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Cloning and Heterologous Expression of the Entire Gene Clusters for PD 116740 from *Streptomyces* Strain WP 4669 and Tetrangulol and Tetrangomycin from *Streptomyces rimosus* NRRL 3016

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The genes for the complete pathways for two polycyclic aromatic polyketides of the angucyclinone class have been cloned and heterologously expressed. Genomic DNAs of *Streptomyces rimosus* NRRL 3016 and *Streptomyces* strain WP 4669 were partially digested with *Mbo*I, and libraries (ca. 40-kb fragments) in *Escherichia coli* XL1-Blue MR were prepared with the cosmid vector pOJ446. Hybridization with the *actI* probe from the actinorhodin polyketide synthase genes identified two clusters of polyketide genes from each organism. After transfer of the four clusters to *Streptomyces lividans* TK24, expression of one cluster from each organism was established through the identification of pathway-specific products by high-performance liquid chromatography with photodiode array detection. Peaks were identified from the *S. rimosus* cluster (pks_{RIM-1}) for tetrangulol, tetrangomycin, and fridamycin E. Peaks were identified from the WP 4669 cluster (pks_{WP-2}) for tetrangulol, 19-hydroxytetrangulol, 8-*O*-methyltetrangulol, 19-hydroxy-8-*O*-methyltetrangulol, and PD 116740. Structures were confirmed by ^{1}H nuclear magnetic resonance spectroscopy and high-resolution mass spectrometry.

Members of the genus *Streptomyces* produce well over half of the known antibiotics of natural origin. Of these, polyketide-derived metabolites are among the most numerous and diverse and include many clinically important members, such as erythromycin, tetracycline, and doxorubicin. Assembly of the skeletons of such compounds by oligomerization of small precursor fatty acid thioesters to form polyketide backbones is carried out by polyketide synthases (PKSs). These are made up of either large multifunctional enzymes (type I PKSs) or multienzyme complexes (type II PKSs). Molecular genetic analyses have revealed that the genes for each PKS are clustered in a relatively small region of DNA (22). The gene clusters for numerous polyketide pathways have been detected by hybridization with probes derived from genes coding for proteins that catalyze equivalent reactions in different pathways (25, 29).

Angucyclinones, a name recently given to naturally occurring benz[*a*]anthraquinones, are a rapidly growing group of polyketide natural products, involving many bioactive compounds (41). They are now the largest class of known aromatic decaketides. Two angucyclinones—tetrangulol (TET) and tetrangomycin—had been isolated from *Streptomyces rimosus* and were the first identified members of this class of antibiotics (26). *Streptomyces* strain WP 4669 produces an angucyclinone, PD 116740, which has activity against L1210 lymphocytic leukemia and HCT-8 colon adenocarcinoma cell lines (47). We have studied the biosynthesis of a number of angucyclinone-derived metabolites (15, 16, 43), including PD 116740 (14). In these pathways, either dehydrorabelomycin (27, 43) or TET (13, 26), plays a key role (Fig. 1). These studies have revealed that the polyketide assembly common to both of these diverges at a prearomatic stage with an additional ketone reduction (at C-6) leading to TET. We have shown TET to be a key intermediate leading to PD 116740, although TET is not accumulated by WP 4669 (14).

We now report the cloning and heterologous expression of the complete gene cluster from WP 4669 (47) for the biosynthesis of the TET-derived PD 116740 and of the complete gene cluster from *S. rimosus* NRRL 3016 (26) for the biosynthesis of TET and tetrangomycin. This is the first report of the production of any angucyclinone in a heterologous host and will allow comparison of numerous secondary metabolic genes and enzymes for the same reaction from different organisms.

MATERIALS AND METHODS

Bacterial strains and plasmids used. *Streptomyces* strain WP 4669 was a gift from James French (Warner-Lambert Co.). *Streptomyces lividans* TK24, used as a recombinant host strain, was obtained from David Hopwood (John Innes Center, Norwich, England). The cosmid pOJ446, used for cosmid library construction, was obtained from Lilly Research Laboratories. *Escherichia coli* XL1-Blue MR and pBluescript II KS$^+$ were purchased from Stratagene. *E. coli* DH5α was purchased from Clontech, and pGEM11zf(+) was purchased from Promega.

Culture conditions for *S. rimosus* NRRL 3016, WP 4669, and *S. lividans*. *S. rimosus* NRRL 3016, WP 4669, or *S. lividans* was propagated on ISPII agar (44) at 27°C for spore preparation or in YEME liquid medium (21) at 27°C for genomic DNA preparation.

