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Cloning and Characterization of a Polyketide Synthase Gene from *Streptomyces fradiae* Tü2717, Which Carries the Genes for Biosynthesis of the Angucycline Antibiotic Urdamycin A and a Gene Probably Involved in Its Oxygenation

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A DNA fragment was cloned as cosmid purd8, which encodes a polyketide synthase involved in the production of the angucycline antibiotic urdamycin from *Streptomyces fradiae* Tü2717. Deletion of the polyketide synthase genes from the chromosome abolished urdamycin production. In addition, purd8 conferred urdamycin resistance on introduction into *Streptomyces lividans* TK24. Sequence analysis of 5.7 kb of purd8 revealed six open reading frames transcribed in the same direction. The deduced amino acid sequences of the six open reading frames strongly resemble proteins from known type II polyketide synthase gene clusters: a ketoacyl synthase, a chain length factor, an acyl carrier protein, a ketoreductase, a cyclase, and an oxygenase. Heterologous expression of the urdamycin genes encoding a ketoacyl synthase and a chain length factor in *Streptomyces glaucescens* tetracenomycin C-nonproducing mutants impaired in either the TcmK ketoacyl synthase or TcmL chain length factor resulted in the production of tetracenomycin C. Heterologous expression of a putative oxygenase gene from the urdamycin gene cluster in *S. glaucescens* GLA.O caused production of the hybrid antibiotic 6-hydroxy tetracenomycin C.

Polyketides form a large group of natural products synthesized especially by bacteria, fungi, and plants. The urdamycins (Fig. 1A) are aromatic polyketides produced by *Streptomyces fradiae* Tü2717 that exhibit antitumor and antibacterial activity (16). They belong to the large group of angucycline antibiotics, which comprise more than 100 natural compounds with many different pharmacological activities (42). Recently, polyketide synthase (PKS) genes responsible for production of actinorhodin (18), tetracenomycin (TCM) C (Fig. 1A) (4), granaticin (48), and doxorubicin (21), to name just a few, have been cloned; they strongly resemble each other with respect to their genetic organization and the deduced amino acid sequences of their polypeptide products responsible for the assembly of the polyketide chain (27). Furthermore, combinations of PKS genes from different gene clusters can form active enzyme complexes and expression of the hybrid PKS in a heterologous host can lead to the production of novel polyketide compounds (30). Isolation of the PKS responsible for production of an angucycline antibiotic would expand the number of PKSs available for the combinatorial design of hybrid PKSs. Like many other aromatic polyketides, urdamycin is made from a series of acetate units (40), but the folding pattern of the nascent polyketide chain differs from the well-analyzed actinorhodin and TCM C (39). It is not clear what determines the apparently unique folding and cyclization pattern of the decaketide precursor of the urdamycins. Furthermore, characterization of the enzymes involved in the post-polyketide formation reactions, e.g., glycosylation with the didexoy sugar D-olivose and the trideoxy sugar L-rhodinose and oxygenation, will provide information about the biosynthesis of urdamycin and subsequently point to ways to generate hybrid antibiotics (26, 30). We in fact have recently isolated two hybrid antibiotics from the urdamycin producer by introduction of *tcm* or elloramycin genes into *S. fradiae* Tü2717; these are 6-hydroxy TCM C (11) (Fig. 1A) and 8-olivosyl-8-demethyl-TCM C (11, 14). As a first step towards analyzing urdamycin biosynthesis genetically and biochemically, we have characterized a 5.7-kb fragment isolated from a genomic cosmid library of *S. fradiae* Tü2717; it carries genes involved in early steps of urdamycin biosynthesis and, possibly, catalyzing a subsequent oxygenation step. During this work, Han et al. (23) published the sequence of genes encoding a PKS involved in production of jadomycin, an angucycline antibiotic produced by *Streptomyces venezuelae* ISP5230.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and other materials. *S. fradiae* Tü2717 and *Streptomyces glaucescens* GLA.O were from the culture collection of the University of Tübingen. *S. glaucescens* WHM1061 (*tcmK*) and *S. glaucescens* WHM1068 (Δ*tcmL*) (32, 49) were obtained from C. R. Hutchinson (Madison, Wis.). *Streptomyces lividans* TK24 was supplied by D. A. Hopwood (Norwich, United Kingdom).

Plasmids pIJ2346 and pIJ4026 were kindly provided by D. A. Hopwood and M. J. Bibb (Norwich, United Kingdom), respectively. We received pKC505 from Eli Lilly (Indianapolis, Ind.) and pGM160 from G. Muth (Tübingen, Germany).

A pure sample of urdamycin A was obtained from H.-P. Fiedler (Tübingen, Germany). Thiostrepton and apramycin were gifts from S. J. Lucania (Squibb Pharmaceutical Research Institute, Princeton, N.J.) and A. Bechthold (Tübingen, Germany), respectively. The plasmids are described in Table 1.

Culture conditions. *Streptomyces* spp. were maintained on 1% malt extract–0.4% yeast extract–0.4% glucose–1 mM $CaCl_2$, pH 7.2 (HA medium). Production of urdamycin was in 2% soybean meal–2% glucose, pH 7.2 (AM medium) (41). For the preparation of protoplasts *S. fradiae* Tü2717 was grown in liquid R2YE for 30 h following the standard procedure (25). The protoplasts were regenerated on R2YE. General methods for the cultivation of *Escherichia coli* DH5α were as previously described (43). *S. glaucescens* was grown in R2YENG for the preparation of protoplasts and production of TCM C, as described elsewhere (50).

Thiostrepton (25 μg/ml in solid media; 10 μg/ml in liquid media), ampicillin (100 μg/ml), apramycin (50 μg/ml for *E. coli*; 25 μg/ml for *Streptomyces* spp.), or erythromycin (50 μg/ml) was used for the selective growth of recombinant strains.

DNA isolation, manipulation, and cloning. Small-scale preparations of *E. coli* plasmid DNA were made by standard procedures (43). Large-scale preparations

