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Minimal *Streptomyces* sp. Strain C5 Daunorubicin Polyketide Biosynthesis Genes Required for Aklanonic Acid Biosynthesis

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The structure of the *Streptomyces* sp. strain C5 daunorubicin type II polyketide synthase (PKS) gene region is different from that of other known type II PKS gene clusters. Directly downstream of the genes encoding ketoacylsynthase α and β (KS_α, KS_β) are two genes (*dpsC*, *dpsD*) encoding proteins of unproven function, both absent from other type II PKS gene clusters. Also in contrast to other type II PKS clusters, the gene encoding the acyl carrier protein (ACP), *dpsG*, is located about 6.8 kbp upstream of the genes encoding the daunorubicin KS_α and KS_β. In this work, we demonstrate that the minimal genes required to produce aklanonic acid in heterologous hosts are *dpsG* (ACP), *dauI* (regulatory activator), *dpsA* (KS_α), *dpsB* (KS_β), *dpsF* (aromatase), *dpsE* (polyketide reductase), and *dauG* (putative deoxyaklanonic acid oxygenase). The two unusual open reading frames, *dpsC* (KASIII homolog lacking a known active site) and *dpsD* (acyltransferase homolog), are not required to synthesize aklanonic acid. Additionally, replacement of *dpsD* or *dpsCD* in *Streptomyces* sp. strain C5 with a neomycin resistance gene (*aphI*) results in mutant strains that still produced anthracyclines.

Daunorubicin (daunomycin) and doxorubicin (adriamycin), clinically important anthracycline chemotherapeutic agents produced by *Streptomyces* sp. strain C5 and *Streptomyces peucetius* ATCC 29050, are synthesized by condensation of nine extender units derived from malonyl coenzyme A (CoA) onto a propionyl moiety to make a theoretical C_{21} polyketide intermediate (16, 33–35) which is reduced at C-9 (34), cyclized, and aromatized to form aklanonic acid (Fig. 1), the first characterized chromophore in these pathways (9, 16, 33–35). Aklanonic acid is converted in four enzymatic steps to ε-rhodomycinone (1, 4, 33–35), which is then glycosylated and modified by a series of reactions to form daunorubicin and doxorubicin (6, 16, 33, 35).

The structures of the *Streptomyces* sp. strain C5 (41) and *S. peucetius* ATCC 29050 (12) daunorubicin type II polyketide synthase (PKS) gene regions differ significantly from those of most other known type II PKS gene clusters (15, 18, 20, 35) (Fig. 1). From a divergently transcribed promoter region reading left are *dpsE* and *dpsF*, encoding polyketide reductase and aromatase, respectively. Reading right from this sequence are five genes (*dauG*, *dpsA*, *dpsB*, *dpsC*, and *dpsD*) encoding, respectively, putative deoxyaklanonic acid oxygenase, ketoacylsynthase (KS_α), ketoacylsynthase homolog (KS_β; described elsewhere as chain length factor; 26), an *Escherichia coli* KASIII homolog lacking an active site, and an acyltransferase (41). The acyltransferase encoded by *dpsD* has been speculated to function as propionyl-SCoA:acyl carrier protein (ACP)-SH acyltransferase (41). The gene encoding ACP, *dpsG*, is located about 6.8 kbp upstream of the genes encoding the daunorubicin KS_α and KS_β (8, 12, 41). Because the daunorubicin/doxorubicin PKS gene cluster of *Streptomyces* sp. strain C5 (41) is virtually identical with that of *Streptomyces peucetius* ATCC 29050 (12) (DNA identity over this region is 93%; 28, 35), the PKS gene names proposed by Grimm et al. (12) have been adopted for the *Streptomyces* sp. strain C5 homologs (33, 35). Similarly, all of the daunorubicin/doxorubicin biosynthesis gene names of *S. peucetius* and *Streptomyces* sp. strain C5 now have been standardized (35). In this work, we describe the minimal gene requirements for aklanonic acid biosynthesis in heterologous hosts and determine the results of disruptions to the unusual genes *dpsC* and *dpsD* in the *Streptomyces* sp. strain C5 chromosome.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *Streptomyces* sp. strain C5, originally obtained from the Frederick Cancer Research Center, has been described in detail elsewhere (1). *Streptomyces lividans* TK24 (14) was obtained from D. A. Hopwood, and *Streptomyces coelicolor* CH999 (26), in which much of the actinorhodin biosynthesis gene cluster has been deleted, was obtained from C. Khosla. Recombinant *S. lividans* TK24 strains were grown in YEME medium (14) containing 1 μg of neomycin and/or 5 μg of thiostrepton per ml as required. Recombinant *S. coelicolor* CH999 strains were grown on plates of solid R2YE medium (14) containing 10 μg of neomycin and/or 50 μg of thiostrepton per ml as required. *Streptomyces* sp. strain C5 and mutants derived from it were grown in nitrate-defined plus yeast extract (NDYE) medium as described previously (1, 4). If required for selection, neomycin was added at a concentration of 1 μg/ml. All strains were routinely maintained on R2YE solid medium. When required for selective pressure, neomycin and/or thiostrepton was added to R2YE medium at 10 or 50 μg/ml, respectively.

E. coli JM83, used to propagate plasmids, was grown in LB medium (31). Plasmids were introduced into *E. coli* by transformation, using standard procedures (31). Ampicillin was added at a concentration of 100 μg/ml to cultures of *E. coli* harboring pUC19 (40), pWHM3 (39), or derivatives made from them.

General genetic manipulations. Procedures for protoplast formation, transformation, and regeneration of protoplasts for *Streptomyces* sp. strain C5 and mutants derived from it have been described elsewhere (23). *S. lividans* was transformed with plasmid DNA as described by Hopwood et al. (14). Plasmids were routinely prepared according to methods described by Carter and Milton (3). Digestion of DNA with restriction endonucleases was carried out according to the manufacturers' directions. Restriction mapping and other routine molecular methods used in this work were as described by Sambrook et al. (31). Plasmids used and constructed in this work are given in Table 1.

