An ABSTRACT OF THE THESIS OF

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Title: Cloning and Characterization of Polyketide Biosynthetic Gene Clusters from *Streptomyces murayamaensis, Streptomyces rimosus,* and *Streptomyces* WP 4669

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Abstract approved:_____
Steven J. Gould

The kinamycin antibiotics produced by *Streptomyces murayamaensis* are derived from an angucycline polyketide intermediate, dehydrorabelomycin. Tetrangulol from *Streptomyces rimosus* and PD 116740 from *Streptomyces* WP 4669 are also angucycline polyketides. In order to clone the polyketide biosynthetic genes of *S. murayamaensis*, a bacteriophage genomic DNA library was constructed and 25 putative PKS gene clones were obtained in previous work. This work started by subcloning the inserts from these lambda clones into either the *Streptomyces* plasmid pIJ941 or the *Streptomyces-Escherichia coli* shuttle vector pKC1218. These subclones were then to be transformed into *Streptomyces lividans* and *Streptomyces parvulus* for expression testing. However, expression was not successful, presumably due to the lack of key genes on the relatively small pieces cloned (15-20 kb). Cosmid genomic DNA libraries of *S. murayamaensis,* as

well as of *S. rimosus* and *S.* WP 4669 were then constructed in *E. coli* XL1-Blue MR using the *Streptomyces-E. coli* bifunctional cosmid, pOJ446. Approximately 6000 individual colonies of the *S. murayamaensis* cosmid library and about 2500 individual colonies of the *S. rimosus* and *S.* WP 4669 cosmid libraries were screened with *actI*-ORF1, the ketosynthase gene from the actinorhodin pathway. From these cosmid libraries 39, 12, and 38 positive clones were identified respectively, from *S. rimosus, S.* WP 4669, and *S. murayamaensis.* Restriction analysis and Southern hybridization showed that there are two polyketide biosynthetic gene clusters in each organism. Transformation of the cosmids into *S. lividans* TK24 followed by HPLC analysis of the fermentation extracts indicated that one of the clusters from *S. murayamaensis* represented the kinamycin pathway, one of the clusters from *S. rimosus* represented the tetrangulol pathway, and one of the clusters from *S.* WP 4669 represented PD116740 pathway. Thus, heterologous expression of a derivative compound of a kinamycin biosynthetic intermediate was obtained in the first case, and expression of tetrangulol and of PD116740 was obtained in the second and third cases, respectively. Expression of these compounds by the *S. lividans* transformants was detected by photodiode array-HPLC analysis of crude extracts and was further confirmed by NMR analysis of purified samples.

Cloning and Characterization of Polyketide Biosynthetic Gene Clusters from *Streptomyces murayamaensis*, *Streptomyces rimosus*, and *Streptomyces* WP 4669

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Seong-Tshool Hong, Author

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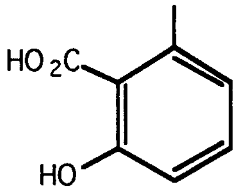
Chapter I

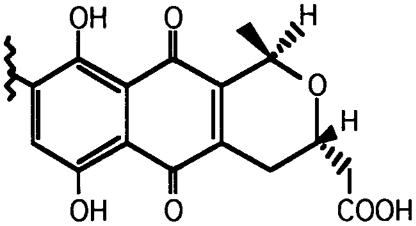
Introduction

The polyketides are a group of organic molecules produced through the successive condensation of small carboxylic acids such as acetate, propionate, or butyrate.[1-3] Even though the polyketides are biosynthesized from the same building blocks and share a common pattern of biosynthesis, their structures are extremely diverse (Figure I-1).

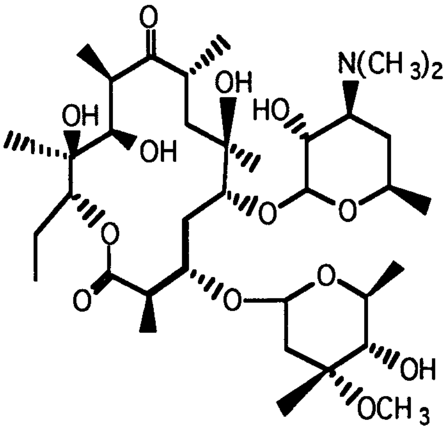
The polyketides are examples of secondary metabolites, so called because the secondary metabolism is not essential for, and plays no part in, growth, in contrast to the primary metabolism that provides the structure and energy requirements of all living cells. The polyketides are found in both prokaryotes and eukaryotes in nature, except for the animal kingdom.[1-3] Typical examples of polyketides are the striking polyketide-derived colors of the flowers and the flavors of higher plants, the phytoalexins that help to defend crop plants against fungal disease, mycotoxins in fungi, and the polyketide-derived antibiotics of *Streptomyces* sp.



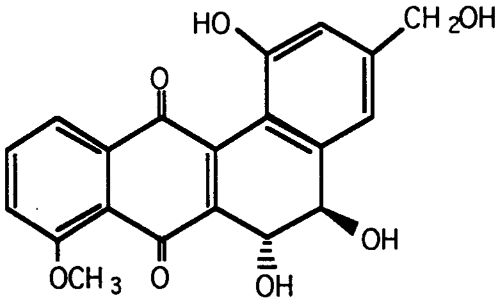
6-methylsalicylic acid



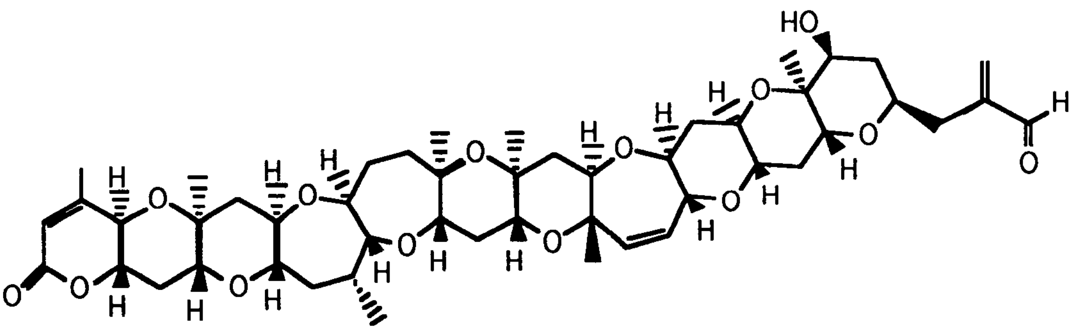
Actinorhodin



Erythromycin A



PD116740



Brevetoxin B

Figure I-1. Structural diversity of polyketides

The polyketide-derived compounds of *Streptomyces,* especially, have attracted attention because they have many significant biological activities such as antifungal, antibacterial, antitumor, and anthelmintic properties. The wide variety of their biological properties reflects the diversity of polyketide structure (Figure I-1). 6-Methylsalicylic acid, which is derived from an eight-carbon chain that cyclized to form an aromatic ring is the smallest polyketide known so far. The largest known polyketide is brevitoxin B, with 50 carbon atoms in its chain.

Mechanism of polyketide biosynthesis

From the results of radio-isotope feeding experiments, Birch suggested that polyketides are synthesized through a mechanism similar to the formation of long-chain fatty acid biosynthesis from carboxylic acid building units.[4] Cloning of the first whole polyketide biosynthetic gene cluster, the one for actinirhodin from *Streptomyces coelicolor* by Hopwood et al, opened a new era for understanding polyketide biosynthetic mechanisms.[5,6] Molecular genetic studies of polyketide biosynthetic pathways[1-3] have confirmed the idea of analogy between formation of long chain fatty acids and synthesis of polyketides.

Fatty acid synthase (FAS) catalyzes repeated decarboxylic condensing reactions of malonyl-CoA to synthesize a fatty acid. In prokaryotes and higher plants, it is a multienzyme complex (so called Type II FAS).[1,2,10] In this case, separate polypeptides catalyze each step in fatty acid biosynthesis. The Type I fatty acid

synthase is a huge multifunctional polypeptide catalyzing all of the steps in fatty acid biosynthesis.[1-3] The Type I FAS is usually active as a homodimer and is typical of animal systems.

Figure I-2 illustrates the general concept of how a fatty acid is biosynthesized. Chain building starts by transferring an acetyl unit from acetyl-CoA to the active-site cysteine residue of the β-ketoacyl synthase (KS) by acetyl-CoA-acyl carrier protein (ACP) transacylase. Next, the building unit, malonate, is transferred from coenzyme A (CoA) to the pantotheine arm on the acyl carrier protein (ACP) by the acyltransferase (AT). Decarboxylative condensation occurs between the ACP-bound malonate and the acetyl unit of KS. The resulting acetoacetyl-ACP usually proceeds through a complete reductive cycle comprising a β-keto-reduction, a dehydration, and an enoyl-reduction, by the action of the β-ketoreductase (KR), dehydrase (DH), and enoylreductase (ER), respectively. The growing chain is then transferred to the KS to start a new cycle. This process is repeated until the desired chain length is reached.

FASs of different organisms always are terminated to produce a long aliphatic linear saturated fatty acid. Even though polyketides are biosynthesized through a mechanism similar to fatty acid biosynthesis, polyketide synthases (PKSs) produce many structurally diverse compounds (Figure I-1). Polyketide biosynthesis differs from fatty acid formation in four aspects (Figure II-2).[1-3] (1) A PKS can sometimes use various building units, such as butyrate and other branched carboxylic acids.

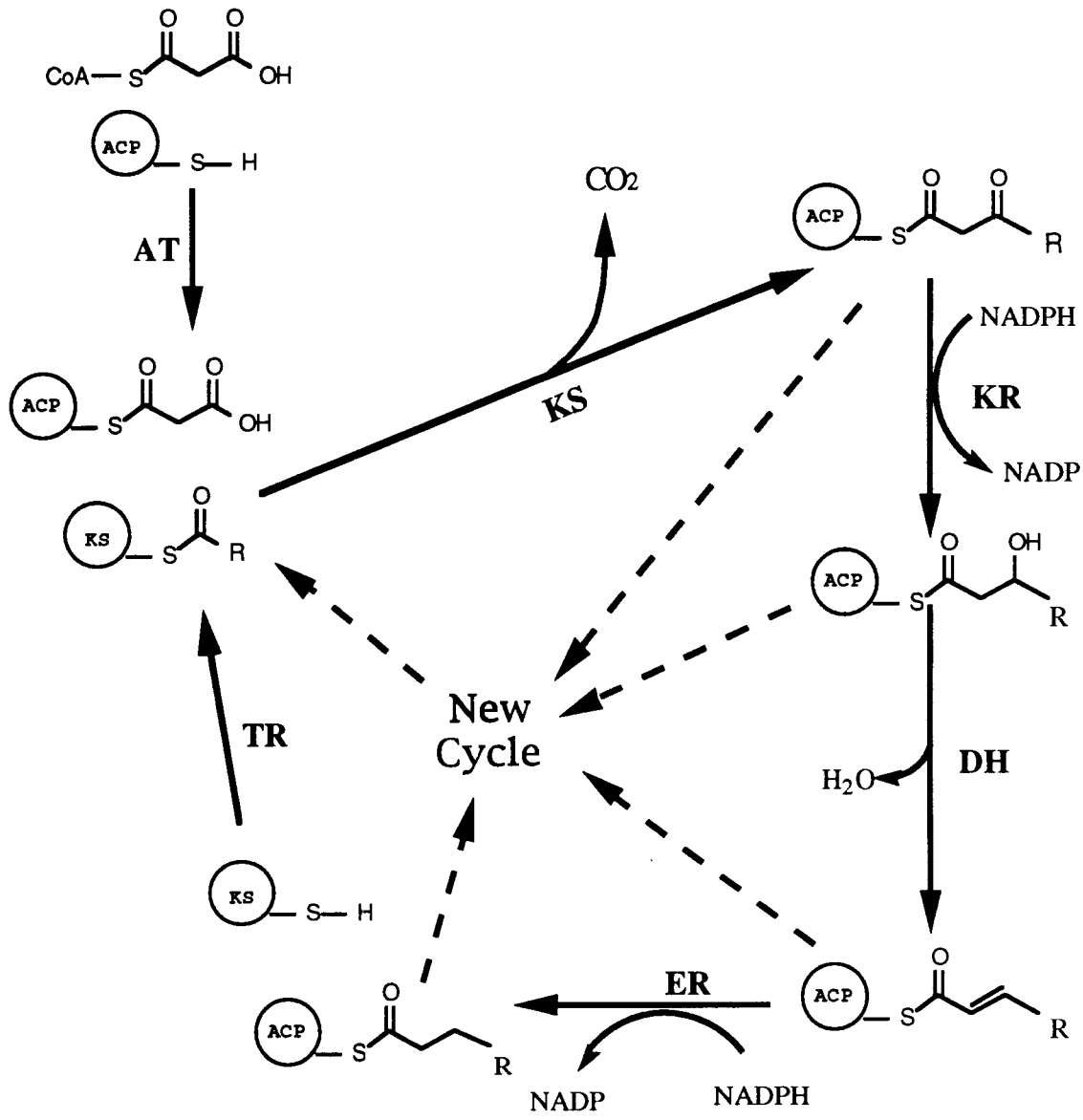


Figure I-2. Biosynthetic mechanism of fatty acid and polyketide formation.

(2) After each decarboxylative condensation, the β-keto-group of the growing chain is not always processed in each cycle of the biosynthesis. A new cycle may be initiated without further

processing of the β-ketone, and an acyl chain containing a β-keto, β-hydroxyl, α,β-olefin, or fully reduced β-carbon can be used by PKS to start a new cycle. (3) Since a PKS sometimes does not always completely process a β-ketone before starting a new cycle, chiral centers may be introduced during the polyketide synthesis. Also, the presence of a side chain on the building unit may end with a chiral center. (4) PKS biosynthesis is accompanied by other processes such as folding and cyclization by carbon-carbon bond formation, lactonization, or formation of an amide bond with an amino acid. Because of this variation in starter and extender units, carbon chain length, substituent functionalization and stereochemistry, the remarkable structurally diverse polyketides can be generated in nature.

Complex Polyketides

Plants have quite different PKSs from bacteria and fungi. The PKSs of plants do not have ACP functionality and catalyze decarboxylative condensing reactions directly on the CoA ester of a carboxylic acid.[1,2,10] Bacterial and fungal PKSs can be classified into two types.[1-3] Type I PKSs are described as multifunctional enzyme complexes like Type I FAS, while Type II PKSs are multienzyme complexes like Type II PKS. Type I PKSs are responsible for production of complex polyketides which consist of polyketide skeletons more highly reduced than those of the poly-β-keto intermediates in aromatic polyketide biosynthesis. The polyketide-derived unit from complex PKSs can end with a linear

chain (e.g. polyethers), a lactone (e.g. macrolides), or as a lactam (e.g. macrolactams).

Feeding experiments for the biosynthesis of bacterial and fungal complex polyketides (i.e. macrolides and polyethers) showed that these pathways involve a stepwise processive mechanism, in which the stereochemistry of the side chains and the appropriate degree of processing of the β-carbons are precisely processed into the acyl chain prior to subsequent elongation cycles.[2,4,10] At each step, the enzyme must determine the correct stereochemistry of the side chain and the degree and stereochemistry of processing of the β-carbon.

The application of molecular genetics to the biosynthetic pathways of complex polyketides by forward genetic approaches has yielded dramatic insights into the organization and function of these biosynthetic systems. The cloning of biosynthetic genes for the macrolide portion for erythromycin, 6-deoxyerythronolide B, by Katz at Abbott Laboratories,[11,12] and Leadlay at University of Cambridge,[13] was truly a landmark development. The *eryA* gene for 6-deoxyerythronolide B biosynthesis is organized into three large (10 kb) open reading frames (ORFs) each containing a series of domains, with each of these responsible for one of the individual steps of polyketide chain assembly (Figure I-3). Each of the three ORFs encode a protein which possesses all of the required activities involved in two methylmalonate-propionate condensation cycles. Each condensation, with the appropriate reducing activities, was designated a module, each of which harbors the appropriate complement of ketosynthase (KS), ketoreductase (KR), dehydrase

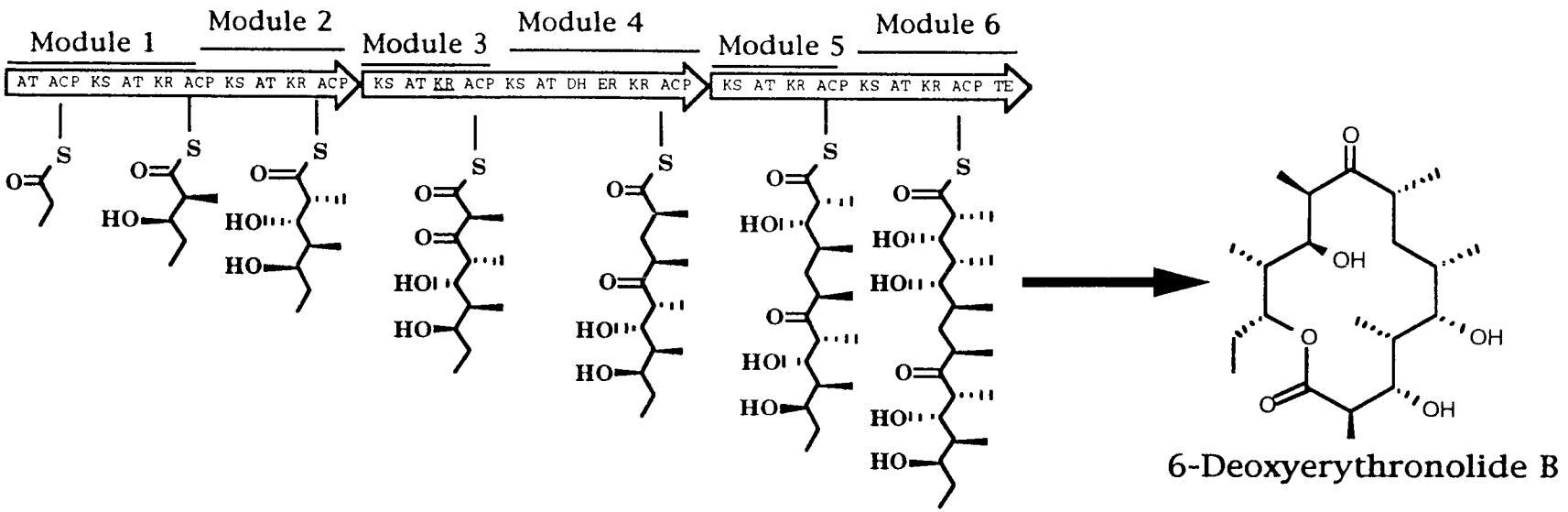


Figure I-3. Biosynthetic pathway for 6-deoxyerythromycin B. 6-Deoxyerythromycin B is the macrolide precursor of erythromycin. Open arrows represent ORFs.

(DH), enoylreductase (ER), acyl carrier protein (ACP) and acyltransferase (AT) domains, strongly reminiscent of an animal fatty acid synthase in both overall size and organization. So, a total of six modules make up the entire 6-deoxyerythronolide B. More recently, the PKS gene clusters for avernectin from *Streptomyces avermitis*,[14] for FK506 from *Streptomyces* sp. MA6548,[15] and a *Bacillus subtilis* gene of unknown function resembling *ery A*,[16] have also been cloned and characterized. The molecular genetic studies on those PKS genes showed that they are indeed multifunctional Type I PKSs, as expected.

Aromatic Polyketides

Type II polyketide synthases, catalyzing the biosynthesis of aromatic polyketides, are multienzyme complexes like Type II FAS. Molecular genetic analysis of a number of PKS gene clusters[1-3] has revealed that each component of the PKS such as ketosynthase, ketoreductase, acyltransferase, dehydrase, acyl carrier protein, etc. is tightly clustered with regulatory gene(s) and self-resistance gene(s) on a relatively small area of chromosomal DNA. The actinorhodin PKS from *Streptomyces coelicolor* and tetracenomycin PKS from *Streptomyces glaucescens* are the best known examples for aromatic PKSs. The entire biosynthetic gene clusters for each of these molecules were cloned and completely sequenced.[17-25] Actinorhodin is biosynthesized by decarboxylative condensing reaction of eight acetate residues, while tetracenomycin is derived from ten acetate residues.

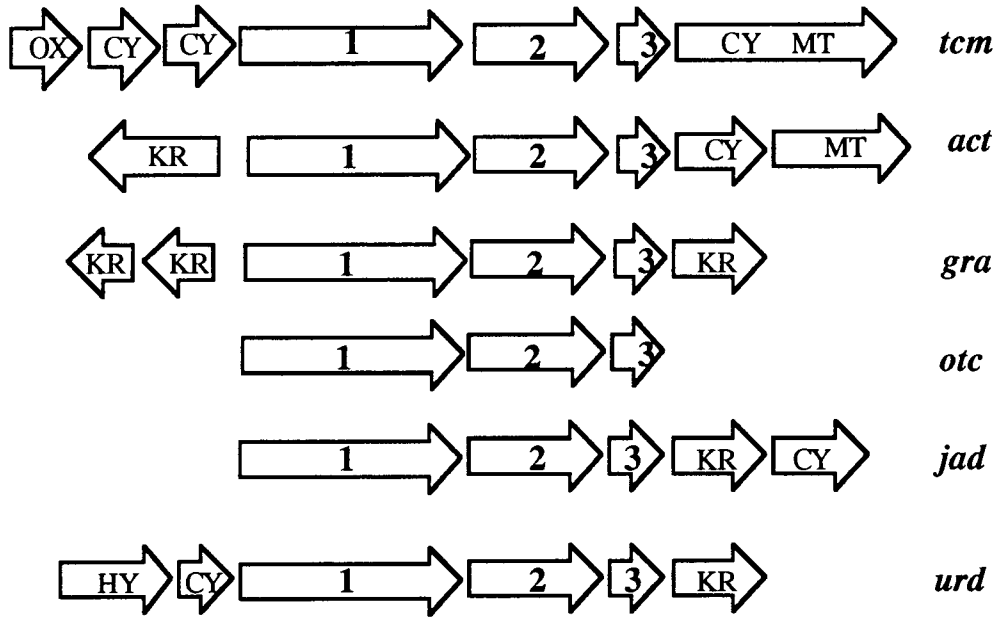


Figure I-4. Organization of aromatic PKS gene clusters in actinorhodin (*act*) in *Streptomyces coelicolor*;[17,18] tetracenomycin (*tcm*) in *S. glaucescens*;[22-24] granaticin (*gra*) in *S. violaceoruber*;[7] oxytetracycline (*otc*) in *S. rimosus*;[8] jadomycin (*jad*) in *S. venezuelae*,[9] urdamycin (*urd*) in *S. fradiae*.[40] Open arrows represent ORFs. KR, ketoreductase; OX, oxidase; CY, cyclase; HY, hydroxylase; MT, methyltransferase.

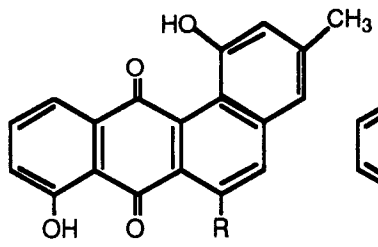
Figure 4 illustrates the several PKS gene clusters that have been cloned and sequenced so far. The PKS-encoding segments thus far characterized showed remarkable organizational similarity. By complementation of actinorhodin blocked mutants of *Streptomyces coelicolor* with the cloned actinorhodin PKS genes, it was possible to partly understand the function of individual gene products of

the PKS gene cluster. The detailed functions and specificity of the individual ORFs were deciphered by the landmark works of Khoslar et al.[26-32] They expressed cassettes of individual ORFs of actinorhodin and tetracenomycin PKS genes in *Streptomyces coelicolor* CH999, which is a PKS minus mutant. From these researches, it was determined that ORF2 (chain length factor) controls the size of a hypothetical nascent poly-β-ketone intermediate made by the KS and ACP component and polyketide cyclases govern the folding and cyclization(s) of the nascent poly-β-ketone intermediate.

