ABSTRACT

Polyketides have been known to display a wide array of functions such as anticancer, antifungal, and antiparasitic activity. The molecule of interest, laulimalide, has been isolated from a marine sponge inhabited by hundreds of symbiotic bacteria. Due to the inability to target the host producer of laulimalide, isolation and characterization of the native laulimalide pathway has proved to be impossible. Proposed biosynthetic pathways for the production of laulimalide incorporating the rearrangement of known modules from pimaricin, rifamycin, amphotericin, and deoxyerythronolide are a possiblity. Modular polyketide synthase genes can be rearranged via intramodular linker regions and intermodular linker regions to facilitate communication between heterologous modules. Restriction sites can be introduced to allow interchange of modules from various pathways. To test the efficiency of the proposed protein-protein interactions, three biosynethic pathways for portions of the core of laulimalide have been proposed. The pathway under study, C13 – C19, contains a linker region created from the module 2 and module 3 interface in the 6-deoxyerythronolide B pathway for the interpolypeptide interface of module 2 of pimaricin and module 12 of amphotericin. A NdeI restriction site has been added at the start of pimaricin module 1 and an EcoRI restriction site has been added after the stop codon of pimaricin module 2. Module 1 and 2 of pimaricin have compatible XbaI and SpeI overlaps. The interpolypeptide linker contains an SpeI site for compatibility with the XbaI site of module 2. Site directed mutagenesis of pUC19 has removed the inherent XbaI site for proper installment of pimaricin module 1, pimaricin module 2, and the interpolypeptide linker.

Aim 1: Install pimaricin module 1 and pimaricin module 2 inserts into pUC 19 vector. Express and check for activity. Continue with biosynthetic pathway for C13 – C19 of the laulimalide core.

INTRODUCTION

Polyketides, functioning as secondary metabolites, are found within a broad spectrum of organisms. Polyketides possess anticancer, immunosuppressive, and antifungal activity. The polyketide of interest, laulimalide, which is a potent anticancer agent, is isolated in trace amounts from a marine sponge inhabited by hundreds of symbiotic bacteria. Due to the small quantity isolated, clinical trials of laulimalide are not likely to occur in the near feature. The problem can be mediated by investigating the nature of laulimalide through total synthesis and the creation of biosynthetic pathways that code for structural portions of the molecule.

Polyketides have been found to be made by modular polyketide synthases, enzymes which consist of proteins with multiple modules. The polyketide synthase pathway for 6-deoxyerythronolide B contains three proteins, each with two modules (1). Figure 1 shows the pathway.

