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Isolation and Sequence Analysis of Polyketide Synthase Genes from the Daunomycin-Producing *Streptomyces* sp. Strain C5

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A contiguous region of about 30 kbp of DNA putatively encoding reactions in daunomycin biosynthesis was isolated from *Streptomyces* sp. strain C5 DNA. The DNA sequence of an 8.1-kbp *Eco*RI fragment, which hybridized with *actI* polyketide synthase (PKS) and *actIII* polyketide reductase (PKR) gene probes, was determined, revealing seven complete open reading frames (ORFs), two in one cluster and five in a divergently transcribed cluster. The former two genes are likely to encode PKR and a bifunctional cyclase/dehydrase. The five latter genes encode: (i) a homolog of TcmH, an oxygenase of the tetracenomycin biosynthesis pathway; (ii) a PKS Orf1 homolog; (iii) a PKS Orf2 homolog (chain length factor); (iv) a product having moderate sequence identity with *Escherichia coli* β-ketoacyl acyl carrier protein synthase III but lacking the conserved active site; and (v) a protein highly similar to several acyltransferases. The DNA within the 8.1-kbp *Eco*RI fragment restored daunomycin production to two *dauA* non-daunomycin-producing mutants of *Streptomyces* sp. strain C5 and restored wild-type antibiotic production to *Streptomyces coelicolor* B40 (*actVII*; nonfunctional cyclase/dehydrase), and to *S. coelicolor* B41 (*actIII*) and *Streptomyces galilaeus* ATCC 31671, strains defective in PKR activity.

Daunomycin (daunorubicin; Fig. 1), doxorubicin (formerly called adriamycin), and aclacinomycin A, commercially important anthracycline anticancer agents, are polyketides produced from a propionyl starter unit and nine malonyl extender units (43). Previous data from our laboratory on the interfunctionality of anthracycline polyketide synthase (PKS) and actinorhodin PKS components suggest that daunomycin PKS is a type II enzyme complex (5, 44). Type II PKS systems are composed of small, unifunctional or bifunctional enzymes (23, 25). The products of type II PKSs are typically aromatic polyketides which are synthesized by using acetyl or propionyl starter units and only a malonyl moiety for extension (23). The sequences of at least eight *Streptomyces* type II PKS gene clusters have been published previously (23, 25). Only in the cases of the tetracenomycin (40) and actinorhodin (5, 19, 34, 55) PKSs, however, have biochemical characterizations also been carried out. Thus, most of the information on type II PKSs is derived from the strong conservation of gene structure among the PKS genes (23, 25) and cross-functionality of the components (5, 26, 34, 41). We describe here the structure of a gene region from *Streptomyces* sp. strain C5 that putatively encodes daunomycin biosynthesis and show that it has a significantly different overall structure from other type II PKS gene regions.

MATERIALS AND METHODS

Bacterial strains and media used. *Streptomyces* sp. strain C5 and mutants derived from it have been described previously (3, 4). *Streptomyces lividans* TK24 (22), used as a recombinant host strain, was obtained from D. A. Hopwood. *Streptomyces coelicolor* mutants, described by Rudd and Hopwood (37), were obtained from H. G. Floss. *Streptomyces galilaeus* ATCC 31671, which lacks a functional polyketide reductase (PKR), has been described previously (5, 50).

For liquid cultures, *S. lividans* and *S. galilaeus* were grown in YEME medium (22) supplemented with 20% (wt/vol) sucrose, and *Streptomyces* sp. strain C5-derived strains were grown in NDYE medium as described previously (10). Streptomycete strains carrying pIJ486 (51) or derivatives of it were grown on

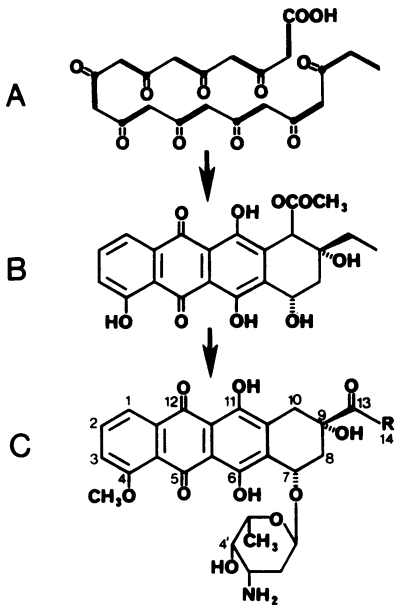

FIG. 1. The aglycone component of daunomycin is synthesized through a theoretical polyketide intermediate (A) from a propionyl starter molecule and nine C_2 units derived from malonyl moieties (43). The intermediate, ε-rhodomycinone (B), is also accumulated by cultures of *Streptomyces* sp. strain C5. The final product, daunomycin (C) (R, $=CH_3$), contains the 2,3,6-trideoxy-4-aminohexose, daunosamine, derived from TDP-D-glucose (48), attached at C-7 of the aglycone. Doxorubicin (R, $=CH_2OH$), produced by *S. peucetius* subsp. *caesius*, is a related anthracycline. References 4, 43, and 44 detail the proposed pathway for daunomycin biosynthesis in *Streptomyces* sp. strain C5.

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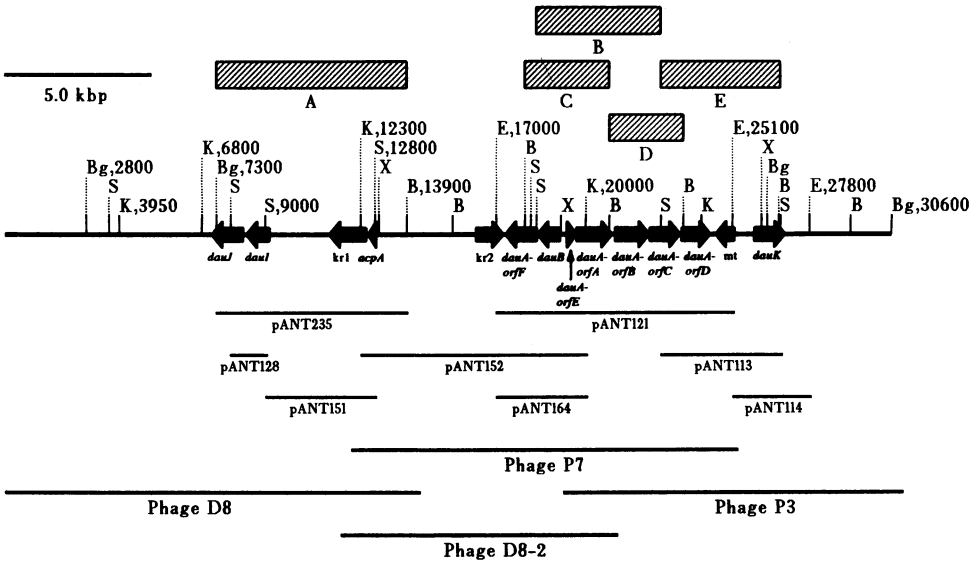