For probes derived from plasmids, the DNA inserts were removed from the plasmid DNA by using the appropriate restriction endonucleases and purified from gel slices using published methods (31). The inserts were then labeled by the ^{32}P-random primer procedure (10), using 50 μCi of [α-^{32}P]dCTP per μg of DNA and random primers (Stratagene, La Jolla, Calif.).

Streptomyces sp. strain C5 chromosomal DNA was extracted and purified through $CsCl_2$ density gradients as described by Hopwood et al. (14). DNA hybridizations were carried out by transferring $CsCl_2$-purified DNA to be assayed to BA-85 nitrocellulose filters (Schleicher and Schuell, Inc., Keene, N.H.), using the Southern blot method (31). Hybridizations were carried out as described by Hopwood et al. (14) using SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer (31).

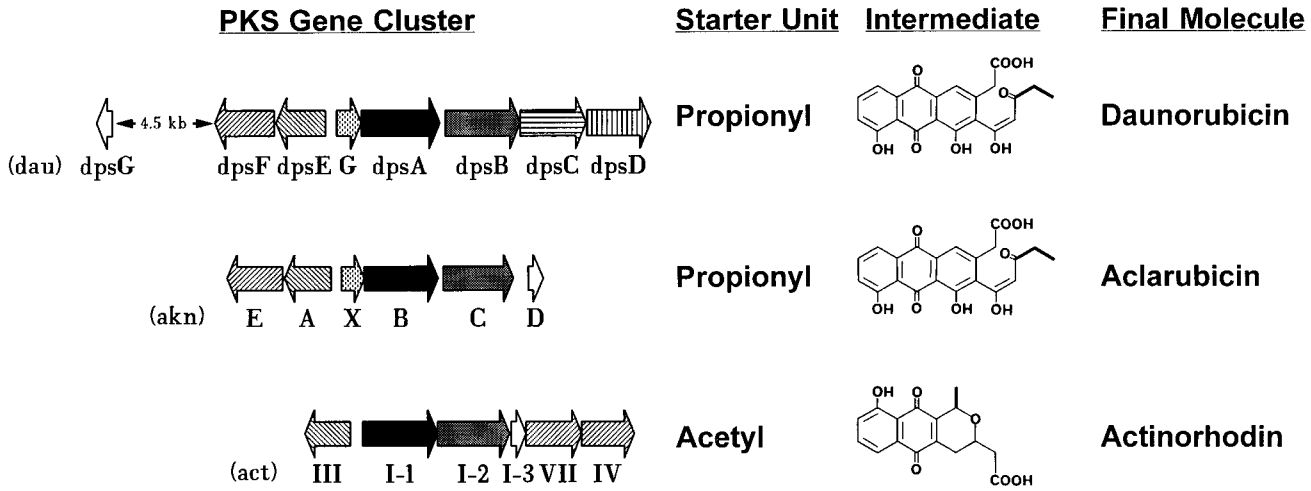

FIG. 1. Model showing PKS gene organization, starter moiety, and an example intermediate for daunorubicin (aklanonic acid; 16, 33), aclarubicin (aklanonic acid; 33), and actinorhodin (dihydrokalafungin; 11) biosynthesis. The genes encoding the following functions are indicated: KS_α, solid arrows; KS_β, shaded arrows; ACP, open arrows; polyketide reductase, arrows with left-slanted lines; cyclases/aromatases, arrows with right-slanted lines; deoxyaklanonic acid oxygenase, dotted arrows; fatty acid KASIII homolog lacking an apparent active site, arrow with horizontal lines; acyl transferase, arrow with vertical lines. The positions of the starter unit moieties within the intermediates are indicated by heavy lines.

Gene disruption methods. For gene replacement experiments, the 3.75-kbp *Bcl*I-*Eco*RI fragment containing the 3′ end of *dpsE* as well as the entire lengths of genes *dpsC* and *dpsD* was subcloned from pANT121 (41) into *Bam*HI-*Eco*RI-digested pUC19 (destroying the *Bam*HI and *Bcl*I sites). The resultant plasmid, pANT716, was the starting point for construction of all subsequent replacement vectors used in this work.

To construct the plasmid in which *dpsD* was replaced with *aphI*, encoding neomycin phosphotransferase from pIJ61 (38), the 616-bp *Bam*HI-*Kpn*I fragment of pANT716 was replaced with a 1.27-kbp *Eco*RI-*Kpn*I fragment containing *aphI* from pKK840 (22). For this experiment, pANT716 and pKK840 were digested with *Bam*HI and *Eco*RI, respectively, and those sites were then end filled using Klenow fragment (31). The plasmids were then digested with *Kpn*I, and the fragments, each containing a blunt end and a *Kpn*I site, were ligated using T4 DNA ligase (31) to generate pANT726. This plasmid, in which the *aphI* gene was flanked to the left and right with 2.05- and 1.08-kbp fragments of homologous *Streptomyces* sp. strain C5 DNA, respectively, was used to transform protoplasts of *Streptomyces* sp. strain C5 as previously described (23). After 24 h, the regenerated protoplasts were challenged with 10 μg of neomycin per ml, and neomycin-resistant transformants were picked over to fresh plates after 7 days.

For replacement of *dpsC* and *dpsD*, the 1,385-bp *Sst*I-*Kpn*I fragment of pANT716, encompassing the 3′ half of *dpsC* and the 5′ half of *dpsD*, was replaced with the 1.27-kbp *Eco*RI-*Kpn*I fragment containing *aphI* from pKK840 (22). In this case, pANT716 and pKK840 were digested with *Sst*I and *Eco*RI, respectively, and those sites were then end filled using Klenow fragment (31). The plasmids were then digested with *Kpn*I, and the fragments, each containing a blunt end and a *Kpn*I site, as described above, were ligated with T4 DNA ligase (31) to generate pANT740. This plasmid, in which the *aphI* gene was flanked to the left and right with 1.28- and 1.08-kbp fragments of homologous *Streptomyces* sp. strain C5 DNA, respectively, was used to transform protoplasts of *Streptomyces* sp. strain C5 as described above.