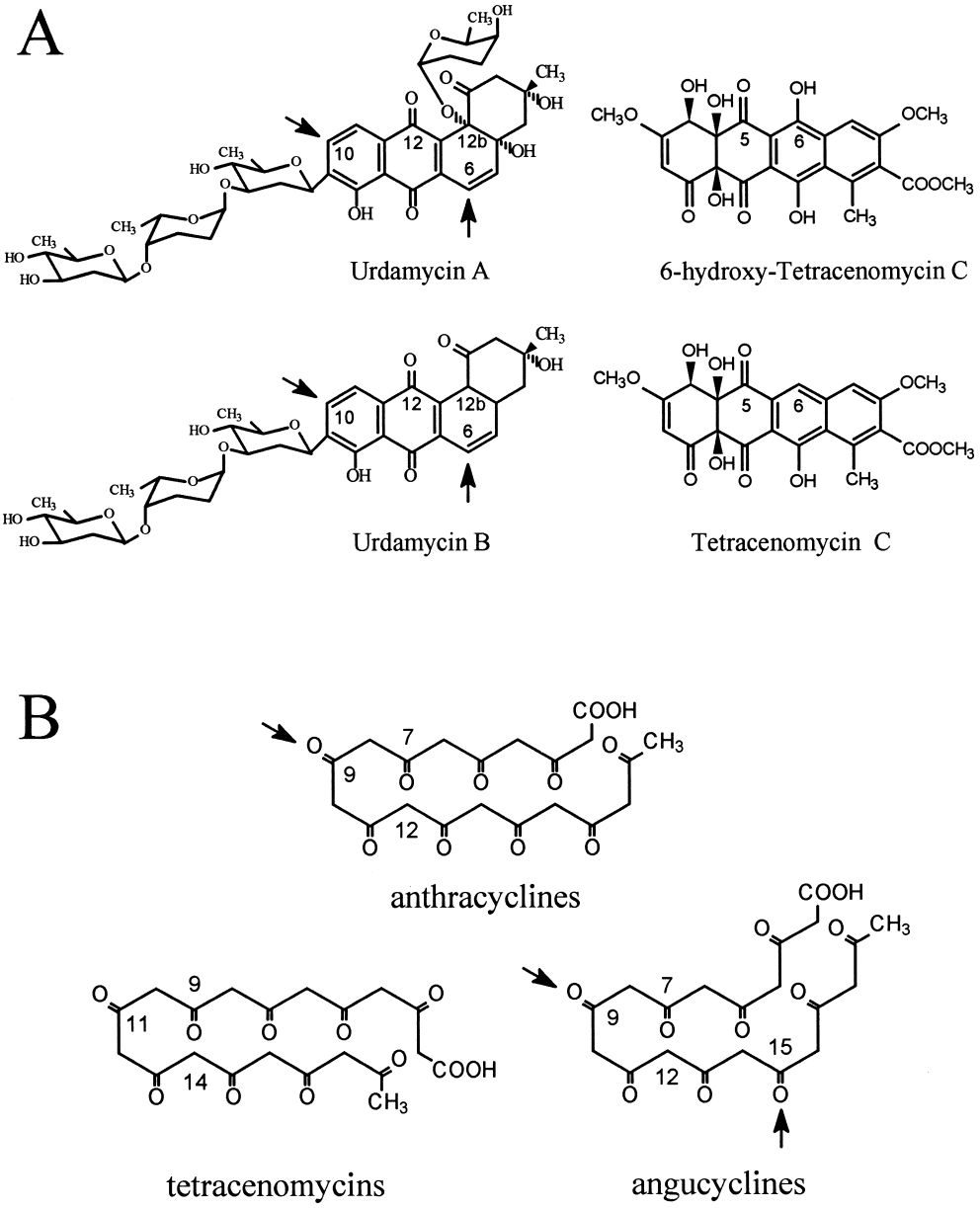

FIG. 1. (A) Structures of the polyketide antibiotics urdamycin A, urdamycin B, TCM C, and 6-hydroxy TCM C. Arrows indicate the positions in urdamycin A and B where ketoreduction presumably occurs. (B) Hypothetical decaketide structures of three different types of tetracyclic antibiotics: anthracyclines, TCMs, and angucyclines. The first intramolecular adol condensation occurs between C-7 and C-12 in the case of anthracyclines and angucylines or between C-9 and C-14 for TCM C. Arrows indicate the positions where ketoreduction occurs.

of *E. coli* plasmid DNA were made with the Nucleobond AX100 kit (Macherey und Nagel, Dürer, Germany). For small-scale plasmid DNA isolations from *Streptomyces* spp. we used the method of Hopwood et al. (25). Large-scale isolations of DNA were carried out with the Nucleobond AX100 kit, increasing the volumes of the buffers S1, S2, and S3 by a factor of 3 for a 20-ml culture (24 to 48 h). Chromosomal DNA of *S. fradiae* Tü2717 was isolated according to the small-scale isolation protocol (25). For the construction of the gene library from *S. fradiae* Tü2717, chromosomal DNA was digested with *Sau*3AI and fragments of 20 to 25 kb were purified on a NaCl gradient (43) and ligated into the *Bam*HI site of pKC505. The DNA was packaged into phage particles by using the Packagene Kit from Promega (Madison, Wis.) and introduced into *E. coli* DH5α. Recombinant *E. coli* cells were selected with apramycin (100 μg/ml). Clones carrying DNA homologous to *tcmKL* were isolated by colony hybridization using nitrocellulose following standard procedures (43) with a digoxigenin-labeled *tcmKL* probe. DNA probes were labeled using the DIG labeling and detection kit (Boehringer-Mannheim, Mannheim, Germany). Southern blot transfers were performed with Hybond N nylon membranes (Amersham, Braunschweig, Germany) by standard techniques (43). Detection of DNA fragments on nylon membranes was done with the chemiluminescence substrate Lumigen PPD (Boehringer-Mannheim). Hybridizations were carried out at 68°C for 16 h (5× SSC [1× SSC is 0.15 M NaCl–0.015 M sodium citrate]–0.1% *N*-lauroylsarcosine–0.02% sodium dodecyl sulfate–1% blocking reagent). DNA fragments were isolated from agarose gels with the Sephaglas Bandprep kit (Pharmacia, Freiburg, Germany).

DNA sequencing. Cosmid purd8 was digested with *Bgl*II, and four fragments (15.5, 6.2, 5, and 0.3 kb) were cloned in the *Bam*HI site of pUC19. Starting from those plasmids, further subclones were made by cloning into pUC19, and the resulting plasmids were sequenced with an automated laser fluorescence sequencer (A.L.F.; Pharmacia). Sequencing reactions were done using the Autoread sequencing kit (Pharmacia) according to the supplier's instructions (quick annealing protocol, chain termination method [44]) with double-stranded templates purified with the Nucleobond AX100 kit. The sequencing reactions were analyzed with an automated laser fluorescence sequencer (A.L.F.; Pharmacia) on a 7 M urea–6% polyacrylamide gel in 1.2× Tris-borate-EDTA, and the

TABLE 1. Plasmids used in this study

Plasmid	Construction and characteristics	Reference or source
pGM160	*E. coli-Streptomyces* shuttle vector	33
pWHM3	*E. coli-Streptomyces* shuttle vector	57
pKC505	*E. coli-Streptomyces* shuttle cosmid	38
pUC19	*E. coli* cloning vector	Pharmacia
pGEM7zf+	*E. coli* cloning vector	Promega
pT7-7	*E. coli* expression vector	53
pIJ2346	pBR329 derivative with a 1.1-kb DNA fragment; *act*III	28
pIJ4026	pUC19 derivative with a 1.7-kb DNA fragment; *ermE*	6, 54
pWHM1026	pWHM3 with entire *tcm* cluster	12
purd2	pKC505 derivative with a 25.0-kb fragment from *S. fradiae* Tü2717 ligated into the *Bam*HI site of cosmid pKC505	This work
purd8	pKC505 derivative with a 27.0-kb fragment from *S. fradiae* Tü2717 ligated into the *Bam*HI site of pKC505	This work
purd10	pUC19 derivative with a 5-kb *Bgl*II DNA fragment from purd8 ligated into the *Bam*HI site of pUC19 (Fig. 2A)	This work
purd11	pUC19 derivative with a 6.0-kb *Bgl*II DNA fragment from purd8 ligated into the *Bam*HI site of pUC19 (Fig. 2A)	This work
purd12	pGEM7zf+ derivative with a 0.3-kb *Kpn*I-*Bam*HI DNA fragment from pIJ4026; *ermE* promoter	This work
purd13	pWHM3 derivative with a 3.0-kb *Bam*HI-*Eco*RI DNA fragment and a 0.3-kb *Xba*I-*Bam*HI DNA fragment from purd12 ligated into the *Eco*RI-*Xba*I site of pWHM3; *urdFAB* under the control of the *ermE* promoter (Fig. 2B)	This work
purd16	pT7-7 derivative with a 1.5-kb *Nde*I-*Bam*HI DNA fragment isolated from purd10; *urdE* (Fig. 2B)	This work
purd17	pUC19 derivative with a 0.3-kb *Kpn*I-*Bam*HI DNA fragment from pIJ4026; *ermE* promoter	This work
purd18	pWHM3 derivative with a 0.3-kb *Eco*RI-*Xba*I DNA fragment from purd17 and a 1.5-kb *Xba*I-*Bam*HI DNA fragment from purd16 ligated into the *Eco*RI-*Bam*HI sites of pWHM3; *urdE* under the control of the *ermE* promoter[a]	This work
purd19	pGM160 derivative with a 5.8-kb DNA fragment ligated into the *Hin*dIII-*Xba*I site of pGM160; plasmid for gene disruption of the urdamycin PKS	This work

[a] The last three amino acids at the C terminus of *urdE* (QQS) preceding the TGA stop codon were cleaved off by the cloning procedure and substituted by a leucine followed by a TAG stop codon.

running buffer was 0.9× Tris-borate-EDTA (spacer 0.35 mm; 50°C). Primers were either universal or reverse from the Autoread sequencing kit.