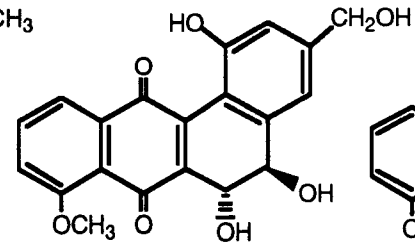
A common feature of the organization of the sequenced aromatic PKS gene clusters is a group of three characteristic ORFs; ORF1-2-3. These ORFs are the so called 'minimal PKS genes' that perform the condensations of a specific acyl coenzyme A (CoA) starter unit with a specific varying number of malonyl-CoA extender units, resulting a specific polyketide backbone. ORF1 encodes both ketosynthase and acyltransferase activities, and contains an especially highly conserved GPXXXXXXXCXSXL motif. This is present at the active site of both Type I and II PKSs, and provides the enzyme residue required for thioester linkage to the acyl chain. ORF2 encodes a chain length determining factor. The deduced protein sequences of ORF1-2 show particularly high similarity with the FabB condensing enzyme of *E. coli* Type II FAS. ORF3 polypeptides, the acyl carrier proteins, are all less than 100 amino acids in length and contain the highly conserved DLXGYDS motif characteristic of the 4-phosphopantotheine binding site of ACPs.

Biosynthetic studies on aromatic polyketides from *Streptomyces* WP 4669, *S. murayamaensis*, and *S. rimosus*

Kinamycins[35] (3) from *S. murayamaensis*, with a 5-diazobenzo[b]fluorene skeleton, are derived from an aromatic benz[a]anthraquinone precursor, dehydrorabelomycin (4).[36] The biosynthetic pathway of kinamycins (Figure II-1) was elucidated mostly by studies of isotope labeling and *S. murayamaensis* mutants which were defective in kinamycins synthesis.[41] Dehydrorabelomycin derives from a single decaketide chain formed on the polyketide biosynthetic pathway. Enzymatic ring closure reactions of the hypothetical decaketide result in dehydrorabelomycin and are followed by a remarkable process of oxidation, ring opening, and ring contraction to convert into kinamycins. These unusual interconversion steps are still under investigation. *S. murayamaensis* produces another aromatic polyketide compound, the phenanthraquinone murayaquinone (Figure II-2).[38]



1, R=H
4, R=OH



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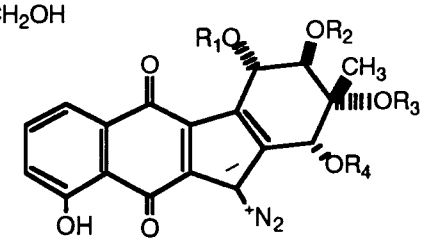

3

Tetrangulol[33] (1) from *S. rimosus* and PD 116740[34] (2) from *S.* WP 4669 are aromatic benz[a]anthraquinone polyketide antibiotics. Because of the obvious structural similarity between PD 116740 and dehydrorabelomycin, a study set out to establish the biosynthesis of PD 116740.[42] Cultures of *S.* WP 4669 were supplemented with [1-$^{18}O_2$] acetate. This gave rise to PD 116740 devoid of any oxygen-18 at C_6. Also cultures supplemented with [2,4,5,9,11] dehydrorabelomycin did not label the resultant PD 116740 with deuterium. However, when cultures were supplemented with [2,4-$^{2}H_4$] tetrangulol, deuterium was incorporated regiospecifically into H-2 and H-4 of PD 116740. These results clearly indicate that tetrangulol is the key intermediate in the biosynthesis of PD 116740 but is not a precursor to dehydrorabelomycin. An enolization of the polyketide intermediate occurs to generate the C-6 oxygen of dehydrorabelomycin whereas a reduction-elimination sequence operates to generate tetrangulol.[37] Presumably the C-5,6 diol of PD 116740 arises after epoxidation and hydrolysis from tetrangulol.

While the biosynthetic studies on tetrangulol, PD 116740, and kinamycins have elucidated the pathways that we know, it became clear that an understanding of the molecular biology of these pathways would be of great value.

Strategies for cloning aromatic PKS biosynthetic gene clusters from *S.* WP 4669, *S. murayamaensis*, and *S. rimosus*

Since polyketide assembly is always likely to involve similar biochemical processes and generate structurally related thioester intermediates, cloned DNA coding for one synthase may serve as a hybridization probe for the isolation of others.[39] The actinorhodin biosynthetic genes of *S. coelicolor* are the most widely used probes to identify other aromatic polyketide biosynthetic genes of *Streptomyces* sp. In an attempt to clone the polyketide biosynthetic genes of *S. murayamaensis*, a genomic DNA library of *S. murayamaensis* was constructed in EMBL4 bacteriophage prior to the work described in this thesis. By screening the library with actinorhodin and granaticin biosynthetic genes, the 25 clones showing homology with either of the genes were identified. *Eco*RI and *Bam*HI restriction enzyme mapping of the clones showed that the 25 lambda clones might be grouped into several independent PKS gene clusters.

The aim of my research has been to clone the biosynthetic genes of kinamycin, tetrangulol, and PDII of *S. murayamaensis, S. rimosus,* and *Streptomyces* WP 4669, respectively. Work began with the 25 clones from the bacteriophage *S. murayamaensis* genomic DNA library. The insert DNAs (15-20 kb) were subcloned into the *Streptomyces* low copy plasmid pIJ941[43] or the *Streptomyces-E.coli* shuttle vector pKC1218[44] and then transformed into *S. lividans* and *S. parvulus*. However, no expression of metabolites identifiable with either the kinamycin or

murayaquinone pathways was observed from any of the *S. lividans* or *S. parvulus* transformants.

Since typical aromatic polyketide biosynthetic gene clusters are roughly 22-34 kb long, we decided to clone much larger pieces of *S. murayamaensis* DNA to improve the chances of expressing recognizable polyketide metabolites. While constructing the new *S. murayamaensis* genomic DNA library in *E. coli* using the *Streptomyces-E. coli* shuttle cosmid vector pOJ446,[44] genomic DNA libraries of *S. rimosus* and *Streptomyces* WP 4669 were prepared in the same manner. By screening each of the cosmid libraries with a probe, *actI*-ORF1, the ketosynthase gene of the actinorhodin biosynthetic gene cluster, two PKS biosynthetic gene clusters from each of the three libraries were identified. These cosmids were then used to transform *S. lividans*. Expression of identifiable metabolites was obtained from one gene cluster from each organism.

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Chapter II

Cloning two polyketide biosynthetic gene clusters from a *Streptomyces murayamaensis* bacteriophage library

Introduction

Streptomycetes are Gram-positive, pseudo-fungal prokaryotes that produce diverse secondary metabolites including antibiotics, chemotherapeutic agents, ionophores and immuno-modulators. Among the nearly 6000 antibiotics of natural origin that have been characterized, more than 60% are produced by members of the genus *Streptomyces*.[1] Interestingly, most *Streptomyces* species produce more than one secondary metabolite.
Streptomyces murayamaensis is a typical example. It has extremely complex secondary metabolism. This organism produces a variety of aromatic polyketide structures, including 5-diazobenzo[b]fluorenes (kinamycins),[2-4] a benz[a]anthraquinone (dehydrorabelomycin),[5-7] phenanthraquinones (murayaquinone[8] and murayalactone[9]) and 4-hydroxy-3-nitrosobenzamide,[10] as well as metabolites based on aliphatic compounds (antimycins[11,12]).

The kinamycins, broad-spectrum antibiotics with modest cytotoxicity, are produced from a polyketide pathway.[5-7]

Biosynthetic studies conducted in our group on the kinamycins showed that they are derived from a single decaketide precursor (Figure II-1). Murayaquinone, having antibacterial activity against *Mycoplasma galliseptia* and *Treponema hyodysenteriae*, is derived from an independent decaketide pathway (Figure II-2).[11]

One of our group's major interests is to understand the biosynthetic pathway for the kinamycins. Much of the present information about the pathway has been obtained from isotope labeling studies and by spectroscopic examination of the accumulated intermediate compounds from kinamycin mutants.[2-17] These traditional approaches have provided a detailed though incomplete outline of the biosynthetic pathway. As a complement to available structural details, a molecular genetic approach is required to address questions pertaining to detailed functions and specificities of the polyketide synthases (PKS).

In order to begin studying the molecular biology of the *S. murayamaensis* polyketide pathways, Professor Gould constructed a *S. murayamaensis* genomic DNA library in EMBL4 (λ) bacteriophage. The bacteriophage library of *S. murayamaensis* has roughly 15 to 20 kb DNA inserts. Plaque lifts representing about 8,000 λ clones were screened with probes made from gra ORF1, the DNA region encoding the ketosynthase gene of the granaticin biosynthetic gene cluster,[37] and with *actIII*, the ketoreductase gene from the actinorhodin biosynthetic PKS gene cluster.[21] A total of 22 clones were tentatively identified as hybridizing with one or both of the probes. I started to work with these 22 clones to transfer the inserts into *Streptomyces* plasmid pIJ941[18] or

Streptomyces-E. coli shuttle vector pKC1218.[19] These subclones were then to be transformed into *S. lividans* and *S. parvulus* for expression testing. *S. lividans* is the most frequently used host in *Streptomyces* molecular genetics, but it has its own polyketide pathway (antinorhodin) and there was a possibility of unpredictable interactions. *S. parvulus* does not have a polyketide pathway, but it is more difficult to work with. It was hoped that the kinamycin PKS, and possibly the murayaquinone PKS, could be identified by expression of these metabolites or recognizable intermediates.

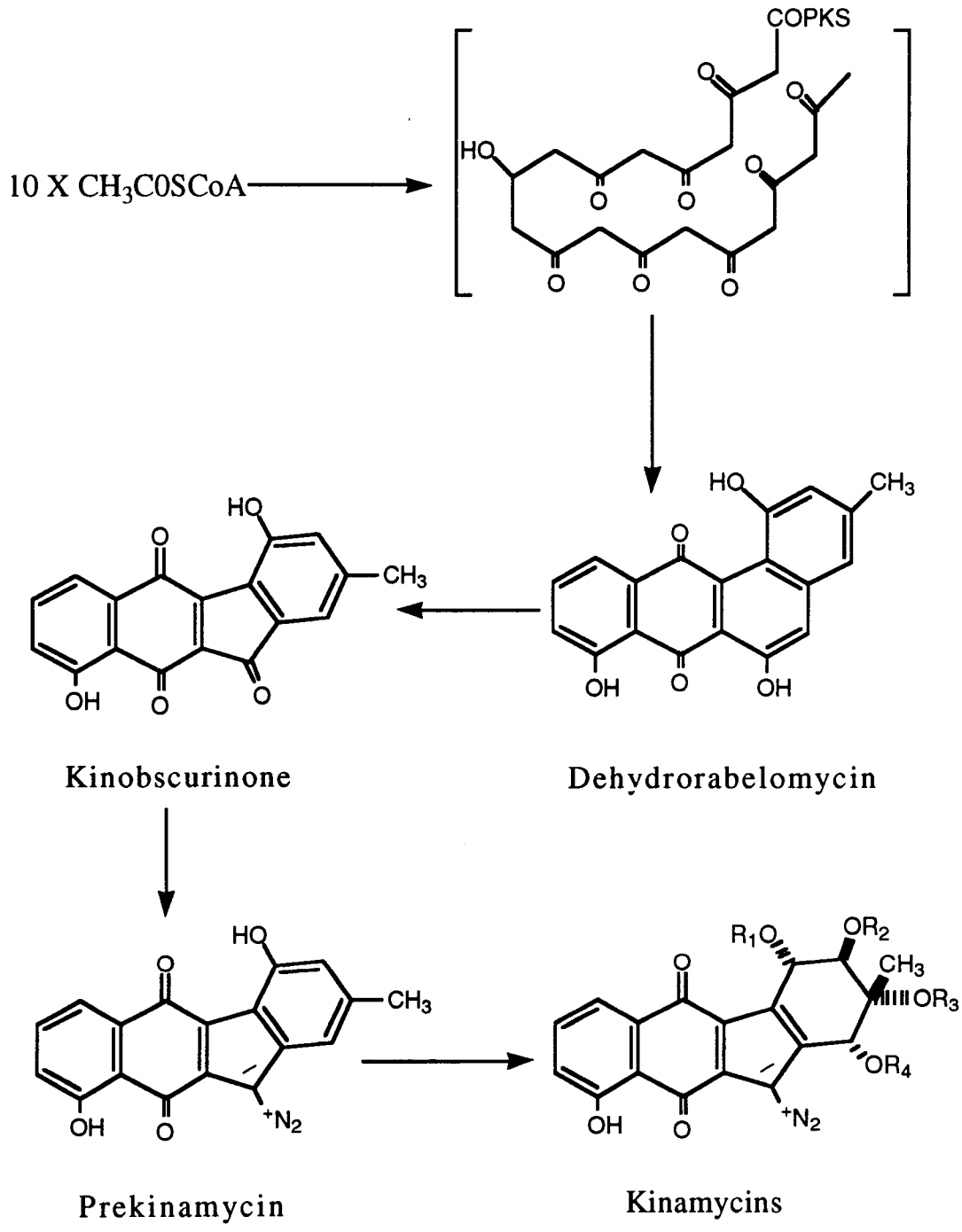


Figure II-1. Kinamycins biosynthetic pathway of *S. murayamaensis*

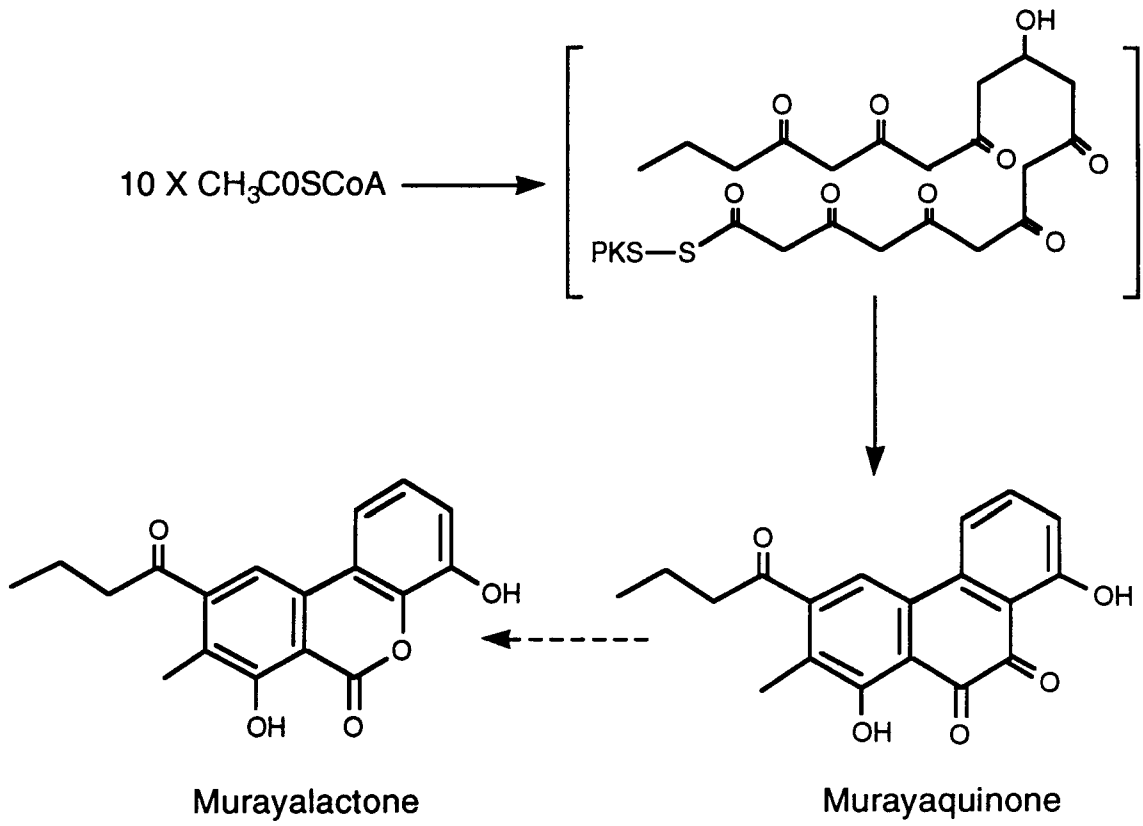


Figure II-2. Murayaquinone biosynthetic pathway of *S. muryamaensis*.

Materials and Methods

Bacterial strains and plasmids used

Streptomyces murayamaensis was a gift from Professors Omura (Kitasato University, Japan) and Hornemann (University of Wisconsin). *Streptomyces lividans* TK24 and *Streptomyces parvulus* (ATCC # 12434), used as recombinant host strains, were obtained from the John Innes Institute (England) and ATCC, respectively. *E. coli* LE392, used as a bacteriophage host, was kindly provided by Dr. C. Mathews (Oregon State University, USA). *E. coli* DH5α, used to propagate plasmids, was a gift from Dr. T. Dreher (Oregon State University, USA). pIJ941 and pKC1218, used as cloning vectors, were obtained from the John Innes Institute (England) and Lilly Research Laboratories, respectively.

Bacteriophage DNA preparation for subcloning

Lambda phage recovery. Plating bacteria (*E. coli* LE392) were prepared by culturing a single bacterial colony in L-broth (50 mL)[20] supplemented with 0.2% maltose in a sterile 250 mL flask (37 °C, 250 rpm, overnight). The bacteria were centrifuged at 2300 rpm, 4 °C, 15 min, then resuspended in 10 mM $MgSO_4$ (10 mL). The lambda phage clones prepared previously were diluted to a 10^{-7} dilution in SM buffer.[20] Lambda phages (50 µl) from each dilution were mixed with the plating bacteria suspension (100 µl) in sterile 1.5 ml microcentrifuge tubes. After a 20 minute incubation at room temperature, melted top agarose (3 ml, 48 °C)

was poured directly into the mixture and the mixture was poured onto dried NZ agar[20] in Petri-dish plates. After overnight culture at 37 °C, well separated single plaques from an appropriate dilution were recovered in SM buffer (100 µl) containing chloroform (0.3-0.5 µl).

Minilysate preparation. Phage mini-lysates were prepared by infecting 1/10-1/2 volume of the phage recovery suspension into an *E. coli* LE392 suspension (2-5 µl) at 37 °C, 1 hour, followed by incubation in NZ broth (5 ml) for 20 min at room temperature, followed by 37 °C, 250 rpm overnight.

Mini-prep of the phage DNAs. After clear lysis was obtained, the phage were purified from the bacterial debris by low speed centrifugation (4000 x g, 20 min) after addition of chloroform (0.5% w/v). Then, solid PEG 8000 and NaCl were added to a final concentration of 10% (w/v) and 1 M, respectively. After overnight incubation at 4 °C, phage particles were sedimented by centrifugation (30 min, 4000 x g). The precipitate was dispersed in SM buffer (0.5 ml), and cellular DNA and RNA were removed by treatment with DNase I (1 $\mu g.ml^{-1}$) and RNase A (10 $\mu g.ml^{-1}$) at 37 °C for 30 min. Then, SDS (10% w/v), EDTA (0.25 M), and proteinase K (20 mg/ml) were added to final concentrations of 0.1% (w/v), 10 mM and 0.1 $mg.ml^{-1}$, respectively, and the mixture was incubated at 55 °C for 30 min. After two phenol/chloroform extractions, DNA was recovered by precipitation with 0.3 M sodium acetate (pH 7.0) and 2.5 volumes of ethanol. Typically, about 20 µg of phage DNA was obtained from a 5 ml culture.

Maxi-Lysates for phage preparation. Phage preparations were made by infecting exponentially growing cultures of *E. coli* LE392 in 200 ml $NZCYM^{20}$ (OD_{600} =0.5) with mini-lysate (2.5 mL). The growth was followed spectrophotometrically. When the OD_{600} dropped to 0.6-0.7, the lysis was completed by addition of $CHCl_3$ (0.5 ml). After further incubation (10 min, 37 °C), the lysate was treated with NaCl (8g). DNase and RNase (to 1 μg/ml) were added to the lysate to remove bacterial DNA and RNA. After further incubation (1 hour), the supernatant was collected by centrifugation (10 min, 4 °C, 14,000 x g). The supernatant was treated with PEG 8000 (20 g) and stored overnight at 4 °C. The precipitated phage were recovered in SM buffer (10 ml) after centrifugation (10 min, 10,000 rpm, 4 °C). The phage were further purified by collecting the phage particles by centrifugation (25,000 rpm, 2 hours, 4 °C) in a Beckman SW41 rotor. After removing the supernatant, SM buffer (1 ml) was added to the glassy pellet of bacteriophage particles and left overnight.

DNA preparation. Phage particles were collected in 10 mM Tris-HCl, 20 mM EDTA, pH 8.0, (3-5 ml) in 17x100 mm Falcon tubes (Falcon #2002). Then, proteinase K (20 mg/ml) and SDS (10% w/v) were added to final concentrations of 0.5 mg/ml and 0.5% w/v, respectively. After 1 hour at 55 °C, the DNA was extracted with phenol/chloroform and dissolved in TE 8 buffer (400-500 μl).

Subcloning *S. murayamaensis* PKS gene inserts into pIJ941

pIJ941 preparation for subcloning.

pIJ941 extraction. Spore suspensions (0.2 ml) of *S. lividans* TK24 containing pIJ941 were used to inoculate four 2 L flasks containing YEME (500 ml) supplemented with 0.5% glycine. Each flask was baffled with a stainless steel spring in order to improve aeration and prevent clumping of mycelia. Cultures were grown for three days at 30 °C with vigorous shaking (250 rpm). The *S. lividans* mycelia were collected by centrifugation (2500 X g, 15 min) in a Beckman table top centrifuge. The mycelia from each flask (2-5 g) were resuspended in 25 ml TEG (50 mM glucose, 10 mM EDTA pH 8.0, 25 mM Tris pH 8.0). After treating with lysozyme (50 mg) and RNase (25 ml of a 5 mg/ml) for 10-30 min at room temperature, the mycelia were lysed by addition of 0.2 M NaOH/1% SDS (50 ml) followed by incubation at room temperature for 30 min. The mixtures were then treated with 3M NaOAc, pH 4.8 (40 ml) and acid phenol/chloroform (5 ml), and then kept on ice for 30 min. After removing the precipitate by centrifugation (10,000 x g, 10 min), an equal volume of isopropanol was added to the supernatant. After a 30 min incubation on ice, the DNA precipitate was recovered in TE (10 ml) by centrifugation (10,000 x g, 10 min). The protein contaminant was removed by phenol/chloroform extraction. DNA was recovered once again in TE buffer (7 ml) after isopropanol precipitation.

pIJ941 purification. The extracted pIJ941 was purified by a standard CsCl ultracentrifugation method.[20]

Preparation of *S. lividans* and *S. parvulus* protoplasts for transformation

S. lividans and *S. parvulus* were propergated on a R2YE medium. The spore suspensions of the *Streptomyces* were prepared by scraping the surface of a sporulating agar culture as described in the *Streptomyces* Lab Mannual.[18] Cultures of *S. lividans* or *S. parvulus* (50 ml) were grown for 40 hours from a spore suspension (0.2 ml) in YEME medium + 0.5% glycine *(S. lividans)* or 1.0% glycine *(S. parvulus)* at 30 °C, 250 rpm in an orbital shaker. Each culture was divided in half and centrifuged in two universal bottles (3000 rpm, 20 min) in a Wifuge desktop centrifuge. The mycelia were collected for protoplast preparations as discussed in the *Streptomyces* Lab Manual.[18] For *S. parvulus* protoplast preparation, 2 mg/ml of lysozyme solution was used instead of 1 mg/ml of lysozyme solution. After making protoplasts, the number of protoplasts were counted by a hemacytometer. 5 X 10^8 protoplasts were devided into each 1.5 ml microcentrifuge tubes.

