Phage P1-Derived Artificial Chromosomes Facilitate Heterologous Expression of the FK506 Gene Cluster

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Abstract

We describe a procedure for the conjugative transfer of phage P1-derived Artificial Chromosome (PAC) library clones containing large natural product gene clusters (≥70 kilobases) to Streptomyces coelicolor strains that have been engineered for improved heterologous production of natural products. This approach is demonstrated using the gene cluster for FK506 (tacrolimus), a clinically important immunosuppressant of high commercial value. The entire 83.5 kb FK506 gene cluster from Streptomyces tsukubaensis NRRL 18488 present in one 130 kb PAC clone was introduced into four different S. coelicolor derivatives and all produced FK506 and smaller amounts of the related compound FK502. FK506 yields were increased by approximately five-fold (from 1.2 mg L⁻¹ to 5.5 mg L⁻¹) in S. coelicolor M1146 containing the FK506 PAC upon over-expression of the FK506 LuxR regulatory gene fkbN. The PAC-based gene cluster conjugation methodology described here provides a tractable means to evaluate and manipulate FK506 biosynthesis and is readily applicable to other large gene clusters encoding natural products of interest to medicine, agriculture and biotechnology.

Introduction

A wealth of microbial gene clusters that encode the biosynthetic pathways to bioactive natural products, also known as secondary or specialized metabolites, are being unveiled with the ever-increasing number of sequenced bacterial genomes [1]. While some clusters correspond to known compounds, a large number (so-called cryptic gene clusters) encode the biosynthesis of previously undiscovered molecules and represent a promising source of new drugs and therapeutics. Many of these gene clusters feature modular type I polyketide synthase (PKS) and/or non-ribosomal peptide synthetase (NRPS) enzymes, and can exceed 100 kilobases (kb) in size and consist of several tens of genes [2], [3].

One of the most useful strategies for advancing the study of natural products is heterologous expression of the genes responsible for biosynthesis in a tractable host organism. This approach has many advantages, including compound yield improvements [4], [5], [6] and the ability to work with a strain that is more amenable to genetic manipulation, e. g. in order to disrupt genes [7], to create analogs [8], to study regulation [9], to determine the minimal gene set necessary for biosynthesis [10], or to aid in the identification of unknown molecules, such as from metagenomic libraries [11], [12].

While heterologous expression has been used successfully with small biosynthetic gene clusters (≤40 kb) [13], standard techniques are not as straightforward with large biosynthetic gene clusters (≥70 kb), largely because of the amount of DNA that needs to be cloned and transferred into a suitable expression host. In frequently used E. coli cosmid libraries, insert sizes are limited to approximately 42 kb. Thus, large clusters are typically fragmented across multiple clones and require often laborious reassembly strategies such as Red/ET-mediated recombination in E. coli [7], [14] or Transformation Associated Recombination (TAR) in yeast [12], [15].

One method that avoids most problems with cluster reassembly is to clone large gene clusters directly into artificial chromosomes that are able to tolerate insert sizes of approximately 200 kb [16], [17]. This size capacity is sufficient for virtually all known bacterial modular gene clusters and should also allow for several kb of adjacent DNA sequence to be included if the cluster boundary regions are unclear. However, techniques for subsequent handling of these artificial chromosomes and their introduction into heterologous expression strains are not always straightforward. Vectors with large inserts that require protoplast transformation [6], [18] are prone to sequence deletions or rearrangements and frequently give very low transformation rates.

In this paper, we provide an expedient, reliable procedure for transfer of large natural product gene clusters using phage P1-derived Artificial Chromosomes (PACs) to Streptomyces coelicolor derivatives that have been engineered for improved heterologous expression. This approach allows for stable integration of PAC DNA via conjugation without any additional modifications, immediately creates a platform for gene cluster analysis, and enables targeted systems and synthetic biology approaches that might not be possible in wild-type strains. To demonstrate the feasibility of this procedure, we have heterologously expressed the 83.5 kb biosynthetic gene cluster for FK506 (tacrolimus), a...
clinically important polyketide from *Streptomyces tsukubaensis* NRRL 18488, using a PAC construct of approximately 130 kb.

FK506 is a high value calcineurin inhibiting immunosuppressant [19], marketed under the trade name Prograf with annual sales of approximately $2 billion (evaluatepharma.com, [20]). It is now used following bone marrow, kidney and liver transplants [21], [22], [23], is effective at treating skin inflammation and eczema [24], [25] and is gaining additional attention for its potential in treating cancer [26] and neurological conditions [27].