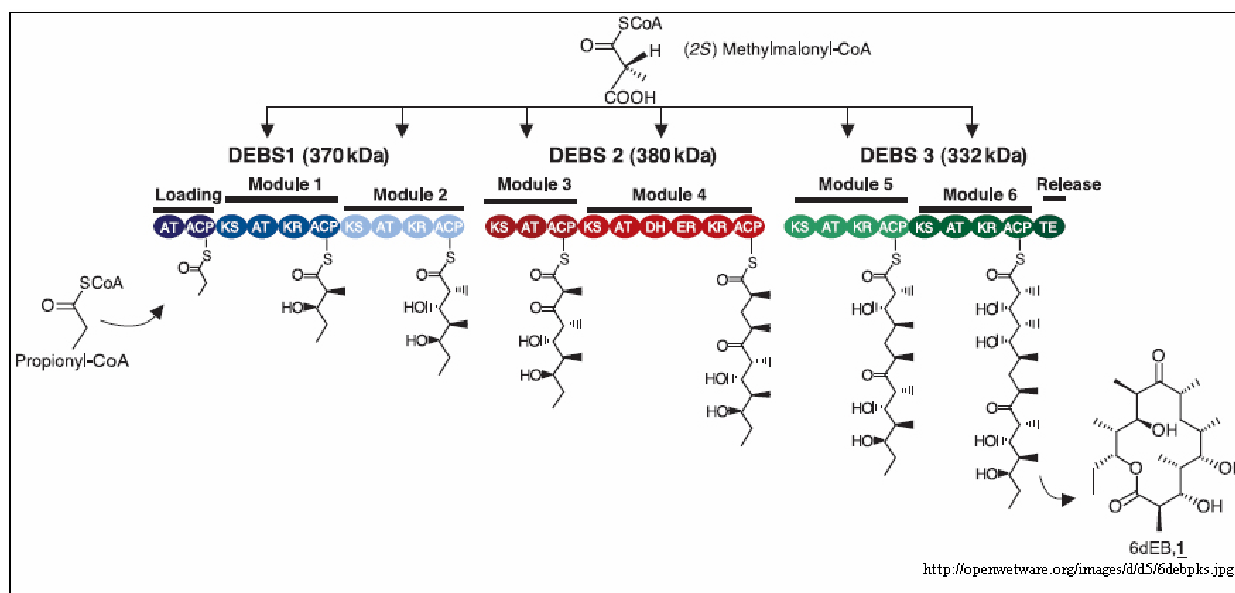


Figure 1

The discovery of clustered genes in genomic DNA encoding modules in polyketide

synthases has led to a new approach in manipulating polyketide biosynthesis. New biosynthetic pathways can be engineered by recombining modular polyketide synthase genes. Alteration or replacement of polyketide synthase modules can lead to new biosynthetic routes, designing 'unnatural' natural products (2). A biosynthetic pathway can be designed for the production of laulimalide using known modules from various pathways such as the rifamycin, pimaricin, amphotericin, and 6-deoxyerthronolide B pathway.

The function of intramodular and intermodular linkers have to be assessed before the significance of modular interchange can be understood. Intrapolypeptide linkers are spacers of ~ 20 amino acids separating the ACP of one module from the ketosynthase of the next (3). Interpolypeptide linkers consist of ~80-130 amino acids at the C-terminal of one module that interacts wuth a cognate ~30-50 amino acid sequence at the N terminus of the downstream module. Introduction of engineered linkers between foreign modules can provide processing of intermediates further downstream along a newly formed biosynthetic pathway (4).

The introduction of restriction sites at the beginning and end of foreign modules, domains and linkers can provide a tractable way of creating new biosynthetic routes. As an example, gene sequences encoding modules from various pathways were aligned to scan for common nucleotide sequences. After commonality was assessed, restriction sites were introduced at positions with minimal alteration of native sequence. Modules were then recombined using the introduced sites. Figure 2 gives an example of restriction sites introduced at the beginning and end of modular domains.

