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Authors(s)	Carmody, Maria, Byrne, Barry, Murphy, Barry, Breen, Ciaran, Lynch, Susan, Flood, Elizabeth, Finnan, Shirley, Caffrey, Patrick
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Analysis and manipulation of amphotericin biosynthetic genes by means of modified phage KC515 transduction techniques.

Key words: KC515-pACYC177 hybrid vector; engineered biosynthesis; novel pentaene.

Maria Carmody, Barry Byrne, Barry Murphy, Ciaran Breen, Susan Lynch¹, Elizabeth Flood², Shirley Finnan³ and Patrick Caffrey*.

Department of Industrial Microbiology, Centre for Synthesis and Chemical Biology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin 4, Ireland.

*Correspondence: Patrick Caffrey, Department of Industrial Microbiology, University College Dublin, Belfield, Dublin 4, Ireland.

Telephone: ++ 353 1 716 1396

FAX: ++ 353 1 716 1183

E-mail: patrick.caffrey@ucd.ie

¹Present address: Department of Microbiology and Immunology, Stanford School of Medicine, Stanford University, California 94305-5124, USA.

²Present address: Eugene McDermott Centre for Human Growth and Development, University of Texas Southwestern Medical Centre at Dallas, Texas 75390-8591, USA.

³Present address: Department of Microbiology, University College Cork, Cork, Ireland.

Non-standard abbreviations: PEG, polyethylene glycol; DMSO, dimethylsulphoxide; HTH, helix-turn-helix; PKS, polyketide synthase; DIG, digoxigenin; PCR, polymerase chain reaction; KS, ketosynthase; AT, acyl transferase; DH, dehydratase; ER, enoyl reductase; KR, ketoreductase; ACP, acyl carrier protein.

Abstract

Amphotericin B is a medically important antifungal antibiotic that is produced by *Streptomyces nodosus*. Genetic manipulation of this organism has led to production of the first amphotericin analogues by engineered biosynthesis. Here, these studies were extended by sequencing the chromosomal regions flanking the amphotericin polyketide synthase genes, and by refining the phage KC515 transduction method for disruption and replacement of *S. nodosus* genes. A hybrid vector was constructed from KC515 DNA and the *Escherichia coli* plasmid pACYC177. This vector replicated as a plasmid in *E. coli* and the purified DNA yielded phage plaques on *Streptomyces lividans* after polyethylene glycol (PEG)-mediated transfection of protoplasts. The left flank of the amphotericin gene cluster was found to include *amphRI*, *RII*, *RIII* and *RIV* genes that are similar to regulatory genes in other polyene biosynthetic gene clusters. One these regulatory genes, *amphRI*, was found to have a homologue, *amphRVI*, located in the right flank at a distance of 127 kbp along the chromosome. However, disruption of *amphRVI* using the hybrid vector had no effect on the yield of amphotericin obtained from cultures grown on production medium.

The hybrid vector was also used for precise deletion of the DNA coding for two modules of the AmphC polyketide synthase protein. Analysis by UV spectrophotometry revealed that the deletion mutant produced a novel pentaene, with reduced antifungal activity but apparently greater water-solubility than amphotericin B. This shows the potential for use of the new vector in engineering of this and other biosynthetic pathways in *Streptomyces*.

1. Introduction

Polyene macrolides are antifungal agents that act by disrupting ergosterol-containing fungal membranes. In general, polyenes are highly active but their medical use is restricted by severe side effects that result from low water-solubility and interactions with cholesterol in mammalian cell membranes. Despite this toxicity, polyenes make up one of the most important classes of systemic antifungal antibiotics (Georgopapadakou and Walsh, 1996). The heptaene amphotericin B (Fig. 1) is the drug of choice for the treatment of life-threatening invasive fungal infections (Abu-Salah, 1996).

Polyenes are synthesised by several species from the *Streptomyces* genus of soil bacteria (Omura and Tanaka, 1986). Chemical modification of the original natural products can yield derivatives with improved therapeutic properties (Hartsel and Bolard, 1996). However, the structural complexity of polyenes has limited the number of analogues that can be made by chemical methods. Recent progress in genetic manipulation of polyene-producing streptomycetes indicates that a wider range of analogues can be produced by engineered biosynthesis. The biosynthetic gene clusters have been sequenced for nystatin, pimarinin, amphotericin, candicidin/FR008 and rimocidin, and methods have been developed for manipulation of the producing organisms (Aparicio et al., 2003; Chen et al., 2003; Seco et al., 2004; Nikodinovic et al., 2003). These advances have led to the construction of the first genetically modified strains that produce novel polyene analogues (Aparicio et al., 2003; Brautaset et al., 2002; Byrne et al., 2003).

Polyenes also have activity against *Leishmania* parasites, enveloped viruses, and prion diseases (Hartsel and Bolard, 1996). Novel polyenes may find application in other areas of clinical medicine, or as probes for investigating the molecular bases of these diseases.

The aim of this study was to complete the sequence analysis of the chromosomal region involved in amphotericin biosynthesis in *Streptomyces nodosus*, and to refine the phage KC515 transduction method for manipulation of these biosynthetic genes. We describe the construction of a new hybrid vector from phage KC515 DNA and the

Escherichia coli plasmid pACYC177. This was used to disrupt a regulatory gene and to modify the polyketide synthase (PKS) to engineer the biosynthesis of a novel pentaene.

2. Materials and Methods

2.1 Microbial strains, plasmids, phages

S. lividans 66 (John Innes strain 1326) was used for transfections and propagation of phage KC515 (Kieser et al., 2000). *S. nodosus* ATCC14899 (Gold et al., 1956) was used for disruption of the *amphRVI* gene and for deleting internal sequences from the *amphC* gene. *E. coli* XL1-Blue was used as a host for cloning. The hybrid vector was constructed from KC515 DNA and plasmid pACYC177 (Chang and Cohen, 1978). The cosmid library of *S. nodosus* DNA was that described previously (Caffrey et al., 2001). *Saccharomyces cerevisiae* was used as an indicator organism to test antifungal activity.