Both FK506 and the related natural product FK520 (ascomycin) are produced by several *Streptomyces* strains. The two molecules differ only in one position with the presence of either an allyl (FK506) or ethyl (FK520) side chain (Figure 1A). Several recent studies have greatly expanded our understanding of FK506 and FK520 biosynthesis, including the identification of the enzyme FkbO as a chorismate hydrolase that generates the first FK506/FK520 precursor [20], the enzymes responsible for constructing allylmalonyl CoA or ethylmalonyl CoA for the two different side chains of FK506 and FK520 [29], [30], [31] and genes whose products positively or negatively regulate FK506 expression [32], [33]. Introduction of precursors into culture media or overexpression of certain components of the FK506 gene cluster have also led to improvements in FK506 yields [34], [35]. Despite these advances, several challenges exist in pursuing FK506 yield improvements in wild-type *Streptomyces* producers. Genomic information for FK506 producing *Streptomyces* has only recently emerged [36], and targeted genetic manipulations in some producing strains are hindered by slow growth or difficulties with introduction of exogenous DNA. In addition, FK506 production is sensitive to a number of culture conditions including pH and the concentrations of lysine, nitrogen, and phosphate [20].

These challenges made FK506 an ideal candidate molecule for heterologous production via PAC based conjugation. Production of FK506 in *S. coelicolor* should enhance the ability to make targeted gene replacements or modifications and potentially provide new avenues for industrial production.

**Results**

**Generation of a PAC library using the P1-derived artificial chromosome pESAC13**

For the generation of a genomic library of *S. tsukubaensis* NRRL 18488, we used the phage P1-derived artificial chromosome vector (= PAC vector) pESAC13, depicted in Figure 1B. This vector was developed by M. Sosio and S. Donadio, NAICONS, Milano, Italy and is used by Bio S&T Inc. (Montreal, Canada; see below) for PAC library construction. pESAC13 contains the phage P1 origin of replication, as well as the phage phiC31 integration system that allows stable insertion into the genomes of most *Streptomyces* species. In contrast to previously developed *E. coli*-*Streptomyces* Artificial Chromosomes, pESAC13 (a derivative of pPAC-S1, [16]) contains an oriT site that allows transfer into *Streptomyces* by conjugation, rather than by time-consuming protoplast transformation. The bla resistance gene in pESAC13 is lost upon library construction; however, the vector also confers kanamycin resistance in *E. coli* and thiostrepton resistance in *Streptomyces*.

A major technical challenge in the generation of large insert libraries is the development of protocols for the isolation of high-molecular weight DNA, especially from uncharacterized actinomycetes where even cell lysis can be problematic. This can now be outsourced to companies specializing in this technique; in our case, Bio S&T Inc. (Montreal, Canada; www.biost.com). Starting from

![Figure 1. Phage P1-derived Artificial Chromosome (PAC)-based approach for heterologous expression of FK506. A) Structures of FK506 and FK520. B) Vector map of PAC pESAC13. C) Region of *Streptomyces tsukubaensis* NRRL 18488 genome included in PAC20N and used for heterologous expression. Gene colors refer to the following functions [30]: Yellow, allylmalonyl-CoA biosynthesis; dark blue, polyketide synthase; green, chorismate hydrolase (starter unit biosynthesis); brown, peptide synthetase; orange, post-PKS modification; purple, FK506 regulation; red, thioesterase; black, other genes. doi:10.1371/journal.pone.0069319.g001](http://www.plosone.org/figure/1.85211.5.69319/0069319.g001)
Materials and Methods) used as a control were moved into the expression studies. The smaller PAC, designated 20N, contained the 109347 bp clone depicted in Figure 1C and was chosen for heterologous expression. Both of these clones were end-sequenced, confirming the absence of insertions. Gel electrophoresis showed amplification with all three primer pairs. 768 PAC clones (≈ two 384 well plates) resulted in two clones showing amplification with all three primer pairs listed in Table 1 and shown in Figure 1C, amplifying fragments flanking and within the cluster. Screening of the first clone, designated 20N, contained the 109347 bp insert depicted in Figure 1C and was chosen for heterologous expression studies.

Identification of PAC clones containing the complete FK506 gene cluster

Comparison of the previously published sequences of the FK506 biosynthetic gene clusters from different producer strains [30], [36] confirmed that all known FK506 clusters contain a core set of genes including allA-allD, the three PKS genes fkbA-fkbC, and fkbG-fkbQ with certain strains also containing orthologs of the genes allMNPOS and the regulatory gene fkbR (Figure 1C). All of these genes are present in an 83.5 kb region of the S. tsukubensis genome. This region therefore contains all genes known to be involved in FK506 biosynthesis [29], [30] as well as allMNPOS, whose functions have not been elucidated. The recently published genome sequence of S. tsukubensis NRRL 18448 [36] allowed for bioinformatic investigation of the DNA regions that flank this core cluster. On the left side of the cluster depicted in Figure 1C a putative NRPS siderophore gene cluster was identified that contained putative regulatory genes, while on the right side no genes were identified with any obvious role in secondary metabolite biosynthesis.