Genomic DNA isolation. Genomic DNAs were prepared from mycelia from 50-ml cultures of *S. rimosus* and of WP 4669 grown in YEME containing 0.5% glycine. Mycelia were collected by centrifugation, washed twice with 10.3% sucrose, and 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 of RNase per ml, and 10 mg of lysozyme per ml. After 5 to 10 min of incubation at room temperature, proteinase K was added to a concentration of 0.5 mg/ml and the mixture was incubated at 50°C overnight. The mixture was then worked up in standard fashion, and the genomic DNA was dissolved in 3 ml of Tris-EDTA buffer.

Preparation of cosmid libraries of *S. rimosus* and WP 4669. Genomic DNA from *S. rimosus* was digested with *Bam*HI, *Kpn*I, *Pst*I, and *Sal*I. The digested DNAs were separated by electrophoresis. Southern blots were probed with ^{32}P-radiolabeled *actI*, the β-ketoacylsynthase gene from the actinorhodin path-

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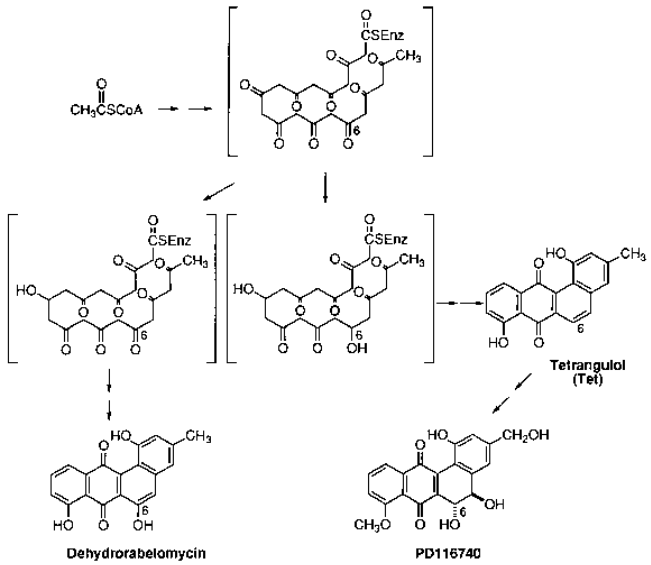

FIG. 1. Biosynthesis of dehydrorabelomycin, TET, and PD 116740.

way of *Streptomyces coelicolor* A3(2) (10), which was present in pIJ2345, and washed at moderate stringency (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–sodium dodecyl sulfate). The probe hybridized with two *Bam*HI DNA fragments (13.7 and 11.2 kb). Two bands also hybridized in the *Kpn*I and *Sal*I digests, while only a single large band (>30 kb) hybridized in the *Pst*I digestion. Genomic DNA from WP 4669 was digested with the same enzymes. Southern blots probed with *actI* yielded three DNA fragments (16.9, 7.9, and 6.3 kb) in the *Bam*HI digest and two each in the *Kpn*I, *Pst*I, and *Sal*I digests.

Genomic DNA from WP 4669 was partially digested with *Mbo*I, and 30- to 40-kb fragments were isolated from a low-melting-point agarose electrophoretic gel. *S. rimosus* genomic DNA was partially digested with *Mbo*I but was not size fractionated. Two to three micrograms of either the 30- to 40-kb WP 4669 genomic DNA fragments or the partially digested *S. rimosus* genomic fragments were ligated with 4 to 6 μg of *Bam*HI- and *Hpa*I-digested pOJ446 by using T4 DNA ligase, and the ligated DNA was packaged in vitro with Giga Pack II XL packaging extract (Stratagene) and then used to transfect *E. coli* XL1-Blue MR. The manufacturer's protocol was followed.

Colony hybridization of the cosmid libraries. Two thousand to three thousand *E. coli* colonies each from the *S. rimosus* and WP 4669 cosmid libraries were spread on dried nylon membranes (138-mm diameter; Amersham) on several filter papers as described by the manufacturer. After the inoculum was spread, the membrane was transferred onto a Luria-Bertani agar plate containing 100 μg of apramycin per ml and incubated overnight at 37°C. The cosmid libraries were replicated to new nylon membranes, lysed, and hybridized as described by the manufacturer, with ^{32}P-labeled *actI*.

Thirty-nine hybridizing clones were obtained from *S. rimosus* DNA, and restriction mapping with *Bam*HI and *Eco*RI showed that they segregated into two nonoverlapping sets: 15 clones for one PKS gene cluster (pks_{RIM-1}) and 24 clones for the other (pks_{RIM-2}). The first contained an 11.2-kb *actI* homologous *Bam*HI fragment, while the second contained a 13.7-kb *actI* homologous *Bam*HI fragment.