2.2 DNA methods

Digestion of DNA with restriction enzymes, agarose gel electrophoresis, ligations and transformation of *E. coli* were carried out by standard methods. The alkaline-SDS method was used to isolate plasmid and cosmid DNA from *E. coli* (Sambrook et al., 1989). Labelling of probes with digoxigenin (DIG)-dUTP and hybridization were carried out as described previously (Caffrey et al., 2001).

Oligonucleotides were ordered from Sigma-GenoSys. The polymerase chain reaction (PCR) was carried out with Deep Vent as thermostable DNA polymerase. Dimethylsulphoxide (DMSO) was included in reaction mixtures to a final concentration of 10% (v/v). This reduced formation of secondary structure in GC-rich primers.

DNA sequencing was carried out to publication standard by MWG Biotech. Sequences were analysed using BLAST (<http://www.ncbi.nlm.nih.gov>). The sequences flanking the left and right ends of the amphotericin cluster have the GenBank accession numbers **AY639386** and **AY636001** respectively.

2. 3 *Streptomyces genetics*

Phage KC515 DNA was extracted from phage particles as described previously (Hopwood et al., 1985). Transfections were carried out as described (Kieser et al., 2000), with a few modifications. Frozen protoplasts were found to give adequate numbers of plaques and were used routinely. Polyethylene glycol 1000 (PEG) was obtained from Fluka. Trace elements were omitted from P buffer used to make 25% (w/v) PEG solution and soft R2 top agar. In addition, DMSO was incorporated in the 25% (w/v) PEG solution to a final concentration of 1% (v/v). Recombinant phages were identified by PCR with oligonucleotide primers specific for the insert (Khaw et al., 2000). A 5 μ l volume of phage suspension was used as a source of template DNA for each of these PCRs.

Genomic DNA was isolated from *S. nodosus* lysogens and mutants as described (Hopwood et al., 1985). Revertants resulting from prophage excision were identified by tooth-picking regenerated protoplasts onto tryptic soy (TS) agar and TS agar containing 50 μ g thiostrepton per ml.

2. 4 *Analysis of polyene production*

To assess polyene production, *S. nodosus* and mutant strains were grown on fructose-dextrin soya medium (McNamara et al., 1998). Culture supernatants or sedimented mycelial cells were extracted with equal volumes of butanol. The UV absorption spectra of the extracts were scanned in the wavelength range 250 to 450 nm. Antifungal activity against *S. cerevisiae* was assessed by agar diffusion assay.

3. Results and discussion

3. 1 *Construction of a bifunctional vector from pACYC177 and KC515 DNA*

It has previously been shown that phage KC515 can be used to introduce DNA into *S. nodosus* for gene disruption and replacement (Byrne et al., 2003; Caffrey et al., 2001). However, extraction of KC515 vector DNA from phage particles is laborious and requires large quantities of *S. lividans* spores for preparation of high-titre phage suspensions. Bifunctional derivatives of Φ C31 have been described that replicate as

phages in streptomycetes and as plasmids in *E. coli*. Chater and co-workers previously inserted pBR322 into the genome of a Φ C31 derivative to generate KC100 (Chater, 1986). This construct was readily isolated from *E. coli* and was biologically active in streptomycetes. However, KC100 contains an *attP* site and is not used for insert-directed homologous recombination. Two *attP*-deleted derivatives of KC100, KC400 and KC200, retain the pBR322 replicon but were also considered unsuitable for genetic manipulation of *S. nodosus*. KC400 has a selectable marker that confers resistance to viomycin, an antibiotic that is not readily available. KC200 has a thiostrepton-resistance marker but also has a temperature-sensitive *cts1* mutation in the phage repressor gene. This raised concerns over whether the temperature-sensitive repressor would maintain stable *S. nodosus* lysogens. We therefore opted to construct a new hybrid vector by fusing KC515 to the low copy number *E. coli* plasmid pACYC177.

A 3021 bp *Bam*HI – *Pst*I fragment of pACYC177 (GenBank accession number = **X06402**) contains the p15 origin of replication, the kanamycin resistance gene and part of a β -lactamase gene. This fragment was purified and ligated to *Bam*HI – *Pst*I cut KC515 DNA (Fig. 2). Ligated DNA was introduced into *S. lividans* protoplasts by transfection. Plaques were picked into Difco nutrient broth and the resulting phage suspensions were screened by PCR with oligonucleotides KanF [5' ACGGGAAACGTCTTGCTCGA 3'] and KanR [5' CATGAGTGACGACTGAATCC 3'] specific for the kanamycin resistance gene sequence of pACYC177. DNA isolated from one positive phage was treated with T4 ligase, to ensure covalent joining of the *cos* ends, and then introduced into *E. coli* XL-Blue by transformation. Plasmid DNA was isolated and subjected to restriction analysis. The construct had the restriction map of the expected KC515-pACYC177 hybrid, except that a 0.3 kb contaminating *Pst*I fragment was present in the *Pst*I site. This fragment was deleted to generate the final construct that was named KC-UCD1. KC515 retains a 758 bp *Eco*RI - *Pst*I fragment of pBR322 (accession number = **J01749**) that contains the 5' end of a β -lactamase gene. Correct ligation of pACYC177 and KC515 results in reassembly of a functional β -lactamase gene spanning the *Pst*I site. KC-UCD1 therefore confers resistance to both ampicillin and kanamycin on *E. coli*. When isolated from *E. coli* this DNA was biologically active in *S. lividans* and gave plaques after transfection of protoplasts.

Attempts to subclone *Bam*HI or *Pst*I fragments in KC-UCD1 in *E. coli* XL-Blue were unsuccessful. However, fragments with compatible ends were readily cloned between the *Bam*HI and *Pst*I sites when *S. lividans* was used as a host. Ligated DNA yielded numerous plaques when introduced into *S. lividans* by transfection.