The S. tsukubensis PAC library was screened with the three primer pairs listed in Table 1 and shown in Figure 1C, amplifying fragments flanking and within the cluster. Screening of the first 768 PAC clones (≈ two 384 well plates) resulted in two clones showing amplification with all three primer pairs. Gel electrophoresis suggested the inserts to be 160 and 110 kb in size, respectively. Both of these clones were end-sequenced, confirming that they spanned the DNA region containing the FK506 cluster. The smaller PAC, designated 20N, contained the 109347 bp insert depicted in Figure 1C and was chosen for heterologous expression studies.

Integration of the FK506 gene cluster into the genomes of engineered S. coelicolor host strains

S. coelicolor is the genetically best-characterized streptomycete, and a large number of tools are available for its genetic manipulation [37], [38], [39]. S. coelicolor M512 [40] has been used previously as a heterologous expression host [13]. Recently, M1146 was developed as an improved host; it lacks all of the genes required for the production of actinorhodin, the prodigines, coelincycin, methylenomycin and the calcium-dependent antibiotic, and thus lacks anti-microbial activity. The deletion of these biosynthetic gene clusters also removes unwanted sinks of carbon and nitrogen, and hence competition for precursors, and prevents potential cross-talk between different biosynthetic pathways that might result in product inactivation [5]. The strain also has a much simplified extracellular metabolite profile, greatly facilitating analysis of culture supernatants by mass spectrometry. Strains M1152 and M1154 both carry additional mutations in RNA polymerase (in the RpoB subunit) and M1154 also has a mutation in ribosomal protein S12. Both mutations were reported previously to increase the production levels of several secondary metabolites [17], [41], [42].

PAC clone 20N containing the FK506 cluster (PAC20N) as well as a corresponding empty pESAC13 vector (pESAC13Apr; see Materials and Methods) used as a control were moved into the non-methylating E. coli ET12567 strain [43] via triparental mating and introduced into S. coelicolor M512, M1146, M1152 and M1154 by conjugation, following the procedure summarized in Figure 2.

Heterologous production of FK506

Upon cultivation in MGm 2.5 medium [20], production of FK506 was detected in all strains containing PAC20N using HPLC-UV and HPLC-MS in comparison to an authentic FK506 standard (Inresa Pharma, Bartenheim, France). Figure 3 shows representative chromatograms from strain M1146/PAC20N. Figure S1 shows confirmation of the identity of FK506 by LCMS-MS analysis. No FK506 was detected after the introduction of pESAC13Apr into host strains.

Average production levels in exconjugants from strains M1146, M1152 and M1154 were 0.75, 2.81 and 2.06 mg L⁻¹, respectively (Figure 4A), clearly exceeding production in M512 (0.16 mg L⁻¹). As observed previously [4], production levels varied among independent exconjugants (e.g. 0.53, 0.68 and 1.05 mg L⁻¹ for three exconjugants of M1146) but were reproducible in repeated cultivations of the same exconjugant strain. A smaller amount of the related compound FK520 was also produced in the strains containing PAC20N, amounting to approximately 10% of FK506 production (Figure 3). This FK506:FK520 production ratio was comparable to that observed in Streptomyces tsukubensis NRRL 18448 under the same growth conditions.

Growth rate and production were most reproducible in strain M1146, therefore S. coelicolor M1146/PAC20N exconjugant #3 (1.05 mg L⁻¹) was chosen for subsequent experiments to determine if FK506 yields in S. coelicolor could be improved by over-expression of FK506 regulatory genes.

Over-expression of fkbN increases FK506 production levels

FK506 production in wild-type Streptomyces strains appears to be positively regulated primarily by the LuxR-type transcriptional regulator FkbN. Over-expression of fkbN increased FK506 yields in S. tsukubensis NRRL 18448 [33] and Streptomyces sp. KCTC 11604BP [32] and fkbN is present in all other identified FK506 gene clusters [30]. We introduced fkbN into S. coelicolor M1146/PAC20N#3 using the replicative, conjugal expression plasmid pUWL-on-T-apr containing the strong constitutive ermEβ promoter [44] and monitored FK506 production levels as above. While growth rates and biomass accumulation were comparable, over-expression of fkbN resulted in approximately five-fold FK506 yield improvements (5.5 mg L⁻¹ compared to 1.2 mg L⁻¹ observed upon introduction of an empty pUWL-on-T-apr plasmid; Figure 4B), demonstrating that fkbN over-expression is also beneficial to FK506 yields when the cluster is expressed heterologously.

The LysR-type regulator FkbR has also been shown to influence FK506 production levels, although this gene or its homologues have not been observed in all FK506 gene clusters [30]. In Streptomyces sp. KCTC 11604BP, over-expression of the fkbR homologue tcs7 decreased FK506 production, while tcs7 deletion elevated FK506 yields [32]. In contrast, in S. tsukubensis NRRL 18448 over-expression of fkbR increased FK506 production levels, while deletion had the opposite effect [33]. We introduced fkbR into S. coelicolor M1146/PAC20N#3 in the same manner as fkbN; the fkbR, but observed a decrease of 44% (0.65 mg L⁻¹, Figure 4B). It appears that constitutive, high fkbR expression in S. coelicolor is detrimental to FK506 yields.