Preparation of *S. lividans* transformants with recombinant plasmids

Preparation of *S. murayamaensis* DNA insert. Lambda phage DNAs (20 µl from 3-4 µg/µl stock) were diluted up to 200 µl and kept several days in a cold room to be completely dispersed. The diluted phage DNAs (200 µl) were digested for 2 minutes with *Eco*RI (20 units) at 37 °C. *S. murayamaensis* DNA inserts were then separated by 0.6 % agarose gel electrophoresis and purified by Sephaglass BP (Pharmacia Co.) by manufacturer's suggestion.

Ligation of *S. murayamaensis* DNA fragment with pIJ941. The ligation mixtures (10 µl) were composed of the

dephosphorylated pIJ941 (0.2-0.3 μg), *S. murayamaensis* DNA insert (0.05-0.1 μg), T4 ligase (1 μl, 1 Weiss unit) to react overnight at 16 °C. The completion of the ligation reaction was confirmed by 0.6% agarose gel electrophoresis.

Transformation of *S. lividans* with the recombinant pIJ941. An aliquot (2 μl) from the ligation mixture (30 μl) was applied to *S. lividans* protoplasts (5 x 10^8) in P-buffer (50 μl) to be transformed by using T buffer (detailed procedure in Hopwood *Streptomyces* Lab Manual).[18] The transformants were detected by overlaying soft nutrient agar (2.5 ml) containing thiostrepton (500 μg/ml) per plate 14-20 hours later. Transformants that were both thiostrepton resistant and hygromycin sensitive were selected[18] and then transformants were spread onto fresh R2YE agar plates containing 50 μg/ml thiostrepton for confluent growth. Spore suspensions were prepared from the agar plates 2-4 weeks later by the standard method.[18]

Analysis of the *S. lividans* transformants. Each spore suspension (0.1 ml) of thiostrepton resistant, hygromycin sensitive *S. lividans* transformants was inoculated into YEME (50 ml) supplemented with glycine (0.5 %) and thiostrepton (5 μg/ml) in a 50 ml flask baffled with a stainless steel spring. Cultures were grown for three days at 30 °C with vigorous shaking (250 rpm). The mycelia were harvested by centrifugation (2500 x g, 10 min) and washed with sucrose (10.3%) prior to storage at -20 °C. The frozen mycelia were thawed at room temperature and resuspended in 4 ml of lysozyme solution (25 mM Tris-HCl (pH 8), 25 mM EDTA (pH 8), 0.3 M sucrose, 2 mg/ml lysozyme, and 2.5

mg/ml RNase) followed by incubation for 15 min at room temperature. The mycelia were completely lysed by adding 0.3 M NaOH/2% SDS followed by incubation at room temperature for 10 min. The mixture was treated with 3 M sodium acetate (4 ml, pH 4.8) and left on ice for 15 min. After removal of the precipitate by centrifugation (3000 x g, 15 min) in a Beckman table top centrifuge, an equal volume of isopropanol was added to the supernatant. After incubating the mixture on ice for 30 min, the precipitated plasmid was recovered by centrifugation (3000 x g, 15 min) in a Beckman table top centrifuge and resuspended in 0.8 ml TE. The plasmid was further purified using Pharmacia Magic Plasmid Prep Kit (Pharmacia, Madison, Wisconsin). The typical yield of the plasmid was 3-5 μg/50 μl.

Transformation of *S. parvulus*.

Aliquots (2 μl) from the *S. lividans* plasmid preps were applied to *S. parvulus* protoplasts (5 x 10^{8}) in 50 μl P buffer and transformed using the T buffer (detailed procedure in Hopwood *Streptomyces* Lab Manual).[18] Transformants were detected by overlaying with 2.5 ml of soft nutrient agar (containing 500 μg/ml thiostrepton) per plate 14-20 hours after incubation. Thiostrepton resistant transformants were selected, and spore suspensions were prepared from single colonies.

Subcloning *S. murayamaensis* PKS gene inserts into pKC1218

Preparation of *S. murayamaensis* PKS gene inserts from *Eco* RI digested phage DNA. Aliquots (10 µl) of the original phage stocks (1-2 µg/µg) were diluted to 495 µl with a mixture consisting of *Eco* RI buffer (50 µl) and ddH_2O (435 µl). After incubation at 4 °C for several hours to completely disperse the phage DNA, *Eco* RI (50 units) was added. The mixture was incubated at 37 °C for 5 min for partial digestion (e.g. clone 14a) and for 3 hours for complete digestion. The digested phage DNA was extracted one time each with phenol/$CHCl_3$ (pH 8.0) and with $CHCl_3$. The $CHCl_3$ was removed from the DNA solution using a Microcon-100 microconcentrator (Amicon Co.).

Ligation of the *S. murayamaensis* inserts into dephosphorylated pKC1218. Ligations of the digested phage DNAs into dephosphorylated pKC1218 were achieved by a two-step ligation. First, the digested phage DNAs (2-5 µg) were mixed with the dephosphorylated pKC1218 (0.8-2 µg) in 9.5 µl ligation mixture containing 1 µl of T4 ligase buffer. After adding T4 ligase (0.5 µl, 2.5 units, USB Co.), the ligation mixtures were incubated for 30 min at 16 °C. In the second step, 90 µl of ligation dilution mixture (9 µl T4 ligase buffer, 1 µl T4 ligase, and 80 µl ddH_2O) was added, and the ligation mixture was incubated overnight at 16 °C.

Transformation of *E. coli* DH5α. For each transformation reaction, a microcentrifuge tube containing *E. coli* DH5α competent cells was thawed in an ice-water bath. A portion of the ligation mixture (8-10 µl) was added to the tube. The tube was kept at 0 °C for 40 min, placed in a 42 °C water bath for 90 seconds, kept again at 0 °C for 2 min, diluted with 0.8 ml SOB^{20} and then

incubated for 1 hour at 37 °C. Aliquots of the transformed *E. coli* were spread onto LB agar plates containing 100 μg/μl apramycin.

Recombinant Plasmid Screening. White colonies carrying recombinant plasmids were inoculated into 2 ml of LB broth containing 15 μg/ml of apramycin in a loosely capped 15 ml culture tube. The culture was incubated overnight at 37 °C, 280 rpm. Plasmid DNAs were isolated from 1.5 ml of the cultures according to standard procedures.[20] Recombinant plasmids having the correct insert were confirmed by restriction enzyme digestion analyses.

Transformation of *S. lividans* with isolated recombinant plasmids. *S. lividans* protoplasts (5 x 10^8 in 50 μl P-buffer) were mixed with the 5 μl of the *E. coli* recombinant plasmid preps (0.01-0.1 μg/μl) by tapping the tube. The protoplasts were transformed by adding 200 μl of T-Buffer as in the standard method.[18] The transformants were detected by overlaying 2.5 mL of soft nutrient agar (600 μg/ml apramycin) per plate 14-20 hours later.

Southern hybridization of the recombinant EMBL4 bacteriophages

Preparation of DNA-transferred nylon membrane. Recombinant EMBL4 phage clones (0.3-0.5 μg) completely digested with *Bam*HI were loaded into individual lanes of a 0.75 % agarose gel for overnight electrophoresis in 0.5 x TBE buffer at 35 V. After visualizing and photographing the DNA bands by UV light after staining with ethidium bromide, the DNA in the gels was

denatured in 0.4 N NaOH/0.6 M NaCl for 30 min incubation at room temperature with gentle agitation. The gels were neutralized by incubating in 1.5 M NaOH/0.5 M Tris-HCl, pH 7.5 for 30 min with gentle agitation. The denatured DNA in the gel was transferred by capillary action onto a positively charged nylon membrane (GeneScreen Plus, Dupont Co.) in 10xSSC for 24 hours. The transferred DNA on the nylon membrane was completely denatured by immersing in an excess of 0.4 N NaOH for 60 seconds. After rinsing with an excess of 0.2 M Tris-HCl, pH 7.5/2xSSC, the membrane was dried at room temperature.

Probe preparation. 10 ng of *actI*-ORFI (ketosynthase gene of actinorhodin biosynthesis) or *actIII* (ketoreductase gene of actinorhodin biosynthesis) were labeled with 10-25 μCi of [α^{32}P]dATP using a Random Primed DNA labeling Kit from Boehringer Mannheim Co. for 1 hour at 37 °C. The unincorporated [α^{32}P]dATP was removed by dialysis several times through a Microcon-30 concentrator (Amicon Co.)

Hybridization with the probe. Prehybridization was carried out in 10 ml of 1 % SDS/1 M NaCl/10 % dextran sulfate and 1 mg of fragmented salmon sperm DNA in a hybridization oven at 65 °C. After several hours of prehybridization, the radiolabeled probe was denatured by boiling and rapidly cooling on ice and then added to the mixture. Incubation was continued overnight at 65 °C.

Washing the membrane and preparation of X-ray film. After hybridization, the membrane was washed two times with 2xSSC/1 % SDS at 65 °C and then placed on Kodak X-ray film for 40-44 hours at -80 °C. The detailed washing procedures for the

hybridized membranes were followed by the manufacturer's instructions.

Expression testing of *S. lividans* and *S. parvulus* transformants

Fermentation of *S. lividans* and *S. parvulus* transformants. An *S. lividans* or *S. parvulus* spore suspension (0.1 ml) was inoculated into 5 ml of YEME containing 6 μg/ml of apramycin for transformants containing pKC1218-derived recombinant plasmids or 10 μg/ml of thiostrepton for transformants containing pIJ941-derived recombinant plasmids. After incubation for 3-4 days at 28 °C, 260 rpm, 0.25 ml of the seed culture was used to inoculate 5 ml of three production media; GPS,[35] glycerol-asparagine,[36] and YEME[18] containing appropriate antibiotics in 18 ml culture tubes. The rest of the cultures were used for plasmid mini-preps to confirm the presence of the correct plasmids. All cultures were incubated at 28 °C in a LabLine Rotary Incubator-Shaker at 300 rpm for 5 and 9 days.

Analysis of the transformant metabolites. The cultures were acidified (pH 2.5-3.0) with 0.1 N HCl, extracted with EtOAc, and the extracts dried using a SpeedVac centrifuge. The dried extracts were taken up in 100 μl of 10 % MeOH/CH_2Cl_2. An aliquot (10 μl) of each extract was analyzed by reverse phase HPLC. The metabolites were separated on a Waters NovaPak C_{18} radial compression column (0.8 x 10 cm) using a gradient of 5-95% acetonitrile in water over period of 30 min at 1.5 ml/min. Detection was by photodiode array.

Other Molecular biological techniques

Plasmid DNA isolations from *E. coli* and preparation of *E. coli* competent cells were performed according to standard procedures.[20] Restriction enzymes, DNA ligase and calf intestinal alkaline phosphatase (CIP) were purchased from Gibco BRL, New England Biolabs, United States Biochemical (USB), Promega and Boehringer Mannheim Co. and used according to the manufacturers' instructions.

Results and Discussion

Southern hybridization of the lambda clones with *actI*-ORF1 and *actIII*

actI-ORFI (ketosynthase) and *actIII* (ketoreductase) probes derived from actinorhodin biosynthetic genes were hybridized separately at moderate stringency to Southern blots of a *Bam*HI restriction digest of each of the lambda DNAs. In the experiment using *actI*-ORFI, the probe hybridized strongly with the 6A1, 10a, 13a, 14a, and 17a clones, moderately with the 1B1, 2A1, 4A1, 5B1, 7c, and 15b clones, and weakly with the rest of the clones (Figure II-3). Using *actIII*, strong hybridization was observed for the 10a, 13a, and 14a clones (Figure II-4). 1B1, 1B2, 2A1, 2B1, 3B1, 5B2, and 18A2 did not hybridize with *actIII* and the rest of the clones hybridized very weakly with the probe.

The *actIII* gene encodes the C-9 ketoreductase, which catalyzes the reduction of the keto group to the corresponding secondary alcohol at C-9, counting from the carboxy terminus of the assembled polyketide.[21,22] Recently, ketoreduction at the C-9 position by *actIII* was further confirmed by engineered biosynthesis of novel compounds produced by expressing a combination of individual genes of the actinorhodin and tetracenomycin PKS gene clusters.[23,24] The *actIII* gene product catalyzes reduction of the C-9 carbonyl of any length nascent polyketide backbone studied so far, and the derived hydroxyl

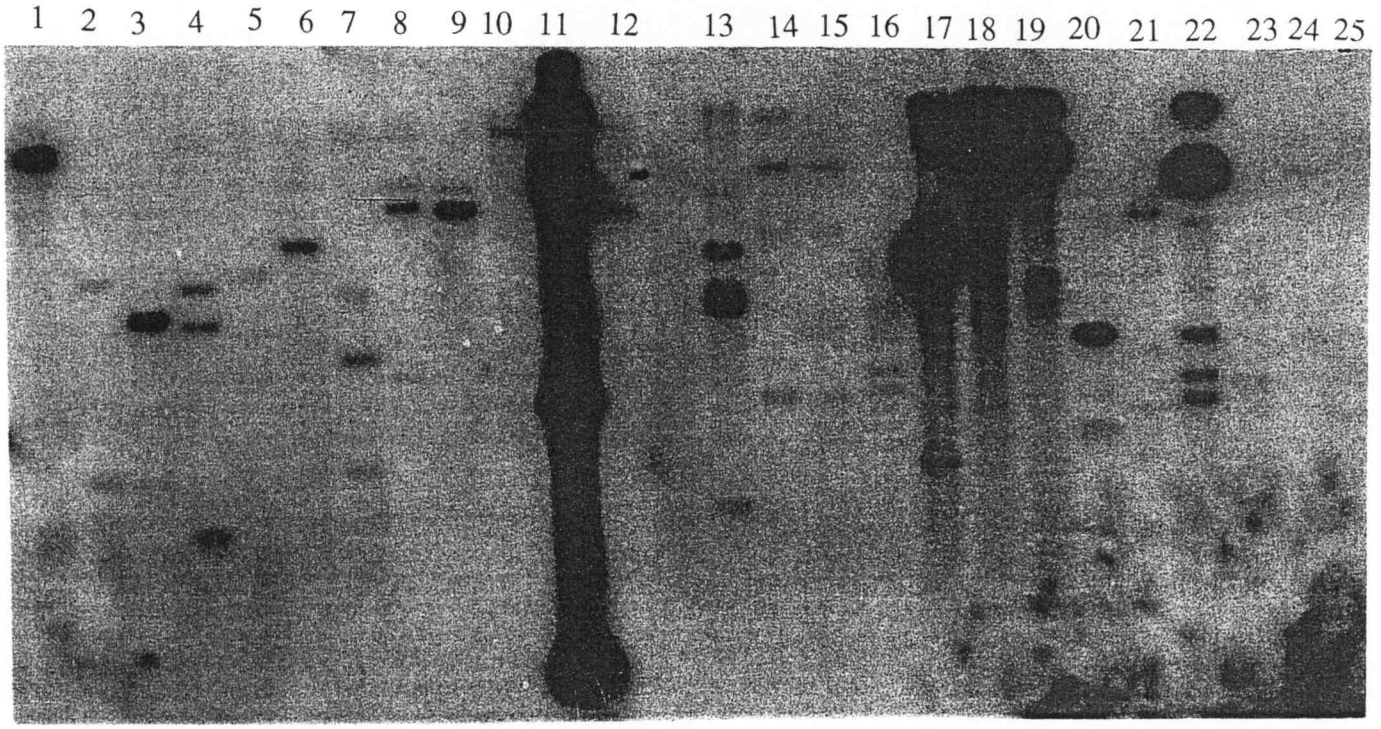


Figure II-3. Southern hybridization of *Bam*HI digested *S. murayamaensis* lambda clones with *actI*-ORF1. Lanes: 1, 1B1; 2, 1B2; 3, 2A1; 4, 2B1; 5, 3B1; 6, 4A1; 7, 4A2; 8, 5A2; 9, 5B1; 10, 5B2; 11, 6A1; 12, 7a; 13, 7c; 14, 8A2; 15, 8B1; 16, 9c; 17, 10a; 18, 13a; 19, 14a; 20, 15b; 21, 15d; 22, 17a; 23, 18A1; 24, 18A2; 25, 19B1. Washing; 2x SSC/1% SDS.

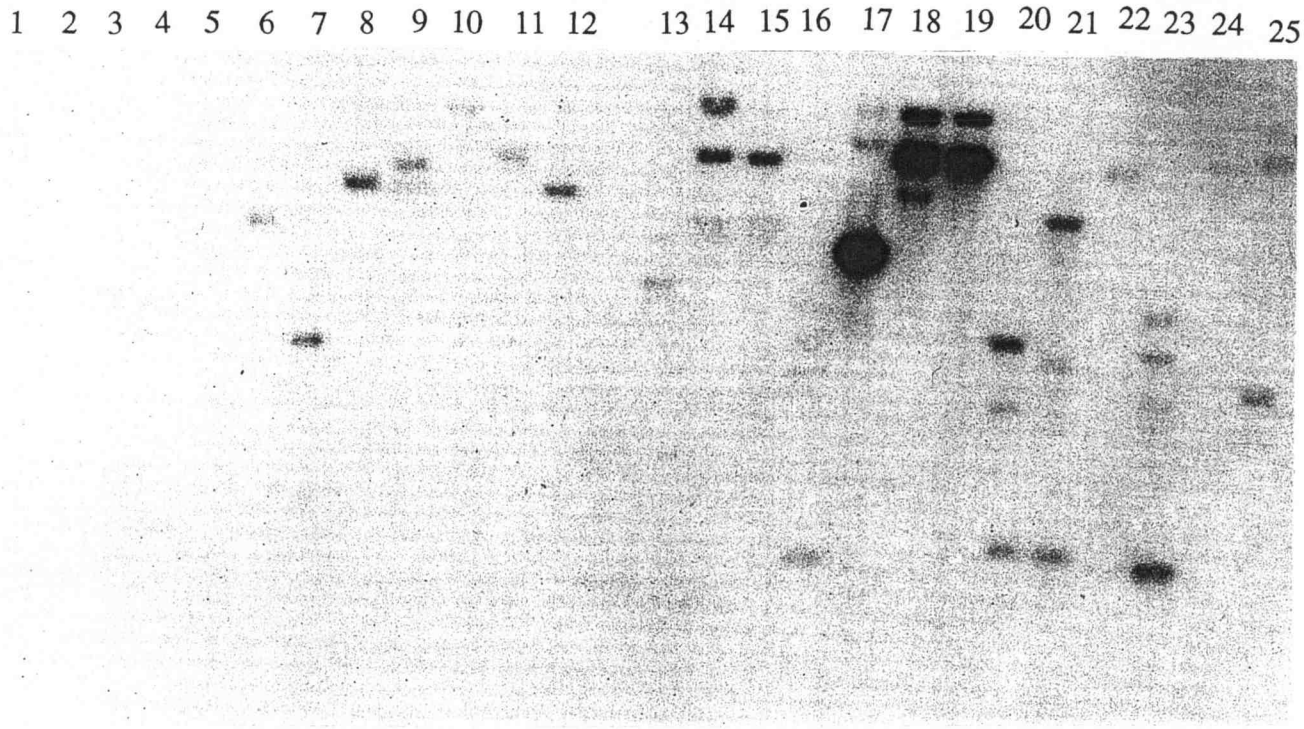


Figure II-4. Southern hybridization of *Bam*HI digested *S. murayamaensis* lambda clones with *actIII*. Lanes: 1, 1B1; 2, 1B2; 3, 2A1; 4, 2B1; 5, 3B1; 6, 4A1; 7, 4A2; 8, 5A2; 9, 5B1; 10, 5B2; 11, 6A1; 12, 7a; 13, 7c; 14, 8A2; 15, 8B1; 16, 9c; 17, 10a; 18, 13a; 19, 14a; 20, 15b; 21, 15d; 22, 17a; 23, 18A1; 24, 18A2; 25, 19B1. Washing; 2x SSC/1% SDS.

group at C-9 is eliminated by subsequent aromatase action. The kinamycin antibiotics, which are 5-diazobenz[b]fluorenes, are biosynthesized from a polyketide origin through an intermediate benz[a]anthraquinone, dehydrorabelomycin, which does not have an oxygen function at the C-9 position.[4,6,7,11] Thus, its PKS gene cluster should contain an *actIII* homologue.

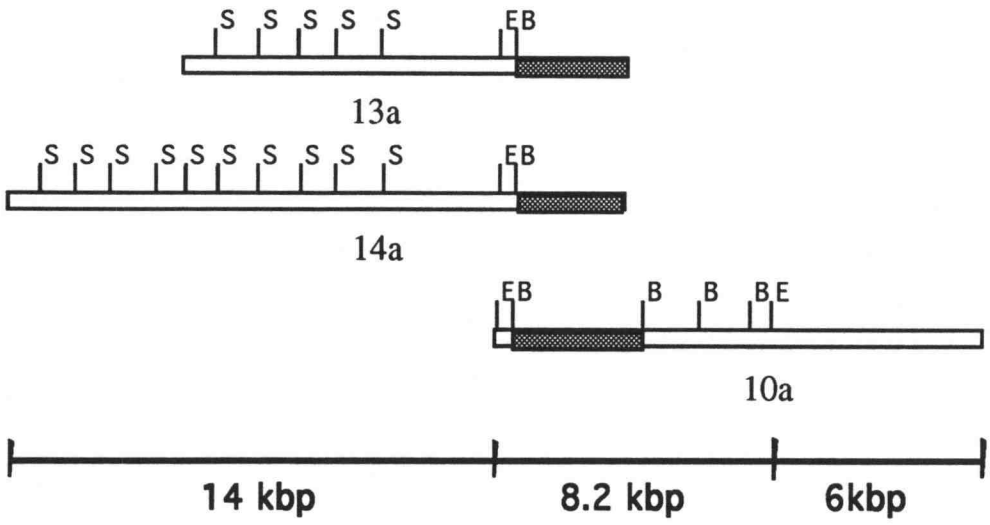


Figure II-5. Restriction map of lambda clones 10a, 13a, and 14a. The shaded area represents 5.6 kb *Bam* HI fragment which has been sequenced.[31] The number of *Sal*I sites on the left end of map could not be counted exactly because so many *Sal*I sites generated the same size of small DNA fragments. Abbreviations for restriction endonuclease sites: B, *Bam*HI; E, *Eco*RI; S, *Sal*I.