3. 2 Effect of DMSO on efficiency of transfection

In initial studies, the efficiency of transfection was extremely variable even when identical solutions were used. It has been reported that the quality of the PEG has a dramatic effect on the efficiency of PEG-mediated transfection and transformation (Hopwood et al., 1985; Kieser et al., 2000). We considered the possibility that problems with transfection might result from reactive oxygen species. PEG and related polyoxyethylene-based polymers like the detergent Triton X-100 frequently contain high levels of hydrogen peroxide that result from the action of oxygen on the polyoxyethylene chain (Lever, 1977; Ashani and Catravas, 1980). For this work, we used Fluka PEG which contained $\leq 0.001\%$ peroxides at the time of analysis by the manufacturers. A H_2O_2 concentration of 0.001% (w/w) is equivalent to 0.294 μmole per gram PEG, or a final concentration of 73.5 μM in the final solution of 25% (w/v) PEG in P buffer. Micromolar concentrations of hydrogen peroxide are sufficient to damage DNA (Nakakura et al., 2003). H_2O_2 generates extremely destructive hydroxyl radicals in the presence of iron via the Fenton reaction [$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\cdot$] (Halliwell and Gutteridge, 1990). P buffer contains Fe^{3+} as part of the trace elements solution. This can be reduced to Fe^{2+} by another reaction with hydrogen peroxide [$2\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow 2\text{Fe}^{2+} + \text{O}_2 + 2\text{H}^+$]. It is conceivable that other metals from the trace elements solution such as copper and manganese could also provide electrons for hydroxyl radical formation. Hydroxyl radicals could impair transfection by causing double strand breaks in transfecting DNA or by initiating lipid peroxidation in protoplast membranes. In an attempt to improve our transfection technique, trace elements were omitted from the P buffer used to prepare the 25% PEG solution and from the soft R2 agar used for plating transfected protoplasts. In addition, DMSO, a scavenger of hydroxyl radicals, was included in the PEG solution at a final concentration of 1% (v/v). These measures increased the efficiency and reproducibility of transfection so that about 1000 plaques

were consistently obtained per 10ng KC-UCD1 DNA. In early studies on *Streptomyces* genetics, 40% (w/v) PEG containing 15% (w/v) DMSO was used to bring about protoplast fusion (Hopwood et al., 1985; Kieser et al., 2000). With more recent protocols, the inclusion of DMSO in PEG solutions has not been considered important. However, our results suggest that DMSO can detoxify deteriorating batches of PEG.

For technical convenience, frozen protoplasts were used for most transfections. This reduced the efficiency by a factor of two. However, this reduction was not critical since when dephosphorylated vector was used, screening of 24 plaques by PCR usually yielded several isolates of the required recombinant phage.

3.3 Analysis of regions flanking the amphotericin PKS genes

The genes required for synthesis and export of amphotericin B have been sequenced (accession number = [AF357202](#)). Chromosome walking was carried out to clone the sequences flanking this 113,193 bp region. The previously-constructed cosmid library of *S. nodosus* genomic DNA was screened with probes derived from the *amphG* and *amphC* genes, which are located at opposite ends of the cluster (Caffrey et al., 2001). Hybridising cosmids were identified and partially characterized. This preliminary analysis indicated that relevant DNA extended for about 16kb along from *amphG* and for about 4 kb along from *amphC*. In keeping with the orientation of the amphotericin cluster in the GenBank database, the region adjacent to *amphG* will be referred to as the left end; the right end is then adjacent to *amphC*. Complete sequencing was carried out for 20832 bp of the *amphG* left flank region, and for 5811 bp of the *amphC* right flank region. Genes identified by homology searches are listed in Table 1 and their organization is shown in Fig. 3.

The left end contains a group of regulatory genes that is separated from *amphG* by an operon involved in activation and catabolism of free β -ketoacids. The Amph ORFs 8 and 9 are homologous to the two subunits of various CoA transferases that catalyse reversible transfer of CoA from one carboxylic acid to another. The most closely related of these (>64% identity) were bacterial β -keto adipate:succinyl-CoA transferase subunits with accession numbers [NP 746081](#) and [CAE40602](#). Transfer of CoA from succinyl CoA to a free β -ketoacid generates succinate and the corresponding β -ketoacyl CoA.

This allows for efficient activation of the β -ketoacids to CoA thioesters. ORF7 encodes a protein homologous to thiolases that catalyse cleavage of a β -ketoacyl CoA to generate acetyl CoA and an acyl CoA that is shortened by two carbon atoms. The most closely related is a β -ketoadipyl CoA thiolase from *S. coelicolor* (accession number = **CAB45575**). ORFs 7, 8 and 9 are adjacent to ORF6, which is in the opposite orientation and encodes a LysR-type transcriptional regulator. β -ketoadipate can be generated from breakdown of benzoate, 4-hydroxybenzoate or protocatechuate (Parales and Harwood, 1992; Iwagami et al., 2000) but it is not known whether the genes required are present in *S. nodosus*. A plausible role for the Amph ORFs 7, 8 and 9 is the formation of acetyl CoA from acetoacetate, which can be derived from catabolism of leucine and phenylalanine. Carbon from these amino acids is efficiently incorporated into ty lactone (Dotzlaef et al., 1984). ORFs 7, 8 and 9 could contribute to generation of acetyl CoA for polyketide chain assembly, or for other purposes. They are unlikely to have a dedicated or essential role in amphotericin biosynthesis. Since homologues have not been found within any of the polyene biosynthetic gene clusters, the embedding of these genes within the amphotericin cluster may not be significant.

The left flank then contains the *amphRI*, *RII*, *RIII* and *RIV* genes that are similar to the *nysRI*, *nysRII*, *nysRIII* and *RIV* regulatory genes of the nystatin cluster (accession number = **AF263912**) and the *fscRIV*, *fscRIII*, *fscRII* and *fscRI* genes of the FR008 / candicidin cluster (accession number = **AY3100323**) (Chen et al., 2003; Aparicio et al., 2003). The AmphR proteins show at least 40% sequence identity with their respective counterparts and all four have HTH motifs at the C-terminus. AmphRI and AmphRIII have Walker A and B type NTP-binding motifs near the N-terminus. The AmphRIV protein has a PAS sensor-binding domain in the N-terminus as well as the HTH motif. Zotchev and co-workers have extensively investigated the roles of the NysRI – RIV proteins in activating transcription of various nystatin biosynthetic genes (Sekurova et al., 2004). The organization of the *amphRI* – *RIV* genes suggests that overall regulation of *amph* gene expression is similar. The order of synthesis and export genes is similar in the nystatin and amphotericin clusters. However, the *amphRI* to *RIV* regulatory genes are located at the left *amphG* end of their cluster whereas the *nysRI* – *RIV* genes are located at the opposite *nysC* end. In addition, the *amphRI* – *RIV* operon includes an upstream

gene (*amph* ORF5) that appears to be translationally coupled to *amphRI*. ORF5 protein is 34% identical to a putative MarR-family transcriptional regulator of *S. coelicolor* (accession number = NP 825297).