We also generated a pUWL-on-T-apr expression plasmid containing tandemly arranged fkbN and fkbR (each with the same ribosome binding site that was used for their individual expression) that resulted in an increase of FK506 production (2.86 mg L⁻¹),...
but clearly less than the highest yields obtained when over-expressing fkbN alone (Figure 4B). This is consistent with the positive regulatory effect of fkbN over-expression being counteracted by a negative effect of constitutive expression of fkbR.

Discussion

FK506 (tacrolimus) is the most important immunosuppressant in clinical use [45]. Substantial effort has led to yield improvements in wild-type producers through regulator manipulation [32], [33], precursor supplementation into culture media [31], [34], [46] or by introducing extra copies of FK506 biosynthetic enzymes [35]. To the best of our knowledge, our PAC-based conjugation strategy has afforded the first example of heterologous production yields. However, FK506 production decreased upon over-expression of fkbR in *S. coelicolor* M1146/PAC20N/3, which is contrary to recent evidence showing the positive effect of fkbR on FK506 production levels in *S. tsukubaensis* NRRL 18488 [33]. This result may indicate that fkbR functions differently in *S. coelicolor*, perhaps by affecting an unknown component of global regulation that has some influence on FK506 biosynthesis. It is also possible that high level expression of fkbR is detrimental to FK506 biosynthesis, and that this regulator might normally be transcribed at lower levels and/or in a time-controlled fashion. This finding may be reflected in the opposing conclusions reported in other studies of fkbR [32], [33].

The triparental mating and conjugation procedure we describe here for stable transfer of PAC DNA should be applicable to many other natural product gene clusters of biomedical and/or biotechnological interest. Actinobacteria are among the most prolific microbial producers of natural products and the majority of known antibiotics are derived from them [47], [48]. Several antibiotics from actinobacteria are encoded by large gene clusters of known antibiotics are derived from them [47], [48]. Several antibiotics from actinobacteria are encoded by large gene clusters [32], [33], [34], [46] and rubradirin (105 kb, [2]), and could also be useful targets for heterologous expression via PAC cloning. With increasing amounts of data available from genome sequencing projects, PAC-based strategies hold significant value and promise in simplifying subsequent biosynthetic investigations. These methods may be especially valuable when large...
unidentified gene clusters appear to be “silent”, only produce trace amounts of compound that complicate or prohibit structural elucidation, or are found in rare or intractable bacterial strains that do not have any genetic techniques established. In addition, PAC-based conjugation could serve as a means of mobilizing large artificially derived natural product gene clusters as gene synthesis technologies become more affordable. Future work with the PAC containing the FK506 gene cluster or with other PACs containing gene clusters of high relevance could also be targeted at improving industrial compound yields by testing a variety of heterologous hosts and eventually lowering production costs.

We envision that large insert DNA libraries will become a standard complement to microbial genome sequencing projects in the near future [6]. The amount of available sequence information outpaces experimental verification of gene function, and although the quality of bioinformatics-based prediction of natural product gene clusters has advanced tremendously over the last decade [50] strategies to accelerate gene cluster identification and structure elucidation and increase compound production are urgently needed. The PAC based methodology described here to mobilize natural product gene clusters of virtually any size to engineered heterologous Streptomyces production strains should contribute to shortening this timeline and remove impediments to genetic or system biology studies aimed at improving access to compounds of interest.

Materials and Methods

Antibiotic selection
Apramycin (50 µg ml⁻¹), carbenicillin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), nalidixic acid (25 µg ml⁻¹) and thiostrepton (10 µg ml⁻¹) were added to growth media for appropriate selection as required unless otherwise noted.

Preparation of Phage P1 Artificial Chromosome (PAC) library
Streptomyces tsukubaensis NRRL 18488 was obtained from the US Department of Agriculture strain collection and grown on ISP4 plates [20]. Mycelia were prepared for PAC library construction by growing S. tsukubaensis in YEME medium (100 ml) inoculated with mycelia from a TSB starter culture (10 ml, [37]). The mycelia were grown in YEME for 72 h at 30°C in a 300 ml baffled flask with a stainless steel spring, and were then centrifuged to remove the media. The mycelial pellets were washed three times (200 mM NaCl, 10 mM Tris-Cl (pH 7.2), 100 mM EDTA (pH 8.0)) and flash frozen in liquid nitrogen before being stored at −80°C. PCR primers (Table 1) were designed for PAC library screening to isolate positive clones containing the entire FK506 gene cluster, and were tested beforehand using S. tsukubaensis genomic DNA as PCR template. Each PCR product was sequenced to verify that the intended regions of the gene cluster were amplified.
Approximately 7 ml of concentrated mycelia were used to construct a phage P-1 artificial chromosome library using a modified E. coli – Streptomyces Artificial Chromosome (ESAC, [16]) vector (pESAC13, Figure 1B). This vector contains an oriT, a phiC31 integrase gene and a phiC31 attP site that permit conjugation into Streptomyces strains and integration at the attB recombination locus.