FIG. 2. Restriction map of part of the daunomycin biosynthesis gene cluster from *Streptomyces* sp. strain C5. The DNA sequence of the 8.1-kbp *EcoRI* fragment spanning the region from E (nucleotide 17000) to E (nucleotide 25100) is described in detail in this paper. The hatched boxes represent fragments to which probes hybridized as follows: a 6.6-kbp *Bam*HI-*Bgl*II fragment of phage D8 to which the *S. peucetius dnrI-dnrJ* probe hybridized (A); a 4.3-kbp *Sst*I fragment of phage P7 to which the *actIII* (PKR) and *actI* (PKS) probes hybridized (B); 2.9- and 2.6-kbp *Bam*HI fragments (C and D), respectively, of phage P7 to which the *actI* probe hybridized (*actIII* hybridized to only fragment C); and a 4.1-kbp *Sst*I fragment of phage P3 to which the methyltransferase oligonucleotide probe (MT probe) hybridized (E). The sequences of *dauA-orfA* (PKS Orf1), *dauA-orfB* (PKS Orf2), *dauA-orfC* (putative daunomycin PKS Orf3), *dauA-orfD* (putative propionyl-CoA:acyl-carrier-protein acyltransferacylase), *dauA-orfE* (*tcmH* homolog; putative aklanonic acid-anthraquinol oxygenase), *dauA-orfF* (*actVII* homolog; cyclase/dehydrase), and *dauB* (*actIII* homolog; PKR) are described in Results and Discussion. The sequences and analyses of the genes encoding acyl carrier protein (*acpA*), carminomycin methyltransferase (*dauK*), and *dauI* and *dauJ* (*dnrI* and *dnrJ* homologs) will be described elsewhere (14, 15, 53). The ORFs marked kr1 and kr2 represent genes encoding ketoreductases other than *dauB* found in this region as mentioned in the text, and mt represents the ORF encoding a methyltransferase-like gene downstream of *dauA-orfD*. The inserts of plasmids pANT113, pANT114, pANT121, pANT128, pANT151, pANT152, pANT164, and pANT235 and phages D8 (14.4-kbp insert), D8-2 (9.5-kbp insert), P7 (13.5-kbp insert), and P3 (12.0-kbp insert) are indicated below the map. Abbreviations for restriction endonuclease sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; S, *Sst*I; X, *Xho*I.

plates of solid R2YE medium (22) containing 40 μg of thiostrepton per ml.

Escherichia coli JM83, used to propagate plasmids for sequencing and restriction analyses, was grown in Luria-Bertani medium (33). Plasmids were introduced into *E. coli* by standard transformation procedures (33). Other *E. coli* strains used in this study are described with their particular applications. Ampicillin was added at a concentration of 100 μg/ml to cultures of *E. coli* harboring pUC19 or derivatives made from it.

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 (30). *S. lividans* was transformed with plasmid DNA as described by Hopwood et al. (22). Procedures used for the preparation of *Streptomyces* plasmid and chromosomal DNAs were described by Hopwood et al. (22). Digestion of DNA with restriction endonucleases was carried out according to the manufacturer's directions. Restriction mapping and other routine molecular methods used in this work were performed as described by Maniatis et al. (33).

Library construction, phage isolation, and screening. A complete genomic library of *Streptomyces* sp. strain C5 DNA was constructed in lambda EMBL3 (20). The streptomycete DNA was partially digested with *Bam*HI, size selected to a range of 9 to 20 kbp on agarose gels, eluted from the gels, and ligated into the phage vector with T4 DNA ligase. The recombinant phages were packaged in vitro and used to infect *E. coli* LE392 (Pharmacia). Phage DNA was prepared from *E. coli* by procedures described by Maniatis et al. (33).

The phage library of *Streptomyces* sp. strain C5 DNA was screened for hybridization with either linear DNA fragments containing genes or with degenerate, high-G+C-biased oligonucleotides constructed for DNA sequences expected to be clustered with the daunomycin PKS biosynthesis genes. For probes derived from plasmids, the DNA inserts were removed from the plasmid DNA by using the appropriate restriction endonucleases and isolated by the phenol-freeze-fracture method (24). The inserts were then labelled by the ^{32}P random primer procedure (18) with 50 μCi of [α-^{32}P]dCTP per μg of DNA and random primers (Stratagene, La Jolla, Calif.). Alternatively, degenerate, consensus G+C-biased oligonucleotides were end labelled with 50 μCi of [γ-^{32}P]ATP (>7,000 Ci/mmol; ICN Biochemicals) and DNA kinase (33).

Colony hybridizations were carried out by transferring well-separated *E. coli* colonies (approximately 300 per plate) containing the phage library to BA-85 nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) by the Southern blot method (33). Hybridizations were carried out as described by Hopwood et al. (22), with SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer for DNA fragments and SSPE (0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA [pH 7.4]) buffer for oligonucleotides.

DNA sequencing. Plasmids containing inserts to be sequenced were isolated by the methods of Kraft et al. (28). The DNA was sequenced in both directions by the dideoxynucleoside chain termination method (38) with Sequenase version 2.0 (United States Biochemical Corp., Cleveland, Ohio), double-stranded templates, and labelling with [α-thio-^{35}S]dCTP (1,000 to 1,500 Ci/mmol; Dupont-New England Nuclear, Boston,