The remaining DNA adjacent to *amphRI* contains an apparently non-coding region of 1113bp followed by genes for a methyl transferase, a hypothetical protein, a protein phosphatase and nitrate reductase. The sequences are included within the GenBank entry for the left flank (accession number = AY639386) but since these genes have no obvious role in amphotericin biosynthesis, they have been omitted from Fig. 3 and Table 1.

The right flank contains the *amphE* gene whose product is homologous to discrete thioesterases that probably remove non-productively decarboxylated extender acyl groups from the PKS (Kim et al., 2002). An adjacent gene, *amphRVI*, is closely related to *amphRI*. The translated protein products show 74% amino acid sequence identity, but the AmphRVI protein is slightly shorter and lacks the C-terminal DNA-binding HTH motif. ORF10, the next gene adjacent to *amphRVI*, encodes a protein that shows up to 85% sequence identity with numerous amino acid permeases and is probably not directly involved in amphotericin biosynthesis.

It has been suggested that protein-protein interactions occur between nystatin regulatory proteins (Sekurova et al., 2004). The absence of a DNA-binding domain from AmphRVI suggested that it might form inactive heterodimers or complexes with other AmphR proteins, and thereby have a negative effect on amphotericin biosynthesis. An internal fragment of the *amphRVI* gene was amplified by PCR with primers R6F [5' AAAACTGCAGCAAGAGCGTTCTCCTCGACACC 3'] and R6R [5' TTTTGGATCCGGTGACCACCGGGTTCAGTACAC 3']. The 2112 bp PCR product was digested with *Bam*HI and *Pst*I and cloned into KC-UCD1. The recombinant phage integrated into the *S. nodosus* chromosome to generate thiostrepton-resistant lysogens. The expected disruption involves integration of the phage into the 5.9 kb chromosomal *Bam*HI fragment covering the *amphRVI* gene, to form 4.1 and 41.5 kb *Bam*HI fragments containing partial *amphRVI* sequences interrupted by prophage DNA. Analysis of chromosomal DNA by Southern hybridization indicated that the phage had integrated correctly (not shown).

The *amphRVI* disruption mutant was grown on fructose-dextrin-soya production medium containing thiostrepton. Butanol extracts of these cultures were analysed by UV spectrophotometry. This revealed the tetraene and heptaene spectra characteristic of amphotericins A and B. The yields were comparable to those obtained from *S. nodosus* ATCC14899. This indicated that inactivation of *amphRVI* had no effect on amphotericin production under these conditions.

3. 4 Deletion of two modules from the *AmphC* polyketide synthase protein

The bifunctional vector was used further to carry out the first redesign of the amphotericin PKS. The aim of this experiment was to delete two modules to engineer biosynthesis of a pentaene. Contracting the ring size might reduce the antifungal activity but also increase water-solubility to give an overall improvement in the therapeutic index. In addition, pentaenes have a characteristic UV absorption spectrum that would allow rapid and sensitive initial detection of a novel product without recourse to purification and chemical characterization.

Early studies on engineering of modular PKSs have been included in a review by Staunton and Weissman (2001). It is known that small deletions from interdomain or intermodular linkers can impair the function of an engineered PKS module, so that product yields are reduced or abolished. This information was taken into account in designing a strategy for modifying the amphotericin PKS.

The *amphC* gene encodes a hexamodular PKS protein that catalyses cycles 3 to 8 in the assembly of the amphotericin polyketide chain. All six modules contain the PKS domains KS-AT-DH-KR-ACP. The third of these modules also contains an ER domain that is thought to be sluggish, sometimes acting during cycle 5 so that amphotericin A may ultimately be produced as well as amphotericin B (Caffrey et al., 2001). The *AmphC* modules are closely related and *KpnI* sites appear at corresponding positions at the ends of the coding sequences for the KS5, KS6, KS7, and KS8 domains. In each case, the *KpnI* site [5' GGTACC 3'] makes up the first two of the codons specifying a highly conserved amino acid sequence [GTNAHVILE] at the extreme C-terminus of the KS domain. Two of these sites were used to fuse the coding sequences for KS5 and AT7, so as to generate an apparently perfect hybrid module with an intact linker between these

domains (Fig. 4). In addition, the natural intermodular linker still precedes the KS5 domain of the hybrid module. The truncated AmphC protein was therefore expected to be functional.

The plasmid pUC-KR6DH7 contains a *Bam*HI fragment representing nucleotides 100297 – 105485 of the amphotericin cluster. This was linearised with *Eco*RI and then partially digested with *Kpn*I. One of the partial digestion products retained the coding sequence for the AT7-DH7 region attached to pUC118 (Fig. 4). This 5340 kb *Eco*RI - *Kpn*I fragment was purified by preparative agarose gel electrophoresis.

The plasmid subclone pUC-KS5 contains a *Kpn*I fragment (nucleotides 90620 – 91702, accession number = [AF357202](#)) that includes most of the KS5 coding sequence. This region was amplified by PCR with oligonucleotides 5F [5' CGGCGAATTCCTGCAGGACGCGGGCGACTTCGACCCCGAC 3'] and universal primer 5' CAGGAAACAGCTATGAC 3'. The resulting KS5 sequence had *Eco*RI and *Pst*I sites at the 5' end and the conserved *Kpn*I site near the 3' end (Fig. 4). The PCR product was cut with *Eco*RI and *Kpn*I and ligated to the *Eco*RI - partial *Kpn*I fragment containing the AT7-DH7 coding sequence and pUC118. The resulting construct (pUCM57) specified a hybrid protein in which KS5 and AT7 were fused *via* an intact KS-AT interdomain linker. This plasmid was digested with *Bam*HI and *Pst*I and the fragment containing the hybrid sequence was ligated to *Bam*HI – *Pst*I cut KC-UCD1 vector. The ligated DNA was introduced into *S. lividans* protoplasts by transfection. Recombinant phage containing the insert were identified by PCR with oligonucleotides 5F and 7R [5' TCTGCTCGGTGAGCAGTTCGACG 3'] as primers. One of these was designated KC-M57.