The pESAC13 library construction was performed by Bio S&T (Montreal, Canada). Vector DNA was digested with BamHI, dephosphorylated and purified using standard procedures. High-molecular-weight (HMW) DNA was isolated by embedding the mycelium in 1% low-melting-point agarose plugs that were treated with proteinase K overnight, and then partially-digested with Sau3AI. The partially digested HMW DNA was size-fractionated on a 1% (w/v) pulsed field agarose gel in 0.5X TBE using a CHEF DRIII (Bio-Rad, Canada). The 100–250 kb DNA fragments were eluted from the gel by PFGE and dialyzed against 1X TE (10 mM Tris-HCl, 1 mM EDTA, (pH 8.0)) prior to ligation with the vector. The ligation mix was used to transform 20 μl of E. coli DH10β cells (Invitrogen, USA) by electroporation using a CellPorator equipped with a voltage booster (Invitrogen, USA). Transformants were selected at 37°C on LB medium supplemented with 20 μg ml⁻¹ kanamycin and 5% sucrose. For quality control, insert size was determined by DraI or NotI digestion of mini-prepped PAC DNA and subsequent PFGE gel separation. The average insert size of the pESAC library was estimated to be 125 kb.

Two PACs (approximated by gel electrophoresis to contain inserts of 160 and 110 kb) were identified by PCR screening as likely to contain the entire FK506 gene cluster. Both PACs were end sequenced, and the smaller of the two PACS (PAC20N, determined by end sequencing to contain a 109347 bp insert) was chosen for heterologous expression (Figure 1C). The DH10β E. coli strain containing PAC20N was used in a triparental mating (Figure 2) with another DH10β E. coli strain that contained the driver plasmid pR9406 (derived from pUB307 [51]), in which the kanamycin marker has been replaced with carbenicillin and the non-methylating E. coli strain ET12567. Using overnight starter cultures, these three strains were grown to an OD₆₀₀ of 0.4 and mixed together on LB plates without antibiotic selection. After 24 h, the resulting E. coli patch was streaked on selective (kan R, carb R, chl R) LB plates. The resulting ET12567 exconjugants were tested by PCR to confirm that they contained the entire PAC20N clone insert. To create a negative control strain to use in parallel to the PAC20N clone, pESAC13 was digested with BamHI and ScaI and religated to remove the bla resistance gene. This empty vector, pESAC13bla, was also transferred to ET12567 by triparental mating for later conjugation into S. coelicolor.

Introduction of PACs into S. coelicolor strains

The ET12567 strains containing pR9406 and either PAC20N or pESAC13bla were conjugated with germlings of S. coelicolor M512, M1146, M1152, and M1154 [5] according to standard protocols [37] (Figure 2). Following conjugation, mixtures of spores and E. coli were plated on R2 medium without sucrose and overlaid after 16-20 h with thiostrepton (60 μg ml⁻¹) and nalidixic acid (25 μg ml⁻¹). Exconjugants were streaked on DNA plates containing thiostrepton and nalidixic acid at the same concentrations, and then on MS (SFM) plates with 10 mM MgCl₂ and nalidixic acid to allow colonies to sporulate. Three sporing colonies from each S. coelicolor strain were spread on new MS+MgCl₂+nalidixic acid plates and allowed to grow for 5–
7 d prior to harvesting spores for inoculating cultures. After harvesting, dilution series of spores were spread on MS plates to obtain concentrations for each spore stock. Spores \((5 \times 10^8)\) of each strain were inoculated into 50 ml 1:1 TSB:YEME cultures containing thiostrepton \((10 \mu \text{g ml}^{-1})\). Each culture was grown for 3 d at 30°C in baffled flasks as described above. The mycelia were collected and frozen overnight in 20% peptone \((7.5 \text{ ml total volume in 50 ml Falcon tubes})\). The mycelia were subsequently homogenized \([52]\) and stored at \(-20°C\) to serve as stocks for inoculation of production medium. To measure heterologous production of FK506, each strain was grown in 24-well microtiter plates \((28°C, 300 \text{ RPM}, [4,52])\) for 6 days in MGm 2.5 medium \([20]\) with minor modification. Each well of the 24-well microtiter plate contained 3 ml of MGm 2.5 media and was inoculated with 75 μl of homogenized mycelia \(3\) wells per culture replicate. For quantification of FK506 production in each \(S.\ coelicolor\) strain, PAC20N and pESAC13Abla integrants were grown in triplicate.