Restriction enzyme digest analysis showed that the 13a and 14a clones overlapped and there was tentative evidence that clone 10a might also do so (Figure II-5). These 3 clones were believed to be

the most strong candidates for the kinamycin PKS gene cluster, since the clones hybridized strongly with both the ketosynthase (*actI*-ORFI and *gra*-ORF1) and ketoreductase (*actIII*) genes. A 5.6 kb *Bam*HI fragment of clone 10a which hybridized with genes for ketosynthase (*actI*-ORF1 and *gra*-ORF1) and ketoreductase (*actIII*) was subcloned and the entire region was sequenced.[31] The insert was found to contain five complete open reading frames which showed high sequence similarity with genes from other aromatic polyketide pathways encoding ketosynthase (KS), chain length factor (CLF), an acyl carrier protein (ACP), a ketoreductase (KR), and a cyclase (CYC) (Figure II-6). The deduced amino acid sequence also contained several key active site residues from polyketide synthase genes.

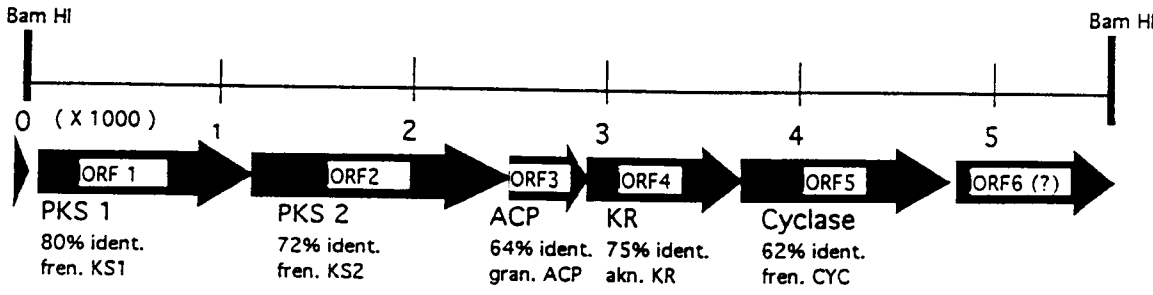


Figure II-6. Organization of the sequenced 5.6 kb *Bam*H I fragment. Abbreviations: fren, frenolicin; gran, granaticin; akn, alkavinone.

Preparation of recombinant phage DNAs

Recombinant phage having the putative PKS gene cluster inserts of *S. murayamaensis* were prepared as described in the experimental section. The phage were purified by precipitation in an ultracentrifuge. Phage DNAs were prepared by extraction with phenol/$CHCl_3$ after removal of protein coats with proteinase K. These were then cut with *Eco*RI and analyzed by gel electrophoresis. This restriction enzyme was only one that would cut at the vector/insert boundary. Unfortunately, many of the insert DNAs were found to also contain *Eco*RI sites. The results are summarized in Table II-1.

Table II-1. The result of the recombinant phage DNA preparation.

Eco RI sites in insert	Recombinant phage clones
zero	1B2, 2A1, 3B1, 5B1, 7a, 15b, 17a, 19B1
one	2B1, 4A1, 4A2, 5B2, 6A1, 7c, 8A2, 10a, 13a, 14a, 15d
two	1B1, 4A2, 9c, 18A1, 18A2
three	5A2

Subcloning lambda clones

Subclonings were initially focused on the clones having no internal *Eco*RI sites or showing strong hybridization to both probes, except the 10a clone which was studied by another member of the group. Intact inserts from these clones were obtained by *Eco*RI digestion and purified fragments were inserted into a very low copy number *Streptomyces* plasmid, pIJ941 (Table II-2). This proved to be very time-consuming. *Streptomyces* grow very slowly, requiring more than 1 week on agar media, compared to overnight growth of *E. coli*. Also, endogenous endonuclease activities and the thick cell wall of *S. lividans* frequently resulted in plasmid preparations of poor yield and quality compared to *E. coli*. After the 6 clones lacking an internal *Eco*RI site were successfully subcloned into pIJ941, a new *Streptomyces-E. coli* shuttle vector, pKC1218, became available.[19] This shuttle vector has many advantages over pIJ941, because preparation of recombinant plasmids is much easier in *E. coli* than in *Streptomyces*. The remaining two clones with no internal *Eco*RI sites and clone 14a with one internal *Eco*RI site were cloned into this shuttle vector.

The *Eco*RI site was the only restriction enzyme site available for removing the inserts from the lambda clones. Unfortunately, clone 14a, which hybridized very strongly to both probes, had one *Eco*RI site within the *S. murayamaensis* DNA insert. The subcloning work was hampered by a very low yield of the insert after partial

Table II-2 Bacterophages and plasmids used and constructed for expression tests

Phages or plamids	Relevant Characteristic (s)	Source
Phages		
EMBL4	Derivative of lambda, bacteriophage vector	John Innes Institute
1B2	EMBL4 containing 15.5 kbp insert of *S. murayamensis* putative PKS genes	This work
2A1	EMBL4 containing 16.0 kbp insert of *S. murayamensis* putative PKS genes	This work
3B1	EMBL4 containing 18.2 kbp insert of *S. murayamensis* putative PKS genes	This work
5B1	EMBL4 containing 19.0 kbp insert of *S. murayamensis* putative PKS genes	This work
7a	EMBL4 containing 18.3 kbp insert of *S. murayamensis* putative PKS genes	This work
15b	EMBL4 containing 17.1 kbp insert of *S. murayamensis* putative PKS genes	This work
17a	EMBL4 containing 16.8 kbp insert of *S. murayamensis* putative PKS genes	This work
19B1	EMBL4 containing 17.5 kbp insert of *S. murayamensis* putative PKS genes	This work
14a	EMBL4 containing 18.0 kbp insert of *S. murayamensis* putative PKS genes	This work
Plasmids		
pIJ941	25.0 kbp; Hyg^r, $Thio^r$ *Streptomyces* very low copy number plasmid	John Innes Institute
pKC1218	5.8 kbp; *E. coli-Streptomyces* Shuttle plasmid having pUC and Scp2* replicons	Eli Lilly Co.
pSH100	pIJ941 with 15.5 kbp *Eco* RI subclone from phage 1B2	This work
pSH110	pIJ941 with 18.2 kbp *Eco* RI subclone from phage 3B1	This work
pSH120	pIJ941 with 19.0 kbp *Eco* RI subclone from phage 5B1	This work
pSH130	pIJ941 with 18.3 kbp *Eco* RI subclone from phage 7a	This work
pSH140	pIJ941 with 17.1 kbp *Eco* RI subclone from phage 15b	This work
pSH150	pIJ941 with 16.8 kbp *Eco* RI subclone from phage 17a	This work
pSH210	pKC1218 with 16.0 kbp *Eco* RI subclone from phage 2A1	This work
pSH220	pKC1218 with 17.5 kbp *Eco* RI subclone from phage 19B1	This work
pSH230	pKC1218 with 15.5 kbp *Eco* RI subclone from phage 14a	This work

digestion and by very low efficiency for the ligation of such a big insert into a plasmid.

Since the plasmid pKC1218 does not easily accept big pieces of foreign DNA, we developed an efficient ligation protocol by using a two-step ligation method.[25] Ligation of one end of DNA to another can be regarded as a bimolecular reaction whose velocity under standard conditions is determined solely by the concentration of compatible DNA termini. This is true no matter whether the termini are located on the same molecule of DNA (intramolecular ligation) or on different molecules (intermolecular ligation).[20] In principle, at low concentration of DNA, recircularization of the plasmid DNA will occur with high efficiency. If the concentration of DNA in the ligation reaction is increased, a given end of DNA is more likely to encounter a terminus located on another molecule before intramolecular ligation occurs. At high DNA concentrations, therefore, the initial products of ligation will be dimers and larger oligomers. As carried out, a very high DNA concentration (1-3 μg/μl) of the digested phage DNA and the dephosphorylated plasmid was first prepared in 9.5 μl of ligation mixture. After adding 0.5 μl of T4 ligase, the ligation mixtures were incubated for 30 min at 16 °C to activate intermolecular ligation. At this step, the very concentrated DNA fragments should prefer intermolecular ligation. Second, 90 μl of ligation dilution mixture (9 μl T4 ligase buffer, 1 μl T4 ligase and 80 μl ddH_2O) was then added and the ligation mixture incubated overnight at 16 °C. In the second ligation step, the dimerized DNA fragment will prefer intramolecular ligation due to the low concentration of DNA.

Clone 14a was partially digested with *Eco*RI and subcloned into pKC1218 by this direct two-step ligation method. Generally, this method was found to be much more efficient in subcloning big inserts (<10 kb) than conventional single step ligation, and the two lambda clones (2A1 and 19B1) were also subcloned into pKC1218 by the two-step method.

Expression test of transformants

Streptomyces plasmid pIJ941 having the Scp2* replicon[34] propagates in a wide range of *Streptomyces* hosts, including *S. lividans* and *S. parvulus*. However, the transformation efficiency of *S. parvulus* with pIJ941 was 100-1000 times lower than with *S. lividans*. The low transformation efficiency of *S. parvulus* seems to be attributable to the restriction system of the organism rather than to cell membrane characteristics. *S. parvulus* protoplasts showed exactly the same microscopic morphology and same degree of sensitivity to SDS as *S. lividans* protoplasts. The pKC1218 vector has only part of the Scp2* replicon[19] and does not have the *par* gene, which is required to stably propagate the plasmid in the host. The pKC1218 and pKC1218-derived plasmids were able to transform only *S. lividans*. These plasmids were not able to transform *S. parvulus*, which made this latter organism unsuitable for expression testing. It appears that the entire Scp2* replicon is essential to propagate pKC1218 in *S. parvulus*.

After confirming the presence of the recombinant plasmids in *S. lividans* and *S. parvulus* transformants, the transformants were

cultured in 3 different production media: GPS,[35] YEME,[18] and Glycerol-Asparagine (Gly-Asn)[36] and then screened for new metabolite production. Each medium has different characteristics. The Gly-Asn medium is a minimal media and is good for production of any secondary metabolites formed in a very nutrient poor condition or in a very late stage of stationary phase of a *Streptomyces* growth curve. The YEME medium contains a large quantity of carbon source (sucrose) and is good for production of secondary metabolites formed in a nutrient rich condition or in an early stage of stationary phase. The GPS is a nutrient rich medium and is good for production of a wide variety of different secondary metabolites at both early and late stages of the stationary phase.

The metabolites of the transformants were extracted from each of the production cultures with ethyl acetate and analyzed by reverse phase HPLC with photodiode array detection. The fact that a compound's retention time matches that of a standard compound under a given set of HPLC conditions is not by itself a sufficient criterion for identification of compounds. The use of the photodiode array detector provides a UV-Visible spectrum of each peak in an HPLC chromatogram. The UV-Visible spectrum depends on the chemical structure of compound. So demonstrating that both the retention time and the UV-Visible spectrum of a compound match a standard would be a strong preliminary evidence for structural assignment of metabolites from the transformants.

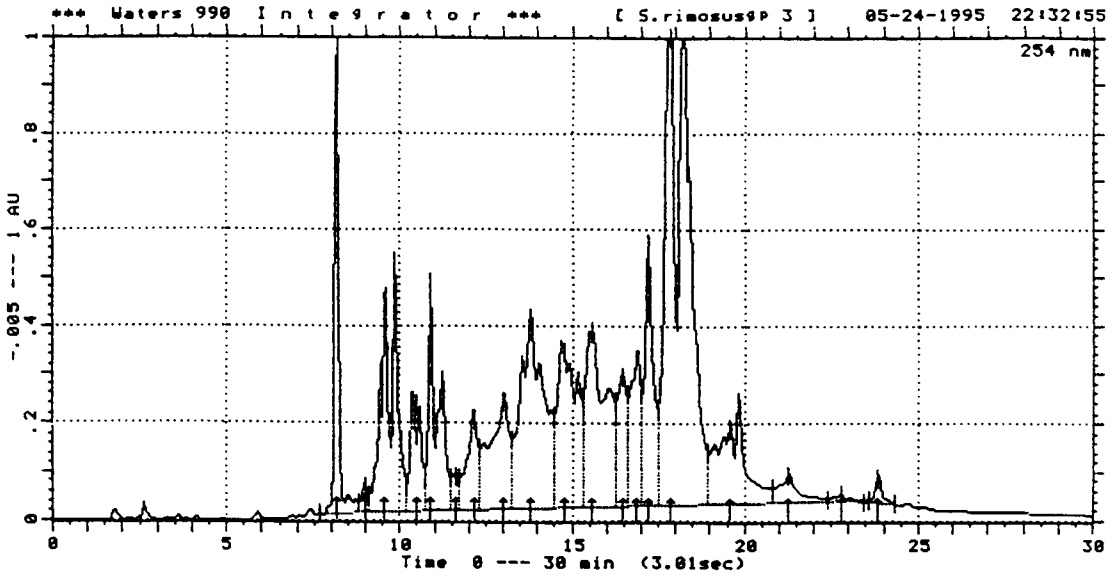
The production media of transformants carrying a recombinant plasmid were sometimes different colors than fermentation of *S.*

lividans or *S. parvulus* carrying the vector alone. However, HPLC analysis showed there were no obvious new peaks in any of the transformants, except for one *S. lividans* transformant, *S. lividans*/pSH210 (Figure II-7).

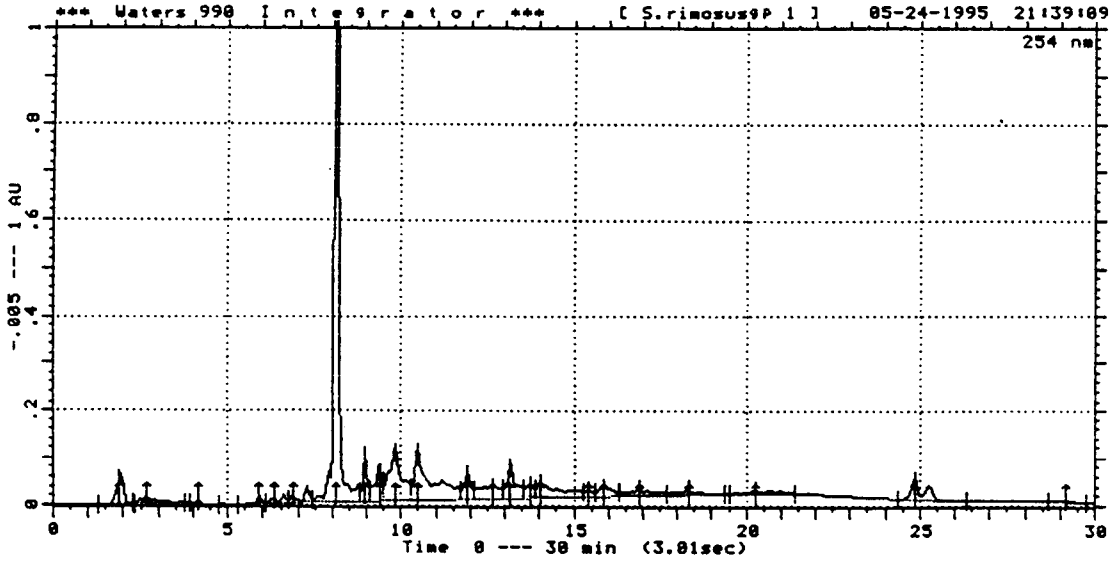
The size of complete aromatic polyketide biosynthetic gene clusters examined to date have been shown to be 22-34 kbp.[26-28] The total size of the bacteriophage inserts which were subcloned for expression tests in this portion of the work was 15-20 kb, which is too small to contain an entire PKS gene cluster. While it was possible that an intermediate compound, such as dehydrorabelomycin, might have been produced even by DNA regions smaller than an entire PKS cluster, this appeared not to be occurring.

For the pSH210 transformants in the GPS production medium, new metabolites were recognized by HPLC (Figure II-7 and II-8). Although most metabolites did not match any of the metabolites produced by *S. lividans,* they also did not match any known metabolites from *S. murayamaensis*. These metabolites was suspected to be stress metabolites of *S. lividans* host. Under normal fermentation conditions, these compounds were never observed from *S. livians*. Later, it was found that these metabolites were repeatedly produced from other *S. lividans* transformants having an unstable plasmid. The instability of some recombinant plasmids may come from intramolecular or intermolecular recombination of the plasmids.[32,33] Especially in the case of big inserts, such as cosmid clones, many plasmids were not stable (Chapter III).

PKS gene clusters of *Streptomyces* sp. usually consist of the biosynthetic genes and associated regulatory and self resistance genes. All of the PKS gene clusters studied to date have proven to be tightly clustered in one region of the chromosome. The DNA fragments compromising a part of PKS gene clusters may express intermediate compounds in a heterologous host such as in case of daunorubicin[9] and tetracenomycin.[10] However, the identification of the PKS genes obtained from *S. murayamaensis* by expression in heterologous hosts was not successful, probably because the *S. murayamaensis* PKS inserts (15-20 kb) were too small even for expression of intermediate compounds. Identification of the genes by other ways such as gene disruption or rescuing mutants is laborious and time-consuming, and ambiguous results[11-12] have often been obtained. So, an *S. murayamaensis* genomic DNA library containing much larger pieces of DNA was constructed in a cosmid vector, pOJ446, (Chapter III) to improve the chances of expressing recognizable polyketide metabolites.



S. lividans/pSH210



S. lividans/pKC1218

Figure II-7. HPLC trace of *S. lividans*/pSH210 compared to *S. lividans*/pKC1218.

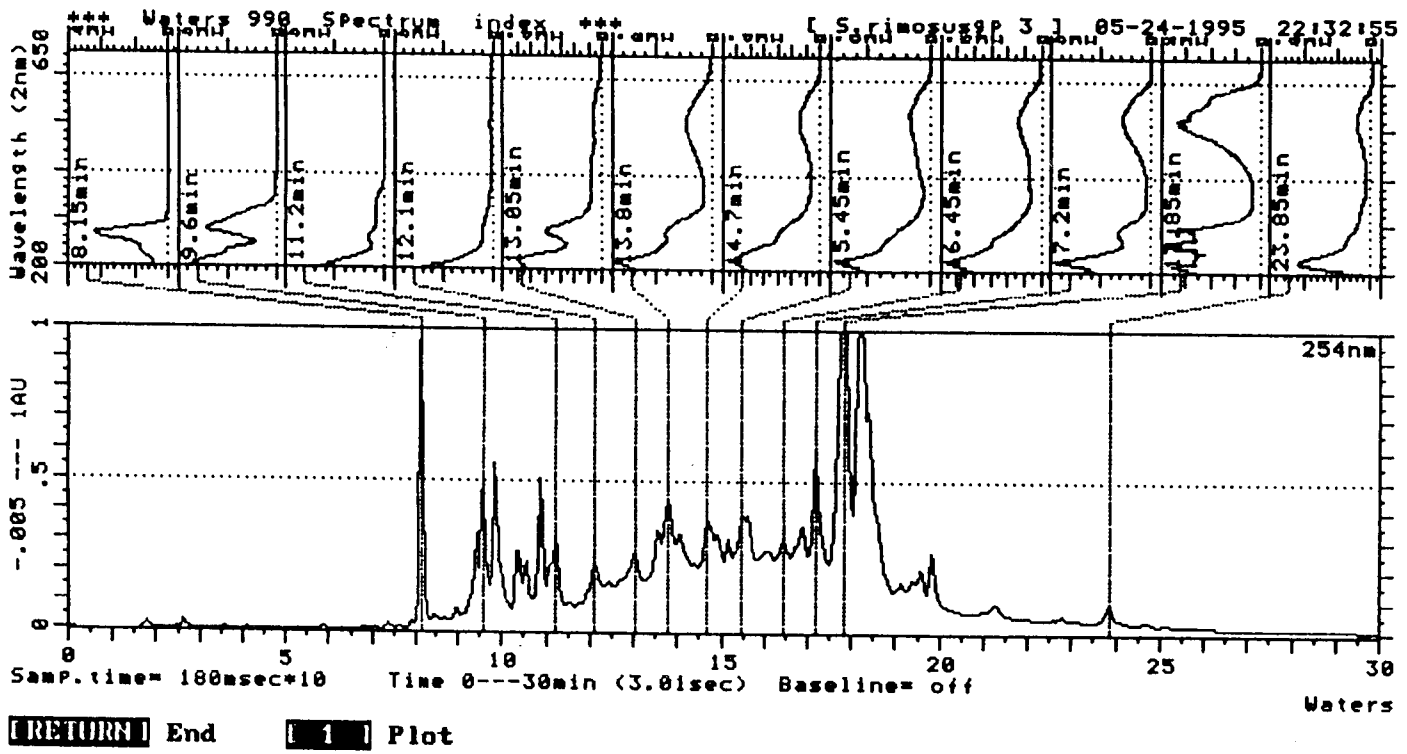


Figure II-8. Spectrum index plot of stress metabolites from *S. lividans*/pSH210

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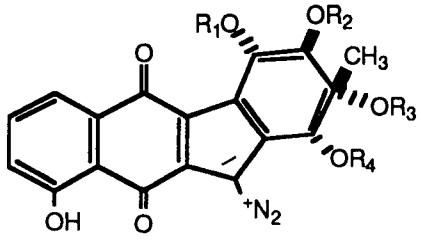
Chapter III

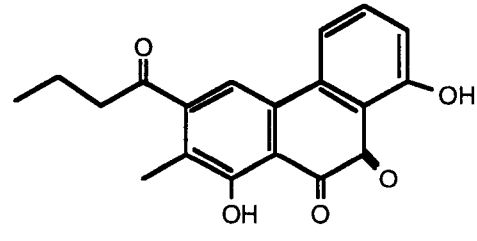
Cloning two PKS gene clusters from *S. murayamaensis* and identification of a putative kinamycin biosynthetic gene cluster

Introduction

S. murayamaensis is known to have at least two independent aromatic polyketide biosynthetic pathways which give rise to the distinct structural classes represented by kinamycins[1] (1) and by murayaquinone[2] (2).



1



2

Kinamycin antibiotics are derived from the angucycline intermediate, dehydrorabelomycin (Figure II-1).[4] The ring folding processes during biosynthesis of the angucycline polyketide

skeleton and for the murayaquinone polyketide phenanthraquinone skeleton are expected to be different from those for linear polycyclic compounds. Cloning the kinamycins and murayaquinone PKS gene clusters may lead us to understand what causes the nascent polyketide backbones to adopt non-linear conformations and generate the angular skeletons of the angucycline and phenanthraquinone structures.

The kinamycin family of metabolites have been extensively studied in our group, and we are especially interested in the kinamycin biosynthetic gene cluster. The region of the S. murayamaensis genome comprising the lambda clones 10, 13a, and 14a (Figure II-5) contains a 5.6 kbp *Bam*HI fragment (Figure II-6) which were initially identified because it hybridized strongly with both ketoreductase and ketosynthase genes. It has recently been sequenced in our group, and was found to contain ACP and dehydrase/cyclase genes,too.[8] Attempts to knock out this PKS gene cluster with several of the sequenced ORFs by single crossover events in an effort to identify the product of this pathway. However the single crossover were unsuccessful, mainly because of the difficulty of introducing foreign DNA into *S. murayamaensis*. A double crossover disruption to knock out the ketoreductase gene of this cluster was then carried out. The ketoreductase gene in *S. murayamaensis* genome was apparently interrupted. However, no detectable change was observed in the product profile of the *S. murayamaensis*. The attempt to identify the PKS gene clusters by expression in heterologous hosts was not also successful (Chapter II).