In the same manner, two sets of nonoverlapping clones were obtained from WP 4669 DNA: five in the first cluster (pks_{WP-1}) with 12.1- and 6.9-kb *Bam*HI fragments hybridizing to *actI* and seven in the second (pks_{WP-2}) with one 16.9-kb *actI* homologous *Bam*HI fragment.

Fermentation of *S. lividans* and transformants. Colonies of each organism were inoculated into 5 ml of YEME containing 6 μg of apramycin per ml for transformants. After incubation for 3 to 4 days at 28°C and 260 rpm, 0.25 ml of the seed culture was used to inoculate 5 ml of three production media in 18-ml culture tubes: glucose-Proflow-salt (GPS) (9), glycerol-asparagine, (6), and YEME, each containing 6 μg of apramycin per ml. The rest of the transformant cultures were used for plasmid minipreps to confirm the presence of the correct plasmids. All cultures were incubated at 28°C and 300 rpm for both 5 and 9 days.

Analysis of the metabolites. The cultures were acidified (pH 2.5 to 3.0) with 0.1 N HCl and extracted with 3 ml of ethyl acetate (EtOAc), and the extracts were dried with 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 high-performance liquid chromatography (HPLC) with a Waters Associated 600 E gradient pump. The metabolites were separated on a Waters NovaPak C_{18} radial compression column (0.8 by 10 cm, 4 μm) with a gradient of 5 to 95% acetonitrile in water containing 0.1% acetic acid over a period of 30 min at 1.5 ml/min. Detection was by photodiode array with a Waters 990$^+$ detector.

Preparation of physical maps of the pks_{RIM-1} and pks_{WP-2} clusters. Clones pSH2020 and pSH2030 containing *S. rimosus* pks_{RIM-1} DNA were digested with *Spe*I, *Xba*I, *Eco*RI, and *Eco*RV, both individually and in pairs. The first two excised the insert DNAs from the polylinker; no sites internal to the inserts were observed. The latter two enzymes were used to generate a rough restriction map. The 11.5-kb *Spe*I-*Eco*RI fragment of pSH2020 was subcloned with pBluescript II KS$^+$ to generate pSH2020.10. The 1.9-kb *Eco*RI fragment of pSH2030 and the 19.3-kb *Eco*RI-*Xba*I fragment of pSH2030 were subcloned with pGEM11zf(+) to generate pSH2030.20 and pSH2030.30, respectively. Each of these was restriction mapped with *Bam*HI and *Pst*I, and Southern blots of the digests were probed with ^{32}P-labeled *actI* and ^{32}P-labeled *actIII* [the 9-ketoreductase gene of *S. coelicolor* A3(2) contained on pIJ2346] (10).

Clones pSH3060 and pSH3071 containing WP 4669 pks_{WP-2} DNA were similarly treated. The 18.8-kb *Spe*I-*Eco*RI fragment of pSH3060 was subcloned with pBluescript II KS$^+$ to generate pSH3060.10. The 13.9-kb *Eco*RI fragment and the 7.1-kb *Eco*RI-*Xba*I fragment of pSH3071 were subcloned with pGEM11zf(+) to generate pSH3071.20 and pSH3071.30, respectively. These were restriction mapped and probed as described above.

Other molecular biological techniques. Plasmid DNA isolations from *E. coli* and preparation of *E. coli* competent cells were performed according to standard procedures (42). Restriction enzymes, DNA ligase, and calf intestinal alkaline phosphatase were purchased from Gibco BRL, New England Biolabs, United States Biochemical, Promega, and Boehringer Mannheim and used according to the manufacturers' instructions. Plasmid isolation and protoplast preparation from *S. lividans*, and transformation of *S. lividans* protoplasts, were carried out according to the method of Hopwood et al. (21).

Southern hybridization. Southern blots of recombinant cosmids were performed with Hybond-N nylon membranes (Amersham), and the blots were hybridized with the *actI* or *actIII* probe, as recommended by the manufacturer.

RESULTS

Genomic DNA from *S. rimosus* and from WP 4669 was digested with a variety of enzymes as described in Materials and Methods, and the Southern blots were probed with *actI*, the β-ketoacylsynthase gene from the actinorhodin pathway of *S. coelicolor* A3(2) (10). These results clearly suggested the presence of two PKS gene clusters in each organism.