**Detection of FK506 and FK520**

Culture samples from each \(S.\ coelicolor\) strain were extracted with ethyl acetate \((1:1)\) and each extract was reduced to dryness using a speed vacuum system. Samples were resuspended in 100 μl of methanol and profiled using HPLC \((25 \mu \text{l injections})\) and LCMS \((2.5 \mu \text{l injections})\) in comparison to standards of FK506 (Inresa Pharma, Bartenheim, France) and FK520 (Sigma). An HPLC solvent system of A) \(\text{H}_2\text{O}\) with 0.01% TFA and B) 20% methyl tert-butyl ether \((\text{MTBE})\) in acetonitrile \((\text{ACN})\) was used to resolve the FK520 and FK506 peaks \((43%-100\% \text{ B:A over } 35 \text{ min}, 0.4 \text{ ml min}^{-1}, 55°C)\) using a ReproSil-Pur Basic C18-HD, 3 μm, 150×3 mm HPLC column \((\text{Dr. Maisch GmbH, Ammerbuch-Entringen, Germany})\). LCMS analysis of these samples was performed with an Agilent HPLC-ESI-MS system \((\text{LC/MSD Ultra Trap System XCT 6330, Waldbronn, Germany})\), using a gradient of A) \(\text{H}_2\text{O}\) with 0.1% formic acid and B) 0.06% formic acid in ACN \((50\%-100\% \text{ B:A over } 20 \text{ min}, 100\% \text{ B to } 22 \text{ min}, 0.4 \text{ ml min}^{-1}, 40°C)\) on a Nucleosil 100 C18 3 μm column \((100 \times 2 \text{ mm ID})\) with a precolumn \((10 \times 2 \text{ mm ID})\) \((\text{Dr. Maisch})\). Detection of \(m/z\) values consistent with FK506 and FK520 was conducted with Agilent DataAnalysis for 6300 Series Ion Trap LC/MS 6.1 ver. 3.4 software \((\text{Bruker-Daltonik GmbH})\).

**Genetic modifications to \(S.\ coelicolor\) M1146 containing the FK506 PAC**

The genes encoding the FK506 regulators FkbN and FkbR were PCR amplified from the FK506 PAC and ligated independently into separate pUWL-oriT-apr vectors containing the \(\text{ermE}^*\) promoter \([44],[53]\). The forward primer for each amplicon \((\text{Table 1})\) contained a \(\text{Streptomyces}\) ribosome binding site \([54]\). These constructs were verified by sequencing and conjugated, along with pUWL-oriT-apr as a control, into \(S.\ coelicolor\) M1146/PAC20N exconjugant #3 using standard protocols \([37],[43]\). This \(S.\ coelicolor\) M1146 FK506 derivative had shown the highest levels of FK506 production when assaying \(S.\ coelicolor\) M1146/PAC20N exconjugants in preliminary experiments. Another pUWL-oriT-apr construct was created containing both fkbN and fkbR, with each gene preceded by the same ribosome binding site as above \([54]\). All plates, preculture media and production media used for...
References

1. Medini D, Serruto D, Parkhill J, Donati C, et al. (2008) Microbiology in the post-genomic era. Nat Rev Microbiol 6:419–430.

2. Kim CG, Lammichhane J, Song Kl, Nguyen VD, Kim DH, et al. (2008) Biosynthesis of rubruradin as an ansamycin antibiotic from Streptomyces achromogenes var. rubruradin NRRL3061. Arch Microbiol 189:463–473.

3. Laureti L, Song L, Huang S, Corre C, Leblond P, et al. (2011) Identification of a bioactive 51-membered macrolide complex by activation of a silent polypeptide synthase in Streptomyces antibioticus. Proc Natl Acad Sci USA 108:6256–6263.

4. Flinspach K, Westrich L, Kaysser L, Siebenburg S, Gomez-Escribano JP, et al. (2010) Heterologous expression of the biosynthetic gene clusters of cenomycin A1, chlorobiocin and caprazamycins in genetically modified Streptomyces coelicolor strains. Biopolymers 93:823–832.

5. Gomez-Escribano JP, Bibb MJ (2011) Engineering Streptomyces coelicolor for heterologous expression of secondary metabolite gene clusters. Microb Biotechnol 4:207–213.

6. Komatsu M, Komatsu K, Koizumi H, Yamada Y, Kosone I, et al. (2013) Engineered Streptomyces avermitilis host for heterologous expression of biosynthetic gene cluster for secondary metabolites. ACS Synth Biol. doi: 10.1021/sb3001003.

7. Gust B (2009) Cloning and analysis of natural product pathways. Meth Enzymol 40:1249–1255.

8. Kim EJ, Lee JH, Choi H, Pereira AR, Ban YH, et al. (2012) Heterologous production of 4-O-demethylbarbamide, a marine cyanobacterial natural product. Org Lett 14:5824–5827.

9. Dangel V, Harle J, Goerke C, Wolz C, Gust B, et al. (2009) Transcriptional regulation of the novobiocin biosynthetic gene cluster. Microbiol 155:4025–4033.

10. Gust B (2009) Cloning and analysis of natural product pathways. Meth Enzymol 40:1249–1255.

11. Kim JH, Feng Z, Bauer JD, Kallifidas D, Calle PY, et al. (2010) Cloning large DNA gene clusters back together with TAR. Biopolymers 93:833–844.