The expected gene replacement is shown schematically in Fig. 5. Phage KC-M57 was propagated on *S. nodosus* and thiostrepton-resistant lysogens were selected. Genomic DNA was isolated from two lysogens and correct integration of the phage was confirmed by Southern blotting (not shown). One lysogen was propagated in the absence of thiostrepton for several generations. Strains that had undergone a second recombination event were identified by screening for loss of thiostrepton resistance. These thiostrepton-sensitive strains were assessed by analyzing genomic DNA by PCR. Primers 5F and 5R [5' TGA~~CTT~~GCCGGAGAGGATCAG 3'] were designed to detect

the wild-type KS5-AT5 region and primers 5F and 7R were designed to detect revertants containing the KS5-AT7 sequence (Fig. 6). Of twenty revertants, only one had undergone the correct gene replacement. This mutant was analysed further by Southern hybridization (Fig. 7).

The required recombination events should result in replacement of 5.0 and 8.9 kb chromosomal *Nco* I fragments with a 2.5 kb *Nco* I fragment (Fig. 5). Samples of genomic DNA from *S. nodosus* ATC14899 and the mutant were digested with *Nco* I and probed with a labelled AT7 fragment. Analysis of the mutant DNA revealed loss of an 8.9 kb fragment and appearance of a 2.5 kb fragment (Fig. 7). Both wild-type and mutant DNA contained a 5.3 kb band that hybridised to the probe. This was tentatively identified as the 5308 bp *Nco* I fragment containing the coding sequence for AT8, which is closely related to that for AT7. The wild-type DNA also contained a 5 kb band that was absent from the deletion mutant. This may represent the 5013 bp *Nco* I fragment that contains the KS5 coding sequence (Fig. 5). These results indicated that gene replacement had occurred correctly.

3. 5 Analysis of products made by M57 mutant

The revertants were grown on production medium and polyenes were extracted with butanol. Extracts were analysed for polyene content by UV spectrophotometry. Amphotericin B has specific absorption maxima at 346, 364, 382 and 405nm, whereas the tetraene amphotericin A absorbs at 280, 292, 305 and 320nm (McNamara et al., 1998). Extracts from wild-type *S. nodosus* control cultures showed mixed spectra indicating production of amphotericin B along with smaller quantities of amphotericin A (not shown). Extracts from the M57 mutant showed a UV absorption spectrum characteristic of a pentaene (Omura and Tanaka, 1984) with absorption maxima of 310, 318, 333, and 352nm (Fig. 8). The yields of this product remained high in spite of the deletion from the PKS. This suggests that the hybrid module is indeed functional and that the downstream modules tolerate the nascent oligoketide intermediates containing a shortened polyene unit.

The pentaene was detected exclusively in culture supernatant fractions, indicating that it had either increased water-solubility or decreased tendency to aggregate.

Amphotericins A and B normally sediment with the mycelial fraction of producing cultures. Aggregation and low water-solubility increase the toxicity of amphotericin B (Kawabata et al., 2001).

The initial butanol extracts typically gave an A^{352} of around 1.0 after 1:20 dilution in butanol. Extracts were tested for antifungal activity in agar diffusion assays with *Saccharomyces cerevisiae* as an indicator organism. Inhibition zones were just visible around wells containing undiluted butanol extracts, indicating that the crude pentaene had low antifungal activity. Further work will be required to purify the pentaene. The availability of purified material will allow determination of the minimum inhibitory concentration as well as initiation of structural studies.

During the late stages of amphotericin biosynthesis, the macrolactone core undergoes three late modifications: oxidation of a C-16 methyl branch to a carboxyl group, glycosylation with mycosamine and hydroxylation at C-8. Fig. 9 shows the predicted structure for the pentaene which assumes that post-PKS modifications proceed normally. Contracting the polyene unit might distort the polyol chain so that the ring no longer has the shape required for interaction with sterols or for stable channel formation. Antifungal activity might be restored by contracting the polyol chain. This could be done by deleting two of modules 13 to 18, or by re-positioning the thioesterase domain.

Deletion of module 5 from the nystatin PKS resulted in synthesis of a novel hexaene (Brautaset *et al.*, 2002). Chemical analysis of this compound indicated that the hexaene underwent late modifications, although glycosylation and polyol chain hydroxylation were less efficient than with the normal 38-membered macrolactone substrate. The hexaene also showed decreased antifungal activity.

4. Conclusions

Vectors based on phage Φ C31 have now been used in engineering biosynthetic genes for several polyketide natural products (Aparicio et al., 2003; Byrne et al., 2003; Kieser et al., 2000; Khaw et al., 1998; Seco et al., 2004). The work described here should assist further use of this system. The KC-UCD1 vector is easily isolated from *E. coli* and appears to perform as well as KC515 DNA extracted from phage particles. The vector has unique sites for *Bam*HI, *Pst*I, and the blunt cutter *Sca*I. These sites are compatible

with the cohesive ends generated by several restriction enzymes. Further development could introduce more restriction sites, or different selectable markers. The inclusion of DMSO in PEG solutions increases the efficiency and reproducibility of transfection procedures. This modification may also be useful for PEG-mediated transformations. The use of PCR to identify recombinant phages with inserts is more rapid than screening by hybridization. Taken together these modifications reduce the labour associated with the phage transduction method for introducing DNA into streptomycetes for gene replacement.

Manipulation of the large amphotericin PKS genes requires replacement of chromosomal sequences via homologous recombination events. The 6kb capacity of KC515 and KC-UCD1 should be sufficient to allow these to occur with adequate efficiency. The first successful redesign of the amphotericin PKS has yielded a novel pentaene. In future work, recombinant phages that yield productive gene replacements could be used combinatorially to construct strains with multiple changes in the PKS and late genes. The efficiency could be further increased by introducing a gene to allow selection of recombinants that have undergone a second cross-over event. The *gkA* glucose kinase gene of *S. coelicolor* is required for sensitivity to 2-deoxyglucose and has been used for counterselection (Kieser et al., 2000). The homologous gene can now be deleted from the *S. nodosus* chromosome and incorporated into KC-UCD1, so that lysogens are 2-deoxyglucose-sensitive as well as thiostrepton-resistant. Revertants that have excised the prophage could be obtained by selecting for resistance to 2-deoxyglucose rather than by laborious screening for loss of thiostrepton resistance.