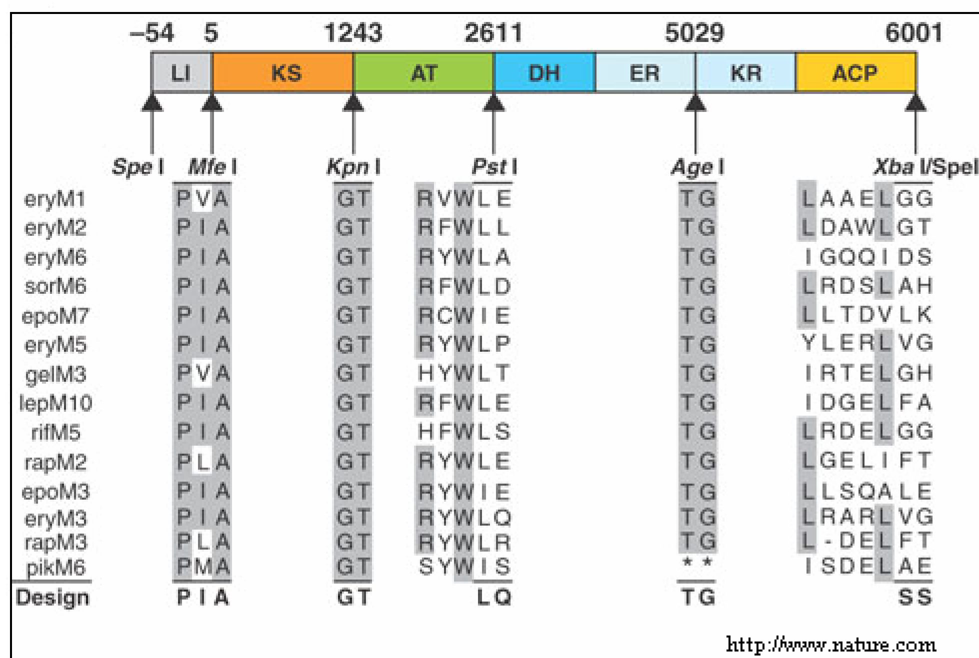


Figure 2

Biosynthetic pathways were proposed for structural portions of laulimalide using modules flanked with specific restriction sites. Each of the pathways will be tested before the total biosynthetic pathway of laulimalide will be proposed. Module 1 and module 2 of pimaricin was used for the beginning of the biosynthetic pathway for C13-C19. The insertion of a intermodular linker derived from the module 2 and module 3 interface of the 6-deoxyerythronolide pathway will provide communication between the C-terminal of Module 2 of pimaricin and the N-terminal of module 12 of amphotericin. If activity of module 1 and module 2 of pimaricin proves to be successful, completion of the route for C13-C19 will be attempted to produce a molecule which can be used in semi-synthesis of laulimalide. The former route will also be used in the total biosynthetic pathway of laulimalide. Modules with similar function will be introduced if the proposed biosynthetic pathway fails.

METHODS AND MATERIALS

Isolation and construction of Module 1 and Module 2 of Pimaricin into pCR® Blunt Vector

Forward and reverse oligonucleotide primers with engineered restriction sites were designed for PCR (polymerase chain reaction) using Streptomyces natalensis genomic DNA as template. Table 1. provides the forward and reverse sequences with the engineered restriction sites in bold.

Table 1. Oligonucleotide primer sequences with engineered restriction sites (in bold) used for cloning module 1 and module 2 of the pimaricin gene sequence

GENE	FORWARD (5' to 3')	REVERSE (3' to 5')	CUT SITES
Module 1	aac tca ***cat atg*** tcg aac gag gag aag ctg c	aag tca ***tct aga*** gag ccg gtt gcg cag gt	NdeI/XbaI
Module 2	aac tca ***cat atg*** aac tca gta ***act agt*** gcg acg ggc ctg cgg ctg c [1]	aac tca ***gaa ttc*** aac tca gta ***tct aga*** gag gcg gtt gcg gag ttc gac [2]	Nde I/SpeI [1] EcoRI/XbaI [2]

Genomic DNA used for PCR was isolated from Streptomyces natalensis using the Promega Wizard® Genomic DNA Purification Kit. Streptomyces natalensis ATCC 27448 was used as the source of genomic DNA (5). Module 1 and Module 2 of the pimaricin gene sequence were targeted for amplification. The thermocycler Mastercycler® Personal was used for all PCR reactions. Pfu Turbo® DNA polymerase and associated buffer were used for PCR reactions during trial 1 with Module 1 and Module 2. PrimeSTAR HS DNA polymerase and associated buffer were used for PCR reactions during trial 2 with Module 1. All primers used in the PCR reaction were diluted to 5µM concentration with water. Touchdown PCR was used for all PCR reactions with the following thermocycler conditions for Module 2: 1 cycle of 95.0 °C for 5 min, 11 cycles of 30 s at 95.0 °C, 11 cycles of 30 s at 72.0 °C (-1 °C per cycle), 11 cycles of 6 min and 30 s at 72.0 °C, 24 cycles of 30 s at 95.0 °C, 24 cycles of 30 s at 57.0 °C, 24 cycles of 6 min and 30 s at 72.0 °C, and 1 cycles of 20 min at 72.0 °C. Final temperature was held at 4 °C overnight. Module 1 PCR conditions were similar except with the decrease of time from 6 min and 30 s to 5 min and 30 s in the above mentioned. 2µl of 50µl PCR reaction was run on an agarose gel to check for desired amplification product at 85V for 5 min and 115V for 40 min. Remaining volume was loaded into larger wells, approximately 47µl per well. The gel was

analyzed under 365nm and the band of interest was excised. DNA of interest was purified using eppendorf Perfectprep® Gel Cleanup Kit. Module 1(MR01) and Module 2(MR02) were then ligated into pCR® -Blunt and stored overnight at 4 ºC. The Zero Blunt® PCR Cloning Kit was used for transformation of ligation product into One shot® TOP10 Competent Cells. The plasmid has kanamycin resistance markers. Transformed cells were recovered in LB for 1 hour at 37 °C with shaking and then plated on LB supplemented with kanamycin. Selected colonies were grown overnight in glass tubes with 2ml LB and 2µl of kanamycin. 1.5ml from each tube was spun down for 2 minutes at 13.2 x1000 rpm in eppendorf tubes and supernatant was decanted. The QIAprep® Spin Miniprep Kit was used to purify plasmid DNA. Plasmid DNA was digested with EcoRI to test for insert (Module 1 and Module 2). If insert was present 50ml culture was grown overnight and plasmid DNA was isolated using the Promega PureYield™ Plasmid Midiprep System.