Detection of anthracyclines and aklanonic acid. *Streptomyces* sp. strain C5 and mutant strains C5-VR2 (*dpsD* null mutant), C5-VR5, C5-VR6, and C5-VR7 (*dpsCD* null mutants) were grown for 6 days at 30°C in 50 ml of NDYE medium (1 μg of neomycin per ml was added to the cultures of the mutant strains) in 250-ml Erlenmeyer flasks with springs for dispersal of the mycelia. At 6 days, the whole broth from each culture was adjusted to pH 8.5 with 5 N NaOH and then extracted with an equal volume of chloroform:methanol (9:1). The anthracyclines in the extracts were reduced to dryness, reconstituted in 100 μl of methanol, and separated on silica gel thin-layer chromatography (TLC) plates as previously described (7), with chloroform:heptane:methanol (10:10:3) as the liquid phase, or by reversed-phase high-pressure liquid chromatography (HPLC), with a solvent system of methanol:water:acetic acid (65:30:5), as previously described (6).

For detection of aklanonic acid from recombinant cultures of *S. lividans* TK24, the strains were grown for 4 days at 30°C in 50 ml of YEME medium as described above. For extraction, the pH of each culture broth was adjusted to 1.5 with 6 N HCl, and the acidified broths were extracted with an equal volume of chloroform, reduced to dryness, and reconstituted in 100 μl of chloroform. Aklanonic acid produced by the recombinant strains was compared with authentic aklanonic acid obtained from K. Eckardt (9) in parallel and by cochromatography, using TLC as described previously (4). Aklanonic acid also was analyzed by HPLC as previously described (4, 7), using a C_{18} reverse-phase column and a solvent system of methanol:water:glacial acetic acid (65:30:5) (4).

For detection of aklanonic acid produced by recombinant *S. coelicolor* CH999 strains, the strains were grown for 7 days on R2YE medium containing 10 and/or 50 μg of neomycin and thiostrepton per ml, respectively, as required for plasmid selection. The agar containing the recombinant *S. coelicolor* CH999 lawns was removed from the plates, mashed, and extracted with 10 ml of methanolic HCl (12 N HCl:methanol; 1:1) per plate. The acidic extracts were filtered through cheesecloth and then extracted with an equal volume of chloroform. The organic layer was removed, dried, reconstituted in 50 μl of chloroform, and analyzed as described above.

Radiolabeling of aklanonic acid. Fifty-milliliter cultures of recombinant *S. lividans* TK24(pWHM3) (negative control) and *S. lividans* TK24(pANT785) were grown for 36 h at 30°C in YEME medium plus 1 μg of thiostrepton per ml. Then 5 μCi of filter-sterilized [1-^{14}C]propionic acid–sodium salt (84 mCi/mmol) was added to each culture, and the cultures were incubated for an additional 48 h with the added label. At the end of incubation, the cultures were extracted, and the radiolabeled aklanonic acid was separated by TLC as described above and analyzed by autoradiography as previously described (7).

Enzymatic conversion of aklanonic acid. Authenticity of the aklanonic acid produced by recombinant strains was also confirmed by conversion of the product in the presence of *S*-adenosyl-L-methionine to aklanonic acid-methyl ester by extracts of *S. lividans* TK24(pANT154), using the methods described by Dickens et al. (7). The products were analyzed by TLC and HPLC (7).

MS analysis. For mass spectrometry (MS) analysis, *S. lividans* TK24 (pANT785) was grown at 30°C for 48 h in 50 ml of YEME medium in 250-ml Erlenmeyer flasks with springs, after which the pH of the whole culture broth was adjusted to 1.5, and the acidified solution was extracted with an equal volume of chloroform. The extracted aklanonic acid was separated from contaminants on TLC plates as described above, scraped from the plates as described previously (6), and extracted again with acidified chloroform. The purified aklanonic acid in chloroform was washed five times with double-distilled water and then dried. The mass spectra were acquired on a Micromass Quattro II (Altrincham) equipped with an atmospheric pressure chemical ionization (APCI) source operating in the positive-ion mode.

RESULTS AND DISCUSSION

PKS gene structure. The gene organization for the daunorubicin PKS gene cluster of *Streptomyces* sp. strain C5 is not typical of other streptomycete type II PKS gene clusters, in which the ACP gene usually is located immediately downstream of the genes for KS_α and its homolog (KS_β) (15, 18, 20) (Fig. 1). It has been hypothesized that this novel PKS gene arrangement in the daunorubicin biosynthesis gene clusters is correlated with the requirement of a propionyl moiety as the

TABLE 1. Bacterial plasmids used and constructed in this study[a]