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

Phage or plasmid	Relevant characteristic(s)	Source or reference
Phages		
EMBL3	Derivative of lambda	Pharmacia
D8	EMBL3 containing ca. 14.4-kbp insert of *Streptomyces* sp. strain C5 DNA containing daunomycin biosynthesis genes	This work
D8-2	EMBL3 containing ca. 9.5-kbp insert of *Streptomyces* sp. strain C5 DNA containing daunomycin biosynthesis genes	This work
P3	EMBL3 containing ca. 12.0-kbp insert of *Streptomyces* sp. strain C5 DNA containing daunomycin biosynthesis genes	This work
P7	EMBL3 containing ca. 13.5-kbp insert of *Streptomyces* sp. strain C5 DNA containing daunomycin biosynthesis genes	This work
Plasmids		
pUC19	2.686 kbp; Amp^r, *E. coli* plasmid	J. N. Reeve
pIJ486	6.2 kbp; derivative of pIJ101; HC, Thio^r	51
pANT12	12.8 kbp; pIJ350 with 8.8-kbp *Pst*I insert from *S. coelicolor* containing *actI*, *actIII*, *actVII*, *actIV*, and *actVb* loci	5
pANT14	3.79 kbp; pUC19 containing 1.1-kbp *Bam*HI fragment with the *actIII* gene from *S. coelicolor*	This work
pANT15	4.9 kbp; pUC19 with 2.2-kbp *Bam*HI fragment containing parts of *actI-orf1* and *actI-orf2* genes of *S. coelicolor*	This work
pANT113	6.8 kbp; pUC19 containing a 4.1-kbp *Sst*I fragment hybridizing to methyltransferase gene probe	This work
pANT114	5.34 kbp; pUC19 with 2.65-kbp *Eco*RI fragment subcloned from phage P3 containing *dauK*	This work
pANT121	10.9 kbp; pUC19 containing 8.2-kbp *Eco*RI DNA insert from *Streptomyces* sp. strain C5 DNA in phage P7	This work
pANT122	14.3 kbp; pIJ486 containing 8.2-kbp *Eco*RI insert from pANT121	This work
pANT128	3.8 kbp; 1.2-kbp *Sst*I subclone of pANT235 in pUC19 containing partial sequences of *Streptomyces* sp. strain C5 homologs of *dnrI* and *dnrJ*	This work
pANT151	7.8 kbp; 5.1-kbp *Sst*I fragment from phage P7 in pUC19	This work
pANT152	10.5 kbp; 7.8-kbp *Kpn*I fragment from phage P7 in pUC19	This work
pANT164	9.2 kbp; pIJ486 containing 3.0-kbp *Eco*RI-*Kpn*I insert from pANT121	This work
pANT233	6.5 kbp; pUC19 containing 3.8-kbp *Bam*HI fragment from pWHM333 containing *dnrI* and *dnrJ* genes of *S. peucetius* (45)	This work
pANT235	9.3 kbp; pUC19 containing a 6.6-kbp *Bam*HI-*Bgl*II DNA fragment from *Streptomyces* sp. strain C5 carrying the *dnrRI*-hybridizing region	This work

[a] Abbreviations: HC, high-copy-number plasmid; Thio^r, thiostrepton resistance; Amp^r, ampicillin resistance; *act*, actinorhodin genetic loci.

Mass.). Conditions for DNA sequencing are described in the brochure accompanying the Sequenase enzyme (United States Biochemical Corp.). Sequencing reactions were carried out with 7-deaza-dGTP nucleotide mixes to reduce compressions. Universal and reverse primers were used to obtain the initial sequences in the inserts before the generation of specific primers for the sequences within the inserts.

DNA and deduced amino acid sequence analyses and database searches. DNA sequence data were analyzed by means of Clone Manager (Science and Educational Software, Inc., Stateline, Pa.), Genepro (Riverside Scientific, Inc., Seattle, Wash.), and the Sequence Analysis Software package of the Genetics Computer Group (Madison, Wis.) (13). The sequenced DNA was analyzed by FRAME (8) and CODON PREFERENCE (52) algorithms and use of an IBM-PC program (27) to determine the presence and direction of potential open reading frames (ORFs). Amino acid sequences of potential gene products were compared with those in the databases by means of BLAST (1).

Detection of anthracyclines. Strains to be tested for antibiotic production were grown for 7 days in 50 ml of NDYE or YEME medium supplemented with thiostrepton (10 μg/ml) in a 250-ml Erlenmeyer flask containing a coiled spring (10). Cultures were extracted, and anthracyclines were analyzed by thin-layer chromatography and high-performance liquid chromatography (HPLC) as described previously (10, 11).

Nucleotide sequence accession number. The DNA sequence data described in this paper have been deposited at GenBank with the accession number L34880.

RESULTS AND DISCUSSION

Construction of probes for *Streptomyces* sp. strain C5 daunomycin biosynthesis gene cluster. A 6.6-kbp *Bam*HI-*Bgl*II DNA fragment from *Streptomyces* sp. strain C5 hybridized at high stringency to a 3.8-kbp *Bam*HI fragment carrying *dnrI* and *dnrJ* from *Streptomyces peucetius* (45). Thus, a partial library of *Bam*HI- and *Bgl*II-digested *Streptomyces* sp. strain C5 DNA of around 6.6 kbp was ligated into *Bam*HI-digested pUC19 and the mixture was used to transform *E. coli* JM83. Plasmid pANT235, containing a 6.6-kbp *Bam*HI-*Bgl*II DNA insert that hybridized at high stringency with the *S. peucetius dnrI-dnrJ* probe (Fig. 2), was isolated by colony hybridization.

A probe for the PKR gene was constructed by subcloning the 1.1-kbp *Bam*HI fragment containing *S. coelicolor actIII* (21) from pANT12 (5) into pUC19 to make pANT14. A PKS probe was constructed by subcloning a 2.2-kbp *Bam*HI DNA fragment (32), containing most of the *S. coelicolor actI-orf1* and *orf2* genes (19), from pANT12 (5) into pUC19 to make pANT15.

In order to isolate the *Streptomyces* sp. strain C5 genes encoding aklanonic acid methyltransferase (10) or carminomycin methyltransferase (12, 31), a consensus, degenerate, high-G+C-biased, 47-mer oligonucleotide probe (5′-GGC[G]-GAC-TTC-TTC-GAG-CCG[C]-CTG[C]-CCG-CGC[G]-AAG-GCC[G]-GAC-GCC-ATC-ATC-CT-3′) (MT probe) was synthesized for a highly conserved region (GDFFEPL-PRKADAIIL) of several methyltransferases, including *S. peucetius* DnrK (31), *Streptomyces glaucescens* TcmO and TcmN

FIG. 3. Nucleotide sequence of the 8.1-kbp *Eco*RI DNA fragment from *Streptomyces* sp. strain C5 containing the daunomycin PKS region. The deduced amino acid sequences of the proposed translation products are given below the nucleotide sequence. For genes reading from right to left, the second strand reading in the opposite direction has been added. Where double-stranded sequence is shown, the top strand reads from 5′ to 3′. The numbers at the right indicate nucleotide positions. Potential ribosome binding sites (rbs) are underlined. Inverted repeats (dashed arrows) and stop codons (*) are indicated.