Efficient engineering of large PKSs like the amphotericin system will increase the diversity of structures that can be obtained by combinatorial biosynthesis. These efforts in synthetic biology should assist development of new antibiotics and other pharmaceuticals.

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Table 1. Genes in the regions flanking the amphotericin PKS genes.

Gene	Protein	Size (AA)	% Identity ^a
Left flank			
ORF5	Transcriptional regulator	148	34
AmphRI	Regulatory protein	948	44
AmphRII	Regulatory protein	930	40
AmphRIII	Regulatory protein	929	46
AmphRIV	Regulatory protein	243	63
ORF6	LysR-type regulator	299	44
ORF7	3-ketoacyl CoA thiolase	401	61
ORF8	CoA transferase subunit A	223	67
ORF9	CoA transferase subunit B	231	64
Right flank			
AmphE	Discrete thioesterase	287	80
AmphRVI	Regulatory protein	889	42
ORF10	Amino acid permease	497	85

^a % Amino acid sequence identity with closest homologue in GenBank database. Accession numbers for these homologous sequences are given in Section 3.3.

Figure Legends

Fig. 1. Structure of amphotericin B. The late stages of amphotericin biosynthesis involve oxidation of a methyl branch at C-16 to a carboxyl group, addition of a mycosamine sugar at C-19 and hydroxylation at C-8. In the co-metabolite amphotericin A the C-28-C29 double bond is reduced.

Fig. 2. Construction of KC-UCD1. The pACYC177 sequence replaces the *Bam*HI – *Pst*I fragment of KC515 that contains the viomycin resistance (*vph*) gene. The KC-UCD1 vector retains a unique site for the blunt cutter *Sca*I that is 237 bp upstream from the *Pst*I site towards the 5' end of the ampicillin resistance gene.

Fig. 3. Organisation of genes in regions flanking the amphotericin PKS genes. The *amphG* gene encodes an ABC transporter protein and is located at the extreme left end of the amphotericin biosynthetic gene cluster sequence (accession number = AF357202). The *amphC* PKS gene is at the right end.

Fig. 4. Construction of coding sequence for a hybrid module in which KS5 is fused to AT7. The 1122 bp KS5 PCR product contains engineered *Eco*RI and *Pst*I sites at the 5' end. The first base of the *Kpn*I site at the 3' end corresponds to 91697 in the sequence. This site is conserved in several KS coding sequences. The PCR product was digested with *Eco*RI and *Kpn*I and ligated to the *Eco*RI – partial *Kpn*I fragment of pUC-KR6DH7. The resulting 3 kb *Bam*HI – *Pst*I fragment containing the hybrid module coding sequence was subcloned from pUC118 into KC-UCD1.

Fig. 5. Replacement of the coding sequences for modules 5, 6 and 7 with the 5-7 hybrid module coding sequence. The junction between the KS5 and AT7 coding sequences in KC-M57 is drawn as an extended broken line. The three *Nco*I sites shown begin with nucleotides 90339, 95385 and 104325 in the sequence of the amphotericin biosynthetic genes. The *Nco*I fragments shown in the wild type chromosomal DNA are 5046 and 8940 bp in length whereas the *Nco*I fragment in the M57 mutant DNA is 2530 bp.

Fig. 6. PCR analysis of KS5-AT5 and KS5-AT7 coding sequences in genomic DNA from *S. nodosus* ATCC14899 and the mutant. A. Schematic representation showing chromosomal regions and PCR primers. B. Agarose gel analysis of PCR products. Primers 5F and 5R amplified the expected 1150 bp KS5-AT5 product from wild-type genomic DNA (lane 1) but not from M57 mutant DNA (lane 3). Primers 5F and 7R amplified the expected 1251 bp KS5-AT7 product from M57 genomic DNA (lane 4) but not from wild type DNA (lane 2).

Fig. 7. Analysis of AT7 region in chromosomal DNA from wild type and mutant. Genomic DNA from both strains was digested with *NcoI* and analysed by Southern hybridization with an AT7 probe. The 8.9 kb *NcoI* fragment in wild-type (lane 1) is absent from the mutant (lane 2) which contains the expected 2.5 kb band. The 5.0 kb band in both tracks is thought to represent the *NcoI* fragment that contains the related AT8 coding sequence. The 5.3 kb band in lane 1 is thought to represent the *NcoI* fragment containing the AT5 sequence. This fragment is deleted from the mutant.

Fig. 8. Analysis of polyene extracted from the replacement mutant by UV spectrophotometry. The heptaene spectrum characteristic of purified amphotericin B (10 µg/ml) is shown in panel A. The absorption spectrum of polyenes extracted from the M57 mutant is shown in panel B. A sample of culture supernatant was extracted with an equal volume of butanol. The initial extract was diluted 1:20 in butanol and scanned to give the spectrum shown. The absorption maxima at 310, 318, 333 and 352 are characteristic of a pentaene.

Fig. 9. Predicted structure of pentaene produced by *S. nodosus* M57.

Figure 1.