Plasmid	Relevant characteristics	Reference
pUC19	2.686 kbp; Amp^r; *E. coli* cloning vector	40
pKK840	4.1 kbp; pIJ2925 (19) containing 1.4-kbp PCR-amplified *aphI* gene from pIJ61	22
pWHM3	7.20 kbp; *E. coli-Streptomyces* shuttle vector constructed from pIJ486 and pUC19; HC; Thio^r	39
pANT841	2.746 kbp; pUC19 containing a polylinker with additional cloning sites optimized for cloning DNA with high G+C content; blue-white selectable; Amp^r	5
pANT121	10.8 kbp[b]; pUC19 containing 8.1-kbp *Eco*RI DNA insert from *Streptomyces* sp. strain C5 DNA in phage P7	41
pANT152	10.36 kbp[b]; 7.67-kbp *Kpn*I fragment from phage P7 in pUC19	41
pANT235	9.2 kbp[b]; pUC19 containing a 6.48-kbp *Bam*HI-*Bgl*II DNA fragment from *Streptomyces* sp. strain C5 carrying the *dnrI*-hybridizing region	41
pANT716	6.41 kbp; pUC19 with 3.74-kbp *BclI* fragment from pANT121 ligated into the *Bam*HI-*Eco*RI sites	This work
pANT726	7.09 kbp; pUC19 with *dpsBC*; *dpsD* replaced by *aphI* cassette	This work
pANT740	6.26 kbp; pUC19 with *dpsB*, partial *dpsCD* with *aphI* cassette	This work
pANT749	7.38 kbp; 4.65-kbp *Kpn*I-*Eco*RI fragment from pANT152 in pUC19	This work
pANT750	12.77 kbp; 4.89-kbp *Kpn*I-*Hin*dIII fragment from pANT235 into pANT749	This work
pANT751	16.74 kbp; 9.54-kbp *Eco*RI-*Hin*dIII fragment from pANT750 into pWHM3 (contains intact *dauI*, *doxA*, *dauV*, *dauU*, *dpsG*, *dauZ*, *dnmT*, and *dauH*)	This work
pANT752	6.45 kbp; 3.74-kbp *Bcl*I-*Eco*RI fragment cloned into *Bam*HI-*Eco*RI-digested pANT841	This work
pANT753	5.83 kbp; 600-bp *Bam*HI-*Kpn*I fragment deleted from pANT752	This work
pANT754	5.06 kbp; 1.36-kbp *Sst*I-*Kpn*I fragment deleted from pANT752	This work
pANT755	10.15 kbp; 3.13-kbp *Bcl*I-*Eco*RI fragment from pANT753 ligated to 4.33-kbp *Eco*RI-*Bcl*I fragment from pANT121 and *Eco*RI-digested pUC19 (reconstructs all PKS genes except *dpsD*)	This work
pANT756	9.38 kbp; 2.3-kbp *Bcl*I-*Eco*RI fragment from pANT754 ligated to 4.33-kbp *Eco*RI-*Bcl*I fragment from pANT121 and *Eco*RI-digested pUC19 (reconstructs all PKS genes except *dpsCD*)	This work
pANT765	7.2 kbp; *Eco*RI and T4 DNA polymerase-blunted *Kpn*I fragment from pKK840 into *Eco*RI and Klenow end-filled *Cla*I of pWHM3; HC; Neo^r	This work
pANT767	15.23 kbp; 8.08-kbp *Eco*RI fragment from pANT121 into pANT765 (contains all daunorubicin PKS genes of pANT121; 41)	This work
pANT770	10.78 kbp; 1.6-kbp *Bam*HI fragment from pANT152 into pANT235 (reconstitutes *dauH* gene)	This work
pANT771	15.29 kbp; 8.09-kbp *Eco*RI-*Hin*dIII fragment from pANT770 into pWHM3	This work
pANT776	3.17 kbp; 442-bp *Xho*I-*Bcl*I fragment from pANT235 into pANT841 (gives intact *dauG* gene)	This work
pANT777	5.37 kbp; 2.62-kbp *Sal*I fragment from pANT776 into pANT841 (yields intact *dauG* and *dauI* genes)	This work
pANT778	5.33 kbp; 1.36-kbp *Bam*HI-*Bcl*I fragment deleted from pANT777 (leaves intact *dauI* activator gene)	This work
pANT779	9.78 kbp; 2.73-kbp *Eco*RI-*Hin*dIII fragment from pANT777 into pWHM3	This work
pANT780	9.85 kbp; 1.37-kbp *Eco*RI-*Hin*dIII fragment from pANT778 into pWHM3	This work
pANT781	7.50 kbp; 540-bp *Eco*RI-*Hin*dIII fragment from pANT776 into pWHM3	This work
pANT782	17.96 kbp; 8.08-kbp *Eco*RI fragment from pANT121 into pANT779	This work
pANT783	15.77 kbp; 8.08-kbp *Eco*RI fragment from pANT121 into pANT781	This work
pANT784	17.93 kbp; 8.08-kbp *Eco*RI fragment from pANT121 into pANT780	This work
pANT785	16.58 kbp; 6.69-kbp *Eco*RI fragment from pANT755 into pANT779	This work
pANT786	16.54 kbp; 6.69-kbp *Eco*RI fragment from pANT755 into pANT780	This work
pANT787	14.39 kbp; 6.69-kbp *Eco*RI fragment from pANT755 into pANT781	This work
pANT788	17.35 kbp; 7.46-kbp *Eco*RI fragment from pANT754 into pANT779	This work
pANT790	3.53 kbp; 817-bp *Nco*I-*Bam*HI fragment from pANT235 into pANT841	This work
pANT791	9.10 kbp; 642-bp *Hin*dIII-*Eco*47III fragment from pANT790 into *Hin*dIII- and *Hpa*I-digested pANT797	This work
pANT795	7.10 kbp; *Eco*RI-digested pANT765 end filled with Klenow fragment and religated; HC; Neo^r	This work
pANT796	5.34 kbp; 1,331-bp *Kpn*I-*Mlu*I fragment from pANT849 (5, 6) into pANT841 (5)	This work
pANT797	8.43 kbp; 1,337-bp *Nru*I fragment and T4 DNA polymerase-blunted *Kpn*I fragment into *Pvu*II-digested pANT795; *E. coli-Streptomyces* shuttle vector; expression of genes cloned into polylinker is driven by SnpR-activated *snpA* promoter (5, 6); HC; Neo^r	This work

[a] Abbreviations: HC, high-copy-number plasmid; Thio^r, thiostrepton resistance; Amp^r, ampicillin resistance; Neo^r, neomycin resistance.
[b] The sizes of this plasmid and its insert have been revised slightly from those reported by Ye et al. (41).

chain initiator unit (12, 27, 41) and that the two unusual genes, *dpsC* and *dpsD* (12, 41), are required for initiation of the C_{21} polyketide chain. The structure of the *Streptomyces galilaeus* PKS gene cluster, encoding assembly of aclacinomycin A (aclarubicin), an anthracycline also initiated with a propionyl moiety, has been reported to have an ACP gene in the location analogous to that occupied by *dpsCD* (18). This brought to light the question of the requirement of *dpsC*, which encodes a homolog of *E. coli* fatty acid KS III (KASIII) but lacks an obvious active site (12, 41), and *dpsD*, which encodes an acyltransferase homolog of as yet unproven function (12, 41).