12. Heide L (2009) Aminocoumarins: Mutasynthesis, chemoenzymatic synthesis, and revision of peptide stereochemistry. Microbiol 151:1507–1523.

13. Heide L (2009) Aminocoumarins: Mutasynthesis, chemoenzymatic synthesis, and revision of peptide stereochemistry. Microbiol 151:1507–1523.

14. Binz TM, Wenzel SC, Apotheke H-JS, Bechthold A, Muller R (2008) Engineered Streptomyces coelicolor as an expression host for heterologous gene clusters. Methods Enzymol 459:437–455.

15. Eustaquio AS, Gust B, Galin U, Li S-M, Chater KF, et al. (2005) Heterologous expression of novobiocin and chlorobiocin biosynthetic gene clusters. Appl Environ Microbiol 71:2452–2459.

16. Pinna XB, White DF (2010) Recent application of metagenomic approaches toward the discovery of antimicrobials and other bioactive small molecules. Curr Opin Microbiol 13:603–609.

17. Kim JH, Zhou Z, Barner JD, Kallifidas D, Calle PY, et al. (2010) Cloning large natural product gene clusters from the environment: Piecing environmental DNA gene clusters back together with TAR. Biopolymers 93:833–844.

18. Heide L (2009) Aminocoumarins: Mutasynthesis, chemoenzymatic synthesis, and metabolic engineering. Methods Enzymol 459:437–455.

19. Binz TM, Wenzel SC, Apotheke H-JS, Bechthold A, Muller R (2008) Engineered Streptomyces coelicolor as an expression host for heterologous gene clusters. Methods Enzymol 459:437–455.

20. Eustaquio AS, Gust B, Galin U, Li S-M, Chater KF, et al. (2005) Heterologous expression of novobiocin and chlorobiocin biosynthetic gene clusters. Appl Environ Microbiol 71:2452–2459.

21. Kosec G, Goranovic D, Mrak P, Fujs S, Kuscer E, et al. (2012) Novel chemobiosynthetic approach for exclusive production of FK506. Metabolites 2:421–435.

22. Dumont FJ (2000) FK506, an immunosuppressant targeting calcineurin function. Curr Med Chem 7:731–748.

23. Kosec G, Goranovic D, Mrak P, Fujs S, Kuscer E, et al. (2012) Novel chemobiosynthetic approach for exclusive production of FK506. Metabolites 2:421–435.

24. Michel G, Menery L, Honeby B, Ruizciza T (1996) FK506 in the treatment of inflammatory skin disease: Promises and perspectives. Immunol Today 17:106–110.

25. Remitz A, Reitano S (2009) Long-term safety of tacrolimus ointment in atopic dermatitis. Expert Opin Drug Saf 8:501–506.

26. Perzyna R, Warnier M, Tillekeratne MPM, Shou W, Sanchez ER (2007) The immunophilin ligands cyclosporin A and FK506 suppress prostate cancer cell growth by androgen receptor-dependent and –independent mechanisms. Endocrinol 148:4716–4726.

27. Littner A, Herdegen T (2003) FK506 and its analog — Therapeutic potential for neurological disorders. Curr Drug Targets CNS Neurol Disord 2:153–162.

28. Andexer JN, Kendrew SG, Nur-e-Alam M, Lazos O, Foster TA, et al. (2011) Biosynthesis of the immunosuppressants FK506, FK520, and rapamycin involves a previously undescribed family of enzymes acting on chorismate. Proc Natl Acad Sci USA 108:4776–4781.

29. Goranovic D, Kosec G, Mrak P, Fujs S, Harvel J, et al. (2010) Origin of the allyl group in FK506 biosynthesis. J Biol Chem 285:14292–14300.

30. Mo S, Kim DH, Lee JH, Park JW, Banet DB, et al. (2011) Biosynthesis of the allylmalonyl-CoA extender unit for the FK506 polypeptide synthase proceeds through a dedicated polypeptide synthase and facilitates the mutasynthesis of analogs. J Am Chem Soc 133:976–985.

31. Kosec G, Goranovic D, Mrak P, Fujs S, Kuscer E, et al. (2012) Novel chemobiosynthetic approach for exclusive production of FK506. Metabolites 2:421–435.

32. Mo S, Yoo YJ, Ban YH, Lee SK, Kim E, et al. (2012) Roles of fkbN in positive regulation and afsR in negative regulation of FK506 biosynthesis in Streptomyces sp. KCTC 11604BP. Appl Environ Microbiol 78:2224–2253.

33. Goranovic D, Bialoz M, Magdevska V, Horvat J, Kuscer E, et al. (2012) FK506 biosynthesis is regulated by two positive regulatory elements in Streptomyces tukhabanatis. BMC Microbiol 12:238.