Computer analysis of DNA and protein sequences. DNA and protein sequences were analyzed with the Genetics Computer Group software, version 7.2 (15). TBlastN and BlastX analyses (1) were run with the GenBank (release 87), EMBL (release 41), and SwissProt (release 30) data banks, respectively.

Gradient plates. Gradient plates were prepared as described by Szybalski (52) with a linear gradient from 0 to 50 μg of urdamycin A per ml on solid HA medium.

Detection of polyketide compounds. Cultures were grown for 72 to 96 h and extracted with an equal volume of ethyl acetate. The organic layer was subjected to thin-layer chromatography (TLC) analysis on silica gel plates (Merck, Darmstadt, Germany) with CH_2Cl_2-methanol (9:1) as the solvent. Quantification of polyketide compounds was done with a high-pressure liquid chromatograph (HPLC) with a diode array detector as described by Fiedler (19) with a Nucleosil 100 C_{18} column (5 μm) using a linear 0.1% phosphoric acid–acetonitrile gradient (100% acetonitrile after 15 min; flow, 2 ml/min).

Construction of *S. fradiae* Tü2717/ΔPKS. A 1.6-kb *Eco*RI-*Hin*dIII fragment (Fig. 2B, fragment i) encoding genes downstream of the urdamycin PKS was ligated with a 2.5-kb *Sph*I-*Xba*I fragment (Fig. 2B, fragment e) encoding genes upstream of the urdamycin PKS and a 1.7-kb *Sph*I-*Eco*RI fragment encoding the *ermE* resistance gene (6, 54) into the temperature-sensitive vector pGM160 (33) digested with *Hin*dIII and *Xba*I. The resulting plasmid was introduced by transformation into *S. fradiae* Tü2717, and recombinant clones were selected with thiostrepton. They were transferred to plates with erythromycin and thiostrepton, and the plates were incubated at 39°C to select for integration of the plasmid. A spore suspension of the clone *S. fradiae* Tü2717/X3 (Tsr^r Ery^r at 39°C) was plated on HA medium with erythromycin and 32 single colonies were transferred on either thiostrepton or erythromycin agar plates to screen for the double crossover product. Forty percent of the clones exhibited an erythromycin-resistant and thiostrepton-sensitive phenotype. Production of urdamycin was assayed after growth in production medium (AM) supplemented with erythromycin.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL nucleotide sequence database under accession number X87093.

RESULTS

Southern analysis of *S. fradiae* Tü2717 and isolation of DNA homologous to *tcmKL*. Chromosomal DNA from *S. fradiae* Tü2717 was digested with *Pst*I, *Bam*HI, or *Bgl*II, and the fragments were separated by electrophoresis. The DNA was transferred to nylon membranes and hybridized at high stringency with a *tcmKL* PKS gene probe (1.8-kb *Bgl*II DNA fragment including *tcmK* and the N-terminal part of the *tcmL* gene; this region was formerly called *tcmI* [4]). The DNA exhibited two hybridizing fragments in each lane (for example, see Fig. 4A, lane 8). Therefore, we concluded that either *S. fradiae* Tü2717 contains two different PKSs or, less likely, the DNA fragment encoding the PKS has a restriction site for all enzymes used. We therefore constructed a library containing *S. fradiae* Tü2717 genomic DNA in cosmid pKC505 (38). The library was screened with the *tcmKL* gene probe, and two hybridizing clones were identified. Restriction mapping of the cosmid DNA revealed two nonoverlapping DNA segments (Fig. 2A). The lengths of the restriction fragments of the DNA homologous to *tcmKL* on cosmids purd2 and purd8 (Fig. 2A) are the same as those of the fragments detected in Southern blots with *S. fradiae* DNA, suggesting that this organism contains two distinct PKSs. From the incorporation pattern of 1-^{13}C- and 1,2-^{13}C-acetate it can be concluded that ketoreduction occurs at positions C-9 and C-15 of the nascent urdamycin decaketide chain (Fig. 1B), resulting in the absence of acetate-derived oxygen at C-6 and C-10 of urdamycin A and B (Fig. 1A) (40). Therefore, we expected to find at least one ketoreductase (KR) gene in the urdamycin gene cluster similar to the *act*III KR gene from the actinorhodin pathway (22). Southern blot analysis of *S. fradiae* Tü2717 chromosomal DNA with the *act*III gene probe (data not shown) and the two isolated cosmids revealed one hybridizing band in *S. fradiae* Tü2717 and one in cosmid purd8 (Fig. 2A) but no hybridizing fragment in cosmid purd2. These results suggested that cosmid purd8 carries the genes responsible for urdamycin biosynthesis.

Introduction of cosmids purd2 and purd8 in *S. lividans* TK24. To gain more evidence that cosmid purd8 carries a DNA fragment including the genes for urdamycin biosynthesis, we introduced the two *tcmKL*-hybridizing cosmids into *S. lividans* TK24 and assayed the broth for the production of polyketide compounds. The transformants were also tested on

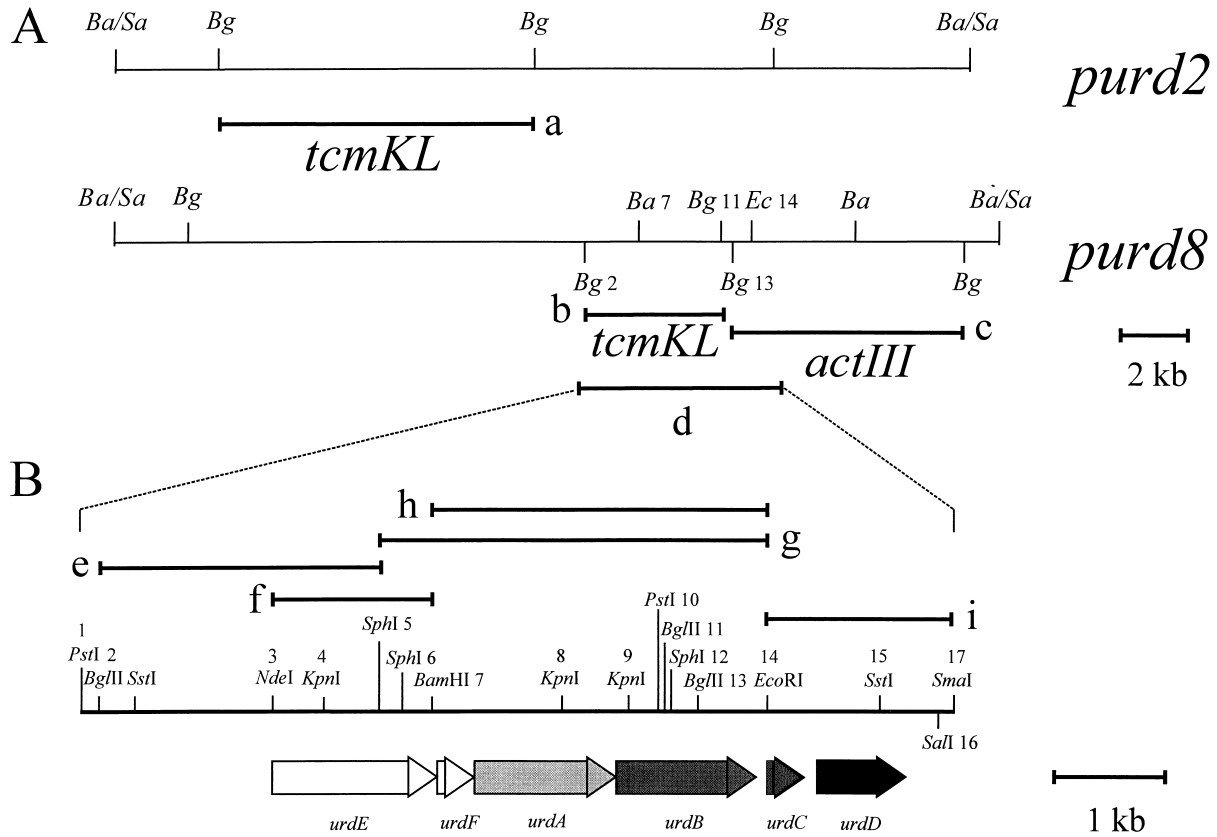

FIG. 2. (A) Restriction map of two different *tcmKL*-hybridizing DNA fragments isolated from a genomic cosmid library of *S. fradiae* Tü2717. a and b, *tcmKL*-hybridizing DNA fragments; c, *act*III-hybridizing DNA fragment; d, DNA fragment shown in panel B; *Ba*, *Bam*HI; *Ec*, *Eco*RI; *Bg*, *Bgl*II; *Sa*, *Sau*3A. (B) DNA fragment isolated from purd8 carrying genes for urdamycin biosynthesis. ORFs are indicated by boxed arrows. e and i, DNA fragments used for construction of the plasmid for the gene disruption experiment; g, fragment deleted from the *S. fradiae* Tü2717 chromosome in the gene disruption experiment; h, DNA fragment carrying *urdFAB*; f, DNA fragment carrying the oxygenase gene *urdE*. Only restriction sites of interest are shown.