Gene replacement. A *dpsD* null mutant was constructed by transforming protoplasts of *Streptomyces* sp. strain C5 with pANT726, a suicide vector in which *dpsD* was replaced with *aphI*. Southern hybridization of several neomycin-resistant transformants, using the integrated DNA as a probe, revealed the presence of both single and double crossovers; a double crossover in which *aphI* replaced *dpsD* in the genome is shown in Fig. 2. This mutant strain, *Streptomyces* sp. strain C5-VR2, was found to produce in NDYE medium copious quantities of various anthracyclines and anthracyclinones similar to those produced by the parental strain. This result was not totally unexpected, however, since Grimm et al. (12) had previously found *S. peucetius dpsD* to be dispensable in aklanonic acid formation in a heterologous strain.

Null mutants of *dpsCD* in *Streptomyces* sp. strain C5 were then constructed by replacement of a 1,385-bp *Sst*I-*Kpn*I DNA fragment, spanning most of both *dpsC* and *dpsD*, with *aphI*.

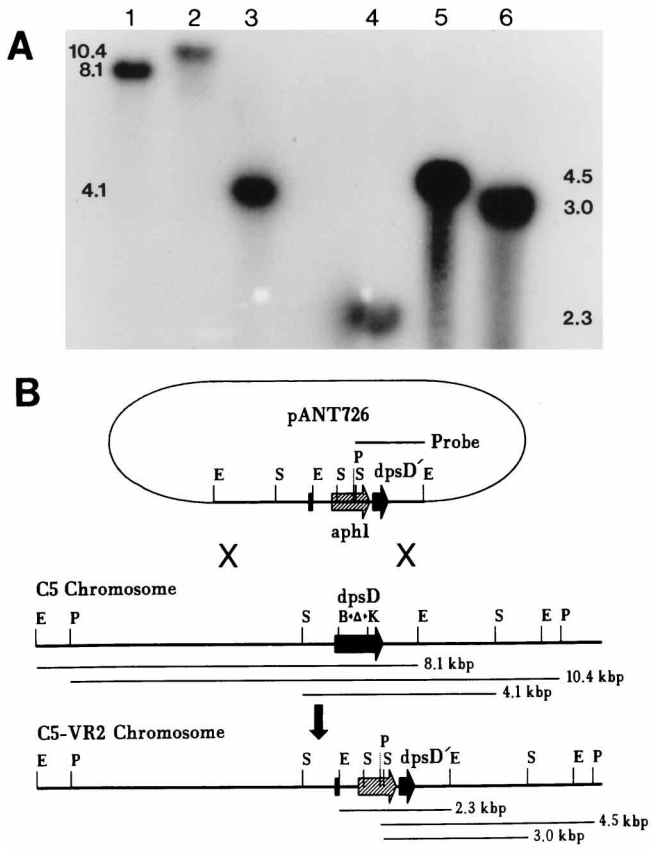

FIG. 2. (A) Southern blot of genomic DNA of strains C5 (parental strain; lanes 1 to 3) and C5-VR2 (mutant; lanes 4 to 6) digested with *Eco*RI, *Pst*I, and *Sst*I, respectively, showing replacement of *dpsD* in the chromosome of *Streptomyces* sp. strain C5 with *aphI*. The *Pst*I-*Eco*RI fragment shown in panel B was used as the probe. (B) Restriction maps of the plasmid, parental chromosome, and mutant chromosome, showing the expected results obtained by the double crossover.

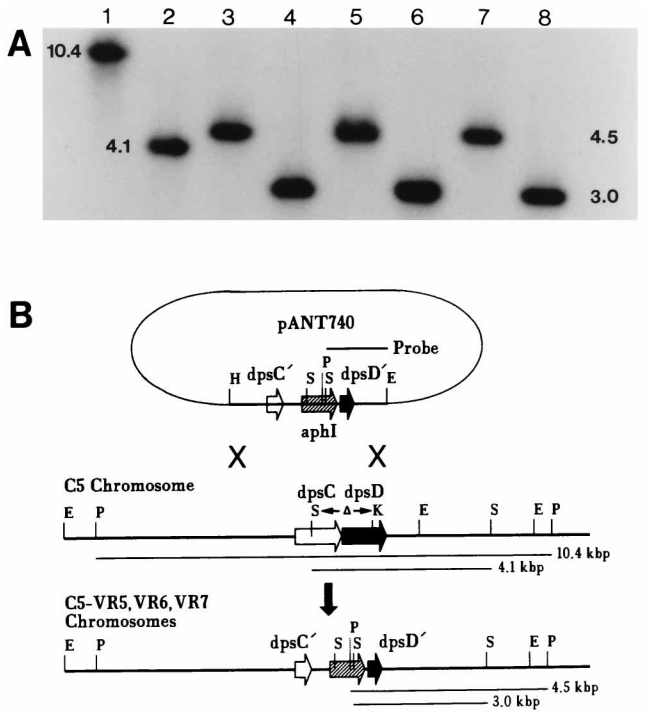

FIG. 3. (A) Southern blot of genomic DNAs from strains C5 (parental strain; lanes 1 and 2) and mutant strains C5-VR5 (lanes 3 and 4), C5-VR6 (lanes 5 and 6), and C5-VR7 (lanes 7 and 8) digested with *Pst*I and *Sst*I, respectively, showing replacement of *dpsC* and *dpsD* in the chromosome of *Streptomyces* sp. strain C5 with *aphI*. The *Pst*I-*Eco*RI fragment shown in panel B was used as a probe. (B) Restriction maps of the plasmid, parental chromosome, and mutant chromosomes, showing the expected results obtained by the double crossover.