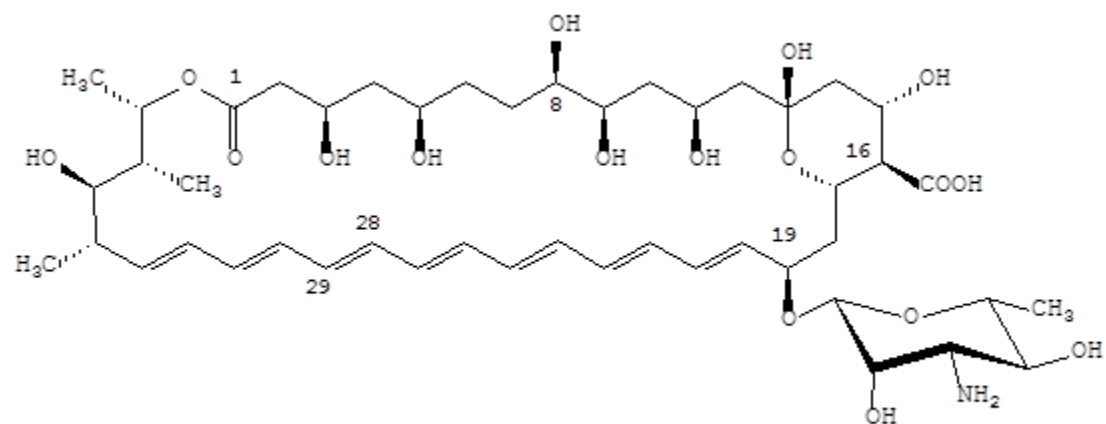


Figure 2

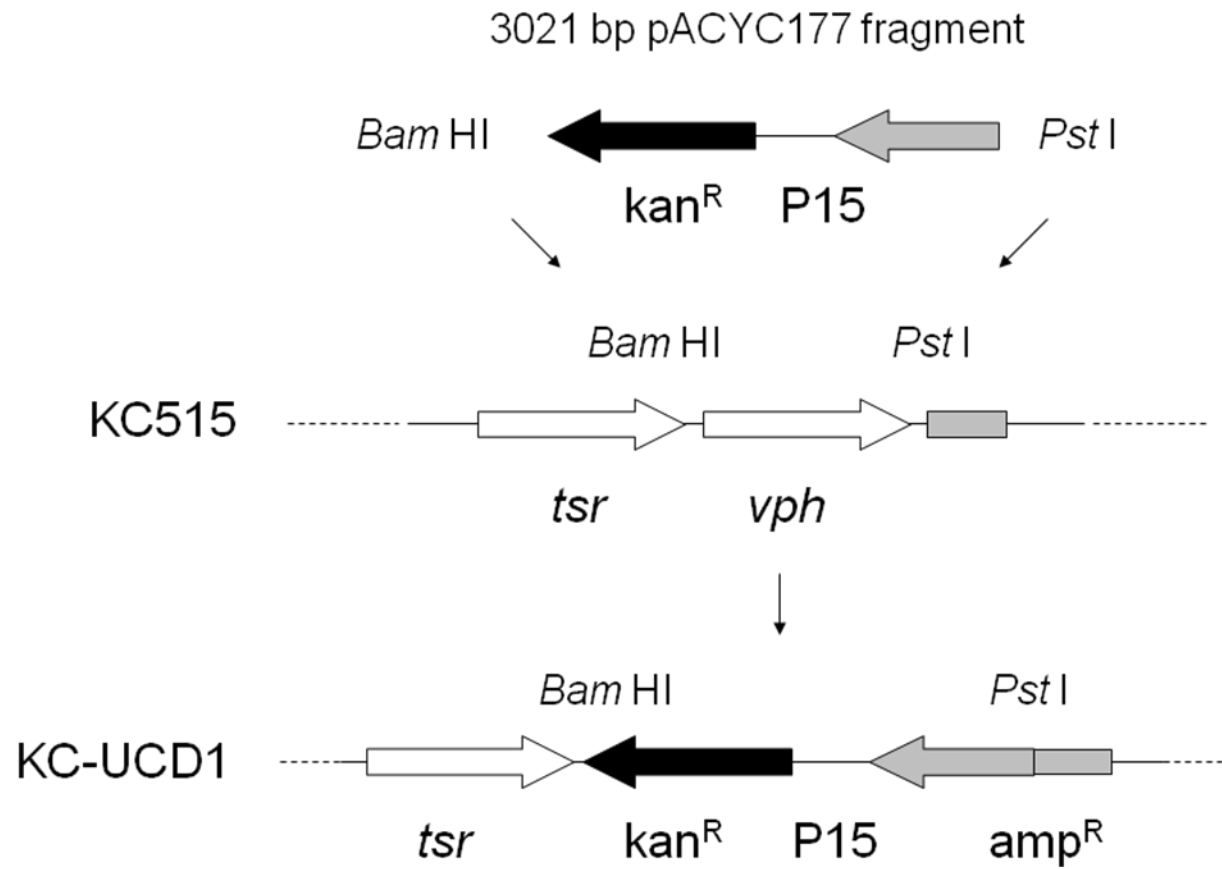
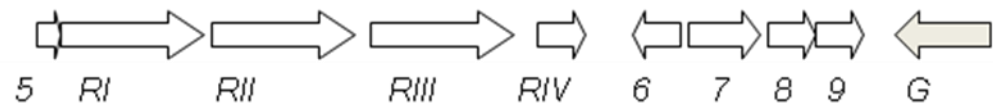