Isolation of two PKS gene clusters from *S. rimosus* and two from WP 4669. Libraries containing fragments of ~30 to 40 kb were constructed from *S. rimosus* and from WP 4669 with partial digests of genomic DNA ligated into a cosmid shuttle vector (1). Screening the *S. rimosus* library with the *actI* probe identified 39 hybridizing clones, and restriction mapping and Southern hybridization results clearly showed that they segregated into two nonoverlapping sets, which were named pks_{RIM-1} and pks_{RIM-2}. Screening the WP 4669 library in the same manner also yielded 12 clones that were divided into two nonoverlapping sets of clones (pks_{WP-1} and pks_{WP-2}).

Expression of a *S. rimosus* PKS gene cluster in *S. lividans* TK24. Eight recombinant cosmids from cluster pks_{RIM-1} and 10 from cluster pks_{RIM-2} obtained from the *S. rimosus* library were introduced by transformation into *S. lividans* TK24. Each transformant was grown in liquid culture, and ethyl acetate extracts of these cultures were analyzed by HPLC with photodiode array detection. Of eight transformants from pks_{RIM-1} that were tested (Table 1), six produced metabolites (Fig. 2) that were not observed in controls of either *S. lividans* or *S. lividans*/pOJ446, but no new metabolites were detected from pks_{RIM-2} transformants. One of the new metabolites from pks_{RIM-1} displayed the same HPLC retention time and UV-visible absorption spectrum (Fig. 2; retention time, 23.81 min) as authentic TET, while a second (Fig. 2; retention time, 14.09 min) matched tetrangomycin. A large-scale fermentation of one of these, clone pSH2020, allowed isolation of these compounds, and their structures were further confirmed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (5). The remaining new metabolite (Fig. 2; retention time, 16.00 min) was the major metabolite of this fermentation. This compound was also purified,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
Streptomyces rimosus	TET and tetrangomycin producer	26
Streptomyces strain WP 4669	PD 116740 producer	47
Escherichia coli		
XL1-Blue MR	Δ(*mcrA*) *183* Δ(*mcrCB-hsdSMR-mrr*) *173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
DH5α	*deoR endA1 gyrA96 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44 thi-1* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*ΔM15 F^- λ^-	Clontech
Streptomyces lividans TK24	Host for expression test ($SLP2^-$ $SLP3^-$)	John Innes Center
Plasmids		
pOJ446	*E. coli-Streptomyces* shuttle cosmid	1
pBluescript II KS^+		Stratagene
pGEM11zf(+)		Promega
pIJ2345	pBR329 with a 2.2-kb *actI* insert	John Innes Center
pIJ2346	pBR329 with a 1.1-kb *actIII* insert	John Innes Center
pSH2010	1 + pOJ446, produces TET and tetrangomycin in *S. lividans*	This work
pSH2020	1 + pOJ446, produces TET and tetrangomycin in *S. lividans*	This work
pSH2030	1 + pOJ446, produces TET and tetrangomycin in *S. lividans*	This work
pSH2040	1 + pOJ446, produces TET and tetrangomycin in *S. lividans*	This work
pSH2050	1 + pOJ446, does not produce TET and tetrangomycin in *S. lividans*	This work
pSH2060	1 + pOJ446, does not produce TET and tetrangomycin in *S. lividans*	This work
pSH2070	1 + pOJ446, produces TET and tetrangomycin in *S. lividans*	This work
pSH2080	1 + pOJ446, produces TET and tetrangomycin in *S. lividans*	This work
pSH3010	2 + pOJ446, produces PD 116740 and intermediate compounds in *S. lividans*	This work
pSH3020	2 + pOJ446, does not produce PD 116740 or intermediate compounds in *S. lividans*	This work
pSH3030	2 + pOJ446, cannot transform *S. lividans*	This work
pSH3040	2 + pOJ446, does not produce PD 116740 or intermediate compounds in *S. lividans*	This work
pSH3050	2 + pOJ446, does not produce PD 116740 or intermediate compounds in *S. lividans*	This work
pSH3060	2 + pOJ446, produces PD 116740 and intermediate compounds in *S. lividans*	This work
pSH3071	2 + pOJ446, produces PD 116740 and intermediate compounds in *S. lividans*	This work
pSH2020.10	pBluescript II KS^+ with 11.5-kb *Spe*I-*Eco*RI fragment of pSH2020	This work
pSH2030.20	pGEM11zf(+) with 1.9-kb *Eco*RI fragment of pSH2030	This work
pSH2030.30	pGEM11zf(+) with 19.3-kb *Eco*RI-*Xba*I fragment of pSH2030	This work
pSH3060.10	pBluescript II KS^+ with 18.8-kb *Spe*I-*Eco*RI fragment of pSH3060	This work
pSH3071.20	pGEM11zf(+) with 13.9-kb *Eco*RI fragment of pSH3071	This work
pSH3071.30	pGEM11zf(+) with 7.1-kb *Eco*RI-*Xba*I fragment of pSH2071	This work

[a] 1, TET biosynthetic gene cluster; 2, PD 116740 biosynthetic gene cluster.

and it was assigned the structure of the previously reported fridamycin E (41), based on spectroscopic data (5). Although this metabolite was not present in *S. rimosus* extracts worked up after 5 days (standard protocol), a subsequent time course study revealed its presence at 21 h and its rapid disappearance during the following 20 h (data not shown).