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GAATTCGCGTGATCGGGGTCGCCCCGGGAGTGATCGAGACGGGCGCGGGAGTCCGGGCCG      60
..I  R  V  I  G  V  A  P  G  V  I  E  T  G  A  G  V  R  A  G

GGATGTCCCAGGAGGCGTACGACGGGTTCCTGGAAGCCATGGGACAACGCGTCCCGCTCG     120
  M  S  Q  E  A  Y  D  G  F  L  E  A  M  G  Q  R  V  P  L  G

GCCGGGTGGGCCGGCCCGAGGACGTCGCGTGGTGGGTGGTGCGGCTCGCCGATCCCGAAG     180
  R  V  G  R  P  E  D  V  A  W  W  V  V  R  L  A  D  P  E  A

CCGCGTACGCCAGCGGTGCCGTGCTCGCCGTGGACGGGGGGCTGTCGGTGACCTGAGAGG     240
  A  Y  A  S  G  A  V  L  A  V  D  G  G  L  S  V  T  *

          ----  ----------->       <---------------
CCCGGTCGGACCCGCACAGGTCGCCCTCGCTCGCCGGAGGGCGACCTGCGGGGTGGTGTC     300

CTGCCGGGGACACGGAGGCCCCCGCCCCGAGGGGGTCGGGACGGGGGTTCCGTACGGGTC     360

GGGACGCTCGTCTCAGGCGCTGGACTCGGCGTACCGTCTGGCCGCTTCCAGGGTCAGCAG     420
          3'-AGTCCGCGACCTGAGCCGCATGGCAGACCGGCGAAGGTCCCAGTCGTC
                *  A  S  S  E  A  Y  R  R  A  A  E  L  T  L  L

ACTGTTGCGGCCCAGGGCCCGGCGGACGAAGTCCACGGCGTCCGTCTCATTCGCCCCCGC     480
TGACAACGCCGGGTCCCGGGCCGCCTGCTTCAGGTGCCGCAGGCAGAGTAAGCGGGGGCG
  S  N  R  G  L  A  R  R  V  F  D  V  A  D  T  E  N  A  G  A

ACCTAGGATGCCGGGGATCGCCGAGCGGGCCACGGACACGGTGTGGGCCGACGTGACGGC     540
TGGATCCTACGGCCCCTAGCGGCTCGCCCGGTGCCTGTGCCACACCCGGCTGCACTGCCG
  G  L  I  G  P  I  A  S  R  A  V  S  V  T  H  A  S  T  V  A

GATCCCGTCCCCGTCGCCGGCGGGACGGACCGACCAGCGGCCGAGATGGAGCGCCAGCAG     600
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  I  G  D  G  D  G  A  P  R  V  S  W  R  G  L  H  L  A  L  L

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GCCGCCGCACGCGTCAACGAACATCTGCTACTCCGCCGCAAGCCCCTTCGTGTGCGCGTG
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GTCGCTGCGATGGAGGAAGCGGTAGATGTCCTCGGCACTGCCACGGACGACGACGGTGTC     840
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  E  F  T  T  P  A  T  G  D  P  G  E  L  A  T  R  L  S  A  L

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  V  I  W  E  G  G  M  A  A  V  P  H  A  S  T  E  Q  R  F  R

GATGCGCCGGACCCGCTCGTCGAGCTCGCGCCGCGACGTCCAGGTACGAAGCGAGCCGTT    1140
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GGCAGTGGCCCAGATGCGGATGCGTTCGCTGGAGTCCGCGCACTCCAACTGTTCGACGTG    1200
CCGTCACCGGGTCTACGCCTACGCAAGCGACCTCAGGCGCGTGAGGTTGACAAGCTGCAC
  A  T  A  W  I  R  I  R  E  S  S  D  A  C  E  L  Q  E  V  H

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  V  S  P  P  F  V  A  P  W  S  R  V  D  A  L  V  A  F  V  A

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CAGGCCGCGCCGGCTCCATACCTAGAGCACGAGCCAGACGACTTCGCCGCTGAGCCTGTA
  D  P  A  A  S  H  T  I  E  H  E  T  Q  Q  L  P  S  E  S fM (dauA-orfF)

CTGCCCTCCTGAGCTCGGGTCGTGGACGGTCAGTAACTGCCGAGGCCACCACAGACATTG    1380
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     (rbs)                  *  Y  S  G  L  G  G  C  V  N

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TACCGGACCCGGCACTAGCTCAGCCGCGACAGCCTCTGGTCCATCACGTGGTAAGGCCGG
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CGGTGAGGGACGAACGGCGGCCATCTCCGCTACAACTACTAGGCCGGGGTCGGCACGAGG
  A  V  G  Q  K  G  G  T  S  A  I  N  I  I  R  G  W  G  H  E

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  R  D  V  A  D  T  S  R  V  D  C  S  T  G  E  V  E  L  G  S

GCCTGAAGTTCCTTCACGGTGCCGGTGACGGCGCTCTCGTCACGAGCGCACAGGAACACC    2040
CGGACTTCAAGGAAGTGCCACGGCCACTGCCGCGAGAGCAGTGCTCGCGTGTCCTTGTGG
  A  Q  L  E  K  V  T  G  T  V  A  S  E  D  R  A  C  L  F  V

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  R  T  G  D  L  A  L  K  R  R  G  V  A  L  G  I  G  S  T  G

CCGGTGACAAGTGCGACCCGGTCGGCTGCGTCGGACATGATCCTCCCTTCGACGGAGGGC    2160
GGCCACTGTTCACGCTGGGCCAGCCGACGCAGCCTGTACTAGGAGGGAAGCTGC-5'
  G  T  V  L  A  V  R  D  A  A  D  S fM   (rbs)  (dauB)

CCACCCTCACACCTCGCGCTCGAGCCGCGCTTGACGACCCGGCTTCCAGAGGAGGTCGAG    2220

CGGCGCCGCAGAACCTCGGAGTCCGGCGTGACCGCACCTGCGGACGCGGCCGCACCGTCG    2280

CACCGACGTCCACCCCACACCTGGACCAACGGACCCGTGGAGCAGCGATGCCTCTGGCCA    2340
                    (dauA-orfE) (rbs)  fM  E  Q  R  C  L  W  P