Transformation of *Streptomyces* sp. strain C5 protoplasts with this plasmid resulted in recovery of several stable neomycin-resistant transformants. Southern hybridization, using the integrated DNA as a probe, confirmed several strains (C5-VR5, C5-VR6, C5-VR7) in which double-crossover events had occurred; Fig. 3 shows the results for three strains in which expected double crossovers occurred, resulting in replacement of *dpsC* and *dpsD* in the genome with *aphI*. These *dpsCD* mutants also were confirmed by Southern hybridization with the homologously integrating suicide plasmid ampicillin resistance marker, which was present in all single crossovers and absent in all double crossovers (28). All three *dpsCD* null mutant strains of *Streptomyces* sp. strain C5 produced copious quantities of anthracyclines as determined by TLC and HPLC (28). Particularly interesting, however, was the fact that ε-rhodomycinone, the major product found in fermentations of parental-type *Streptomyces* sp. strain C5, was virtually absent in fermentations of C5-VR5, C5-VR6, and C5-VR7; instead, the major products were several glycones, including daunorubicin, 13-dihydrodaunorubicin, and feudomycin C (28).

Heterologous biosynthesis of aklanonic acid by minimal PKS. In parallel with the gene disruption experiments described above, we tested daunorubicin biosynthesis PKS genes for their ability to synthesize aklanonic acid in heterologous strains, using an approach similar to that used by Grimm et al. (12).

Our first experiments showed that pANT767, containing the *Streptomyces* sp. strain daunorubicin PKS gene cluster but not including *dpsG* (encoding ACP), did not confer aklanonic acid biosynthesis on *S. lividans* TK24. This result confirms a similar experiment by Ye et al. (41), in which *S. lividans* TK24 (pANT122) (pANT122 contains the same insert as pANT767) did not produce aklanonic acid as possibly expected. Cotransformation of either *S. lividans* TK24 (14) or *S. coelicolor* CH999 (26) with pANT767 (conferring neomycin resistance) and pANT751 (thiostrepton resistance) (the latter plasmid contains eight intact genes, of which one is *dpsG* and another is *dauI* [6], a gene highly homologous to *dnrI*, a transcriptional activator gene for daunorubicin biosynthesis in *S. peucetius* [36]) resulted in the biosynthesis of a compound with the TLC and HPLC characteristics of aklanonic acid (4). This indicated that certain daunorubicin biosynthesis genes in pANT751 were apparently required in addition to the PKS genes in pANT767 to confer the capacity for biosynthesis of the putative aklanonic acid. Similarly, *S. lividans* TK24(pANT767:pANT771) produced a compound chromatographically identical to authentic aklanonic acid (4).

Using the preliminary results shown by Grimm et al. (12), we then constructed a set of single plasmids (pANT782-pANT788; Table 1) in which DNA fragments containing intact *dpsG* (encoding ACP) and *dauI* (transcriptional activator) were subcloned from pANT751 and incorporated into pANT767 and derivatives were made from it. A bright yellow compound chromatographically identical to authentic aklanonic acid (Ta-

TABLE 2. Characteristics of products of daunorubicin PKS genes in heterologous hosts

Sample	Results of:		
	TLC (R_f)[a]	HPLC (RT in min)[b]	APCI-MS (M + 1)[c]
S. lividans TK24(pANT785) product	0.44	6.55	335.16
Authentic aklanonic acid	0.44	6.55	335.16

[a] Solvent system was heptane:$CHCl_3$:CH_3OH (50:50:25). Compounds were viewed under UV light with a wavelength of 260 nm.
[b] RT, retention time. Solvent system was methanol:water:acetic acid (65:30:5).
[c] Peaks resulting from mass spectral analysis using APCI-MS as described in Materials and Methods. M + 1 indicates a mass of 334 for the decarboxy-anhydro-aklanonic acid.

ble 2) was produced in large quantities by *S. lividans* TK24(pANT785), which contains only *dpsG* (ACP), *dauI* (regulatory activator), *dpsA* (KS_α), *dpsB* (KS_β), *dpsE* (polyketide reductase), *dpsF* (aromatase), and *dauG* (deoxyaklanonic acid oxygenase) (Fig. 4). This experiment confirmed that the two unusual open reading frames, *dpsC* and *dpsD*, were not required to synthesize the yellow product, nor did their inclusion increase overall product formation in the heterologous hosts. As mentioned previously, Grimm et al. (12) found that *S. peucetius dpsD* was not required for aklanonic acid biosynthesis in *S. lividans.*

Identity of the product. When the bright yellow compound produced by *S. lividans* TK24(pANT785) was isolated from TLC plates and incubated in vitro with mycelial extracts containing aklanonic acid methyltransferase (encoded by *dauC*) by procedures we have described previously (7), it was converted to aklanonic acid methyl ester (28), indicating that it was biologically active aklanonic acid. Similarly, when incubated with [1-^{14}C]propionate, cultures of *S. lividans* TK24(pANT785) produced a radioactive compound that cochromatographed on TLC with authentic aklanonic acid (28), indicating that the putative aklanonic acid product incorporated propionate as expected (Fig. 1). Finally, purified compound from culture broths of *S. lividans* TK24(pANT785) that was subjected to APCI-MS analysis gave an M + 1 of 335.16 (Table 2), which is identical to that obtained with authentic aklanonic acid obtained from K. Eckardt. This corresponds to the expected M + 1 for decarboxy-anhydroaklanonic acid (M_r, 334), which is similar to the chemical breakdown products observed in mass spectral analyses of aklanonic acid by Grimm et al. (12). Eckardt et al. (9) also had stated that they were unable to obtain a true M + 1 for aklanonic acid, probably because of its instability upon ionization.