Figure 3

Left flank



Right flank

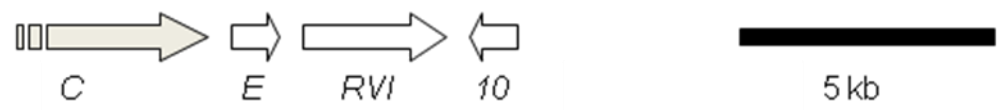


Figure 4

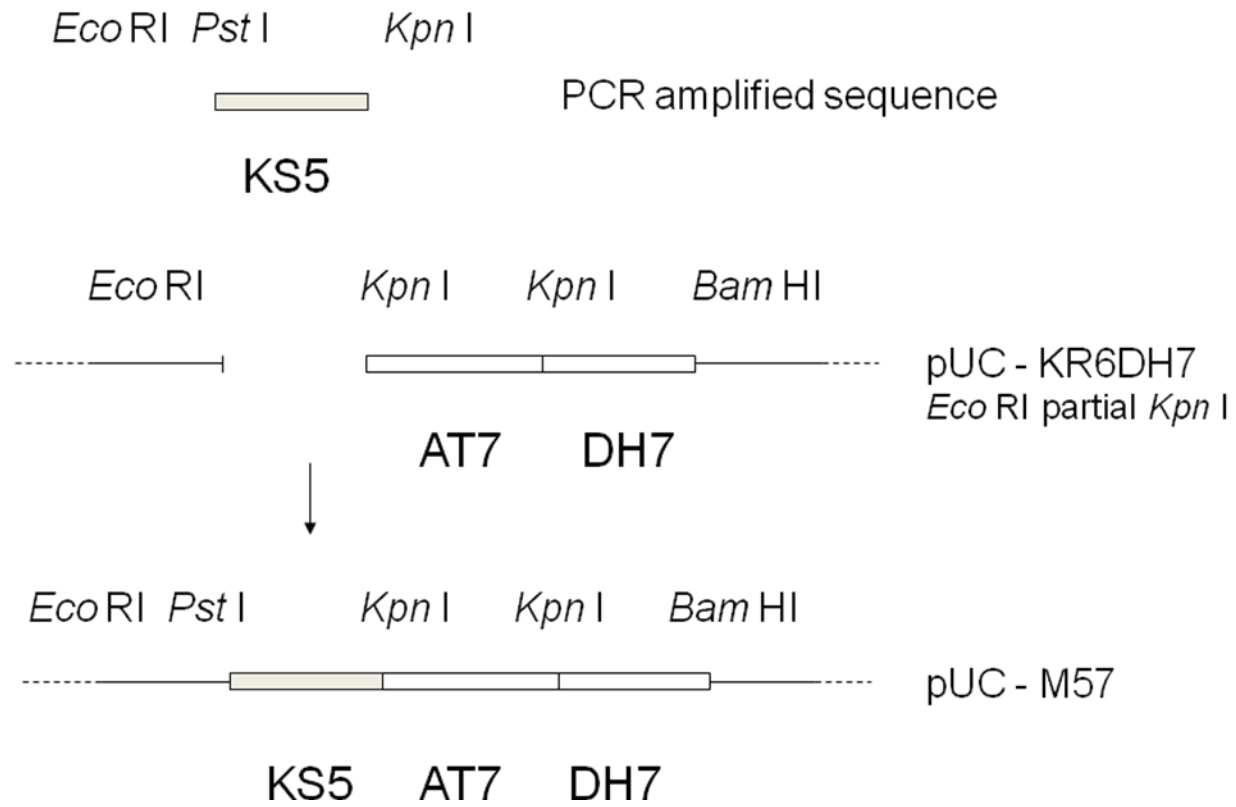


Figure 6

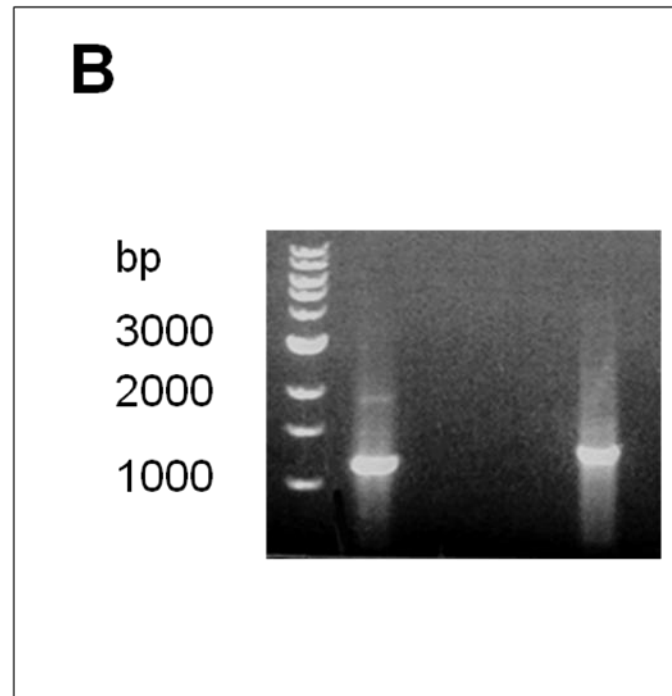
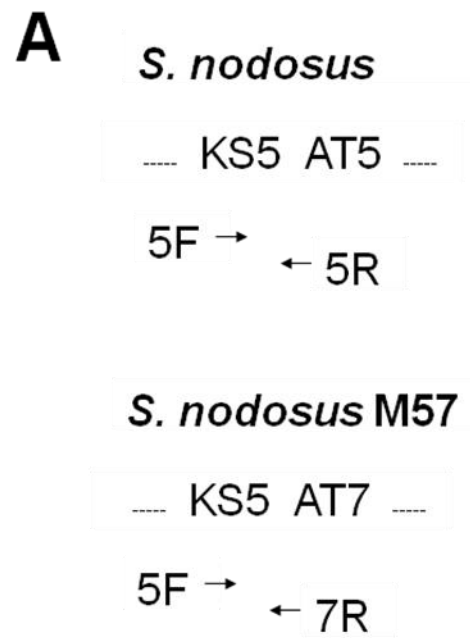


Figure 7

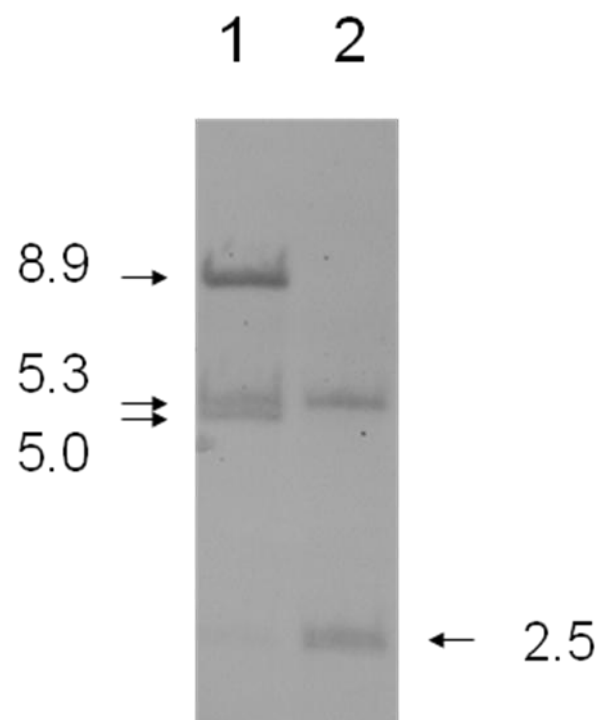


Figure 8