By using two cosmid clones from the cluster that yielded TET, tetrangomycin, and fridamycin E, three subclones that spanned the overlapping region that contained the essential genes were prepared. A physical map of each was generated from digests with *Bam*HI and *Pst*I, and the regions containing DNA homologous to *actI* and to *actIII*, the 9-ketoreductase gene from the actinorhodin pathway of *S. coelicolor* A3(2), were identified by hybridization experiments (Fig. 3A).

Expression of a WP 4669 PKS gene cluster in *S. lividans* TK24. Cosmids containing the two PKS gene clusters from WP 4669 were also introduced into *S. lividans* TK24, and expression tests were conducted in three different production media. HPLC analysis of extracts from the $pks_{WP\text{-}1}$ cultures in all three media showed that no new recognizable compounds were produced.

HPLC analysis of *S. lividans* transformants from $pks_{WP\text{-}2}$ grown in one medium (GPS) showed that three of the six clones produced the metabolites of the PD 116740 pathway in *S. lividans* (Table 1). HPLC peaks (Fig. 4) were observed that matched both retention time and UV-visible spectra for TET and PD 116740, as well as peaks for the potential biosynthetic intermediates 8-*O*-methyltetrangulol (MT) (28, 36), 19-hydroxytetrangulol (HT) (5), and 19-hydroxy-8-*O*-methyltetrangulol (HMT) (4), as shown in Fig. 5. In YEME medium, only intermediate compounds (HT, MT, and HMT) were observed. In glycerol-asparagine medium, neither PD 116740, TET, nor any of the potential biosynthetic intermediates between them were produced by either WP 4669 or the transformants. Scaled-up fermentations of clones pSH3071 and pSH3060 allowed isolation of the metabolite corresponding to PD 116740, and its structure was further confirmed by ^{1}H NMR spectroscopy and high-resolution mass spectrometry (5).

By using two cosmid clones from this cluster, three subclones that spanned the overlapping region that contained the essential genes were prepared. A physical map of each was prepared as described above for $pks_{RIM\text{-}1}$ (Fig. 3B).

DISCUSSION

The studies reported here have revealed that *S. rimosus* NRRL 3016 and WP 4669 each contain two clusters of PKS genes. *S. coelicolor* has PKS gene clusters for actinorhodin (31) and for a spore pigment (7). The genes for the latter product are not expressed during actinorhodin production (48). We