gradient plates containing urdamycin A. Cosmid purd8 conferred urdamycin A resistance to *S. lividans* TK24 (30 μg/ml). Whereas *S. lividans* TK24/purd2 and *S. lividans* TK24 itself were urdamycin A sensitive, in addition, both *S. lividans* TK24/purd2 and *S. lividans* TK24/purd8 produced colored compounds at a low yield. These were not identical by TLC and HPLC analysis, using authentic samples as references, to any known metabolite of the urdamycin pathway. Because of the low yield and the complex mixture of metabolites produced, we did not attempt their isolation.

Sequence analysis of the urdamycin PKS. We sequenced the entire 5.5-kb *Bgl*II *tcmKL*-hybridizing fragment (Fig. 2B, fragment b) and also the adjacent 0.3- and 6-kb *Bgl*II fragments (Fig. 2B, fragment c). Within a 5.7-kb segment we identified six open reading frames (ORFs) (Fig. 2B and 3) with the characteristics of *Streptomyces* genes (overall G+C content, 69%; high bias toward G and C in the third codon position, using the CODON PREFERENCE program [15]). These genes are all transcribed in the same direction, and their translational start sites were predicted from the location of a start codon preceded by a potential ribosome binding site (5) and a high end-to-end similarity of their deduced proteins to known proteins from the databases. The first ORF (urdE) starts at nucleotide (nt) 22 with an ATG start codon and ends at position 1509 with a TGA stop codon, encoding a putative protein with 495 amino acids (aa) (M_r = 53,399). The next ORF (*urdF*) begins at nt 1523 with an ATG start codon, continuing to a TGA stop codon at position 1849. This ORF would encode a protein with 108 aa (M_r = 12,620). The third ORF (*urdA*) starts at nt 1846 with a GTG start codon and extends to nt 3126 with a TGA stop codon. This ORF would encode a polypeptide with 426 aa (M_r = 44,932). The fourth ORF (*urdB*) starts at nt 3123 with a GTG codon and stops at nt 4349 with a TAG codon. The deduced protein would be 408 aa long (M_r = 42,676). The 3′ end of *urdF* overlaps by 4 bp with the 5′ end of *urdA*, and the same is true for the 3′ end of *urdA* and the 5′ end of *urdB*. This suggests translational coupling of the three ORFs. The fifth ORF (*urdC*) encodes a protein of 89 aa (M_r = 9,621) and begins at nt 4457 with an ATG start codon and ends at nt 4726 with a TGA stop codon. The sixth ORF (*urdD*) starts with an ATG at nt 4899 and continues to the TGA codon at nt 5681, encoding a polypeptide of 261 aa (M_r = 27,520). *urdB* and *urdC* would be separated by a stretch of 107 nt and *urdC* and *urdD* would be separated by a stretch of 172 nt of noncoding DNA. The *urdC-urdD* intergenic region exhibits two inverted repeats that could form stem-loop structures (Fig. 3). These structures might play a role as transcriptional termination signals or mRNA stability determinants (ΔG° of −38.6 kcal/mol [ca. −162 kJ/mol] calculated for the putative stem-loop from nt 4743 to 4798; ΔG° of −31.8 kcal/mol [ca. −133 kJ/mol] calculated for the putative stem-loop from nt 4823 to 4861 [55]).

Disruption of the presumed urdamycin PKS gene cluster. To test whether we had in fact cloned genes responsible for production of urdamycin, we deleted the *tcmKL*-hybridizing region contained in purd8 from the *S. fradiae* Tü2717 chromo-

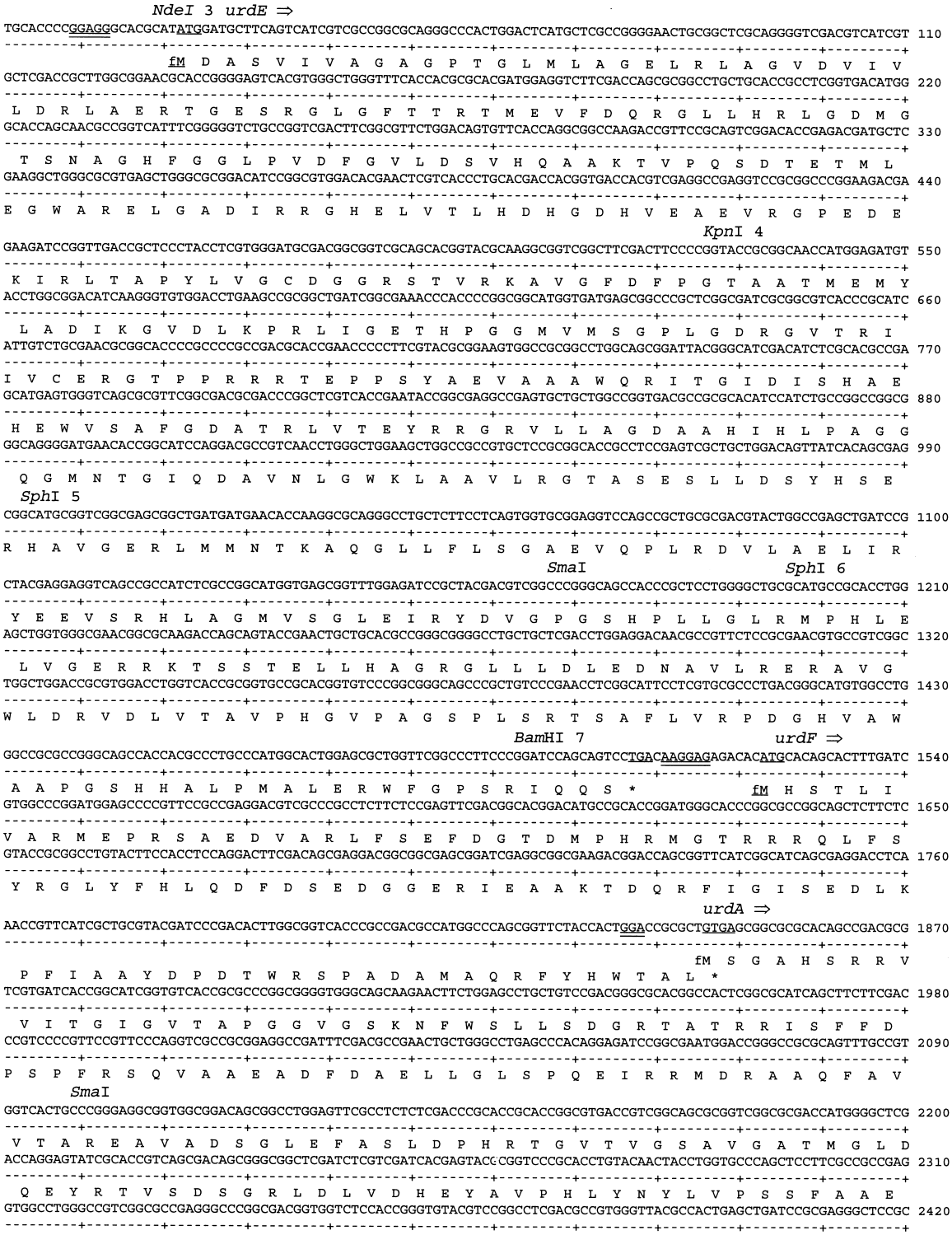

FIG. 3. Nucleotide sequence of a 5.7-kb DNA fragment carrying genes for urdamycin biosynthesis. The proposed translational start sites for *urdE*, *urdF*, *urdA*, *urdB*, *urdC*, and *urdD* and the potential ribosome binding sites are singly or doubly underlined, respectively. Stop codons are underlined and marked by asterisks. Amino acid translations are given for all gene products, and the N termini of the putative proteins are marked by open arrows. Putative stem-loop structures in the *urdC-urdD* intergenic region are underlined and indicated by arrows. Only restriction sites of interest are shown and are numbered corresponding to Fig. 2B.