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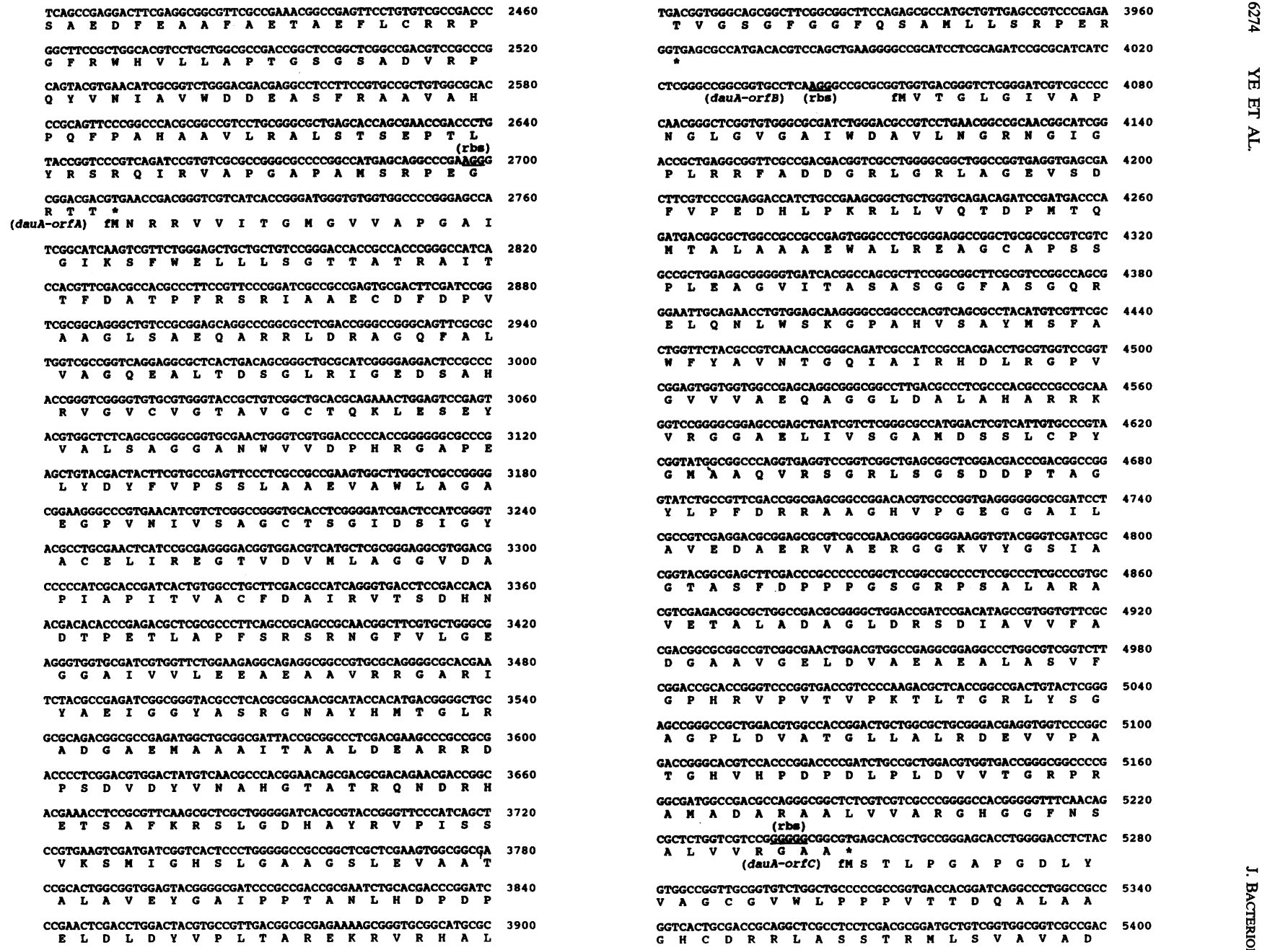
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G F R W H V L L A P T G S G S A D V R P
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(rbs)
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*
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(dauA-orfB) (rbs) fM V T G L G I V A P
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N G L G V G A I W D A V L N G R N G I G
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P L R R F A D D G R L G R L A G E V S D
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F V P E D H L P K R L L V Q T D P M T Q
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M T A L A A A E W A L R E A G C A P S S
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P L E A G V I T A S A S G G F A S G Q R
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(rbs)
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A L V V R G A A *
(dauA-orfC) fM S T L P G A P G D L Y
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V A G C G V W L P P P V T T D Q A L A A
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G H C D R R L A S S T R M L S V A V A D