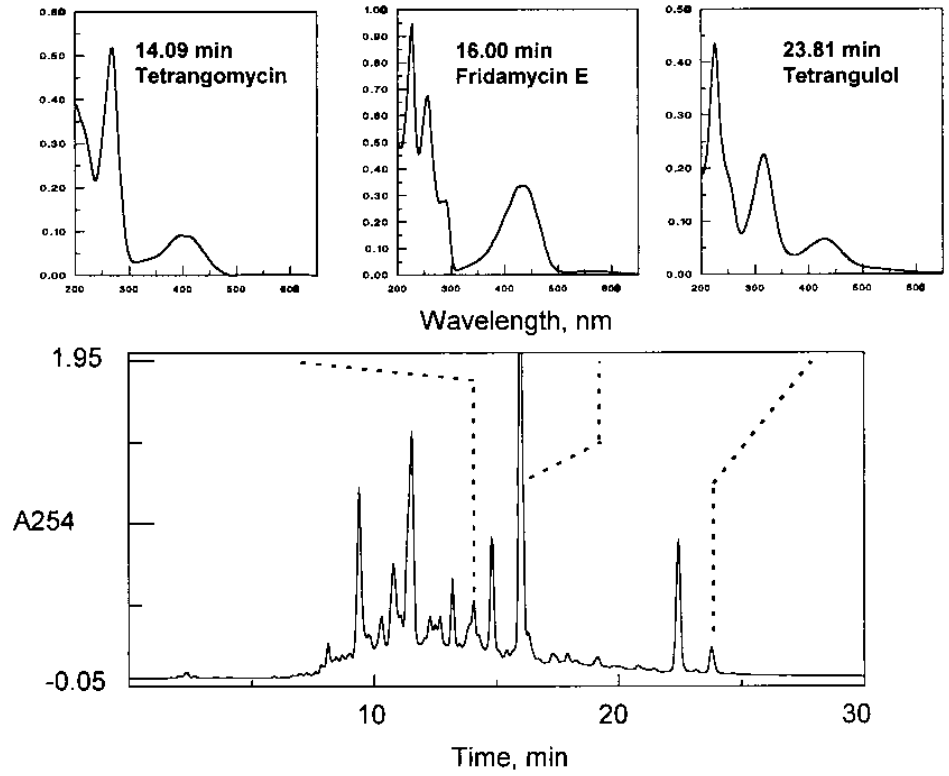

FIG. 2. HPLC trace of an ethyl acetate extract of *S. lividans*/pSH2020 with UV-visible spectra of the tetrangomycin, fridamycin E, and TET peaks obtained by photodiode array detection.

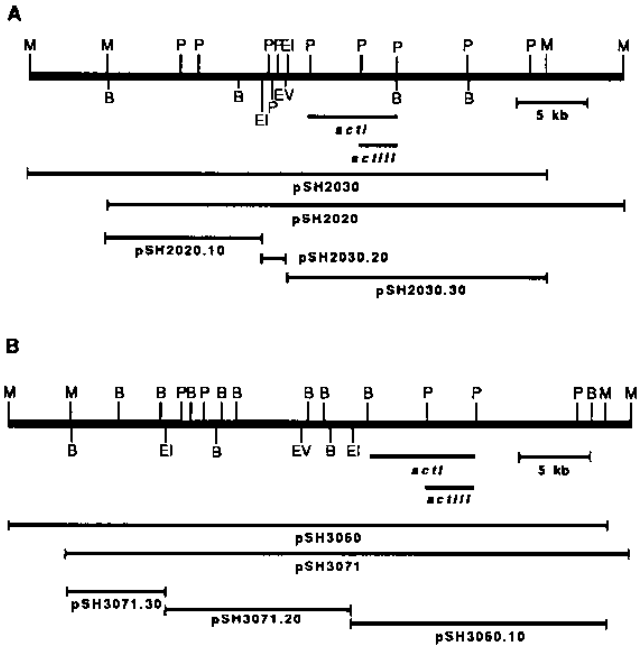

FIG. 3. Physical maps of the pks_{RIM-1} (A) and pks_{WP-2} (B) biosynthetic gene clusters. B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; M, *Mbo*I; P, *Pst*I. Cosmids and subclones used for mapping are shown below the genomic DNA. Regions hybridizing with either *actI* or *actIII* are indicated; the DNA orientation is arbitrarily chosen to conform to that previously observed for other angucyclinone PKS genes.

have also found two apparent PKS clusters in the kinamycin producer *Streptomyces murayamaensis* (20), and the urdamycin producer *Streptomyces fradiae* Tü 2717 also contains two (8). It is possible that the unexpressed clusters from *S. rimosus* NRRL 3016 and WP 4669 may code for spore pigments.

Heterologous production of the metabolites of one *S. rimosus* NRRL 3016 polyketide pathway was obtained, and it yielded both TET and tetrangomycin. To this point, the precise biogenetic relationship between TET and tetrangomycin has not been established, but it appears likely either that they branch from a very closely related intermediate or that one is precursor to the other, and the relevant genes for both have now been shown to be clustered with those of the angucyclinone PKS. In addition to these, a new compound that resulted from oxidative cleavage of an angucyclinone was observed. Spectroscopic characterization identified it as fridamycin E (41). This was the major metabolite produced by *S. lividans* transformed with pks_{RIM-1} and was at first suspected to be a hybrid metabolite involving *S. lividans* enzymes, too. Although it was subsequently shown to accumulate transiently in *S. rimosus* NRRL 3016 grown in GPS, it is not clear why larger quantities are produced by the transformants. This may be due to relaxed regulation of the oxygenase gene in the foreign host. As shown in Fig. 6, it may be derived by a dioxygenase cleavage of TET or a biological Baeyer-Villiger oxidation of tetrangomycin. This represents a very unusual metabolism not previously known to occur in *S. rimosus*. A similar metabolism is presumably involved in the biosynthesis of vineomycin B_2 (24) and of aureolic acid (3).

The aromatic polyketide oxytetracycline (OTC) is produced by a different *S. rimosus* strain (39), and the PKS gene cluster for the OTC pathway has been cloned by Binnie et al. (2).

