace desosamine by feeding 6dEB (or maybe MEB)
  - TDP-D-desosamine and TDP-D-mycaminose (positive controls) and extend to rest of the sugars
- Employ both LC-MS and tandem LC-MS/MS to analyze potential analogues

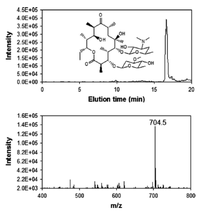


Figure 6. Olivose erythryomycin analogue formation using 6dEB as a substrate

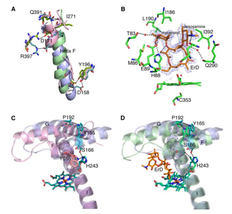


Figure 7. Substrate recognition and locking within the active site of EryK. A, a close up of the helix F network in superposed open EryK (blue), ligand-free closed EryK (pink), and ErD-EryK (green) (Montemiglio et al. 2011)

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I would like to thank Lei Fang and Dr. Blaine Pfeifer of the Pfeifer Group in the Chemical and Biological Engineering Department at the University at Buffalo for their support in this research.

This research would not be possible without the financial support granted by the University Honors College and the Center for Undergraduate Research and Creative Activities.