Are DauZ and DpsG or their homologs required for efficient aklanonic acid biosynthesis? Homologs of *dauZ*, a gene described as "*orf2*" that is divergently transcribed from *dpsG* (Fig. 4) (8), has been found in the PKS gene clusters of *Streptomyces roseofulvus* (frenolicin biosynthesis; 2), *Streptomyces argillaceus* (mithramycin biosynthesis; 25), and *Saccharopolyspora hirsuta* (spore pigment biosynthesis; 24), suggesting that *dauZ* might play a role in polyketide synthesis and assembly (17). A *dauZ* homolog, *actVI-orfA* (11), also has been found in the actinorhodin biosynthesis pathway and is presumed to be present in both *S. lividans* TK24 and *S. coelicolor* CH999. Thus, we tested the effect of overproduction of *dauZ* on the formation of aklanonic acid in *S. lividans* TK24 and *S. coelicolor* CH999. Cultures of *S. lividans* TK24(pANT782/pANT791) and *S. coelicolor* CH999(pANT782/pANT791) contained the minimal genes required for aklanonic acid biosynthesis on one plasmid (Fig. 4) and *dauZ*, expressed from the SnpR-activated *snpA* promoter (5, 6), on the other plasmid. Both of these cultures produced aklanonic acid but in quantities not significantly different from those produced in these strains by pANT782 alone

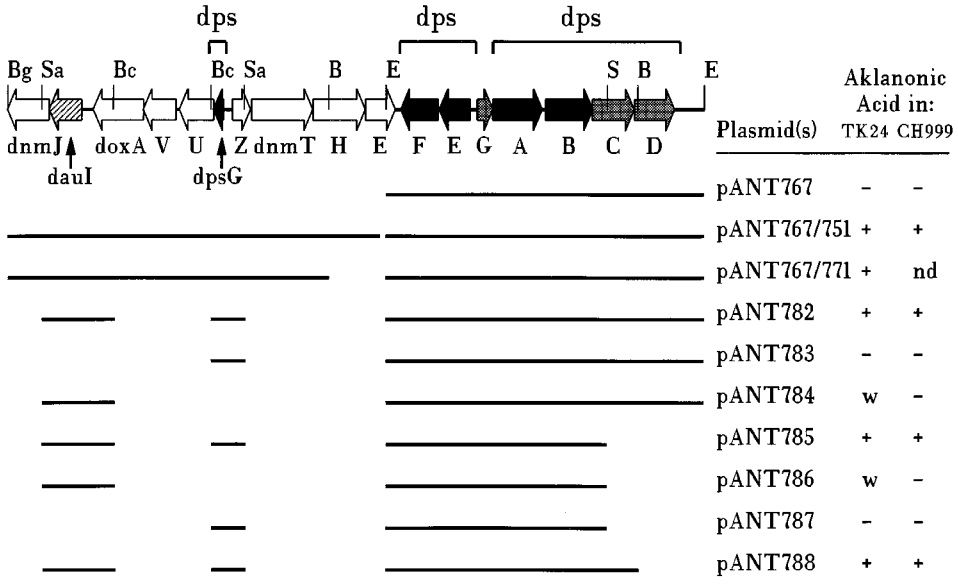

FIG. 4. Restriction map, generated by complete nucleotide sequence data (6, 8, 41), of part of the daunorubicin biosynthesis gene cluster from *Streptomyces* sp. strain C5. The genes depicted by black arrows are the minimal biosynthesis genes required for aklanonic acid biosynthesis in heterologous strains as described in the text. The transcriptional activator gene, *dauI*, is indicated by slanted lines. The daunorubicin PKS biosynthesis genes, *dpsC* and *dpsD*, proven in this work not to be required for anthracycline biosynthesis, are indicated by the shaded arrows. The inserts in plasmids conferring or unable to confer aklanonic acid biosynthesis on *S. lividans* TK24 and *S. coelicolor* CH999 are shown below. These inserts are present in the plasmids in the same orientation that is shown in this figure, and the genes are transcribed from their natural promoters. Complete details for construction of the plasmids are given in Table 1. A bar indicating 2.0 kbp is given. Abbreviations: B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; S, *Sst*I; Sa, *Sal*I; +, aklanonic acid produced in substantial quantities; −, aklanonic acid not produced; w, only traces of aklanonic acid produced; nd, not done.

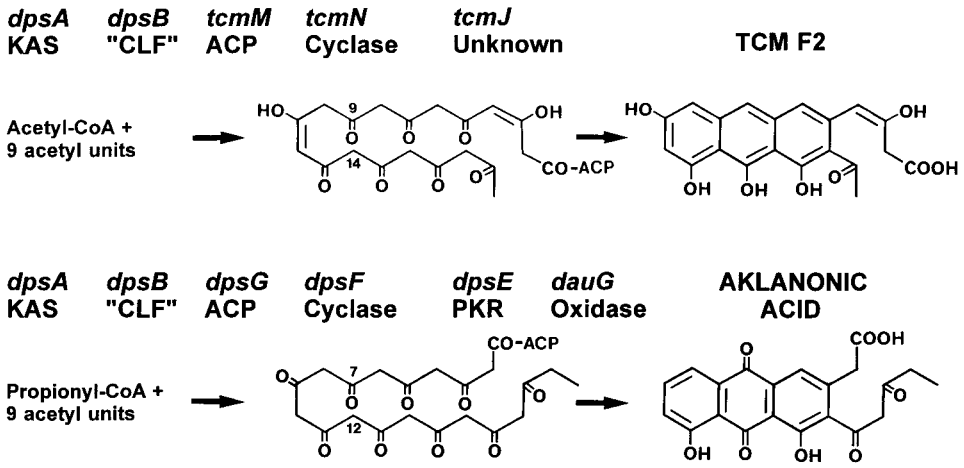

FIG. 5. Biosynthesis of tetracenomycin F2 by *dpsABtcmMNJ* (27), in which the starter unit is acetyl-CoA and the first linkage is a C-9–C-12 linkage, compared to biosynthesis of aklanonic acid by *dpsABFGdauG* (this work), in which propionyl-CoA is the starter unit and the first linkage made is C-7–C-12 (34, 35).