some and tested the mutants for their ability to produce urdamycin. For this experiment we used the broad-host-range shuttle vector pGM160 (33), which has a temperature-sensitive replicon. A 1.7-kb DNA fragment carrying the *ermE* resistance gene (6, 54) was cloned in place of a 3.5-kb DNA fragment (Fig. 2B, fragment g), and the resulting plasmid was introduced into *S. fradiae* Tü2717 by protoplast transformation. Further manipulations were done as described in Materials and Meth-

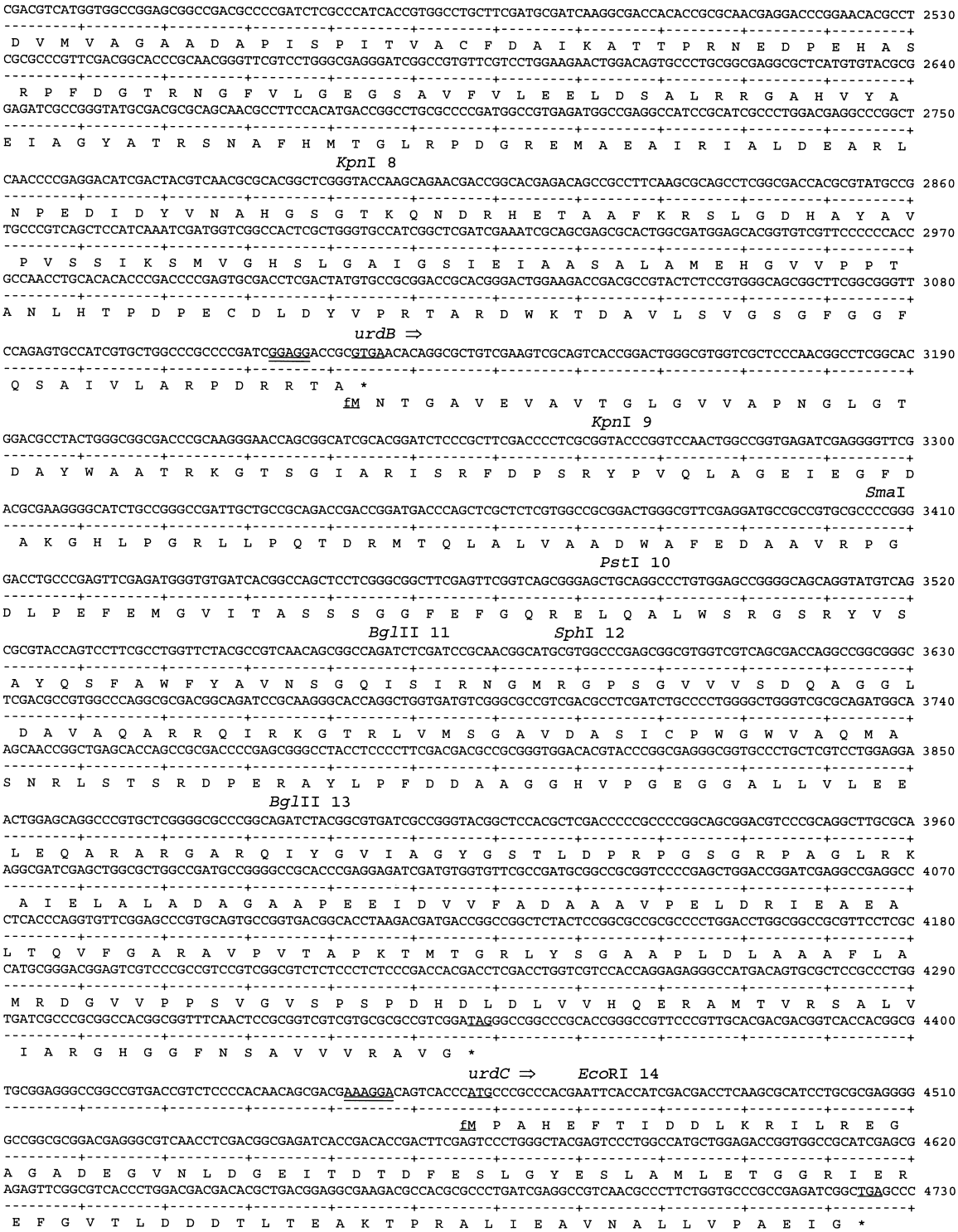

FIG. 3—*Continued.*

ods to obtain *S. fradiae* Tü2717 with a Tsr^s Ery^r phenotype. Southern analysis of five representative Tsr^s Ery^r clones and one Tsr^r Ery^r clone was done to confirm that the putative urdamycin PKS genes had been deleted from the chromosome of *S. fradiae* (Fig. 4A, lanes 3 to 7). *S. fradiae* Tü2717/X3 (Tsr^r Ery^r) still exhibited a *tcmKL*-hybridizing fragment (Fig. 4A, lane 2) 0.9 kb larger than that of the *S. fradiae* wild-type strain (Fig. 4A, lane 8). This is consistent with the integration of purd19 at the 2.5-kb *Bgl*II-*Bam*HI locus (Fig. 2B, fragment e; Fig. 4B).

In addition, we grew the *S. fradiae* Tü2717 Tsr^s Ery^r strains in urdamycin production medium for 96 h but did not detect

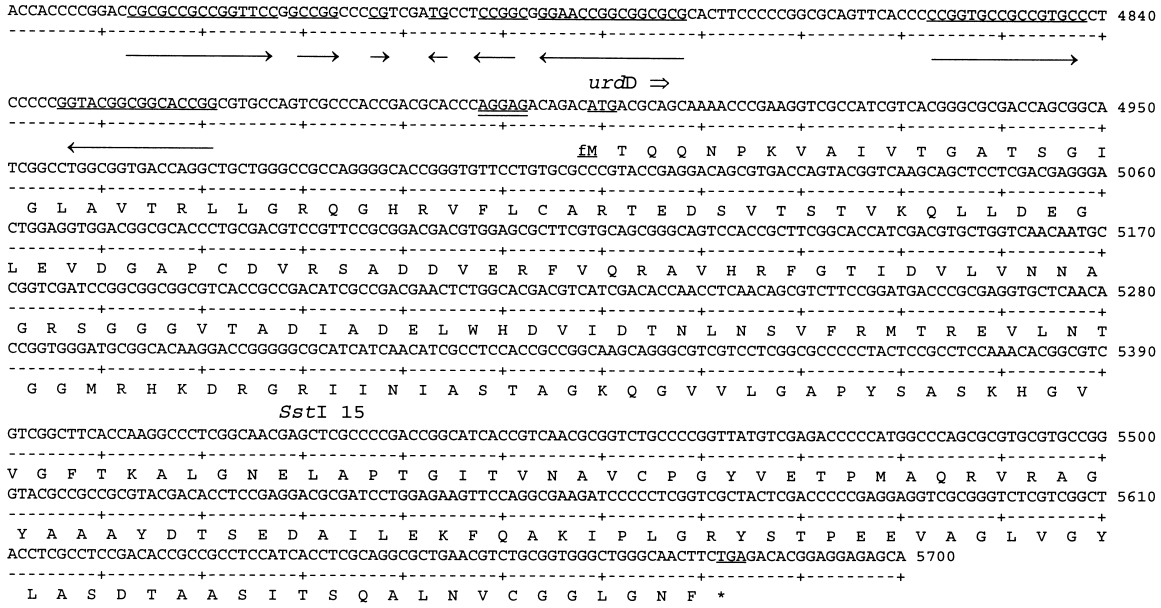

FIG. 3—*Continued.*

any urdamycin compounds by TLC and HPLC. These results clearly demonstrate the involvement of the cloned PKS genes isolated on cosmid purd8 in urdamycin biosynthesis.