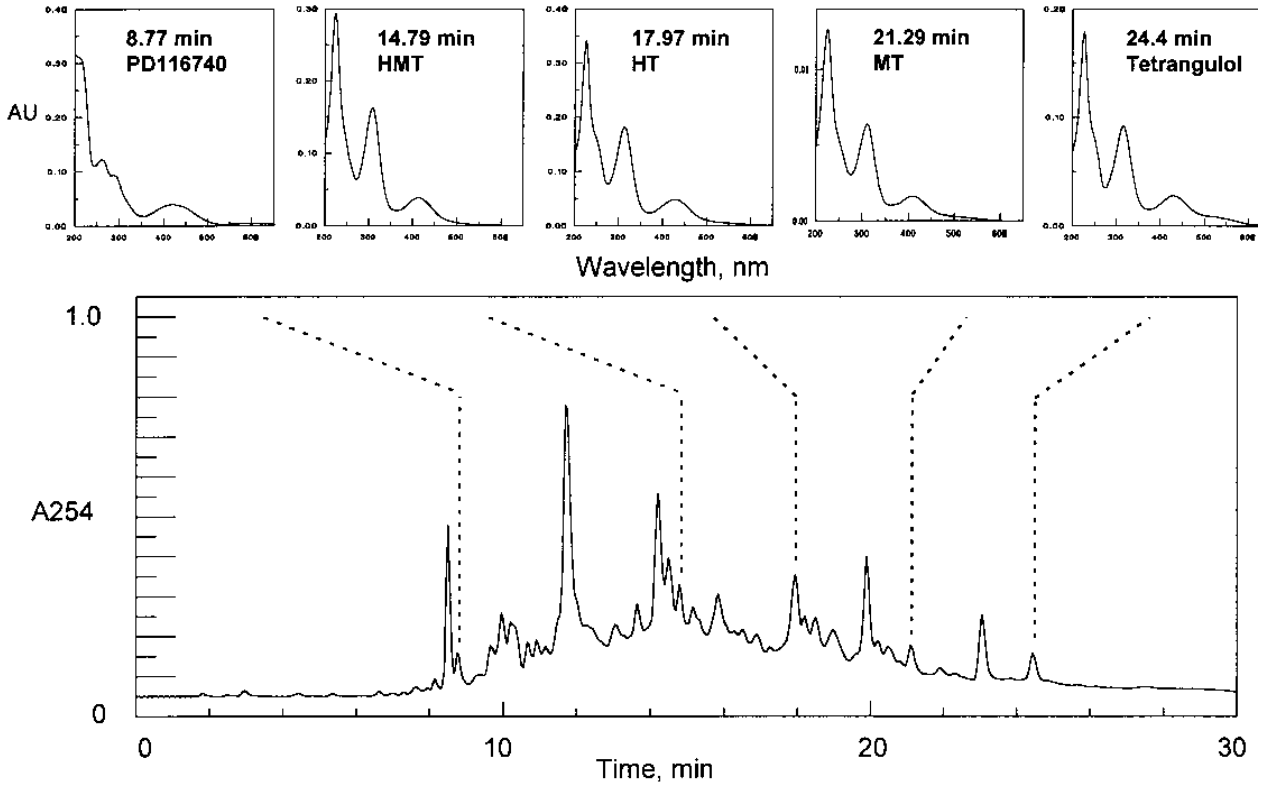

FIG. 4. HPLC trace of an ethyl acetate extract of *S. lividans*/pSH3071 with UV-visible spectra of the PD 116740, HMT, HT, MT, and TET peaks obtained by photodiode array detection.

Interestingly, while it was found that the *S. rimosus* strain used in the current study is clearly resistant to OTC (up to 500 μg/ml), OTC was not produced from the second PKS gene cluster cloned, and restriction maps of both of the PKS clusters isolated from *S. rimosus* NRRL 3016 were clearly different from the reported OTC PKS cluster (data not shown). The OTC biosynthetic genes are notoriously unstable in an industrial strain of *S. rimosus* (17), and the NRRL strain may have lost some or all of these genes.