(28). While this does not prove the requirement or lack thereof for *dauZ* or its homologs in PKS function, it does indicate that overproduction of DauZ has no significant impact on product formation.

S. lividans TK24 transformed with either pANT784 or pANT786 also made minute quantities of aklanonic acid, but *S. coelicolor* CH999 transformed with the same plasmids did not. These results suggest that the actinorhodin biosynthesis ACP in *S. lividans* TK24 may substitute, albeit weakly, for the daunorubicin ACP. ACPs from several different polyketide biosynthesis gene clusters appear to be functionally interchangeable (21, 26). While the small amounts of aklanonic acid produced by *S. lividans* TK24(pANT784) may reflect the copy number difference between the recombinant daunorubicin PKS genes and the host actinorhodin gene encoding ACP, we have also observed that recombinant actinorhodin ACP at a high copy number also did not substitute efficiently for DpsG in aklanonic acid biosynthesis (28). Similarly, Hutchinson and his colleagues recently found that *tcmM*, encoding the ACP for tetracenomycin biosynthesis, did not substitute for *dpsG* with constructs containing *S. peucetius dpsABEFdnrI* (*dpsG* or *tcmM*) to produce aklanonic acid in a heterologous system (17).

Mechanistic and evolutionary implications. Meurer and Hutchinson (27) recently carried out experiments in which *S. peucetius dpsA* and *dpsB* were expressed together with *tcmMNJ* (encoding, respectively, ACP, aromatase, and a protein of unknown function from tetracenomycin C biosynthesis; 27) to synthesize tetracenomycin F2, a compound that is initiated with an acetyl moiety (Fig. 5). This result led them to speculate that *dpsA* and *dpsB* did not contain the information necessary to dictate starter unit specificity (27). They suggested that *dpsC* and *dpsD* together were the primary contributing factors for starter unit specificity (27).

Our results show that *Streptomyces* sp. strain C5 *dpsC* and *dpsD* are not required to specify a propionyl moiety as the starter unit in vivo in either *S. lividans* TK24 or *S. coelicolor* CH999 (Fig. 5). In light of the results shown by Meurer and Hutchinson (27), which indicate that DpsA and DpsB alone do not specify the propionyl starter unit, we propose that the productive protein-protein interactions of multiple proteins within the homologous PKS, including KS_α, KS_β (described elsewhere as chain length factor; 26), "cyclase/aromatase," and polyketide reductase together specify the propionyl starter unit. If this is the case, then it suggests that caution should be observed when interpreting the results of "mix and match" experiments with PKS components (18, 26, 27), since nonhomologous interactions may lead to unexpected results.

We have shown by both gene replacement approaches and heterologous expression that the products of *dpsC* and *dpsD* are not required for anthracycline biosynthesis in *Streptomyces* sp. strain C5. Thus, the information required to confer priming of the daunorubicin polyketide with a propionyl moiety does not lie within these two proteins, as originally hypothesized (12, 27, 41). In recent experiments, however, we determined that the *Streptomyces* sp. strain C5 *dpsCD* mutants C5-VR5, C5-VR6, and C5-VR7 produce methyl side chain anthracyclines initiated with an acetyl moiety as well as C_2 side chain anthracyclines. Similarly, recent preliminary data indicate that *S. lividans* TK24(pANT785), containing *dpsABEFGdauGI* (lacking *dpsCD*), produces not only aklanonic acid but also a methyl side chain analog of aklanonic acid (28, 35). Thus, while *dpsCD* are clearly not required for incorporation of propionyl-CoA as the starter unit, their presence may ensure that the propionyl moiety will be used to initiate polyketide biosynthesis.

Although *dpsD* was previously hypothesized to encode a propionyl-SCoA:ACP acyltransferase, the deduced proteins of *dpsD* from both *Streptomyces* sp. strain C5 (41) and *S. peucetius* ATCC 29050 (12) have sequences that are slightly more related to sequences of enzymes that carry out acetyl-SCoA:ACP acyltransferase reactions than to those that are proposed to carry out propionyl-SCoA:ACP acyltransferase reactions (13). Thus, until the product of *dpsD* is studied biochemically, its actual function in daunorubicin biosynthesis remains unclear.

As mentioned previously, the PKS gene cluster in *S. galilaeus* that encodes aclacinomycin A, an anthracycline also primed with a propionyl moiety, is more analogous to the PKS gene clusters for actinorhodin biosynthesis and other aromatic polyketides initiated with acetyl moieties (18) (Fig. 1). In all of these type II PKS biosynthesis gene clusters, the genes encoding KS_α, KS_β, and ACP are linked in apparent operons (15, 18, 20). It is not known if homologs of *dpsC* or *dpsD* are present in the aclacinomycin A PKS gene cluster of *S. galilaeus* (17).

Alternatively, it is conceivable that the functions of these proteins are utilized when they are present, but alternative enzymes, present in *Streptomyces* sp. strain C5, *S. lividans* TK24, and *S. coelicolor* CH999, may contribute the required activities when these proteins are absent. For example, *S. coeli-*

color was recently shown to possess a KASIII homolog, encoded by *fabH* (29, 30), which might provide the function of *dpsC*. Considering that *S. coelicolor fabH* and *Streptomyces* sp. strain C5 *dpsC* contain only 23% sequence identity (28), however, this is probably not the case. Similarly, malonyl-CoA acyltransferase required for fatty acid biosynthesis, and probably also used in polyketide biosynthesis in *S. coelicolor* and *S. glaucescens* (30, 37), may provide the function of the *dpsD* gene product. Finally, as mentioned previously, the product of resident *actVI-orfA* may provide the function for the *dauZ* product, if this gene were required. Just as with other heterologous PKS expression results (26, 27), these types of questions will be best answered by reconstitution of minimal PKS systems in vitro with purified enzymes.

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