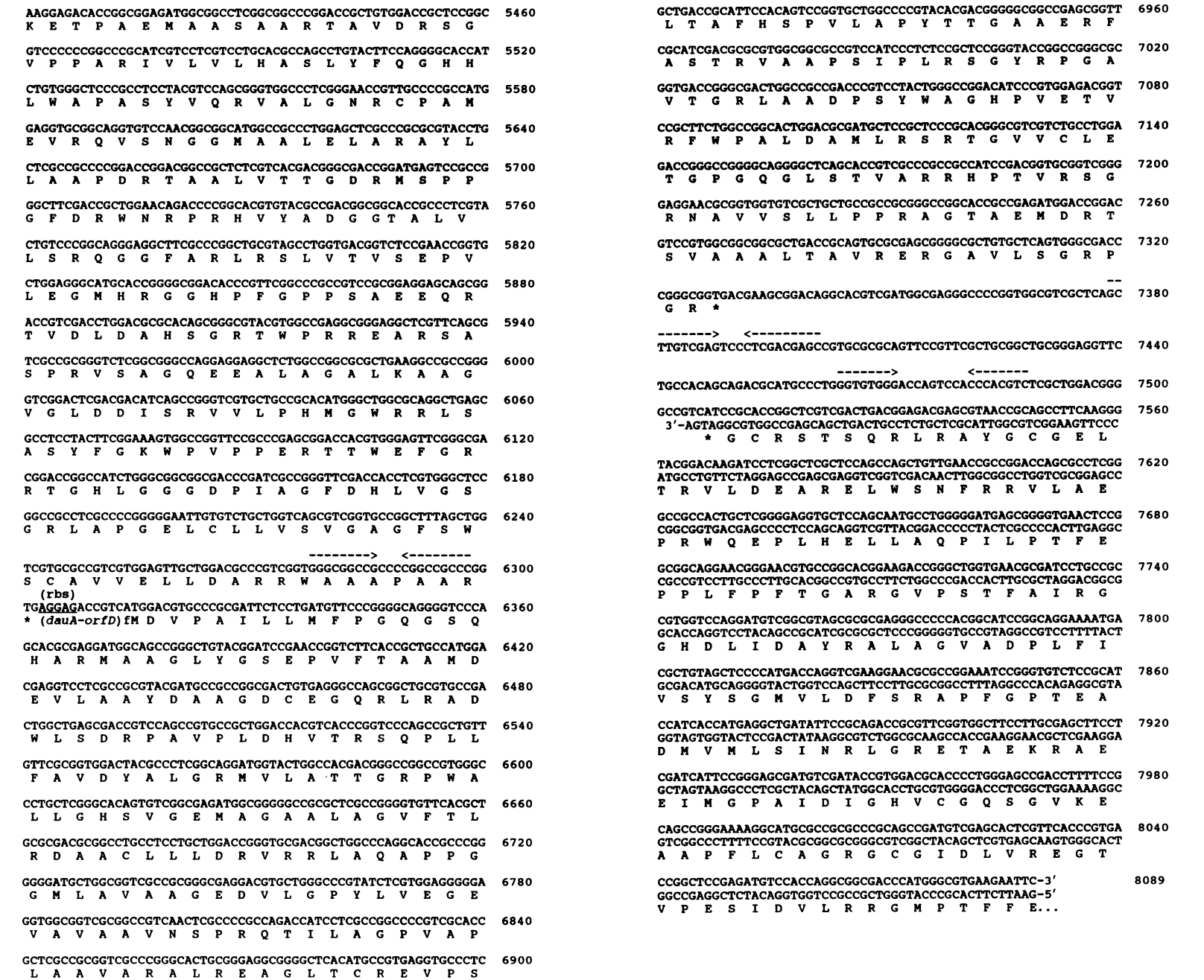

FIG. 3—*Continued.*

TABLE 2. Characteristics of predicted gene products of *dauA* and *dauB* loci

ORF	Length (amino acids)	Predicted M_r	Probable function	Closest homolog (% identity)	Reference
DauA-OrfA	418	43,709	PKS	*S. glaucescens* TcmK (65%)	7
DauA-OrfB	398	40,563	Chain length factor (34)	*S. glaucescens* TcmL (58%)	7
DauA-OrfC	352	37,315	Ketoacyl synthase	*E. coli* FabH (25%)	49
DauA-OrfD	338	35,248	Acyltransferase	*S. erythraea* EryA[a] (33%)	6, 16
DauA-OrfE	131	14,288	Oxygenase	*S. glaucescens* TcmH (29%)	39
DauA-OrfF	315	34,764	Cyclase/dehydrase	*S. coelicolor* ActVII (44%)	19
DauB	262	27,167	PKR	*S. galilaeus* AknA (68%)	50

[a] Identity with acyltransferase domain 1 of EryA-Orf3, the erythromycin PKS of *Saccharopolyspora erythraea*.

(46), hydroxyindole methyltransferase (17), and *Streptomyces alboniger* DmpM (29), and was used to probe *Streptomyces* sp. strain C5 DNA. A 4.1-kbp *Sst*I DNA fragment from *Streptomyces* sp. strain C5 hybridized to the MT probe at very high stringency (Fig. 2). Thus, a partial library of *Sst*I-digested *Streptomyces* sp. strain C5 DNA of around 4.1 kbp was constructed in pUC19 and screened by colony hybridization with the oligonucleotide probe. A plasmid (pANT113) containing the 4.1-kbp *Sst*I fragment in pUC19 was isolated (Fig. 2).

Isolation of DNA from EMBL3 library containing putative *Streptomyces* sp. strain C5 daunomycin biosynthesis genes. Plasmids pANT14, pANT15, pANT113, and pANT235 (Table 1) were used to screen the complete EMBL3 phage library of *Streptomyces* sp. strain C5 DNA. Three phages which hybridized to one or more of the probes were obtained. Phage D8 DNA hybridized with the insert from pANT235; phage P7 DNA hybridized strongly with the inserts of pANT14, pANT15, and pANT113 and weakly with the insert of pANT235; and phage P3 DNA hybridized strongly with the inserts of pANT15 and pANT113 (Fig. 2). Phage D8-2 was subsequently isolated by a "walking" technique using the left end of phage P3. The DNA inserts of the phages were mapped in detail by restriction analysis as shown in Fig. 2 and were analyzed further by hybridization of the phage inserts to the various plasmid inserts described above. Subsequent hybridization and insert end sequence analysis proved the presence of the overlapped sequences of phage D8, D8-2, P7, and P3 (Fig. 2).

Sequence analysis. The 8.1-kbp *Eco*RI fragment from the phage P7 insert was subcloned into the *Eco*RI site of pUC19 to make pANT121 and was sequenced completely in both directions (Fig. 3). The G+C content of the entire 8,089-bp DNA fragment was 72 mol%. FRAME (8) and CODON PREFERENCE (52) analysis indicated seven complete ORFs (each of which is preceded by a plausible ribosome binding site), a partial C-terminal region of an ORF reading into the 5′ end of the sequence shown, and an ORF reading into the 3′ end of the given sequence (data not shown). The characteristics of the predicted gene products are listed in Table 2.

Deduced functions of the gene products. DauA-OrfA and DauA-OrfB appear to encode PKS enzymes (Fig. 4). DauA-OrfA contains the highly conserved putative condensing enzyme active site cysteine (GCTSGID [19, 25]), as well as the highly conserved putative active acyltransferase site (GHSLG) in the C-terminal portion of the deduced amino acid sequence (19).

DauA-OrfC appears to encode a ketoacyl synthase but lacks a highly conserved cysteine active site (e.g., AAACAGF [*E. coli* FabH sequence shown in Fig. 5]) shared by KASIII and thiolase enzymes (36) and at which the condensations apparently take place (49). The two regions of highest amino acid similarities between DauA-OrfC and the β-ketoacyl acyl carrier protein synthase III KASIII proteins are sequences also similarly conserved in chalcone synthases (49), i.e., residues 163 to 173 and the C-terminal 80 residues of DauA-OrfC (Fig. 5).

DauA-OrfD includes a conserved acyltransferase domain containing a serine active site motif (LGHSVGEM) in residues 90 to 97, indicating that it is probably an acyltransferase (Fig. 6A). Considering that daunomycin biosynthesis and actinorhodin biosynthesis appear to occur similarly (44) and that the daunomycin polyketide is primed with a propionyl moiety (Fig. 1 [5, 43, 44]), whereas the actinorhodin, granati-