34. Mo S, Ban-HY, Park JW, Yoo YJ, Yoon YJ (2009) Enhanced FK506 production in Streptomyces clavuligerus CKD1119 by engineering the supply of methylmalonyl-CoA precursor. J Ind Microbiol Biotechnol 36:1473–1482.

35. Chen D, Zhang Q, Zhang Q, Chen P, Xu Z, et al. (2012) Improvement of FK506 production in Streptomyces tukhabanatis by genetic enhancement of the supply of unusual polypeptide extender units via utilization of two distinct site-specific recombination systems: Appl Environ Microbiol 76:5095–5103.

36. Barreiro C, Prieto C, Solá-Landa A, Solera E, Martínez-Castro M, et al. (2012) Draft genome of Streptomyces tukhabanatis NRRL 18481, the producer of the clinically important immunosuppressant tacrolimus (FK506). J Bacteriol 194:3756–3757.

37. Kieser T, Bibb MJ, Buttern MJ, Chater KF, Hopwood DA (2000) Practical Streptomyces Genetics, 2nd edition, Norwich, UK: John Innes Foundation.

38. Benefield SD, Chater KF, Cerdeño-Tarraga AM, Challis GL, Thomson NR, et al. (2002) Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). Nature 417:141–147.

39. Kieser T, Bibb MJ, Buttern MJ, Chater KF, Hopwood DA (2000) Practical Streptomyces Genetics, 2nd edition, Norwich, UK: John Innes Foundation.

40. Andexer JN, Kendrew SG, Nur-e-Alam M, Lazos O, Foster TA, et al. (2011) Biosynthesis of the immunosuppressants FK506, FK520, and rapamycin involves a previously undescribed family of enzymes acting on chorismate. Proc Natl Acad Sci USA 108:4776–4781.

41. Andexer JN, Kendrew SG, Nur-e-Alam M, Lazos O, Foster TA, et al. (2011) Biosynthesis of the immunosuppressants FK506, FK520, and rapamycin involves a previously undescribed family of enzymes acting on chorismate. Proc Natl Acad Sci USA 108:4776–4781.
41. Hu H, Zhang Q, Ochi K (2002) Activation of antibiotic biosynthesis by specified mutations in the rpoB gene (encoding the RNA polymerase beta subunit) of Streptomyces lividans. J Bacteriol 184:3984–3991.
42. Shima J, Heskerh A, Okamoto S, Kawamoto S, Ochi K (1996) Induction of actinorhodin production by rpsL (encoding ribosomal protein S12) mutations that confer streptomycin resistance in Streptomyces lividans and Streptomyces coelicolor A3(2). J Bacteriol 178:7276–7284.
43. Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted Streptomyces gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 100:1541–1546.
44. Erb A, Luzhetskyy A, Harder U, Bechthold A (2009) Cloning and sequencing of the biosynthetic gene cluster for saquayamycin Z and galtamycin B and the elucidation of the assembly of their saccharide chains. ChemBioChem 10:1392–1401.
45. Polvino W (2012) Reformulations must provide added value: Novel drugs must really improve on the original; dosing convenience alone is not enough. Genetic Engineering and Biotechnology News 32: Feb 15.
46. Turlo J, Gazierska W, Klimaszewska M, Król M, Daszewska M (2012) Enhancement of tacrolimus productivity in Streptomyces tsukubaensis by the use of novel precursors for biosynthesis. Enzyme Microb Technol 51:388–395.
47. Baltz RH (2008) Renaissance in antibacterial discovery from actinomycetes. Curr Opin Pharmacol 8:557–563.
48. Nett M, Ikeda H, Moore BS (2009) Genomic basis for natural product biosynthetic diversity in the actinomycetes. Nat Prod Rep 26:1362–1384.
49. Yin X, Zabriskie TM (2006) The enduracidin biosynthetic gene cluster from Streptomyces fungidicus. Microbiol 152:2969–2983.
50. Medema MH, Blin K, Cimermancic P, de Jager V, Zakrewski P, et al. (2011) antiSMASH: Rapid identification, annotation, and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 39 (suppl 2):W339–W346.
51. Piffaretti J-C, Arini A, Frey J (1988) pUB307 mobilizes resistance plasmids from Escherichia coli into Neisseria gonorrhoeae. Mol Gen Genet 212:215–218.
52. Siebenberg S, Bapat PM, Lantz AE, Gust B, Heide L (2010) Reducing the variability of antibiotic production in Streptomyces by cultivation in 24-square deepwell plates. J Biotechnol Bioeng 109:230–234.
53. Bibb MJ, White J, Ward JM, Janssen GR (1994) The mRNA for the 23S rRNA methylase encoded by the ermE gene of Saccharopolyspora erythraea is translated in the absence of a conventional ribosome-binding site. Mol Microbiol 14:533–545.
54. Heazi S, Hashimoto Y, Higashibata H, Maseda H, Ikeda H, et al. (2004) Hyper-inducible expression system for streptomycetes. Proc Natl Acad Sci USA 101:14031–14035.