Deduced functions of the proteins. The deduced amino acid sequence encoded by *urdA* exhibits high similarity to β-ketoacyl synthases from different PKS gene clusters. The highest identity was found for β-ketoacyl synthases (ORF1, 83% identity) from *Kibdelosporangium aridum* (37), which produces an unidentified polyketide-derived metabolite, and from *S. venezuelae* (ORF1, 83%), the jadomycin producer (23). Also, *urdB* clearly resembles its counterparts in other aromatic PKS gene clusters that are believed to influence the length of the polyketide chain (30). The highest identities observed were to the *S. venezuelae* (ORF2, 75%) and *K. aridum* (ORF2, 65%) proteins (23, 37). The deduced amino acid sequences of the *urdA* and *urdB* products are 36% identical, but UrdB lacks the active-site cysteine present in UrdA at position 178. The deduced *urdC* protein exhibits high similarity to acyl carrier proteins from many polyketide producers: 71% identity to the putative acyl carrier protein from *S. venezuelae* (23) and 66% to that from *S. griseus* (61). The deduced *urdD* protein has high similarity to KRs from aromatic polyketide gene clusters that are involved in reduction of the C-9 keto group of the nascent polyketide chain (Fig. 1B). The *act*III KR from the actinorhodin pathway (22) has been studied in more detail, and the function of the gene has been analyzed by complementation of mutants and by the ability of the *act*III gene to complement a 2-hydroxy-aklavinone-producing *Streptomyces galilaeus* ATCC 31671 mutant to yield aklavinone on introduction of the *act*III gene (3). In addition, the catalytic specificity of the *act*III KR was elucidated by combinatorial expression of this gene with different PKSs and subsequent structure determination of the resulting polyketide compounds (20). The putative UrdD protein shows 83% identity to the deduced KR from *S. venezuelae* (23), 83% identity to the KR from *Streptomyces griseus* (61) and 60% identity to the KR from *Streptomyces coelicolor* (22). The protein encoded by *urdE* resembles several flavin-type hydroxylases from other type II polyketide gene clusters, e.g., *S. glaucescens* TcmG (13) (30% identity) and a hydroxylase from *Streptomyces halstedii* (8) (31% identity). UrdE has two motifs common to flavin adenine dinucleotide- and NADPH-dependent enzymes (17, 59, 60). One is near the N terminus and is responsible for the binding of the ADP moiety of flavin adenine dinucleotide; the other is believed to be involved in the binding of the ribityl chain of flavin adenine dinucleotide (Fig. 5). The putative protein encoded by *urdF* has high similarity to ORFVII from the *S. coelicolor whiE* locus (9) (48% identity) and with the *S. glaucescens* TcmI protein (51) (39% identity). The TcmI protein catalyzes the cyclization of the fourth ring of Tcm F2 to yield TCM F1 (46).

Complementation analysis. McDaniel et al. (30) showed that components of PKSs of different origins can be combined functionally to produce novel PKS-derived metabolites. TCM C and urdamycin A are synthesized from 10 acetate units, but the fate of the nascent polyketide chain is quite different. The first aldol condensation occurs between C-7 and C-12 for urdamycin A and between C-9 and C-14 for TCM C (Fig. 1B) (39). Therefore, we were interested in whether introduction of the urdamycin PKS into a *tcmK* (ketoacyl synthase) and a *tcmL* (chain length factor) mutant (32, 49) would complement the *tcm* mutations to produce TCM C or result in the production of novel polyketide compounds with a folding pattern corresponding to urdamycin. We ligated a 3.0-kb DNA fragment (Fig. 2B, fragment h) carrying *urdFAB* into pWHM3 under the control of the *ermE* promoter. The resulting plasmid, purd13, was introduced into *S. glaucescens* WHM1061 (*tcmK*) and *S. glaucescens* WHM1068 (Δ*tcmL*), and the recombinant *S. glaucescens* clones were grown in R2YENG supplemented with thiostrepton. TCM C production was assayed by TLC and HPLC. Interestingly, both the *S. glaucescens tcmK* and the *tcmL* mutants were complemented by their respective urdamycin homologs and TCM C was produced, but no production of novel metabolites occurred.

Production of 6-hydroxy TCM C. We previously demonstrated that introduction of the entire *tcm* gene cluster on pWHM1026 into *S. fradiae* Tü2717 resulted in the production of the novel polyketide 6-hydroxy TCM C (11) (Fig. 1A). In fact, *S. fradiae* Tü2717/pWHM1026 produced a mixture of