The total size of biosynthetic gene clusters in *Streptomyces* for aromatic polyketide compounds have been 22-34 kbp.[5-7] A new approach was undertaken to find the kinamycin and murayaquinone gene clusters. Larger pieces of DNA (30-45 kbp) from *S. murayamaensis* were cloned by using the cosmid vector, pOJ446, constructed with dual replication origins for propagation in *Streptomyces* and *E. coli* (Figure III-1).[13] Clones containing PKS genes were again identified by hybridization with *actI*-ORF1. The positive clones were then used for expression experiments to identify the product of each PKS gene cluster.

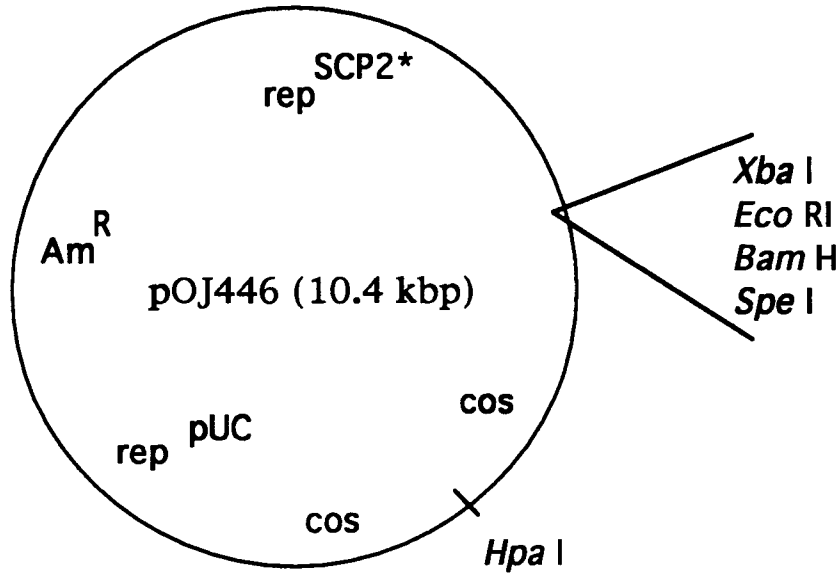


Figure III-1 Cosmid cloning vector used to prepare a library of *S. murayamaensis* genomic DNA.

Materials and Methods

Bacterial strains and plasmids used. The strain of *Streptomyces murayamaensis* was a gift from Professors Omura (Kitasato University, Japan) and Hornemann (University of Wisconsin). *Streptomyces lividans* TK24, used as a recombinant host strain, were obtained from John Innes Institute (England). *E. coli* XL1-BlueMR, used for propagation of cosmid pOJ446 was purchased from Stratagene Co. The cosmid pOJ446, used for a cosmid library construction, was obtained from Lilly Research Laboratories.

***S. murayamaensis* genomic DNA isolation.** Genomic DNA was prepared from *S. murayamaensis* as follows: mycelia from a 50 ml culture grown in YEME containing 0.5 % glycine were collected by centrifugation at 3000 x g. After twice washing the pellet with 10.3 % sucrose, it was resuspended in 10 ml of a solution containing: 25 mM Tris (pH 8.0), 50 mM glucose, 10 mM EDTA (pH 8.0), 20 μg/ml RNase, 10 mg/ml lysozyme and 10 mg/ml acromopeptidase (Sigma, catalog number; A-3547) and incubated 5-10 minutes at room temperature. After the cell walls were partially degraded, proteinase K was added up to 0.5 mg/ml and the mixture incubated at 50 ^{o}C overnight. The DNA was extracted once with phenol (pH 8.0), once with phenol/$CHCl_3$, and once with $CHCl_3$, then precipitated by adding 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 7.0). The tube was kept at room

temperature for 5-10 min while gently mixing. The liquid was decanted carefully and the precipitated genomic DNA adhered to the wall of the tube. The precipitated genomic DNA was washed one time with 70% ethanol, and then air-dried for10 min after inverting the tube. The genomic DNA was dissolved in 3 ml TE.

Southern hybridization. Southern blotting and hybridization methods were basically the same as described in the Materials and Methods section of Chapter II.

Partial digestion of genomic DNA. A series of tubes containing 26 µl of genomic DNAs (1 µg/µl) were each treated with 3 µl of *Mbo* I buffer for partial digestion tests. After keeping in a cold room for at least several hours to completely disperse the genomic DNA, 1 µl (2 unit/µl) of *Mbo*I (10 times diluted stock) was added to each. The mixtures were then incubated at 37 °C for 0, 1, 2, 4, 8, 20, 40, and 60 min to find the condition which gave the best yield of DNA in the 30-40 kb size range. The digested DNAs were examined by electrophoresis through a 0.3 % agarose gel poured on a 1 % agarose support. After identifying the most suitable digestion time, a 10 fold larger scale partial digestion (300 µl) was carried out.

Ligation of genomic DNA to cosmid pOJ446. *Mbo*I partially digested *S. murayamaensis* genomic DNA (10-20 µg/7.5 µl TE8) was mixed with1/10 volume of T4 ligase buffer and 4-6 µg of cosmid pOJ446 that had been digested with *Bam*HI and *Hpa*I.

After adding 0.5 μl (2.5 units) of T4 ligase to the ligation mixture, it was incubated overnight at 16 °C.

Packaging cosmid libraries. A 4 μl aliquot from the 10 μl ligation mixture was used for packaging with Gigapack II XL Packaging Extract (Stratagene). The manufacturer's protocol was followed exactly.

Colony blotting. 2000-3000 *E. coli* colonies of the *S.murayamaensis* cosmid library in LB broth were spread by pipetting on a dried large nylon membrane(Amersham Co., 138 mm diameter) on several dried filter papers as described by the manufacturer. After spreading the inoculum, the membrane was transferred onto LB-agar plate containing 100 μg/ml of apramycin and incubated at 37 °C overnight. After colonies were grown well, the membrane was replicated onto several other membranes by pressing down the original membrane to new membranes on a sterile glass plate with another glass plate. The orientation of original and replica membranes were marked with holes in the pair of filters with an 18-gauge needle. The replica membranes were grown again on LB-apramycin plates overnight. One of the replica membranes was placed colonies side up on a pad of Whatman 3MM absorbent filter paper soaked in denaturing solution (1.5 M NaCl/ 0.5 M NaOH) for 7 minutes. The membrane was then transferred colonies side up on a pad of Whatman 3MM absorbent filter paper soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 0.001 M EDTA) for 3 minutes. After

repeating this step once more, the membranes were rinsed briefly in 2 x SSC, dried in air, and the DNA was then fixed in an oven at 80 °C for 2 hours.

Colony hybridization. The fixed membrane was prehybridized with 30 ml of prehybridization solution (5 x SSPE/5 x Denhardt's solution/0.5% SDS/20 μg per ml of denatured salmon sperm DNA) in a 150 mm glass crystallizing dish at 65 °C without shaking. After one hour, 10^5 dpm of denatured probe was added into the prehybridization solution and the hybridization was carried out at 65 °C overnight without shaking. After hybridization, the filters were washed 1) two times in 2 x SSPE/0.1% SDS at room temperature for 10 minutes, and 2) one time in 1 x SSPE/0.1% SDS at 65 °C for 15 minutes, followed by autoradiography.

Isolation of positive clones. After identification of the positive clones, they were picked from the original membrane and inoculated onto LB-apramycin (100 μg/ml) agar plates to isolate single colonies. After overnight incubation of the plate at 37 °C, the individual single colonies were inoculated into 2 ml of LB containing 50 μg/ml of apramycin and incubated at 37 °C, 280 rpm overnight. Twenty microliters of the overnight cultures were spotted onto a dried Amersham nylon membrane. The DNA of the dot-blotted *E. coli* cultures were fixed and hybridized as previously described in colony blotting and hybridization.

Southern hybridization of the recombinant cosmids, transformation of *S.lividans*, and expression test of the transformed *S.lividans*. These procedures were discussed in the Materials and Methods Section of Chapter II.

Other Molecular biological techniques. Plasmid DNA isolations from *E. coli* and preparation of *E. coli* competent cells were performed according to standard procedures.[14] Restriction enzymes, DNA ligase and calf intestinal alkaline phosphatase (CIP) were purchased from Gibco BRL, New England Biolabs, United States Biochemical (USB), Promega and Boehringer Mannheim Co. and used according to the manufacturers' instruction.

Results and Discussion

Southern hybridization of *S. murayamaensis* genomic DNA with *actI*-ORF1

The genomic DNA isolation from *Streptomyces* is problematic due to thick cell wall and strong endonuclease activity. The method discussed in the *Streptomyces* Lab Manual[15] worked fine only for some *Streptomyces* species such as *S. lividans* or *S. coelicolor*. This method extracts genomic DNA from broken cells which were prepared by treatment with lysozyme treatment followed by SDS treatment. However, many *Streptomyces*, such as *S. murayamaensis*, require a long incubation step (up to 1 hour) with lysozyme at 30-37 ^{o}C, this method always ends up with significant genomic DNA degradation by endogenous endonuclease activity during this long incubation. During this research, a very simple and efficient genomic DNA isolation method for *Streptomyces* was developed. In this method, *Streptomyces* mycelia were briefly treated with lysozyme and achromopeptidase to weaken the cell wall. Completely digested cells were then prepared by treatment with proteinase K overnight at 50 ^{o}C. At this temperature, the DNase activity is almost completely inhibited while the proteinase K eventually destroys these proteins. This method gave very good quality genomic DNA for every *Streptomyces* strain so far tested in our laboratory.

The DNA sequence of ketosynthase genes such as *actI*-ORF1 is strongly conserved among the Type II PKS genes without exception

(Figure I-4).[6,7] So, in this work, *actI*-ORF1 was choosen as a probe to identify aromatic PKS gene clusters of *S. murayamaensis*.

The *actI*-ORF1 DNA fragment was labeled with a ^{32}P random labeling kit and hybridized at moderate stringency (2 x SSC/1% SDS) against Southern blots of digested chromosomal DNA from *S. murayamaensis* (Figure III-2). The *Bam*HI digested genomic DNA gave signals for fragments of ca. 5.6 kbp, 4.3 kbp, 2.6 kbp and 0.5 kbp. The four bands were expected from Southern hybridization results with the lambda clones discussed in Chapter II. The 5.6 kbp *Bam*HI fragment is exactly equivalent to the 5.6 kbp *Bam*HI fragment of clone 10a (Figure II-6). The remaining three matched the *actI* homologous bands of 4.3 kbp, 2.6 kbp and 0.5 kbp of lambda clone 6A1. The Southern hybridization against *Kpn*I and *Pst*I digested genomic DNA showed only two bands each. All of this strongly suggested that there were only two *actI* homologous PKS gene clusters in *S. murayamaensis*.

Isolation of *actI* homologous clones from an *S. murayamaensis* cosmid library

Cosmid pOJ446 was constructed with dual replication origins for propagation in either *Streptomyces* sp. (Scp2*) or *E. coli* (pUC), and the *amR* gene confers resistance to apramycin upon both bacteria. It also contains OriT, a region of DNA that permits conjugal transfer from *E. coli* to *Streptomyces* sp. *E. coli* XL1-Blue MR, used as a cloning host, is a homologous recombination deficient (*recA*$^-$) strain so that recombinant cosmids can propagate stably in the *E. coli* without losing their insert by an intramolecular or intermolecular

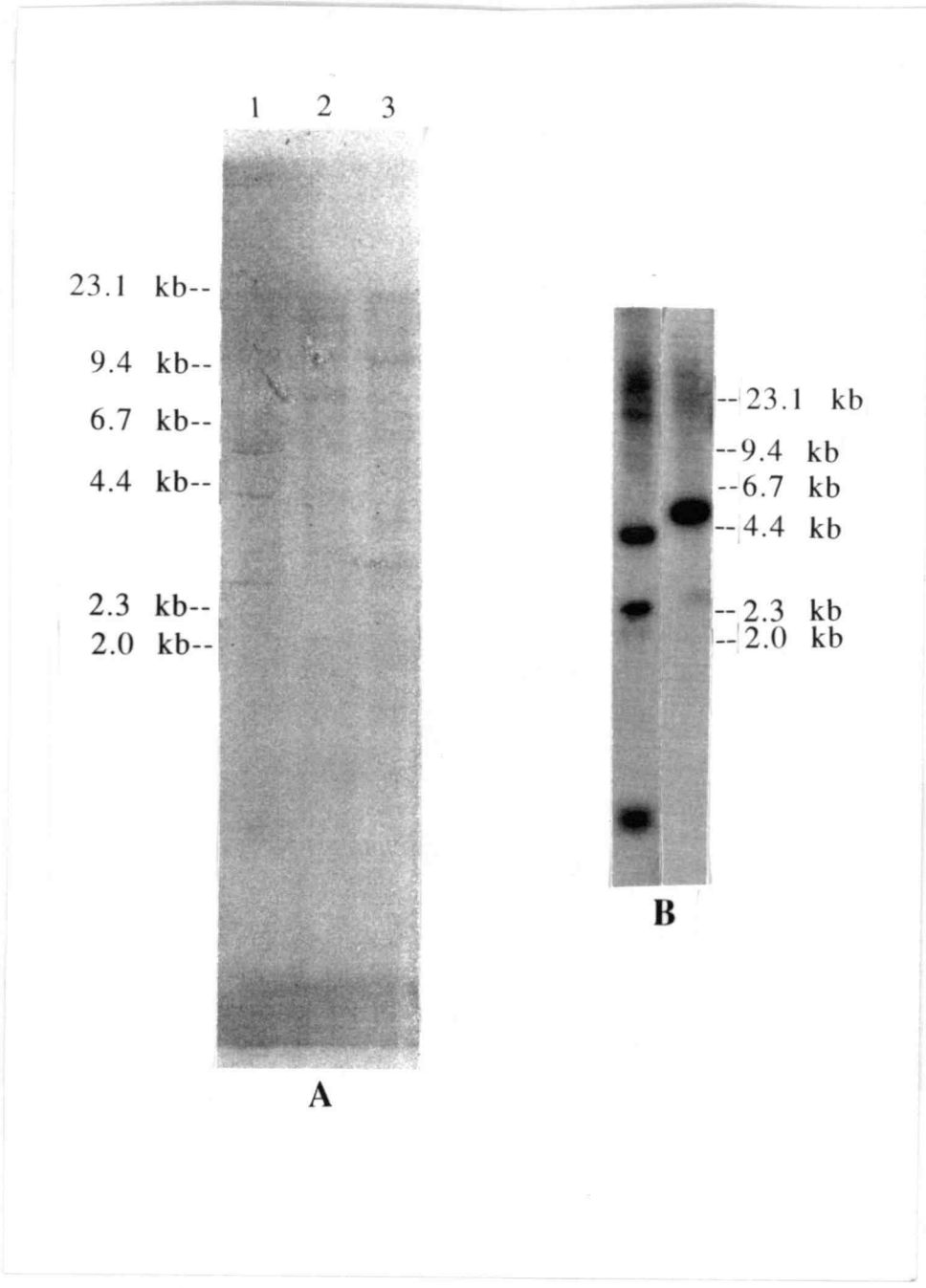


Figure III-2. Southern hybridization of digested *Streptomyces murayamaensis* chromosomal DNA (A) and the two corresponding cloned PKS gene clusters prepared by *Bam*HI digestion (B). A) Lane 1, *Bam*HI digestion; lane 2, *Kpn*I digestion; lane 3, *Pst*I digestion.

recombination process. A cosmid library containing *S. murayamaensis* genomic DNA fragments of ca. 40 kbp from partial *Mbo*I digestion was constructed using the pOJ446 in the *E. coli* XL1-Blue MR.

Since the lambda PKS gene clones (Chapter II) originally suggested the presence of several PKS gene clusters, about 6000 individual *E. coli* colonies were thoroughly screened with the *actI*-ORF1 probe. This led to the identification of 38 positive clones. Based on restriction digestion followed by Southern hybridization, it was found that the 38 clones could be grouped into just two clusters. Of these, 23 clones were grouped into Cluster I, and 15 clones were grouped into the Cluster II. The DNA of Cluster I contains the 5.6 kbp *Bam*HI DNA fragment that hybridized with *actI*-ORF1 (Figure III-2). The Cluster II DNA contains the three *Bam*HI DNA fragments that hybridized with *actI*-ORF1 (Figure III-2). Figure III-2 (B) shows Southern hybridization with *Bam*HI digested DNA from one cosmid clone from each of the two clusters. This clearly demonstrated that the two PKS gene clusters of *S. murayamaensis* were successfully cloned.

Cloning *S. murayamaensis* PKS gene clusters through a cosmid library potentially has a number of advantages over cloning through a bacteriophage library(Chapter II). The large DNA fragments (30-45 kb) can increase the chance of expressing a recognizable product from the cloned PKS genes. Also, the larger cosmid inserts of the genomic DNA made it easier to correctly group PKS clones. Due to the relatively small size of the lambda inserts, it was more likely that the 15-20 kbp fragments would not

include the full set of genes being probed. The only lambda clones 6A1, 10a, 13a, 14a, and 17a seems to have real PKS gene inserts. The restriction map of other lambda clones did not match with either Cluster I or Cluster II obtained from the cosmid clones. The weak Southern hybridization signals from these lambda clones may have come from non-specific binding of the probe to genes which are slightly homologous to PKS genes, such as those for fatty acid biosynthesis.

Heterologous expression of a putative kinamycin biosynthetic gene

Ten cosmid clones from Cluster I and 8 clones from Cluster II were introduced into *S. lividans* by transformation to identify the product of the PKS genes by heterologous expression (Table III-1). The transformation efficiency of *S. lividans* with either cluster was about 100 times lower than the transformation efficiency of *S. lividans* with the cosmid itself. Also, transformants showed the typical phenotype of *S. lividans* growing in a stress condition. Based on the lower transformation efficiency and the stress phenotype, it was considered that both types of recombinant plasmids led to heterologous expression of secondary metabolites which may be potentially harmful to the host strain. *S. lividans* transformants with two of the clones (pSH1500 and pSH1580) of Cluster II produced a green pigment in R2YE and grew especially slowly.

Table III-1. Bacterial strains and plasmids used in this study

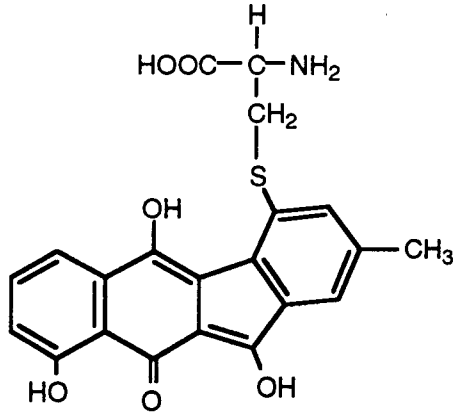
Strains or plasmids	Relevant characteristics	Source or reference
Bacterial strains		
S. murayamaensis	Murayaquinone and Kinamycins producer	2
E. coli XL1-BlueMR	*Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gryA96,relA1, lac*	Stratagene
S. lividans TK24	Host for expression test ($SLP2^-$, $SLP3^-$)	John Innes Institute
Plasmids		
pOJ446	*E.coli-Streptomyces* Shuttle cosmid	5
pSH1500	Cluster II+pOJ446, produce PK1 and PK2 in *S. lividans*	This work
pSH1510	Cluster II+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1520	Cluster II+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1530	Cluster II+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1540	Cluster II+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1550	Cluster II+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1560	Cluster II+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1570	Cluster II+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1580	Cluster II+pOJ446, produce PK1 and PK2 in *S. lividans*	This work
pSH1000	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1010	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1020	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1030	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1040	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1050	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1060	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1070	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1080	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work
pSH1090	Cluster I+pOJ446, did not produce any new metabolite in *S. lividans*	This work

It has been well known that *Streptomyces lividans* TK24 sometimes eliminates foreign DNA inserts by intramolecular recombination.[22,23] Because of this possibility, multiple colonies were inoculated into liquid media instead of single colonies. After growing the transformants on R2YE agar for 7-10 days, multiple colonies were scraped off from the plates to inoculate 5 ml of YEME media. A large quantity of cells was used for inoculation to reduce the chance of intramolecular recombination, since fewer cell divisions would be required to reach log-phase. If we assume the probability of losing the insert is 0.1 % at each cell division, it is obvious that fewer cell divisions are much better for the expression test. After growing the seed culture for 3-4 days, depending on growth of the transformants, 0.3 ml of the seed culture was used to inoculate 5 ml each of GPS and YEME for production, and these were incubated for 7 days. The cultures were extracted with ethyl acetate. The extracts were analyzed using photodiode array HPLC.

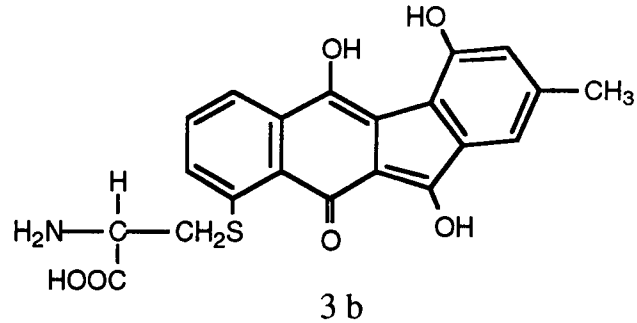
The HPLC analysis showed that the Cluster I transformants did not produce any new metabolites in *S. lividans*. The cosmids were re-isolated from these *S. lividans* strains to see whether they still had an intact insert. Restriction analysis of the cosmid DNA showed that all of clones had lost more than half of their insert, presumably by an intramolecular recombination process. As shown in Figure II-3, the right end of the cluster I PKS gene has an unusually large number of *Sal*I sites, indicating that the region may be a repetitive area. Repetitive areas very easily lose insert by intramolecular recombination.[14] The instability of this PKS

gene cluster may explain why there was no expression of the PKS genes in *S. lividans* TK24. A different host, such as *S. lividans* JT46[22] or ZX7[24] having no intramolecular recombination activity, will be needed for an expression test of this cluster.