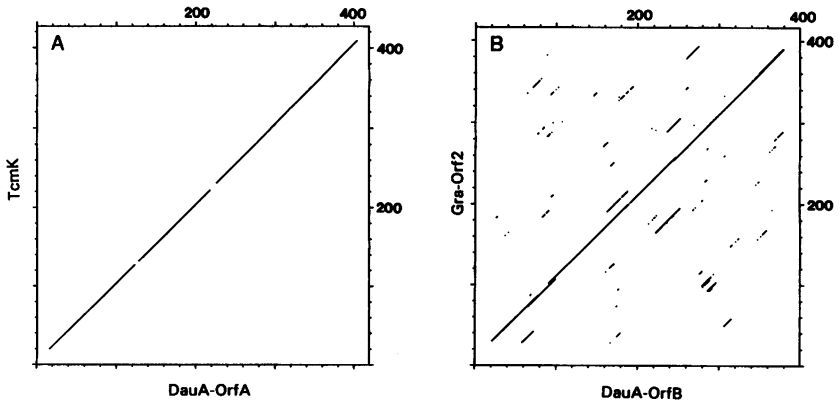

FIG. 4. (A) Dot plot analysis of the PILEUP comparison (13) of the deduced amino acid sequences of *Streptomyces* sp. strain C5 DauA-OrfA with *S. glaucescens* TcmK (stringency, 21.0; window, 30 amino acids). (B) Dot plot analysis of the PILEUP comparison of the deduced *Streptomyces* sp. strain C5 DauA-OrfB with *Streptomyces violaceoruber* Gra-Orf2 (stringency, 19.0; window, 40 amino acids). The amino acid numbers are indicated on the top and right axes.

```
FabH (Ec)  DEWIVTRTGI RERHIAAPNE TVSTMGFEAA TRAIEMAGIE KDQIGLIVVA TT--SATHAF PSAACQIQSM LGIKGCP-AF 106
FabH (Sp)  DEWIATRTGI RQRHVLSGKD SLVDLAAEAA RNALQMANVN PDDIDLILMC TS--TPEDLF GSAP-QVQRA LGCSRTPLSY 175
DauA-OrfC  DRRLASSTRM LSVAV-ADKE TPAEMAASAA RTAVDRSGVP PARIVLVLHA SLYFQGHHLW APASYVQRVA LG-NRCP-AM 112
Consensus  DewiatrTgi r.rhv.a.ke t...maaeAA r.A..magv. pd.I.Lil.a t......hlf .sA..q.q.a LG..rcP.a.

FabH (Ec)  DVAAACAGFT YALSVADQYV KSGAVK-YAL VVGSDVLART C-DPTDRGTI IIFGDGAGAA VLA-----A- -SEEPGIIST 177
FabH (Sp)  DITAACSGFM LGLVSAACHV RGGGFK-NVL VIGADALSRF V-DWTDRGTC ILFGDAAGAV VVQ-----AC DSEEDGMFAF 248
DauA-OrfC  EVRQVSNGGM AALELARAYL LAAPDRTAAL VTTGDRMSPP GFDRWNRPRH V-YADGGTAL VLSRQGGFAR LRSLVTVSEP 191
Consensus  dv.aac.Gfm .aL..A..yv ..g..k..aL V.g.D.lsr. ..D.tdRgt. i.fgDgagA. Vl......A. .see.g....

FabH (Ec)  HLHADGSYGE LLTLPNADRV NPENSIHLT- ---------- --------MA GNEVFKVAVT ELAHIVDETL AANNLDRSQL 238
FabH (Sp)  DLHSDGGGGR HL---NASLL NDETDAAIGN NGAVTGFPPK RPSYSCINMN GKEVFRFAVR CVPQSIEAAL QKAGLTSSNI 325
DauA-OrfC  VLEGMHRGGH PFGPPSAE-- -EQRTVDLDA HSGRTWPRRE ARSASPRVSA GQE------- ---EALAGAL KAAGVGLDDI 258
Consensus  .Lh.dg.gG. .l..pnA... n.e....l.. ....t..... ..s.s...ma G.Evf..av. ........aL .aagl..s.i

FabH (Ec)  DWLVPHQANL RIISATA-KK LGMSMDNVVV TLDRHGNTSA ASVPCA-LDE AVRDGRIKPG QLVLLEAFGG GFTWGSALVR 315
FabH (Sp)  DWLLLHQANQ RIIDAVA-TR LEVPSERVLS NLANYGNTSA ASIPLA-LDE AVRSGKVKPG NIIATSGFGA GLTWGSSIIR 403
DauA-OrfC  SRVVLPHMGW RRLSASYFGK WPVPPERTTW EFGRRTGHLG GGDPIAGFDH LVGSGRLAPG ELCLLVSVGA GFSWSCAVVE 337
Consensus  dwlvlhqan. RiisA.a..k l.vp.erv.. .l.r.gntsa as.P.A.lDe aVrsGr.kPG .l.ll..fGa GftWgsa.vr
```

FIG. 5. LINEUP analysis of the PILEUP comparison (13) of the deduced amino acid sequences of DauA-OrfC with *E. coli* (Ec [49]) and spinach (Sp [47]) FabH (β-ketoacyl synthase III) enzymes. Only the regions of extensive amino acid similarities are shown; these include the putative active site cysteine residues present in the FabH proteins (bullets) but missing in DauA-OrfC, a region which is also conserved with chalcone synthases (box) (see reference 49 for comparison), and the conserved C-terminal regions of the deduced proteins. Dashes indicate gaps generated by PILEUP, and the consensus sequence shown was determined by LINEUP.

cin, and tetracenomycin polyketides are primed with acetyl moieties, we speculate that DauA-OrfD may be propionyl-CoA:acyl-carrier-protein acyltransferase.

TcmH, a homolog of DauA-OrfE, has been shown to insert an oxygen into tetracenomycin F1, a naphthacenone, to produce TcmD3, the 5,12-naphthacenequinone (39). An analogous reaction, conversion of the anthraquinol precursor to aklanonic acid (step G_1 [Fig. 5 of reference 44]), has been proposed to occur in daunomycin biosynthesis. ActVa-Orf6, another homolog of DauA-OrfE, has been proposed to carry out a hydroxylase-like function in the biosynthesis of actinorhodin (9).

On the basis of sequence comparisons, DauA-OrfF is likely to encode a bifunctional cyclase/dehydrase (Fig. 6B). The deduced *dauB* gene product has greatest similarity to *S. galilaeus* AknA, the aklavinone biosynthesis PKR (50). The 3′ end of a gene producing a protein having 34% amino acid sequence identity to the C-terminal ends of *E. coli* 7α-hydroxysteroid dehydrogenase (54) and a putative dehydrogenase from *Leishmania tarentolae* (35) reads from the *Eco*RI site at nucleotide 1 to nucleotide 236.

Organization of *Streptomyces* sp. strain C5 PKS genes. The contiguous *dauA-orfE*, *dauA-orfA*, *dauA-orfB*, *dauA-orfC*, and *dauA-orfD* genes are read divergently from *dauB* and *dauA-orfF*. The larger cluster may be organized as an operon, since *dauA-orfE* and *dauA-orfA*, appear to be translationally coupled, as are *dauA-orfB* and *dauA-orfC*.