Heterologous production of the metabolites of one PKS pathway from WP 4669 yielded the TET-derived PD 116740. This compound is biosynthetically interesting because of the unusual *trans*-diol present in its C ring (12, 46, 47). We have shown that the C-5 hydroxyl is derived from molecular oxygen and that the C-6 hydroxyl is apparently derived from water (14), indicating that the 5,6-*trans*-diol moiety is generated by enzymatic epoxidation of the K-region double bond followed by action of an epoxide hydrolase. However, the intermediates from TET to PD 116740 had not been established, although on chemical grounds it was believed that epoxidation and hydrolysis would be the last steps. Regardless, the overall conversion

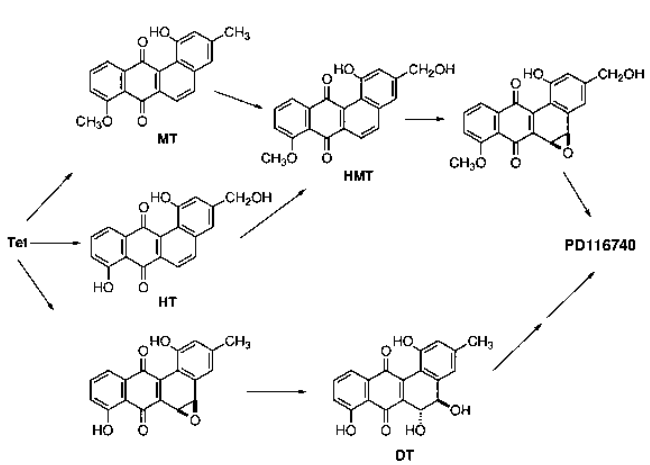

FIG. 5. Potential intermediates in the biosynthesis of PD 116740 from TET. DT, dihydro-5,6-dihydroxytetrangulol.

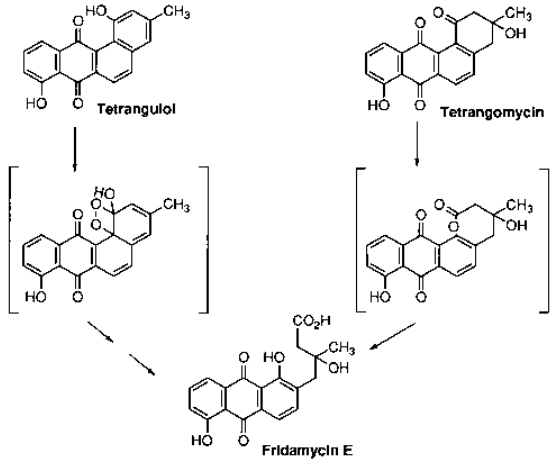

FIG. 6. Possible biosynthesis of fridamycin E from either tetrangomycin or TET.

requires a minimum of four steps, and possible candidates for intermediate compounds included MT, HT, HMT, or dihydro-5,6-dihydroxytetrangulol. HMT had been isolated by Brinkman et al. from the wild-type WP 4669 (4). We have prepared authentic samples of MT (28, 36), HT (5), and HMT (5). Surprisingly, all three of these were then identified in extracts of *S. lividans*/pSH3071 transformants, suggesting either that MT or HT is a branch product or that a metabolic matrix operates in this pathway to produce HMT. Nonetheless, the data support the idea that epoxidase and hydrolase activities are the last steps in PD 116740 biosynthesis. Identification of the correct pathway will require gene disruption and/or biochemical studies; the latter are in progress.

HPLC analysis showed that expression of the PD 116740 pathway in *S. lividans* led to accumulation of substantially larger quantities of intermediate angucyclinones than observed for WP 4669. Indeed, HMT was almost unidentifiable in extracts from WP 4669, while the compound was very easily recognizable in extracts from the *S. lividans* transformants. It is possible that the genes for the last modifying enzyme(s) leading to PD 116740 may not be well expressed in the foreign host.

Numerous PKS gene clusters have now been isolated from a variety of actinomycetes. However, in only some cases has heterologous production of a complete pathway been obtained (18, 30, 37, 38), while in a number of cases the product of the reported PKS genes is entirely unknown (25). During the course of our studies, cloning of portions of gene clusters for two other angucyclinones has been reported, as evidenced by gene disruption experiments (8, 19). Our strategy has been to clone fragments of DNA large enough to maximize the likelihood of obtaining heterologous production of the target metabolites as a means of unequivocally confirming the identity of the genes.

Recently, the detailed functions of individual enzymes of a number of polyketides have been deciphered by expressing combinations of different PKS genes (11, 23, 32–35). This has demonstrated the possibility of rationally designing new polyketides by genetic engineering. These genes have also been used in the combinatorial biosynthesis of novel polyketides (34, 40, 45). The ring folding pattern for angucyclinones, leading to an angular ring system, should be different from that leading to linear systems. Further molecular genetic research on the TET-tetrangomycin and PD 116740 biosynthetic gene clusters may give information about what causes formation of an angular aromatic ring system.

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