Two clones (pSH1500 and pSH1580) of Cluster II produced two new metabolites (PK1, putative kinamycin 1 and PK2, putative kinamycin 2) in *S. lividans*. Figure III-3 shows the HPLC traces of *S. lividans* with just the vector, pOJ446, and with the recombinant cosmid, pSH1580. The UV-visible absorption spectra, obtained by photodiode array detection, are shown in Figure III-4. PK1 started to be detected at an early stage of the fermentation (starting from 2 days in GPS), while PK2 was produced in the late stage of fermentation (usually after 5 days in GPS). In YEME, only PK1 was produced after 5 days of growth. The UV-visible spectrum and retention time of PK1 (Figure III-4) exactly matched one of metabolites previously observed to be produced by *S. murayamaensis* MC1, a kinamycin-deficient mutant generated by NTG treatment.[25] A large fermentation of *S. lividans*/pSH1580 (1L, GPS) was carried out and compound PK1 was isolated and purified. The chemical structure of this compound has been examined by ^{1}H NM, ^{13}C NM and high resolution mass spectrometry. It appears to be either 3a or 3b, and this work is continuing.[26]



3 a



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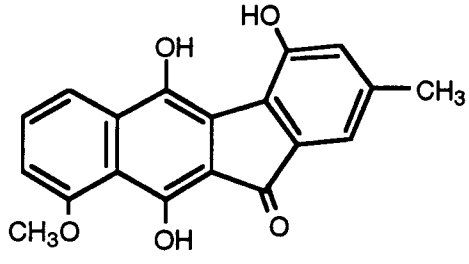

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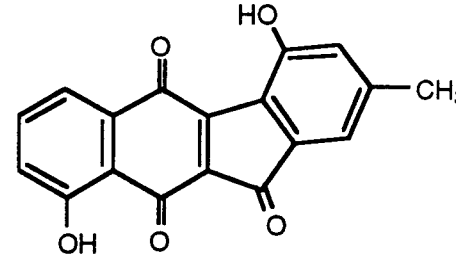
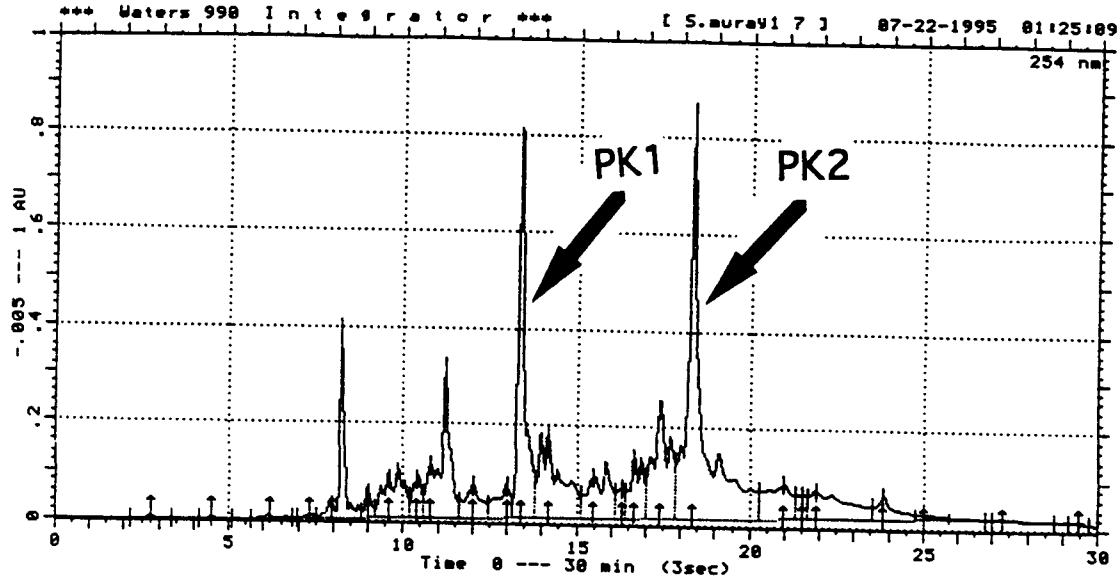
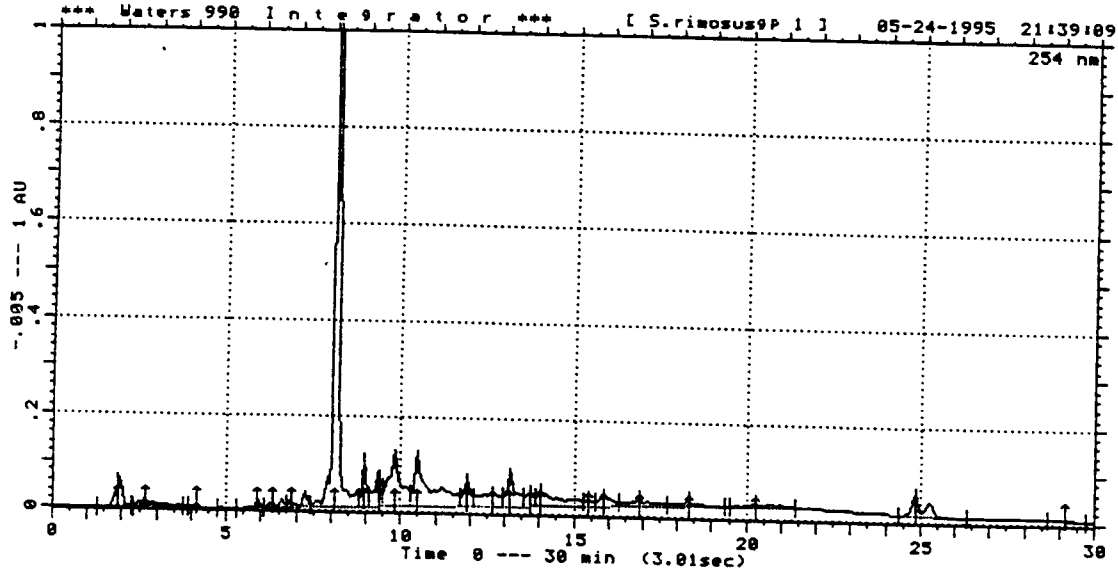


5

While elucidation of the structure of PK1 is not yet complete, this compound is clearly related to kinamycin intermediates. It was also observed in the kinamycin-negative mutant strain MC1, which produces kinafluorenone, (4), a shunt metabolite derived from the kinamycin biosynthetic intermediate kinobscurinone (5). Thus, PK1 appears to also be a shunt product from this pathway.

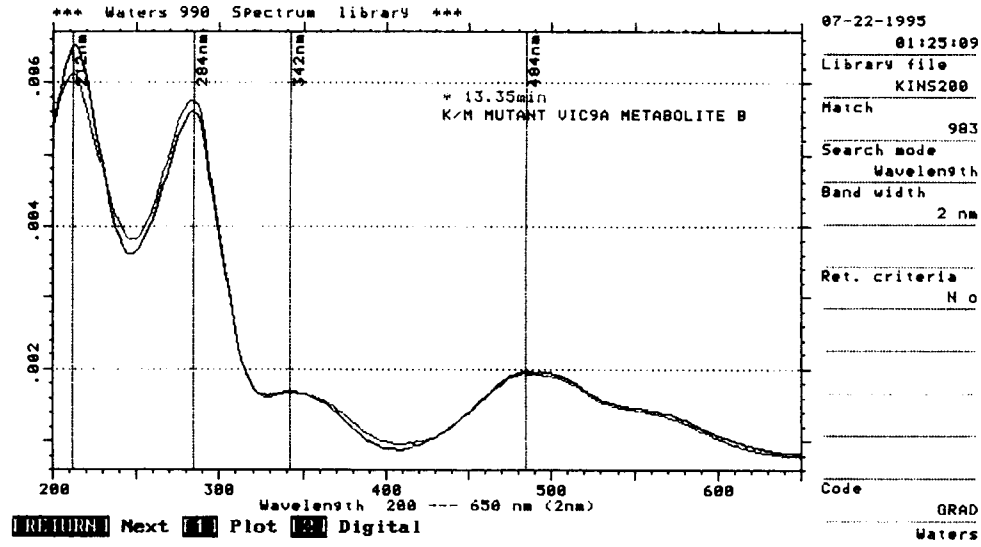


S. lividans/pSH1580

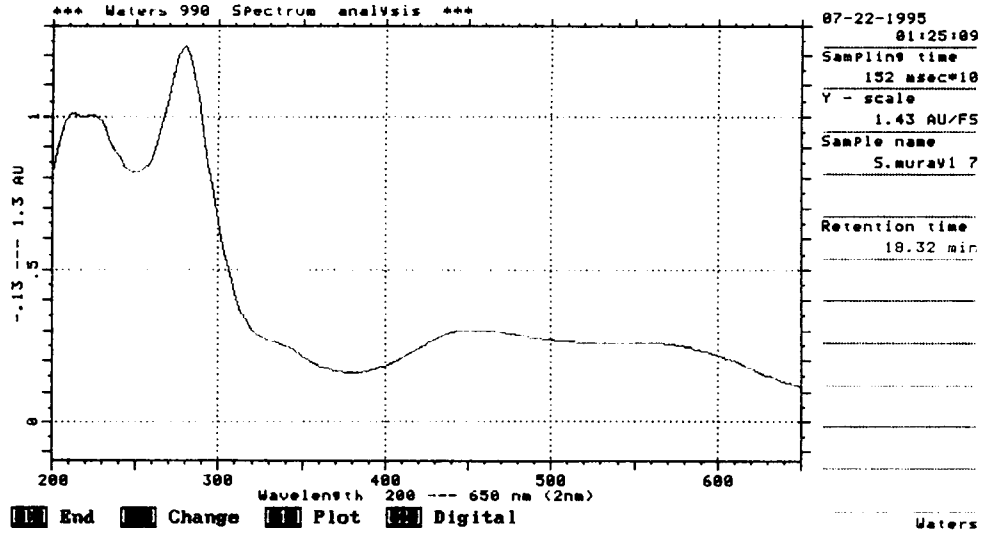


S. lividans/pOJ446

Figure III-3. HPLC trace of *S. lividans*/pSH1580 compared to *S. lividans*/pOJ446. The metabolites were extracted from 5 day culture in GPS. The two metabolites, PK1 and PK2, are indicated by arrow.



PK1



PK2

Figure III-4. UV-Visible spectra of two new metabolites, PK1 and PK2 produced by *S. lividans*/pSH1580 . The red lines indicate the two *S. lividans*/pSH1580 metabolites while the black line represents the spectrum of PK1 from *S. murayamaensis* mutant MC1.

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Chapter IV

Cloning and complete heterologous expression of the tetrangulol biosynthetic gene cluster from *Streptomyces rimosus* and the PD 116740 biosynthetic gene cluster from *Streptomyces* WP 4669

Introduction

Angucyclines, a name recently given to naturally occurring benz[a]anthraquinones, are a rapidly growing group of polyketide natural products, involving many bioactive compounds.[21] Tetrangulol and tetrangomycin, isolated from *Streptomyces rimosus*, were the first identified members of this class of antibiotics.[22] *Streptomyces* WP 4669 produces an angucycline, PD 116740, which has activity against L1210 lymphocytic leukemia and HCT-8 colon adenocarcinoma cell lines.[1] Dehydrorabelomycin, the 6-hydroxy analog of tetrangulol, has been isolated from *Streptomyces murayamaensis* and is an intermediate in the biosynthesis of the kinamycin antibiotics (Figure II-1).[23]

All of the three compounds, dehydrorabelomycin, PD 116740, and tetrangulol, are derived biosynthetically from the predictable folding of a decaketide precursor.[2,23] The hydroxyl oxygen at C-6 of dehydrorabelomycin was shown to be derived from the original

acetate precursor, while the corresponding oxygen in the PD116740 was shown to be derived from water.[2] Biosynthetic research in our group during recent years has shown that tetrangulol is the key intermediate in the biosynthesis of PD 116740 in *S.* WP 4669.[17] In addition, the data clearly indicated that its 6-hydroxy analog, dehydrorabelomycin, was not an intermediate. These results indicated that deoxygenation at C-6 to yield tetrangulol is a prearomatic process. Conversion of tetrangulol to PD 116740 in *S.* WP 4669 required a minimum of four steps in an undefined order: O-methylation, arylmethyl-oxygenation, and a sequence of epoxidation and hydrolysis to generate the *trans*-diol in the K-region of the angular structure (Figure IV-1).

Molecular genetic studies have provided the detailed functions of individual enzymes of aromatic PKS, particularly those governing the early steps of polyketide biosynthesis. This work became possible by expressing cassettes of individual genes of aromatic PKS on a suitable vector into *S. coelicolor* which was devoid of the normal type II PKS.[11] Linear cyclic polyketides and angular cyclic polyketides (angucyclines) are formed by unknown mechanisms folding polyketide backbones produced from the minimal PKSs. We were interested in which factor(s) direct polyketide folding to produce the angular ring systems, as well as in the enzymology of unusual oxygenations, such as those occurring in the biosynthetic steps from tetrangulol to PD 116740. This curiosity led us to investigate the genetics of the tetrangulol and PD 116740 pathways. We describe here the cloning of the whole

tetrangulol biosynthetic gene cluster from *S. rimosus* and the whole PD 116740 biosynthetic gene cluster from *S.* WP 4669, as demonstrated by complete expression of these pathways in a heterologous host.

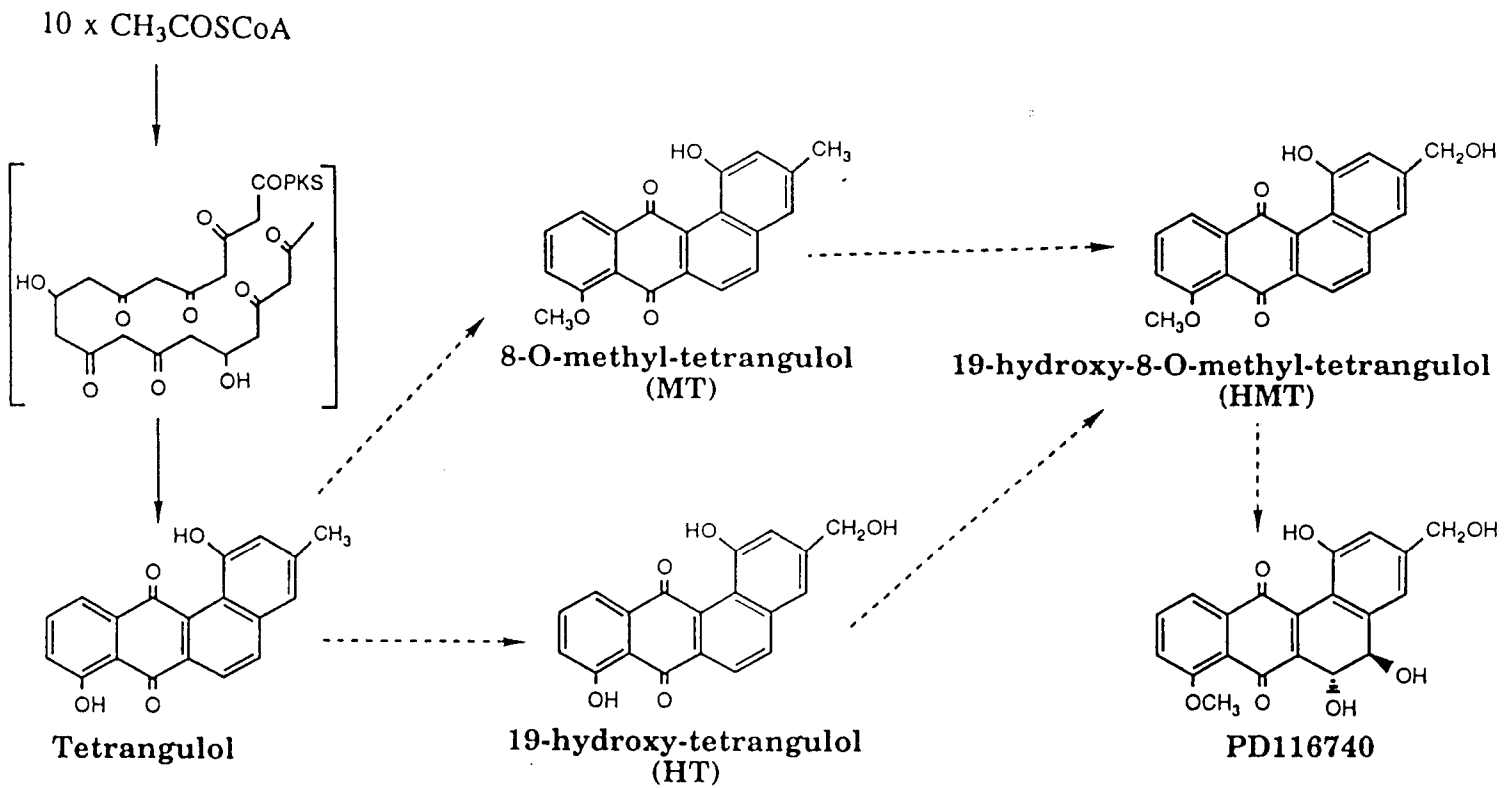


Figure IV-1. proposed biosynthetic pathway for PD 116740

Materials and Methods

All of the experimental procedures were basically same as in the Materials and Methods section of Chapter II or Chapter III except the following:

Culture condition for *S. rimosus* or *Streptomyces* WP 4669. *S. rimosus* or *S. WP 4669* were propagated on ISPII agar medium[32] in a 27 °C incubator for spore preparation or in YEME liquid media at 27 °C for genomic DNA preparation.

***S. rimosus* and *S.* WP 4669 genomic DNA isolation.** The genomic DNA preparations for these two strains were basically same as discussed in Chapter III except that the achromopeptidase was not necessary to destroy the cell walls.

Isolation of 30-40 kb DNA fragments of *S.* WP 4669 genomic DNA. The *Mbo*I partial digested genomic DNA (300 μg) was loaded onto a 0.3 % gel of low-melting agarose (USB Co.). After overnight electrophoresis in TAE buffer, the 30-40 kb fragments were cut out from the gel, and the agarose slices were dissolved by incubation for 10 minutes at 70 °C. After extraction of agarose with phenol (pH 8.0), phenol/$CHCl_3$ (pH 8.0), and $CHCl_3$, the DNA was concentrated by ethanol precipitation. The typical yield of the isolated DNA was 2-4 μg in 9 μl TE.

Ligation of genomic DNA to cosmid pOJ446. 7 µl of the isolated 30-40 kb insert (2-3 µg) was mixed with 1.5 µl of *Bam*HI and *Hpa*I digested pOJ446 (4-6 µg) in a 9.5 µl ligation mixture. After adding 0.5 µl (2.5 unit) of T_4 ligase to the ligation mixture, it was incubated for overnight at 16 ^{o}C.

Results

I. Cloning and Complete Heterologous Expression of Tetrangulol Biosynthetic Gene Cluster from *S. rimosus*

Detection of *actI* homologous DNA regions from the *S. rimosus* genome

S. rimosus genomic DNA was prepared using the proteinase K method described in Chapter III. The *actI*-ORF1 DNA fragment, coding for the ketosynthase gene, is highly conserved among all of known aromatic PKS genes.[4,5] The genomic DNA of *S. rimosus* was digested with *Bam*HI, *Kpn*I, *Pst*I and *Sal*I. The digested DNAs were separated by electrophoresis and probed with ^{32}P-radiolabeled *actI*-ORF1 at moderate stringency (2 x SSC/1% SDS, Figure IV-2, A). The probe hybridized with two *Bam*HI DNA fragments (13.7 kb and 11.2 kb). The Southern hybridization results from the *Kpn*I and *Sal*I genomic DNA digests showed that two DNA bands were also homologous to *actI* in each case. This suggested that there were two PKS gene clusters in *S. rimosus*. *Pst*I digestion yielded a single large band (30 > kb), indicating that *Pst*I sites are very rare in both PKS gene clusters.

The presence of two PKS gene clusters in *S. rimosus* is not surprising. Many *Streptomyces* sp. have more than one PKS gene cluster. For example, *S. murayamaensis* contains the two PKS gene clusters (Chapter III) for kinamycins and an unidentified PKS, *S.* WP 4669 has two PKS gene clusters for PD 116740 and an

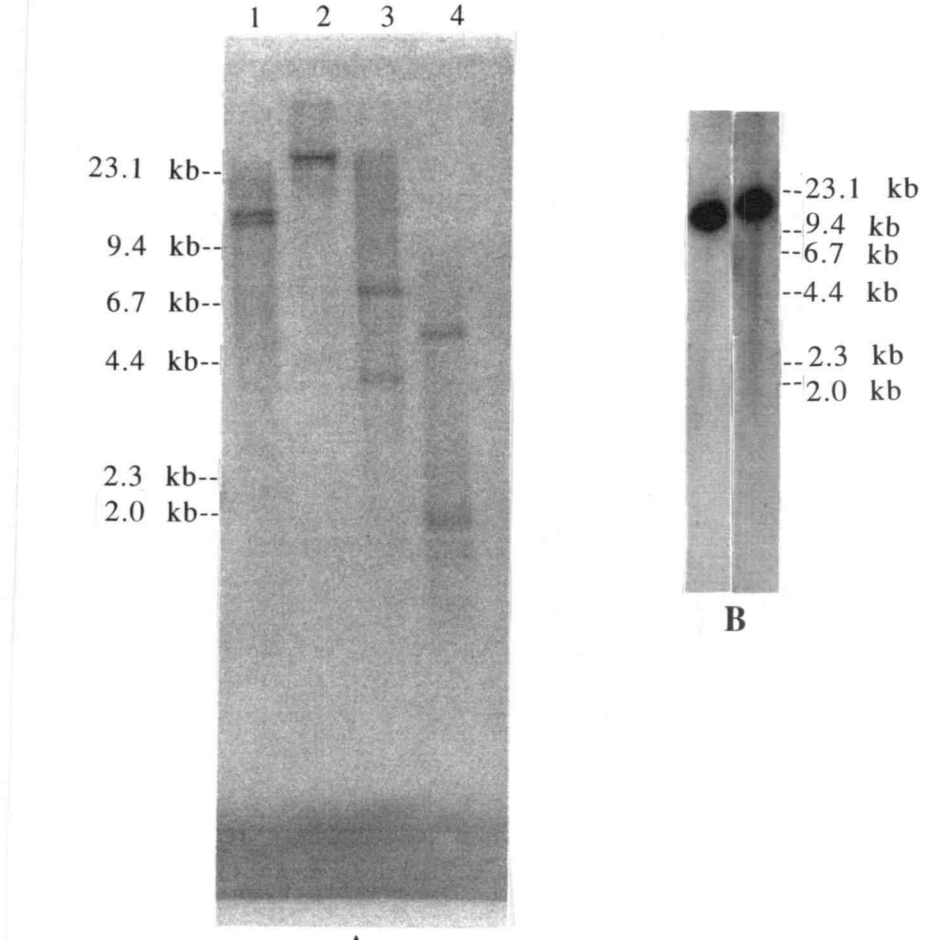


Figure IV-2. Southern hybridization of digested *S. rimosus* chromosomal DNA (A) and the two corresponding cloned PKS gene clusters prepared by *Bam*HI digestion (B). A) Lane1, *Bam*HI digestion; lane 2, *Kpn*I digestion; lane 3, *Pst*I digestion; lane 4, *Sal*I digestion.

unidentified PKS, and *S. coelicolor* has PKS gene clusters for actinorhodin[22] and for a spore pigment.[23]

Isolation of the two PKS gene clusters from *S. rimosus*

A cosmid library containing genomic DNA fragments of 30-43 kb was constructed from *Streptomyces rimosus* as described in the Materials and Methods section. About 2,500 colonies from the *Streptomyces rimosus* cosmid library were screened with the *actI*-ORF1 probe. Screening of the library with the probe led to the identification of 39 hybridizing clones. Each of the 39 recombinant cosmids was digested with *Bam*HI and *Eco*RI, and then hybridized with *actI*-ORF1. Restriction mapping and Southern hybridization results clearly showed that the 39 clones could be divided into two nonoverlapping sets, 15 clones for one PKS gene cluster (Cluster I) and 24 clones for the other cluster (Cluster II). As shown in Figure IV-2, B, the first PKS gene cluster, comprising 24 clones, contains one *actI* homologous *Bam*HI fragment (13.7 kb). The DNA from second cluster contains an 11.2 kb *actI* homologous *Bam*HI fragment. The two *actI* homologous *Bam*HI fragments (Figure IV-2, B) were exactly equivalent to the two *actI* homologous *Bam*HI bands observed in Southern hybridization analysis of *S. rimosus* genomic DNA (Figure IV-2, A). This provides strong evidence that the two PKS gene clusters detected initially by the Southern hybridization of *S. rimosus* genomic DNA were successfully cloned without rearrangement or deletion.

Expression test of the two PKS gene clusters in *S. lividans*

Eight recombinant cosmids from Cluster I and 10 from Cluster II were introduced by transformation into *S. lividans* TK24. The *S. lividans* transformants showed about 100 times lower transformation efficiency than *S. lividans* transformed with the cosmid itself. Also, transformants showed the typical phenotype of *S. lividans* growing under stress conditions. The observed lowering of transformation efficiency and change in phenotype might indicate that both clusters express a gene product which is harmful to the host strain. In *Streptomyces*, gene products harmful to the host strain are most likely to be secondary metabolites.