Construction of modified pUC19 plasmid

Forward and reverse oligonucleotide primers with a modified XbaI site were created for a point mutation of the XbaI site on the pUC19 plasmid. The pUC19 plasmid was used as template. The thymine in the XbaI restriction site was altered to an adenine, changing the sequence from TCT AGA to TCA AGA. The primers were designed with a melting temperature of 68.6 ºC for the forward primer and 68.6ºC for the reverse primer. Table 2 provides the forward and reverse sequences.

Table 2. Engineerd forward and reverse oligonucleotide primers with modified XbaI site (altered XbaI restriction site is bold)

pUC19 plasmid	FORWARD (5' TO 3')	REVERSE (3' TO 5')
	acc cgg gga **tca aga** gtc gac ctg cag	ctg cag gtc gac tct tga gga tcc ccg ggt

The point mutation was carried out using protocol from the QuikChange® Site-Directed Mutagenesis Kit. A PCR reaction was carried out using Pfu Turbo DNA polymerase with the following thermocycler conditions for point mutations: 1 cycle of 30 s at 95.0 ºC, 12 cycles of 30 s at 95.0 ºC, 12 cycles of 1 min at 55.0 ºC, and 12 cycles of 3.5 minutes at 68.0 ºC. 1µl of DpnI digestion enzyme was added to the amplification products for digestion of the non mutated supercoiled DNA. XL1-Blue chemically competent cells were transformed with the DpnI-treated DNA. XL1-blue cells were plated on LB with ampicillin. pUC19 plasmid has an ampicillin resistance marker. Colonies were grown overnight and pUC19 plasmid was purified using the QIAprep® kit. Non modified pUC19 plasmid was used as a control during an XbaI digestion. 10µl of culture from which plasmid DNA was found to have a modified XbaI site was streaked on LB with ampicillin. Colony that was found was grown in 50ml LB with 50µl of ampicillin, and plasmid DNA (MR03) was isolated using the the Promega PureYield^{TM} Plasmid Midiprep System.

Design of LC^{eryM2} intermodular linker

An intermodular linker was designed by engineering forward and reverse primers with a SpeI site and a EcoRI site. Table 3 shows the sequence for the 5' to 3' primer and the 3' to 5' primer. The restriction sites are bold.

Table 3. Engineered forward and reverse oligonucleotide primers of the LC^{eryM2} linker
FORWARD (5' to 3'): agg tca cat ***act agt*** gag gtc cga gga gag gcg ccg tcg gcc ctc gcc ggg ctg gac gcg ctg gaa ggc gcc ctg ccc gag gtg ccc gca acc gag cgg gaa gag ctg gta cag cgc ttg gaa cgg atg ctc gcc gcg cta cgc ccg gtc **SpeI**
REVERSE (3' to 5') ggc gac cat ***gaa ttc*** tca atc gcc gtc gag ctc ccg gcc gag tgc ttc gag cag ttc gtc cac gcc cgc ctc gcc cag gtc gtc gcc gga cgg gtt ggc gcc ggt ccc gga ggc gtc ggc ggc ctg ggc gac cgg gcg tag cgc ggc gag **EcoRI**

Forward and reverse primers were diluted with water to a final concentration of 50µM and placed in the -20 ºC refrigerator.

Digestion of pUC19 muta (modified pUC19 plasmid) and Module 2 in pCR® Blunt Vector

pUC19 muta plasmid was digested with NdeI restriction enzyme for 3 hours and then with EcoRI restriction enzyme for 3 hours. The total volume was 20µl for each digestion and appropriate buffers were used with NdeI and EcoRI. Module 2 in *pCR® Blunt* was digested with NdeI and EcoRI with the above time mentioned for pUC19 muta. The total volume was 50µl for each digestion due to faint band present when stock module 2 in *pCR® Blunt* was tested. All digestions were run on an agarose gel with 40µl loaded into each well. pUC19 muta and module 2 were purified out of the agarose gel using the eppendorf Perfectprep® Gel Cleanup Kit. A 10 µl ligation reaction between module 2 and pUC19 muta was constructed using T4 DNA ligase at 4 ºC overnight. XL1-blue cells were transformed using the ligation mixture. Cultures grown overnight were analyzed for the desired 8.0 Kb band. Plasmid DNA of combined module 2 and pUC19 muta (MR04) was digested with EcoRI to check for the desired product. Same procedure was followed for isolation of stock plasmid DNA of module 2 and pUC19 muta as for MR01, MR02, and MR03.