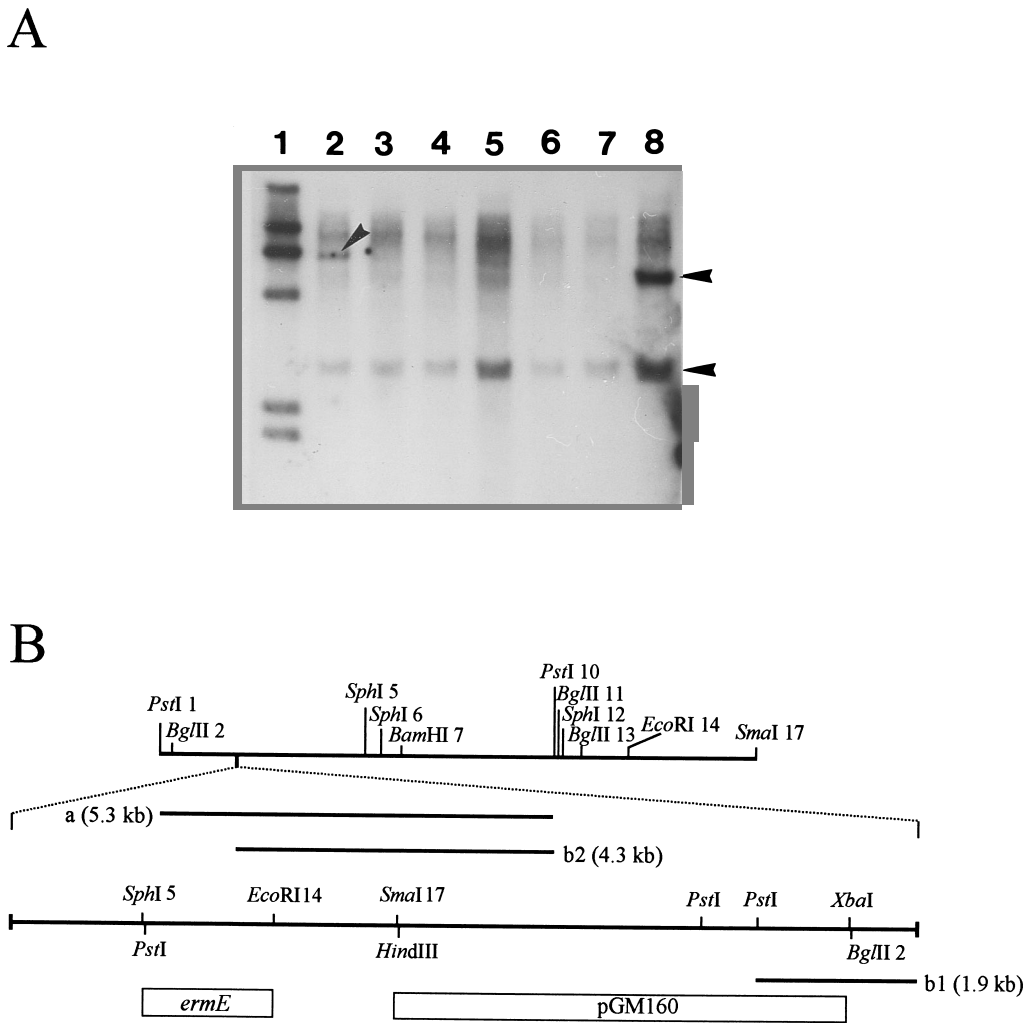

FIG. 4. (A) Southern hybridization of genomic *S. fradiae* Tü2717 DNA digested with *Pst*I using a 1.8-kb *Bgl*II fragment encoding a *tcmKL* PKS gene probe (4). Lane 1, λ-*Hin*dIII markers; lane 2, *S. fradiae* Tü2717/X3 Tsr^r Ery^r (the upper PKS-hybridizing DNA fragment is 0.9 kb larger than that of *S. fradiae* Tü2717); lanes 3 to 7, 5 different clones of *S. fradiae* Tü2717/X3 Tsr^s Ery^r (the upper PKS-hybridizing DNA fragment present in the wild type is missing); lane 8, *S. fradiae* Tü2717. Arrowheads indicate PKS-hybridizing DNA fragments in the wild-type strain and the larger fragment in *S. fradiae* Tü2717/X3 Tsr^r Ery^r. (B) Integration of purd19 into the *S. fradiae* Tü2717 chromosome between the *Bgl*II 2 and *Sph*I 5 restriction sites. The restriction sites are numbered according to Fig. 2B. a, 5.3-kb *Pst*I DNA fragment that hybridizes with the *tcmKL* PKS gene probe in *S. fradiae* Tü2717; b1 (1.9 kb) plus b2 (4.3 kb), 6.2-kb *Pst*I DNA fragment that hybridizes with the *tcmKL* gene probe in *S. fradiae* Tü2717/X3 Tsr^r Ery^r. Open boxes indicate the locations of *ermE* (1.7 kb) and plasmid pGM160 (6.2 kb).

known compounds from the urdamycin (urdamycin A, 25 mg/liter) and tetracenomycin (TCM C, 40 mg/liter) pathways and 6-hydroxy TCM C as a minor product (10 mg/liter). Incorporation of labeled 1-^{13}C- and 1,2-^{13}C-acetate and fermentation of *S. fradiae* Tü2717 under ^{18}O-labeled molecular oxygen indicate that the oxygens in positions C-12 and C-12b of urdamycin A are derived from molecular oxygen (40, 56) (Fig. 1A). Therefore, we expected that two different enzymes in the urdamycin gene cluster catalyze these oxygenation reactions. Upstream of the urdamycin PKS we identified a gene encoding the putative UrdE protein that strongly resembles enzymes involved in introducing oxygen into aromatic rings. Therefore, we concluded that this protein might catalyze one of the oxygenation reactions leading to urdamycin A and could also be responsible for the C-6 oxidation of TCM C. In order to test this hypothesis, we cloned a 1.5-kb *Nde*I-*Bam*HI fragment (Fig. 2B, fragment f) encoding the putative hydroxylase under the control of the *ermE* promoter and introduced the resulting plasmid, purd18, into *S. glaucescens* GLA.O. Transformants were isolated and subsequently cultivated in R2YENG. The cultures were harvested after 72 h, and the metabolites were extracted with ethyl acetate. TLC and HPLC analyses were performed to analyze the production of metabolites by comparison with authentic samples of TCM C, 6-hydroxy TCM C, and other TCM C biosynthetic intermediates. Interestingly, *S. glaucescens*/purd18 produced 6-hydroxy TCM C (40 mg/liter) as the main metabolite and TCM C (1 mg/liter) and intermediates of the TCM C biosynthesis only in minor amounts. The UV spectra of TCM C and 6-hydroxy TCM C and the corresponding HPLC chromatogram are shown in Fig. 6.

DISCUSSION

Southern analysis of *S. fradiae* Tü2717 genomic DNA with a PKS probe from the *tcm* cluster revealed two distinct PKS gene sets. Gene disruption of one of the clusters confirmed their involvement in the biosynthesis of urdamycin A, but the function of the other PKS-like genes remains unclear. The latter

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SCHC      1   ..MNARADRAGDTVHRVPVLVVGGSLVGLSTSVFLGRLGVRHMLVERHAG 48
TCMG      1   MPVSDRPKGCILSTEEVPVLIVGGGLTGLSAALFLSQHGVSCRLVEKHRG 50
DNRF      1   ...........MNDHEVDVLVVGAGLGGLSTAMFLARQGVRVLVVERRPG 39
TFDB      1   ..........MALTIETDVLVVGTGPAGASAGALLARYGVRTMLINKYNW 40
PCPB      1   ..MSTYPINAPGQSADAAVLIVGGGPTGLIAANELLRRGVSCRMIDRLPV 48
URDE      1   ..............MDASVIVAGAGPTGLMLAGELRLAGVDVIVLDRLAE 36
consensus                      VLVVG G  GLS A  L R GV