After 7 days, multiple colonies from each transformant were scraped from the R2YE agar plates and used to inoculate 5 ml of YEME medium. After growing the seed culture for 3-4 days (depending on growth of the transformants), a portion of the seed culture (0.3 ml) was used to inoculate GPS[13] and Glycerol-Asparagine (Gly-Asn)[15] media, respectively, for secondary metabolite production. The cultures were then incubated for 5 more days.

The cultures were extracted with ethyl acetate, then analyzed using photodiode array HPLC. This analysis provided evidence that PKS gene Cluster I produces tetrangulol and two unidentified compounds one of which might be tetrangomycin (Table IV-1). Thus, chromatography on a reverse-phase HPLC column (Figure IV-3) identified a compound matching the retention time

Table IV-1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source or reference
Bacterial strains		
S. rimosus	tetrangulol producer	14
E. coli XL1-BlueMR	*Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gryA96,relA1, lac*	Stratagene
S. lividans TK24	Host for expression test (SLP2$^-$, SLP3$^-$)	John Innes Institute
Plasmids		
pOJ446	*E.coli-Streptomyces* Shuttle cosmid	20
pSH2010	1+pOJ446, produces tetrangulol, PT1, and PH1 in *S.lividans*	This work
pSH2020	1+pOJ446, produces tetrangulol, PT1, and PH1 in *S.lividans*	
pSH2030	1+pOJ446, produces tetrangulol, PT1, and PH1 in *S.lividans*	This work
pSH2040	1+pOJ446, produces tetrangulol, PT1, and PH1 in *S.lividans*	This work
pSH2050	1+pOJ446, does not produces tetrangulol, PT1, and PH1 in *S.lividans*	This work
pSH2060	1+pOJ446, does not produces tetrangulol, PT1, and PH1 in *S.lividans*	This work
pSH2070	1+pOJ446, produces tetrangulol, PT1, and PH1 in *S.lividans*	This work
pSH2080	1+pOJ446, produces tetrangulol, PT1, and PH1 in *S.lividans*	This work
pSH2510	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2520	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2530	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2540	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2550	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2560	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2570	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2580	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2590	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH2600	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work

1; tetrangulol biosynthetic gene cluster

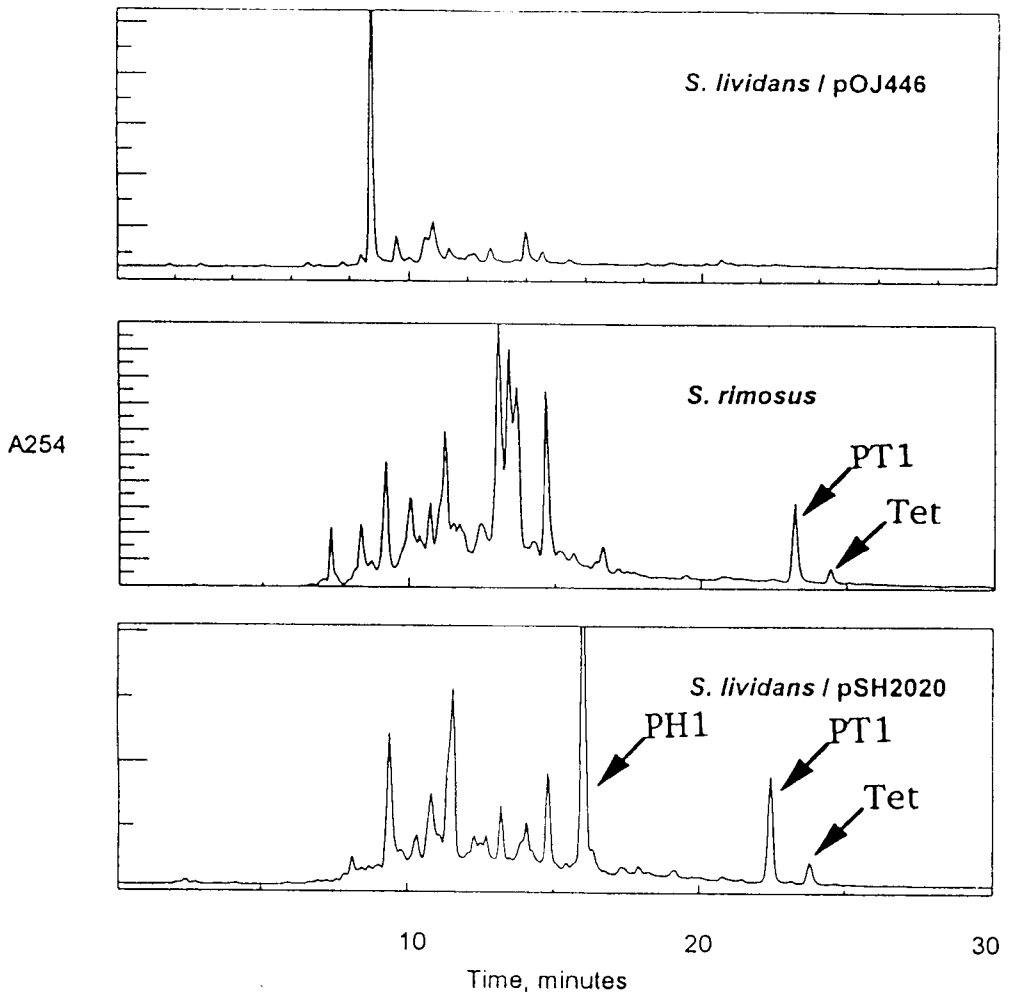


Figure IV-3. HPLC trace of *S. lividans* transformant pSH2020 compared to *S. lividans*/pOJ446 and *S. rimosus*. The peaks of tetrangulol (Tet), PH1, and PT1 are indicated by arrows.

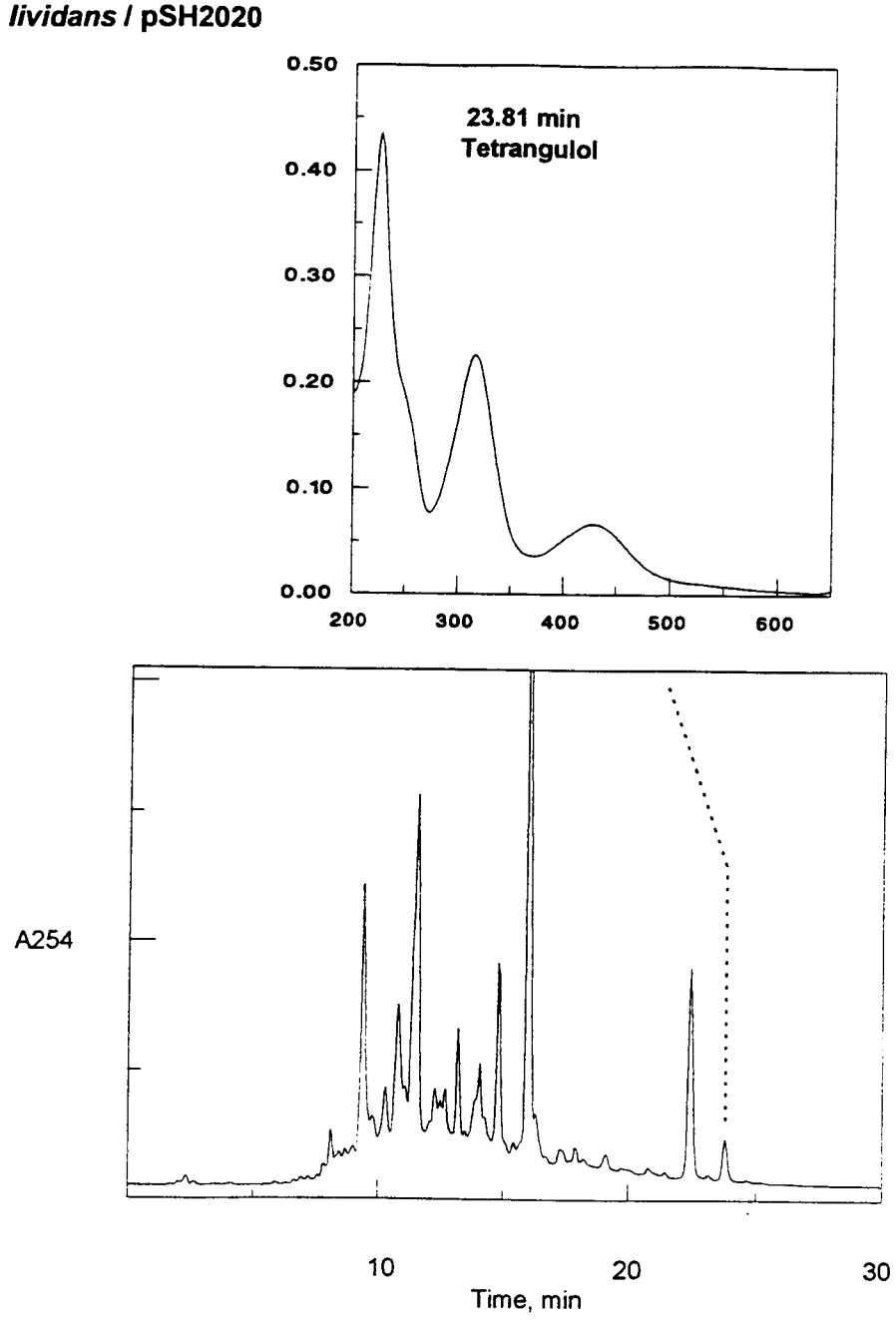
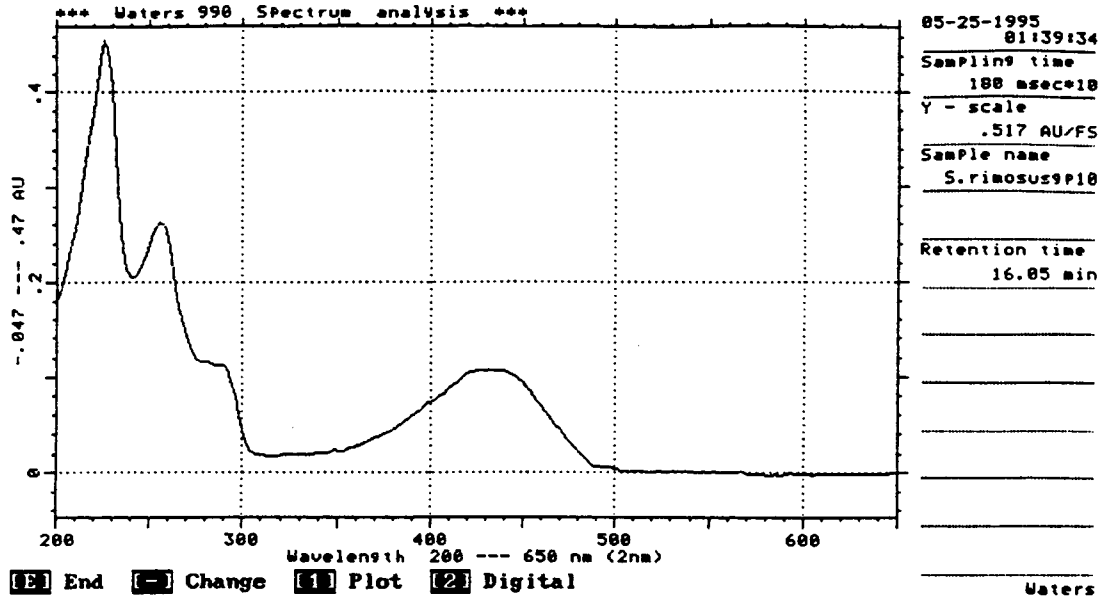
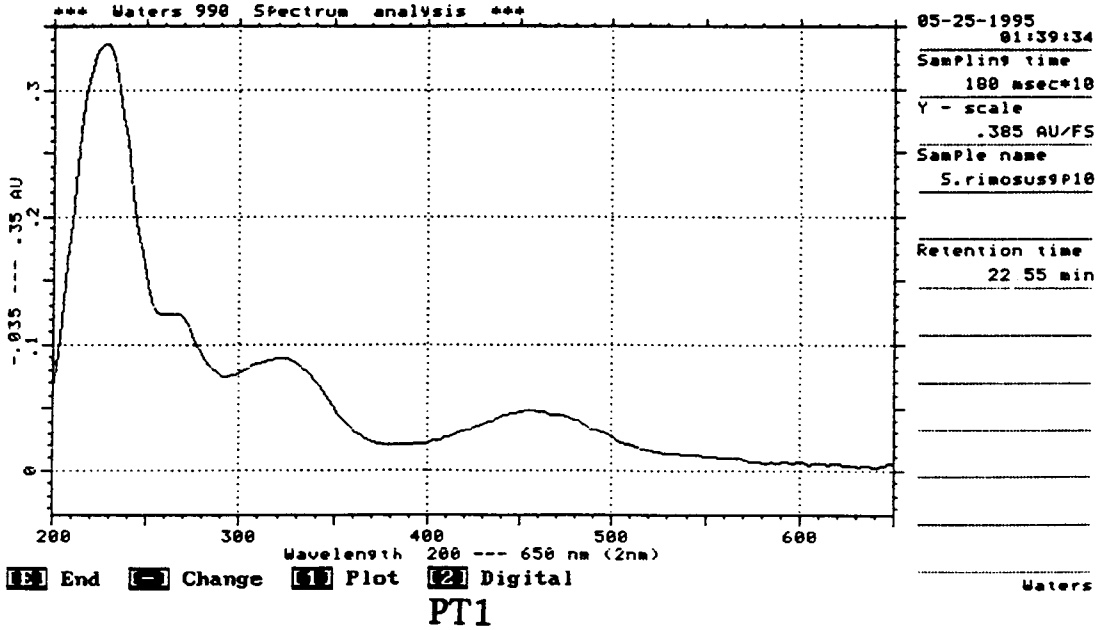


Figure IV-4. Spectrum index plot of tetrangulol produced by *S. lividans* transformant pSH2020. Tetrangulol sample was prepared by unambiguous synthesis.



PH1



PT1

Figure IV-5. UV-Visible spectra of two unidentified metabolites, PT1 and PH1 produced by *S. lividans* pSH2020.

and displaying the same UV-Visible spectrum as tetrangulol (Figure IV-4). In order to confirm the assignment, tetrangulol was isolated from a 500 ml fermentation (clone pSH2020) and its structure was confirmed by ^{1}H NM spectroscopy.[24]

Two new compounds were identifiable from the tetrangulol-producing *S. lividans* transformants (Figure IV-3 and IV-5). One compound PT1 (PT=putative tetrangomycin) with a retention time of 22.6 min was produced both by wild-type *S. rimosus* and by tetrangulol-producing *S. lividans* transformants. PT1 is most likely tetrangomycin because *S. rimosus* is known to produce both tetrangulol and tetrangomycin. *S. lividans* transformants producing tetrangulol produced another new major metabolite PH1 (PH=putative hybrid), retention time at 16.1 min in HPLC profile. PH1 was not observed in extracts of either *S. rimosus* or *S. lividans*. Although the complete structure has not yet been determined, ^{1}H-NM and ^{13}C-NM spectra of the compound clearly indicate that it is an angucycline.[24] Since there is no evidence that *S. lividans*, the most well-studied *Streptomyces* species, contains an angucycline pathway, the presence of PH1 suggests that its formation involves genes derived from the tetrangulol biosynthetic pathway. Thus, this compound may be a hybrid metabolite between the tetrangulol biosynthetic pathway and *S. lividans* metabolism.

HPLC analysis of extracts of cultures of *S. lividans* transformants with Cluster II showed that the cluster did not express any identifiable *S. rimosus* metabolites in *S. lividans*. The altered colony morphology of the *S. lividans* transformants and the transformation efficiency with Cluster II suggests that this DNA

also codes for production of polyketide compound in *S. lividans* that cause stress to the host. However, the pathway seems like unstable in the *S. lividans* host. The tiny amount of polyketide production from the unstable pathway seemed like giving enough stress to the *S. lividans* host but not enough to be detected by the HPLC analysis.

Tetrangulol biosynthetic gene cluster

A restriction map of the tetrangulol biosynthetic PKS gene cluster is shown in Figure V-6. *S. lividans*/pSH2020 and *S. lividans*/pSH2030 produce the same quantity of tetrangulol as wild type *S. rimosus*. The overlapping inserts in pSH2020 and pSH2030 represent about 30 kb of contiguous DNA from *Streptomyces rimosus*, and this region is apparently sufficient to encode the entire tetrangulol biosynthetic pathway.

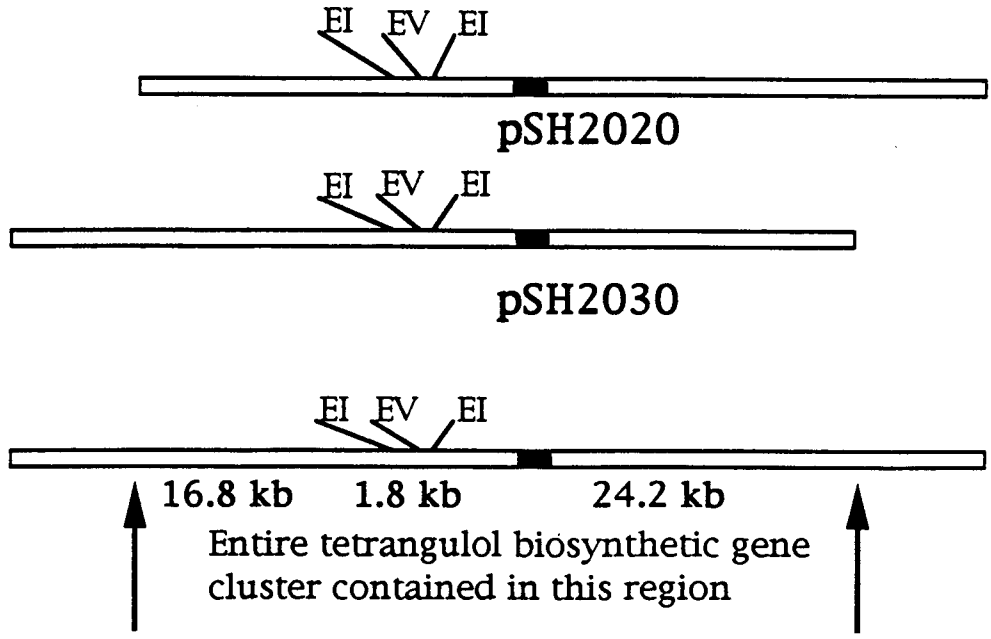


Figure IV-6. Restriction map of the entire tetrangulol biosynthetic gene cluster from *S. rimosus*. The black boxes represent the fragment to which *actI*-ORF1 hybridized. Abbreviation of restriction enzymes: EI; *Eco*RI. EV; *Eco*RV.

II. Cloning and Complete Heterologous Expression of PD 116740 Biosynthetic Gene Cluster from *S.* WP 4669

Detection of *actI* homologous DNA regions in *S.* WP 4669

S. WP 4669 genomic DNA was prepared by using proteinase K as descried in Chapter III. Portions of genomic DNA from *S.* WP 4669 was digested with *Bam*HI, *Kpn*I, *Pst*I and *Sal*I. The digested DNA fragments were separated by electrophoresis and hybridized with *actI*-ORF1 at moderate stringency (2 x SSC/1% SDS, Figure IV-7, A). The probe hybridized with three *Bam*HI digested DNA fragments (16.9 kb, 12.1 kb and 6.9 kb). The southern hybridization results for genomic DNA digested by *Kpn*I, *Pst*I and by *Sal*I showed that two DNA fragments are homologous to *actI* in each case. This clearly suggested the presence of two PKS gene clusters in which one cluster has one internal *Bam*HI site in the *actI*-ORF1 homologous region.

Isolation of the two PKS gene clusters from *S.* WP 4669

A cosmid library containing genomic DNA fragments of 30-43 kb was constructed from *S.* WP 4669 as described in the Materials and Methods section. About 2500 colonies from the *S.* WP 4669 cosmid library were screened with the *actI*-ORF1 probe. On screening of the library with the probe, 12 hybridizing clones were identified. The 12 recombinant cosmids were each digested with

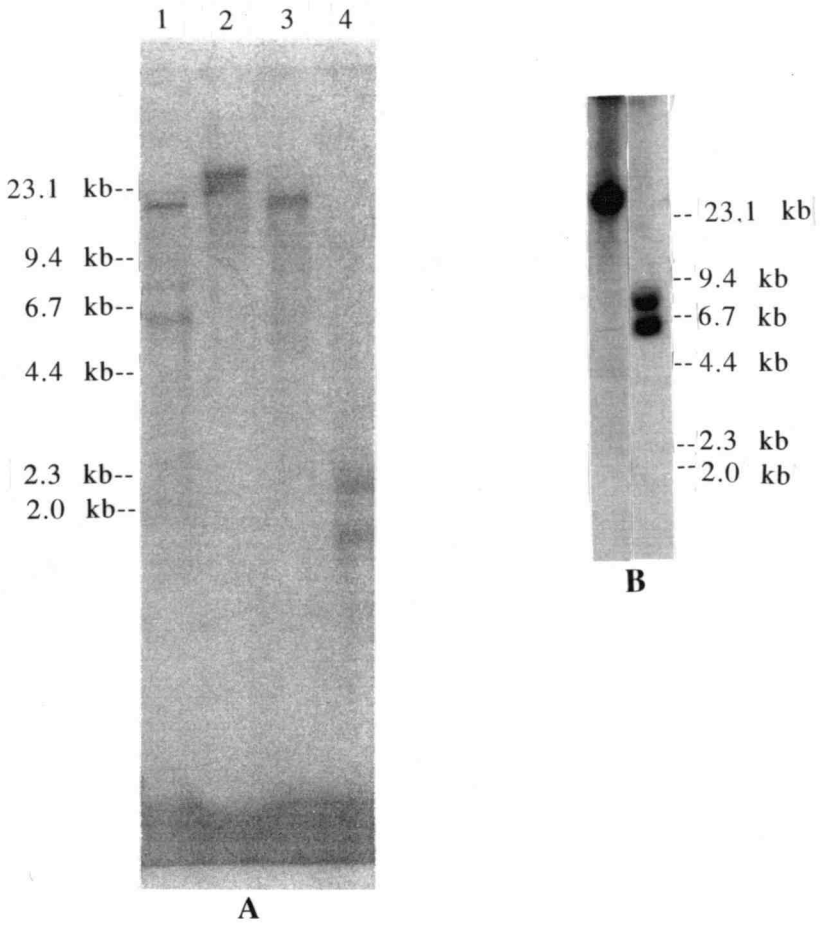


Figure IV-7. Southern hybridization of digested *S.* WP 4669 chromosomal DNA (A) and the two corresponding cloned PKS gene clusters prepared by *Bam*HI digestion (B). A) Lane 1, *Bam*HI digestion; lane 2, *Kpn*I digestion; lane 3, *Pst*I digestion; lane 4, *Sal*I digestion.