Digestion of stock MR01 (Module 1) and MR04 (module 2 and pUC19 muta)

Module 1 was digested for 3 hours with NdeI and for 3 hours with XbaI. MR04 (module 2 and pUC19 muta was digested with NdeI for 3 hours and with SpeI for 3 hours. Both were purified from the agarose gel and ligated overnight using T4 DNA ligase. XL1-blue cells were used for the transformation. Analysis of transformants via plasmid purification and digestion did not reveal desired insert and vector length.

RESULTS

Amplification of module 1 and module 2 of pimaricin proved to be successful.

Figure 3 shows the desired 4.5 Kb band of pimaricin module 1 after PCR reaction and Figure 4 shows the desired 5.6 Kb band of pimaricin module 2 after PCR reaction.

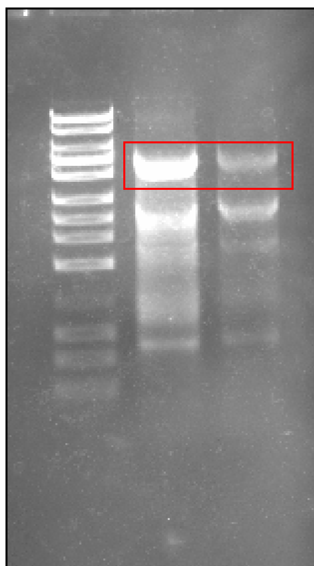


Figure 3

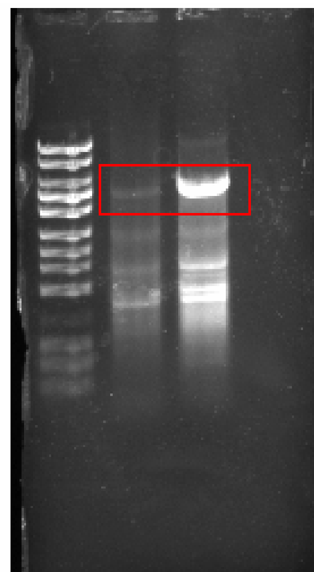


Figure 4

Figure 5 displays an example of module 1 insert after gel purification:

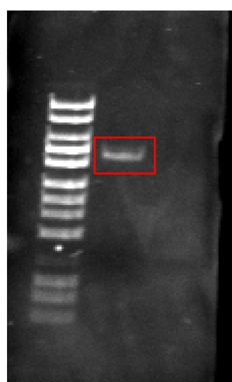
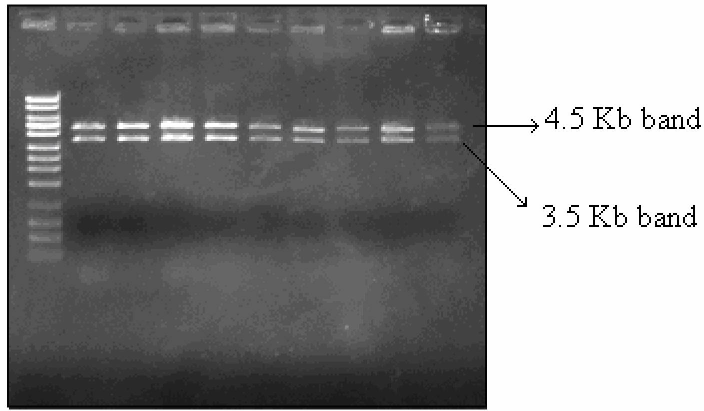


Figure 5

Analysis of transformed One shot® TOP10 Competent Cells with module 1 and module 2 after digestion with EcoRI provided a 3.5 Kb band for the vector (*pCR® Blunt)*, 4.5 Kb band for module 1 insert and ~5.6 Kb band for module 2 insert. Figure 6 displays a 20µl digestion per well of module 1 in *pCR® Blunt*. Isolated module 1 insert was amplified during trial 2 of PCR

reaction with PrimeSTAR HS DNA polymerase. Figure 7 displays the results of digestion with module 2 in *pCR® Blunt*.