SCHC    303   YRAGRVFLAGDSAHEMSPTGAFGSNTGIQDAHNLAWKLAAVLGGWAGDGL 353
TCMG    323   YRSGRVFLAGDAAHVHPPAGAFGANGGIQDAHNLAWKLAAVLKGTAGDAL 373
DNRF    300   WREGRVFLAGDAAKVTPPTGGMSGNAAVADGFDLAWKLAAVLQGQAGAGL 350
TFDB    301   LQQGRVFCAGDAVHRHPPTNGLGSNTSIQDSFNLAWKIAMVLNGTADESL 351
PCPB    287   YRKGNVFLAGDAAHCHSPSGGSGMNVGMQDAFNLGWKIAMVERGEAKPDL 337
URDE    267   YRRGRVLLAGDAAHIHLPAGGQGMNTGIQDAVNLGWKLAAVLRGTASESL 317
consensus     YR GRVFLAGDAAH H P GG G N GIQDA NLAWKLAAVL G A   L
```

FIG. 5. Alignment of the putative nucleotide binding site residues of UrdE with the corresponding region from other hydroxylases by using the PILEUP program. SchC, hydroxylase from *S. halstedii* (8); TcmG, TCM C hydroxylase from *S. glaucescens* (13); DnrF, hydroxylase from the daunorubicin biosynthesis of *S. peucetius* (24); TfdB, 2,4-dichlorophenol hydroxylase from *A. eutrophus* (36); PcbB, pentachlorophenol-4-monooxygenase from a *Flavobacterium* sp. (35). The consensus sequence indicates amino acid residues present in at least four of six proteins. Highly conserved amino acids are underlined.

PKS could be responsible for the production either of a spore pigment or of a polyketide compound that was not detected under the growth conditions used for production of urdamycins. *S. coelicolor* A3(2) contains two PKSs, one for production of the blue antibiotic actinorhodin and the other for the production of a spore pigment (9, 18).

The strong resemblance of the urdamycin genes that we characterized to other type II PKS genes is very striking. The gene organization is similar to that found in *S. griseus* (61), *Streptomyces cinnamonensis* (2), *S. venezuelae* (23), and *Streptomyces roseofulvus* (7), except that we did not identify a putative cyclase/dehydrase-encoding gene downstream of the urdamycin KR (10). We sequenced 1 kb downstream of *urdD* and 2.5 kb upstream of *urdE* and identified a putative urdamycin resistance gene upstream of *urdE* and two ORFs that do not resemble any known proteins in the databases (10). The highest similarity of the polypeptides of the putative urdamycin PKS was with the PKS gene products of *S. griseus*, *S. cinnamonensis*, *S. venezuelae*, and *K. aridum* (37). The close relationship of the urdamycin PKS with the jadomycin PKS might reflect the similarity of the polyketide compounds produced by these *Streptomyces* spp., because both belong to the angucycline group of antibiotics. In contrast, griseusin is not an angucycline and the structures of the aromatic polyketide compounds produced by *K. aridum* and *S. cinnamonensis* are not yet known. CODON PREFERENCE analysis has revealed incomplete coding regions just upstream of the β-ketoacyl synthase in *S. griseus*, *S. cinnamonensis*, *S. venezuelae*, and *K. aridum*, each of which appears to encode a protein similar to UrdF, a putative cyclase. Such a gene has already been identified in the *tcm* (TcmI [51]) and *whiE* (ORFVII [9]) clusters. The function of the TcmI protein has been clarified biochemically: it catalyzes the fourth ring closure in the biosynthesis of TCM C (46). The hypothesis that UrdF catalyzes the fourth ring closure of the urdamycins, based on the resemblance of UrdF to TcmI, is appealing, but experimental data are lacking.

The folding patterns of the TCMs, anthracyclines, and angucyclines are all different (Fig. 1B), and it is not fully understood how the linear polyketide is folded and cyclized correctly to yield a distinct product (39). Since the folding pattern is likely to influence the regiochemistry of the subsequent intramolecular aldol condensations, the fact that the urdamycin PKS genes *urdAB* complement mutations in the corresponding genes in the TCM C producer, *S. glaucescens*, favors the hypothesis that the regiospecificity of the first cyclization of the linear polyketide is not determined by the enzymes UrdA,

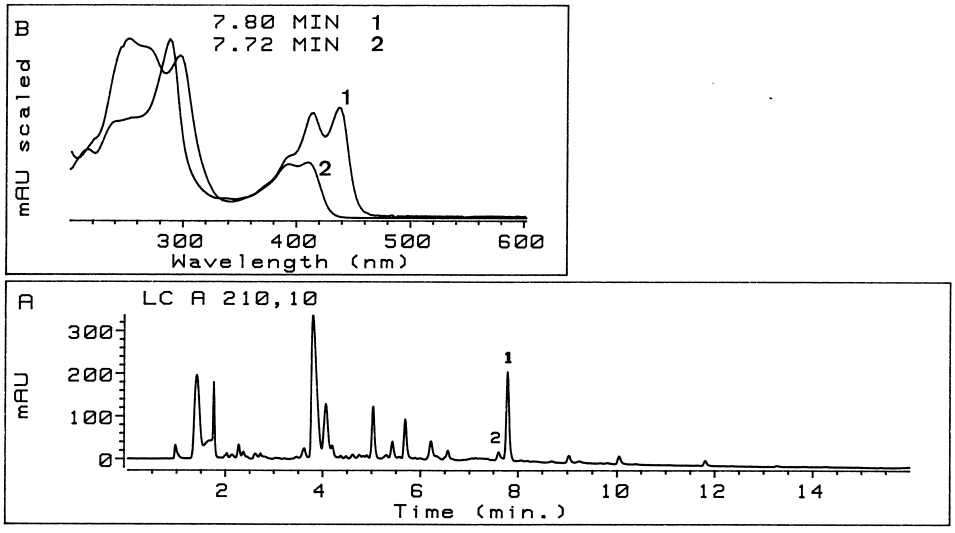

FIG. 6. (A) HPLC elution profile of a sample from a culture of *S. glaucescens*/purd18 recorded at 210 nm. Peak 1, 6-hydroxy TCM C; peak 2, TCM C. (B) Overlaid UV spectra of peak 1 (retention time, 7.80 min) and peak 2 (retention time, 7.72). mAU, milli-absorbance units.

UrdB, and UrdC presumed to form the linear, acyclic poly-β-ketone. Since the first cyclization occurs at C-9 and C-14 in the case of TCM C and at C-7 and C-12 in urdamycin (Fig. 1B), we propose that specific enzymes, e.g., cyclases present in the *tcm* cluster, catalyze this reaction and that the minimal urdamycin PKS (29, 30) consisting of UrdA, UrdB, and UrdC proteins is responsible for the assembly of the polyketide chain from 10 acetate units. This idea is consistent with the work of Shen et al. (47) and McDaniel et al. (31), who proposed recently that TcmN, a multifunctional cyclase-*O*-methyl-transferase from the *tcm* cluster, controls the regiospecificity of the first cyclization of the nascent decaketide. McDaniel et al. (29, 30) have suggested that the minimal PKS controls the regiospecificity of the first ring cyclization but reported the inability of the minimal *tcm* PKS to accurately control this reaction (29). Thus, the intrinsic cyclization pattern determined by the urdamycin minimal PKS may be overwritten by an enzyme present in the *tcm* cluster, most likely TcmN (31, 47). In addition, we speculate that the angucycline structure of the urdamycins is determined not by the minimal PKS itself but by a specific enzyme of the *urd* gene cluster or by the interaction of this enzyme with the urdamycin minimal PKS. Sequence analysis of additional *urd* genes may eventually reveal such a protein.

The absence of oxygen at positions C-6 and C-10 of urdamycin A and B is consistent with the identification of a KR in the *urd* gene cluster (Fig. 1A), and further sequence analysis of cosmid purd8 may reveal another KR. The KR identified downstream of the PKS could catalyze the reduction of either the carbonyl at C-6 or at C-10 (Fig. 1A) in the biosynthetic intermediates or, less likely, both carbonyls. Because of the high sequence similarity to KRs from other PKS gene clusters, we favor the idea that *urdD* catalyzes the ketoreduction at C-10 of urdamycins A and B (Fig. 1A), but this hypothesis has to be confirmed by gene disruption experiments.

The presence of an oxygenase gene, *urdE*, in the urdamycin gene cluster is consistent with the origin of two oxygens at C-12 and C-12b in urdamycin A from molecular oxygen (56). We propose that the *urdE* gene product is involved in the oxygenation at C-12b and not in the oxygenation at C-12 for several reasons. The *urdE* gene has considerable sequence resemblance to an enzyme that introduces hydroxy groups in TCM C (*tcmG* [13]) and to enzymes in a *Flavobacterium* sp. (35) or *Alcaligenes eutrophus* (36) involved in hydroxylation of halogenated aromatic rings. In addition, *urdE* resembles both *dnrF* from *Streptomyces peucetius* subsp. *caesius* (24) and *rdmE* from *Streptomyces purpurascens* (34), which are proposed to be an aklavinone 11-hydroxylase that oxidizes an aromatic ring at the position corresponding to C-6 of TCM C. In contrast, *urdE* does not exhibit significant sequence similarity to the *tcmH* gene (51), which encodes a monooxygenase of the TCM C biosynthesis that catalyzes the conversion of the naphthacene intermediate TCM F1 to the naphthacenequinone TCM D3 (45). TcmH is a small polypeptide (M_r = 12,700) in comparison to the UrdE protein (M_r = 53,399). Anthrones have been postulated as intermediates in the biosynthesis of anthraquinones, tetracyclines, and the angucylines (45). Therefore, we expect that a *tcmH*-homologous gene in the urdamycin gene cluster is responsible for oxygenation at position 12, converting the hypothetical urdamycin anthrone to an anthraquinone.

Introduction of the entire *tcm* cluster into *S. fradiae* Tü2717 resulted in the production of the hybrid antibiotic 6-hydroxy TCM C (11). We had postulated that an enzyme of urdamycin biosynthesis catalyzed this reaction. Sequence analysis has now revealed *urdE* as a hydroxylase gene-like gene. If its product is indeed responsible for introducing the angular oxygen at C-12b, the substrate specificity of UrdE would be relaxed, because we have shown that introduction of the *urdE* gene into *S. glaucescens* under the control of the *ermE* promoter results in the exclusive production of 6-hydroxy TCM C. Gene disruption experiments will be needed to clarify the participation of *urdE* in the biosynthesis of urdamycin A. Preliminary experiments have shown that 6-hydroxy TCM C has antibacterial activity against TCM C-sensitive gram-positive bacteria (10, 58). Because of the ease in producing 6-hydroxy TCM C in larger quantities by using the recombinant strain *S. glaucescens*/purd18, this antibacterial compound can now be evaluated for its antimicrobial and antitumor activity. In addition, the conversion of TCM C to 6-hydroxy TCM C in *S. glaucescens*/purd18 further supports the feasibility of this approach to produce hybrid antibiotics by genetic engineering.

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