*Bam*HI and *Eco*RI, and then hybridized with *actI*-ORF1. The restriction mapping and Southern hybridization results clearly showed that the 12 clones divided into two nonoverlapping sets, 5 clones for one PKS gene cluster and 7 clones for the other cluster. As shown in Figure IV-7, B, the first PKS gene cluster (Cluster I) comprising 5 clones contains characteristically two *actI* homologous *Bam*HI fragments (12.1 kb and 6.9 kb). The DNA from the second cluster (Cluster II) contained a 16.9 kb *actI* homologous *Bam*HI fragment. These three *actI* homologous *Bam*HI fragments were exactly equivalent to the ones observed in Southern hybridization analysis of *S.* WP 4669 genomic DNA. This means the two PKS gene clusters shown by the Southern hybridization of *S.* WP 4669 were successfully cloned.

Expression test of the two PKS gene clusters in *S. lividans*

All of the 12 isolated cosmids containing two PKS gene clusters were introduced by transformation into *S. lividans* TK24, as described in the previous sections. Multiple colonies of *S. lividans* transformants were scraped from each plate for expression tests. The expression tests were conducted in three different media: GPS,[13] YEME[14] and Glycerol-Asparagine (Gly-Asn).[15]

The *S. lividans* transformants from Cluster I showed normal transformation efficiency. The transformant colonies grow very easily and were well-sporulated. HPLC analysis of the transformant culture in GPS, YEME, and Gly-Asn media showed that no new identifiable compounds were produced from these

cultures. Based on the colony morphology, transformation efficiency, and HPLC analysis, the PKS gene cluster might code for just a null-PKS compound or a compound specifically expressed at very late stage of life cycle of the organism, like the*whi* gene in *S. coelicolor,* which is expressed to help sporulation.[16] This idea is supported by the fact that *S.* WP 4669 is extremely well sporulated in any media.

S. lividans transformants from Cluster II showed about 100 times lower transformation efficiency compared to the vector itself. One cosmid clone (pSH3030) was never able to transform *S. lividans*. The transformant colonies grew extremely slowly. Also, the transformants generally sporulated very poorly and produced extremely poor pigment in R2YE agar. The lower transformation efficiency of the recombinant cosmids containing Cluster II might come from a gene dosage effect that over-expression of the foreign secondary metabolites kill the host strain.

HPLC analysis of the cultures grown in GPS medium from the transformants from Cluster II showed that 3 out of the 6 clones tested were able to yield heterologous expression of the PD 116740 pathway in *S. lividans* (Table IV-2). In YEME medium, only intermediate compounds of the PD 116740 biosynthetic pathway were observed. In Gly-Asn medium, no PD 116740 or its intermediates were produced from both *S.* WP 4669 wild type strain and the transformants. PD 116740 is produced from *S.* WP 4669 only in the early stationary phase in nutrient rich media and it disappears

Table IV-2. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source or reference
Bacterial strains		
Streptomyces WP4669	PD 116740 producer	1
E. coli XL1-BlueMR	*Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gryA96,relA1, lac*	Stratagene
S. lividans TK24	Host for expression test (SLP2$^-$, SLP3$^-$)	John Innes Institute
Plasmids		
pOJ446	*E.coli-Streptomyces* Shuttle cosmid	20
pSH3010	PD 116740 biosynthetic gene cluster+pOJ446, produces PD 116740 and intermediate compounds in *S.lividans*	This work
pSH3020	PD 116740 biosynthetic gene cluster+pOJ446, does not produce PD 116740 or intermediate compounds in *S. lividans*	This work
pSH3030	PD 116740 biosynthetic gene cluster+pOJ446, can not transform *S. lividans*	This work
pSH3040	PD 116740 biosynthetic gene cluster+pOJ446, does not produce PD 116740 or intermediate compounds in *S. lividans*	This work
pSH3050	PD 116740 biosynthetic gene cluster+pOJ446, does not produce PD 116740 or intermediate compounds in *S. lividans*	This work
pSH3060	PD 116740 biosynthetic gene cluster+pOJ446, produces PD 116740 and intermediate compounds in *S. lividans*	This work
pSH3071	PD 116740 biosynthetic gene cluster+pOJ446, produces PD 116740 and intermediate compounds in *S. lividans*	This work
pSH3510	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH3520	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH3530	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH3540	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work
pSH3550	Unidentified polyketide biosynthetic gene cluster+pOJ446	This work

rapidly after passing through this phase. The pathway was never expressed in minimal media by the original organism. Thus, the lack of production of PD 116740 in Gly-Asn in *S. lividans* is consistent with the early metabolic characteristic of PD 116740. This means the PD 116740 biosynthetic pathway in a heterologous host, i.e. *S. lividans*, may be regulated exactly same as in its original host.

The formation of PD 116740 by the *S. lividans* transformants was confirmed using reversed-phase HPLC (Figure IV-8), equipped with a photodiode array detector (Figures IV-9). Also, PD 116740 was isolated from 500 ml fermentations (clones pSH3071 and pSH3060) and its structure was confirmed by ^{1}H NM spectroscopy.[24]

The HPLC analysis showed that the PD 116740 pathway in *S. lividans* accumulated more intermediate compounds than PD 116740. Wild type *S.* WP 4669 produces much larger quantities of PD 116740 than intermediate compounds, and one compound, 19-hydroxy-8-O-methyl-tetrangulol (HMT, Figure IV-1) was almost unidentifiable in extracts from *S.* WP 4669 while the compound was very easily recognizable in extracts from the *S.lividans* transformants. We can explain the phenomenon in two different ways. First, the last modifying enzyme leading to PD 116740 may not be well-expressed in the heterologous host. Alternatively, the

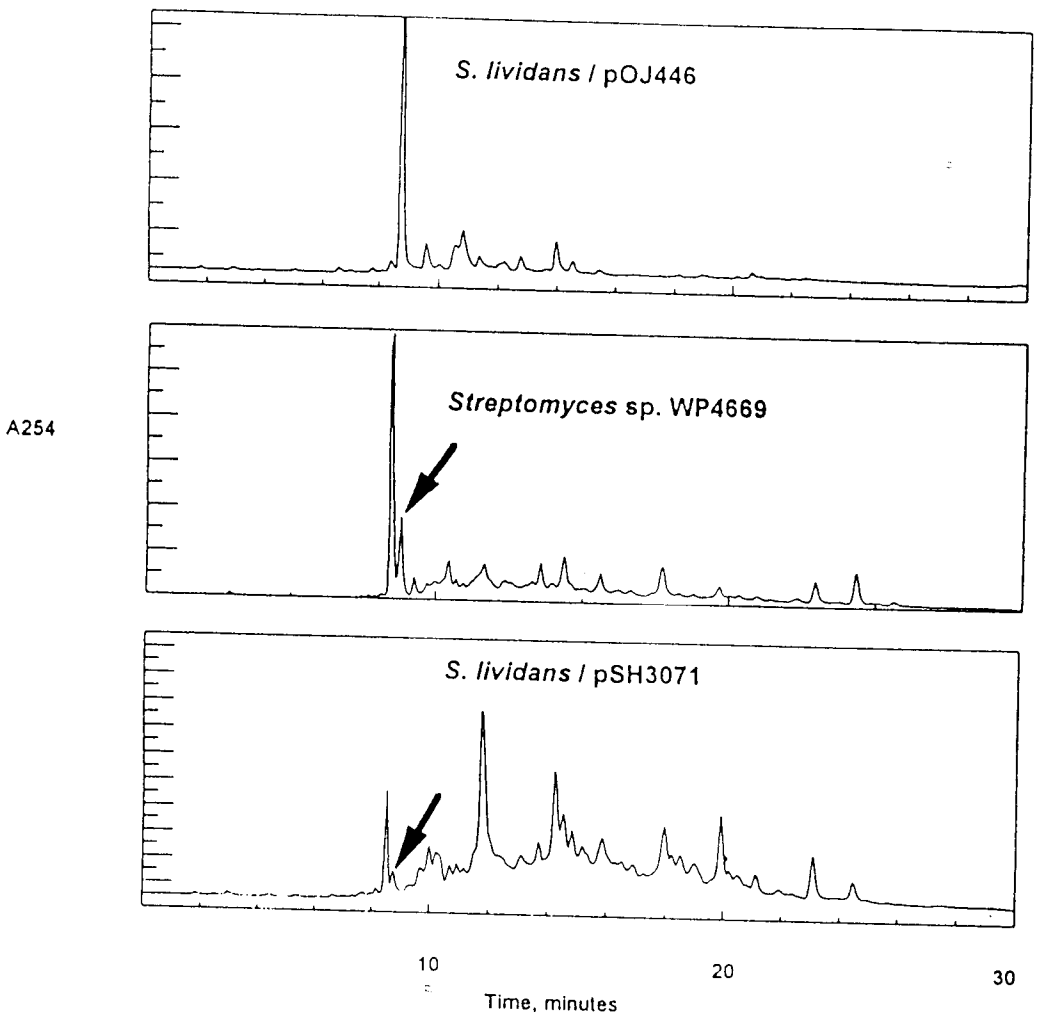


Figure IV-8. HPLC trace of *S. lividans* transformant pSH3071 compared to *S. lividans*/pOJ446 and *Streptomyces* WP 4669. The PD 116740 peaks are indicated by arrows.

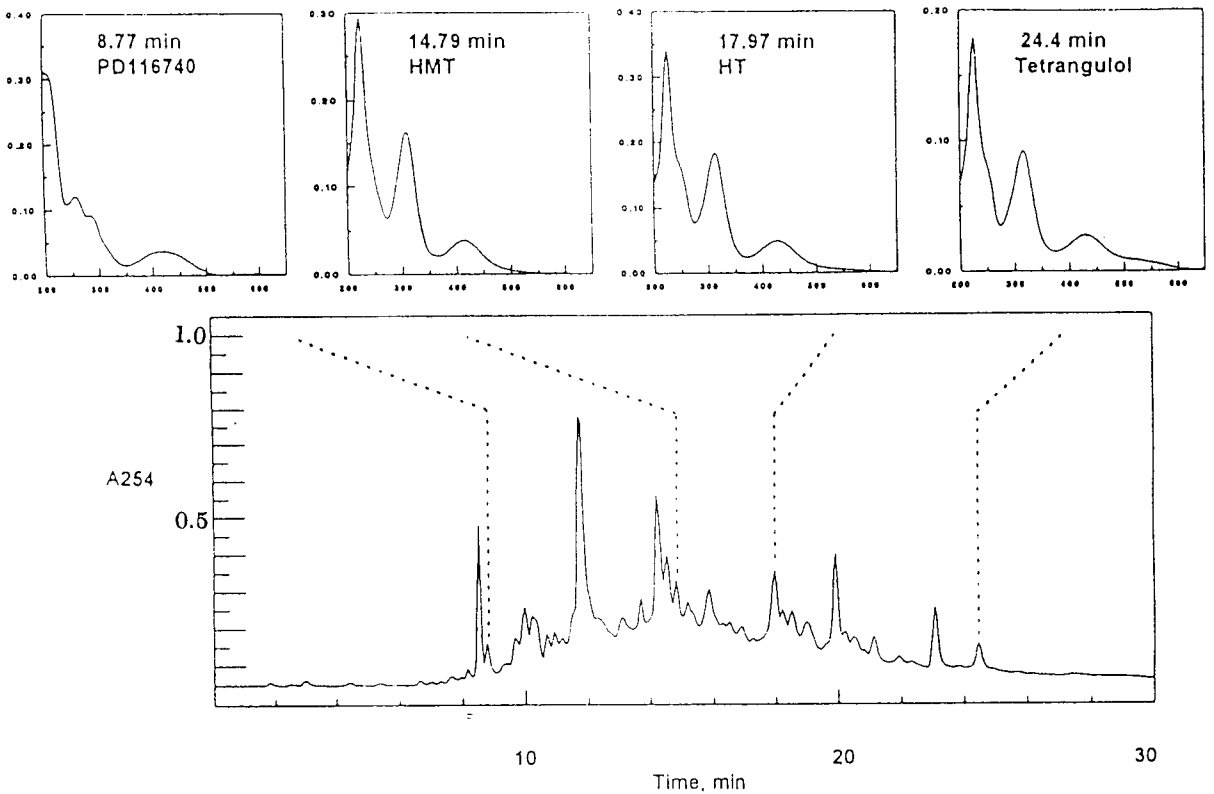


Figure IV-9. Spectrum index plot of PD 116740 and intermediate compounds produced by *S. lividans* transformant pSH3071. UV-Visible spectra of the compounds of the *S. lividans* transformant were confirmed by matching the spectra and retention times with standard compounds. Samples of intermediate compounds were prepared by unambiguous synthesis.

modifying enzyme may be less active under the different physiological conditions in the heterologous host.

PD 116740 biosynthetic gene cluster

A restriction map of the PD 116740 biosynthetic PKS gene cluster is shown in Figure IV-10. Since the overlapping insert in pSH3060 and pSH3071 represents about 30 kb of contiguous DNA from *S.* WP 4669, this is apparently enough to encode the entire PD 116740 biosynthetic pathway. The plasmid pSH3030 was never able to transform *S. lividans*. This means that the 14-17 kb area, right end of the pSH3060 and pSH3071 insert, may encode a kind of resistance gene(s) which protect the host strain from its own antibiotic (PD116740).

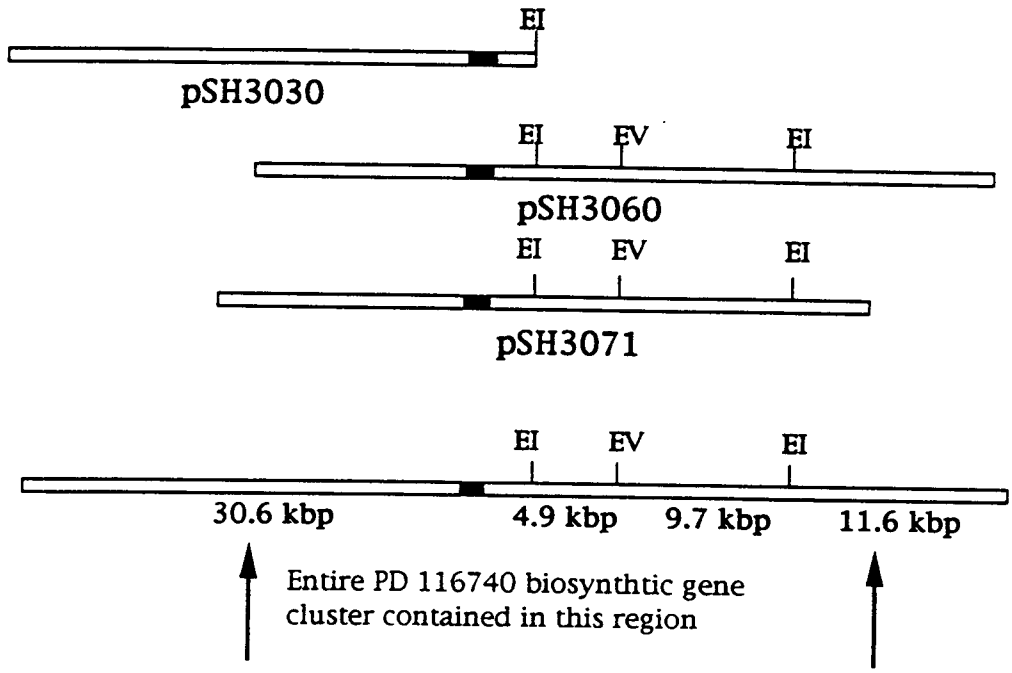


Figure IV-10. Restriction map of the entire PD 116740 biosynthetic gene cluster from *Streptomyces* WP 4669. The black boxes represent fragment to which *actI* hybridized. Abbreviation of restriction enzymes: EI; *Eco*RI. EV; *Eco*R V

Discussion

The aromatic polyketide oxytetracycline (OTC) is produced by a different *S. rimosus* strain than the one used in our studies.[25] The PKS gene cluster for the OTC pathway was cloned by Binnie *et al.*[26] Interestingly, the *S. rimosus* strain used in our work is clearly resistant to OTC (up to 500 μg/ml), which led us to consider whether our strain may have the OTC pathway. However, both of the restriction maps of the two PKS clusters isolated from our *S.rimosus* strain were completely different from the reported OTC PKS cluster (data not shown). The OTC biosynthetic genes are notoriously unstable in an industrial strain of *S. rimosus*. When the industrial strain was propagated as spores, 80 percent of the spores naturally lost the OTC biosynthetic genes.[27] The *S. rimosus* strain used in our work may harbor only the OTC resistance gene while the biosynthetic genes have been lost.

An unexpectedly large number of apparent positive clones were observed upon screening of the *S. rimosus* cosmid library with the *actI*-ORF1 probe. There are several possible reasons. Both PKS gene clusters may be present as multicopy genes. A second possibility is that both PKS clusters in *S. rimosus* might may be located on a giant linear plasmid which is present as several copies. Several hundred kb giant linear plasmids are common in *Streptomyces*, including *S. rimosus* which has been reported to have several different giant linear plasmids.[28-30] The detailed biological functions including copy number of the plasmids are not

yet known. The copy number of one giant linear plasmid in *S. coelicolor* was determined to be 3.7.[31] The copy number of the giant plasmid in *S. coelicolor* suggests that copy number of the giant plasmid of closely related strains, such as *S. rimosus*, should be also 3-4. This strongly supports the idea of the plasmid-borne PKS gene clusters because three to four times as many positive clones as expected were obtained.

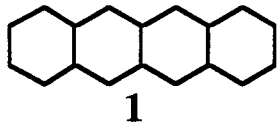
PD 116740 is formed from the cyclization of a decaketide intermediate folded in a manner to generate the angular cyclic skeleton.[2] The compound is biosynthetically interesting because a unique 5,6-dihydroxy functionality is present in its C ring. Recent biosynthetic studies in this group showed that the C-6 hydroxyl group did not originate from acetate-derived oxygen.[17] However, the C-5 hydroxyl was labeled by molecular oxygen. These results indicated that generation of the 5,6-*trans*-diol moiety occurred by enzymatic epoxidation of the K-region double bond followed by action of an epoxide hydrolase. These biosynthetic studies also showed that tetrangulol is a key intermediate in the biosynthesis of PD 116740 (Figure IV-1). However, the intermediates from tetrangulol to PD 116740 were not clear. Based on the chemical structures of PD 116740 and tetrangulol, the possible candidates for intermediate compounds were believed to be 8-O-methyltetrangulol (MT), 19-hydroxy-tetrangulol (HT), 19-hydroxy-8-O-methyltetrangulol (HMT) or dihydro-5,6-dihydroxytetrangulol (DT). One of the proposed intermediates, HMT, was isolated by Brinkman et al. from the wild-type *S.* WP 4669.[18] However, the

rest of the putative intermediates were only considered as potential precursors to PD 116740.

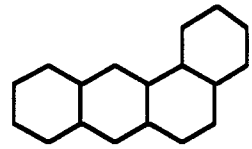
The *S. lividans* transformants containing the PD 116740 biosynthetic gene cluster accumulated an unusually large quantity of potential biosynthetic intermediates compared to the wild-type strain, as shown in Figures IV-8 and IV-9. The possible intermediates of PD 116740 biosynthesis were unambiguously synthesized.[24] By comparison of UV-Visible spectrum and retention time and by coinjection of the authentic samples, we were able to identify HT, MT, HMT, and chlorotetrangulol (CT) from the *S. lividans* transformant. We do not know the reason of presence of CT in *S.* WP 4669 and in the *S. lividans* transformant. Biosynthetic investigations with this product are in progress. So, from the *S. lividans* transformant, all of the suggested intermediate compounds except DT and its methylated or hydroxylated DT were identified. The data suggest that epoxidation and hydrolysis of the epoxide to 5,6-diol are the last steps in PD116740 biosynthesis. Thus, the original hypothesis that PD 116740 may be derived from HMT (Figure IV-1), has gained considerable support from molecular genetics. The identification of enzymes governing each of the late stage steps should clarify the details of the correct sequence of steps tailoring tetrangulol.

Recently, the detailed functions of individual enzymes of a PKS have been being deciphered by expressing combinations of different PKS genes.[7-11] This suggests it may be possible to rationally design new polyketides by genetic engineering. The ring folding pattern for angucyclines, leading to an angular ring system

(2) should be different from that leading to linear systems such as 1.



1



2

Further molecular genetic research on the tetrangulol and PD 116740 biosynthetic gene clusters may give information about what causes the formation of an angular aromatic ring. This would add a new dimension for engineering unnatural hybrid angular secondary metabolites.

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Chapter V

Conclusion and Prospects

Angucyclines are a subclass of the class of natural products known as aromatic polyketides. Many of these compounds exhibit a variety of important biological activities. The nature of the genetic PKS programming for aromatic polyketides is beginning to be deciphered. However, the number of currently available aromatic PKS gene sets is limited. The PKS gene clusters of angucyclines (e.g. those for the kinamycin precursor dehydrorabelomycin, tetrangulol, and PD 116740) as well as the cluster for phenanthraquinones (for murayaquinone) may be important additions for engineering new polyketides. They may also provide further clues into the genetic programming of structurally more complex polyketides.

The initial attempts in this research focused on the heterologous expression of PKS gene clusters from an *S. murayamaensis* lambda library. However, the approach was not successful, presumably due to the lack of key genes on the relatively small pieces cloned (15-20 kb). Identification of PKS genes by heterologous expression after cloning large pieces of DNA (30-45 kb) in a cosmid vector was successful. The entire gene clusters for production of the aromatic polyketides tetrangulol from *S. rimosus* and PD 116740 from *S.* WP 4669 have been successfully cloned in this manner and then

expressed in a heterologous host. Two PKS gene clusters from *S. murayamaensis* were also cloned by constructing a cosmid library. One of these was identified as apparently containing many of the genes for the kinamycin pathway, since it yielded a derivative of the known kinamycin biosynthetic intermediate kinobscurinone.

The future efforts on PKS genetics of *S. murayamaensis*, *S. rimosus*, and *S.* WP 4669 will focus on a number of interesting aspects of this biologically important class, as follows.

a) Sequencing of the cloned PKS genes for tetrangulol, PD 116740, and the kinamycins will provide a clue about what causes a polyketide backbone, synthesized by minimal PKS gene, to yield an angular instead linear polycyclic aromatic product. This understanding may eventually lead us to engineer a variety of unnatural hybrid angular secondary metabolites.

b) We could not identify cryptic PKS gene clusters from *S. rimosus* and *S.* WP 4669, probably because of the background activity of the host *S. lividans*. A possibly better approach to identify these PKS gene clusters will be to express them in *S. coelicolor* CH999, from which two independent secondary metabolisms, actinorhodin and undecylprodigiosin, were deleted. This approach will minimize the background activities and uncertainties associated with the host secondary metabolism.

c) Tetrangulol is derived from a decaketide precursor after only slight modification. So, the introduction of the tetrangulol biosynthetic gene cluster to other *Streptomyces* has a good potential to generate hybrid compounds by the action of the tailoring enzymes of host strains.

d) Sub-cloning the tailoring enzymes such as O-methyltransferase, methylhydroxylase, or epoxidase from the cloned PD 116740 biosynthetic cluster and the tailoring enzymes from the kinamycin biosynthetic gene cluster will eventually lead to overexpression of these enzymes and each enzyme can be purified in an active form. Therefore, cell-free systems prepared from recombinant strains that overproduce these tailoring enzymes are an attractive means to study the enzyme mechanisms of the pathways.

e) Introduction of the DNA of Cluster I of *S. murayamaensis* into *S. lividans* ZX7 and *S. lividans* JT46, which lack of homologous recombination, may produce an identifiable gene product. This may lead us to identify what Cluster I codes for.

f) Confirming the chemical structure of PK1 and determination of the chemical structure of PK2 may be useful to help in developing a more complete description of the kinamycin biosynthetic pathway.

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