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 MboI, 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 (pksRIM-1) for tetrangulol, tetrangomycin, and fridamycin E. Peaks were identified from the WP 4669 cluster (pksRWP-1) for tetrangulol, 19-hydroxytetrangulol, 8-O-methyltetragulol, 19-hydroxy-8-O-methyltetragulol, and PD 116740. Structures were confirmed by 1H 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 prearromatic 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. The gene clusters for PD 116740 from Streptomyces rimosus NRRL 3016 and Tetrangulol and Tetrangomycin from Streptomyces rimosus NRRL 3016

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way of Streptomyces coelicolor A3(2) (10), which was present in pIJ2345, and was used as a probe for Southern hybridization (11). The probe hybridized with two BamHI DNA fragments (13.7 and 11.2 kb). Two bands also hybridized in the KpnI and SalI digests, while only a single large band (>30 kb) hybridized in the PstI digest. 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 BamHI digest and two each in the KpnI, PstI, and SalI digests. Genomic DNA from WP 4669 was partially digested with MboI, and 30- to 40-kb fragments were isolated from a low-melting-point agarose electrophoretic gel. S. rimosus genomic DNA was partially digested with MboI 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 BamHI- and HindIII-digested pOJ446 by using T4 DNA ligase, and the ligated DNA was packaged in vitro with GigaPack II XL packaging extract (Stratagene) and then used to transfect E. coli XL1-Blue M (Stratagene). The manufacturer’s protocol was followed.

Colonies of each organism were inoculated into 5 ml of YEME containing 0.1% (pH 7.0) of ampicillin 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-Pro/flow-salt (GPS) (9), glycerol-asparagine, (6), and YEME, each containing 6 μg of ampicillin 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-CH2Cl2. An aliquot (10 μl) of each extract was analyzed by reverse-phase high-performance liquid chromatography (HPLC) with a Waters Associates 600 E gradient pump. The metabolites were separated on a Waters NovaPak C18 radial compression column (0.8 by 10 cm, 4 μm) with a gradient of 0 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 pksRIM-1 and pksWP-2 clusters. Clones pSH2020 and pSH2030 containing S. rimosus pksWP-1 DNA were digested with SpeI, HindIII, EcoRI, and EcoRV, both individually and in parallel. 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 SpeI-EcoRI fragment of pSH2020 was subcloned with pBlueScript II KS+ to generate pSH2020.10. The 13.9-kb EcoRI fragment of pSH2030 and the 19.3-kb EcoRI-XbaI fragment of pSH2030 were subcloned with pGEMEl1zf(+) to generate pSH2030.20 and pSH2030.30, respectively. Each of these was restriction mappd with BamHI and PstI, and Southern blots of the digests were probed with 3P-labeled actI and 3P-labeled actIII [the 9-ketoadecyshydroxylase gene of S. coelicolor A3(2)] (10).

Clones pSH3060 and pSH3071 containing WP 4669 pksWP-2 DNA were similarly treated. The 18.9-kb SpeI-EcoRI fragment of pSH3060 was subcloned with pBlueScript II KS+ to generate pSH3060.10. The 13.9-kb EcoRI fragment and the 7.1-kb EcoRI-XbaI fragment of pSH3071 were subcloned with pGEMEl1zf(+) 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 Hybrid-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, and the β-ketoacylshydratase 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 pksRIM-1 and pksRIM-2. Screening the WP 4669 library in the same manner also yielded 12 clones that were divided into two nonoverlapping sets of clones (pksWP-1 and pksWP-2).

Expression of a S. rimosus PKS gene cluster in S. lividans TK24. Eight recombinant cosmids from cluster pksRIM-2, and 10 from cluster pksWP-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 pksRIM-2 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 pksWP-2 transformants. One of the new metabolites from pksRIM-2 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 "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.
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 spanning the overlapping region that contained the essential TET, tetrangomycin, and fridamycin E, three subclones that during the following 20 h (data not shown).

**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*<sub>WP-2</sub> cultures in all three media showed that no new recognizable compounds were produced.

HPLC analysis of *S. lividans* transformants from *pks*<sub>WP-2</sub> 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-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 H<sup>1</sup>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*<sub>REM-1</sub> (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
have also found two apparent PKS clusters in the kinamycin producer *Streptomyces murayamaensis* (20), and the urdamycin producer *Streptomyces fradiae* Tu 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* 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 *pksRIM-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 B₂ (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).

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**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 *pksRIM-1* (A) and *pksWP-2* (B) biosynthetic gene clusters. B, *Bam*HI; E1, *Eco*RI; E2, *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.
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