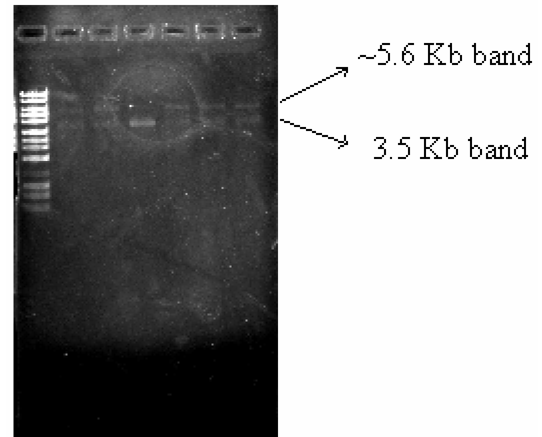


Figure 6 **Figure 7**

pUC19 control plasmid was diluted 10x with water from its 50µM stock concentration and digested with Xba I. Modified pUC19 was digested with XbaI and was ran on an agarose gel next to the control plasmid for comparison. pUC19 was cut at the XbaI site forming a 2.5 Kb linear fragment. pUC19 muta (MR03) did not provide a 2.5 Kb band, instead supercoiled DNA provided a lower band. Results of the point mutation are shown in figure 8.

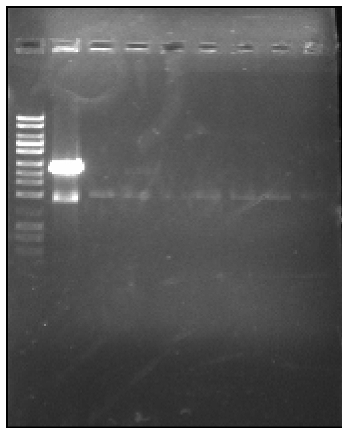


Figure 8

After digestion with NdeI and EcoRI of Module 2 and pUC19 muta, ligation and transformation with XL1- blue cells provided a 8.0 Kb plasmid. The plasmid was digested with EcoRI to confirm the length of the fragment. Figure 9 provides an image of the 8.0 Kb fragment of module 2 and pUC19 muta (MR04) ligated together.

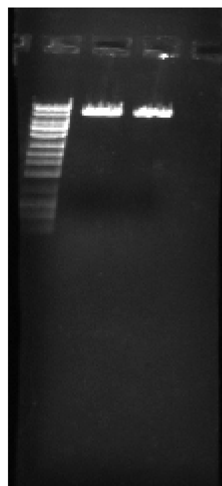


Figure 9

Module 2 and pUC19 muta was digested with NdeI and SpeI and Module 1 in *pCR® Blunt* with NdeI and XbaI. Ligation was induced with T4 DNA ligase. The desired 12.6 Kb band was not present probably due to higher concentration of vector as opposed to insert (Module1). High number of transformed colonies led to the conclusion that the vector closed without module 1 being inserted.

DISCUSSION

Restriction sites introduced into pimaricin module 1 and pimaricin module 2 has facilitated the construction of the biosynthetic pathway for C13-C19 of the core of laulimalide. The NdeI site and EcoRI site of Module 2 was compatible with the NdeI site and EcoRI site found in pUC19 vector. The XbaI site of the vector was modified due to the XbaI restriction sites introduced into module 1 and module 2.

Forms of troubleshooting:

Developing a stock of module 2 in *pCR® Blunt* failed during purification of first 50 ml culture. After a digestion was run with EcoRI, a 5.6 Kb band and a 3.5 Kb band did not appear on the gel. Transformation into XL1-blue cells was repeated using plasmid DNA of module 2 in *pCR® Blunt* stored in the -20 ºC refrigerator. A second digestion of the 50 ml culture developed from the transformation with XL1-blue cells provided the proper insert and vector fragment size.

Digestion of stock module 2 in *pCR® Blunt* provided a faint band when viewed under 365nm. After module 2 was digested, ethanol precipitation was necessary to produce a higher concentration of insert. After the module 2 DNA was more concentrated, ligation with pUC19 muta provided the expected 8.0 Kb plasmid.

Insertion of module 1 into module 2 and pUC19 muta proved unsuccessful due to higher concentration of vector than insert. Ethanol precipitation will be performed before the next ligation.

Checking pimaricin module 1 and 2 for activity

When insertion of module 1 will prove to be successful, a PCR reaction will be performed using the engineered intermodular linker from the interface of module 2 and module 3 in the 6-deoxyerythronolide pathway. Module 1 and 2 will be expressed and assayed for activity. The biosynthetic pathway will be continued if the result is positive.

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