Complementation experiments. The 8.1-kbp *Eco*RI fragment was subcloned into the *Eco*RI site of pIJ486 (51) to construct pANT122, which was used to transform several *Streptomyces* sp. strain C5 *dauA* mutants (Table 3), proposed to be dysfunctional in the polyketide assembly portion of the daunomycin biosynthesis pathway (3, 4). The DNA in pANT122 complemented *Streptomyces* sp. strain SC5-68 (*dauA68*) and *Streptomyces* sp. strain SC5-74 (*dauA74*) to red pigmentation. *Streptomyces* sp. strain SC5-74(pANT122) was grown in NDYE liquid medium, and the red pigments were extracted and analyzed by thin-layer chromatography and HPLC. Daunomycin and ε-rhodomycinone were produced in large quantities by the transformed strains but were not produced by the control transformant, *Streptomyces* sp. strain SC5-74(pIJ486) (Table 3). *Streptomyces* sp. strain SC5-68(pANT122) was unstable and lost the insert from the plasmid at a high frequency, so chromatographic confirmation that the red pigment produced by that recombinant was daunomycin could not be carried out. It is likely that the instability was at least partially due to the high copy number of the vector (pIJ486 [51]) used to carry the inserts.

The DNA in pANT122 heterologously complemented the 2-hydroxyaklavinone-producing, PKR-minus strain, *S. galilaeus* ATCC 31671 (5), to produce aklavinone and aclacinomycin A. No heterologous complementation of *S. coelicolor* B18 (*actI*) or B78 (*actI*) by pANT122 was observed (Table 3). *S. coelicolor* B41 (*actIII*)(pANT122) produced a blue pigment on plates of

TABLE 3. Complementation of mutants with DNA fragments from the *Streptomyces* sp. strain C5 daunomycin PKS gene cluster region

Species and strain	Genotype[a]	Restoration of antibiotic production by[b,c]:	
		pANT122	pANT164
Streptomyces sp.			
SC5-8	*dauA* mutant	−	ND
SC5-11	*dauA* mutant	−	ND
SC5-64	*dauA* mutant	−	ND
SC5-68	*dauA* mutant	+[d]	ND
SC5-74	*dauA* mutant	+	−
SC5-75	*dauA* mutant	−	ND
SC5-79	*dauA* mutant	−	ND
S. galilaeus 31671	"*dauB*" mutant	+	+
S. coelicolor			
B18	*actI* mutant	−	ND
B40	*actVII* mutant	w	+
B41	*actIII* mutant	+	+[d]
B78	*actI* mutant	−	ND

[a] *dauA* mutants make no anthracyclines and are colorless but still sporulate and are able to convert daunomycin precursors produced by other mutants (3, 4); the "*dauB*" mutant lacks a functional PKR (5) which is complemented by *actIII*; *actI*, *actIII*, and *actVII* mutants of *S. coelicolor* are blocked in the production of actinorhodin (19, 37). The *actI* and *actIII* mutants do not accumulate any known intermediates, whereas the *actVII* mutant accumulates mutactin (55).

[b] In all cases, the strains listed transformed with pIJ486 were used as negative controls. The integrity of the plasmid was confirmed in each case.

[c] For *Streptomyces* sp. strain C5 mutants, restoration (positive result) yielded daunomycin production; for *S. galilaeus* 31671, restoration yielded aklavinone and aclacinomycin A production, and for *S. coelicolor* mutants, restoration yielded actinorhodin production. Symbols: +, complementation to wild-type antibiotic production; −, no apparent complementation; w, weak complementation; ND, not done.

[d] Complementation to wild-type antibiotic pigmentation (red for daunomycin; blue for actinorhodin) was obtained reproducibly, but repeated transfer of the cultures resulted in a loss of the complementing phenotype.

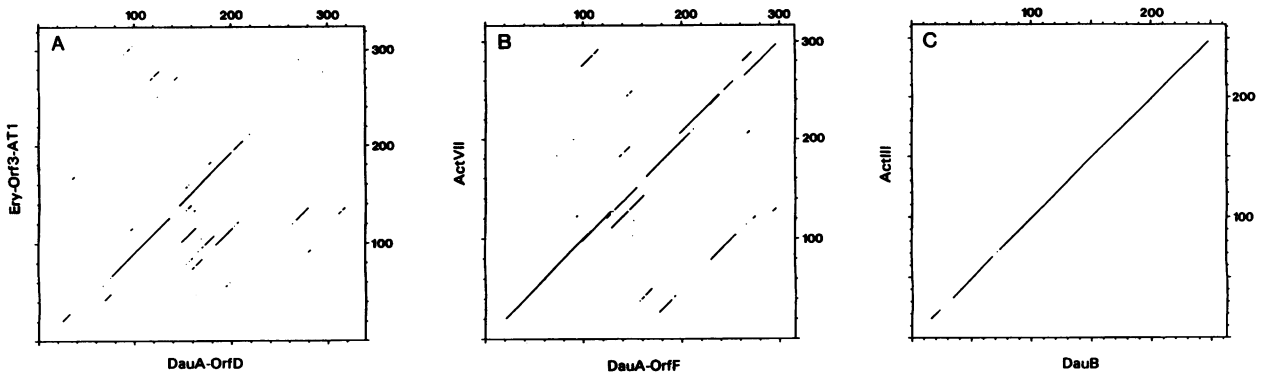

FIG. 6. (A) Dot plot analysis of the PILEUP comparison (13) of the deduced amino acid sequence of the putative acyltransferase, DauA-OrfD, with the acyltransferase domain 1 of EryA-Orf3 (stringency, 19.0; window, 40 amino acids). (B) Dot plot analysis of the PILEUP comparison of the deduced amino acid sequences of DauA-OrfF with *S. coelicolor* ActVII (actinorhodin biosynthesis bifunctional cyclase/dehydrase [19]) (stringency, 19.0; window, 40 amino acids). (C) Dot plot analysis of the PILEUP comparison of the deduced amino acid sequences of DauB with *S. coelicolor* ActIII (actinorhodin PKR [21]) (stringency, 21.0; window, 30 amino acids). The amino acid numbers are indicated on the top and right axes.

R2YE medium, which was confirmed by extraction and chromatographic comparison to be actinorhodin (data not shown). *S. coelicolor* B40 (*actVII*)(pANT122) transiently produced a weak blue pigment, suggesting that it was weakly but unstably complemented by the DNA insert. The amount of antibiotic produced was not sufficient to extract.

A 3.0-kbp *Eco*RI-*Kpn*I subclone of pANT122, containing the putative daunomycin cyclase/dehydrase and PKR genes

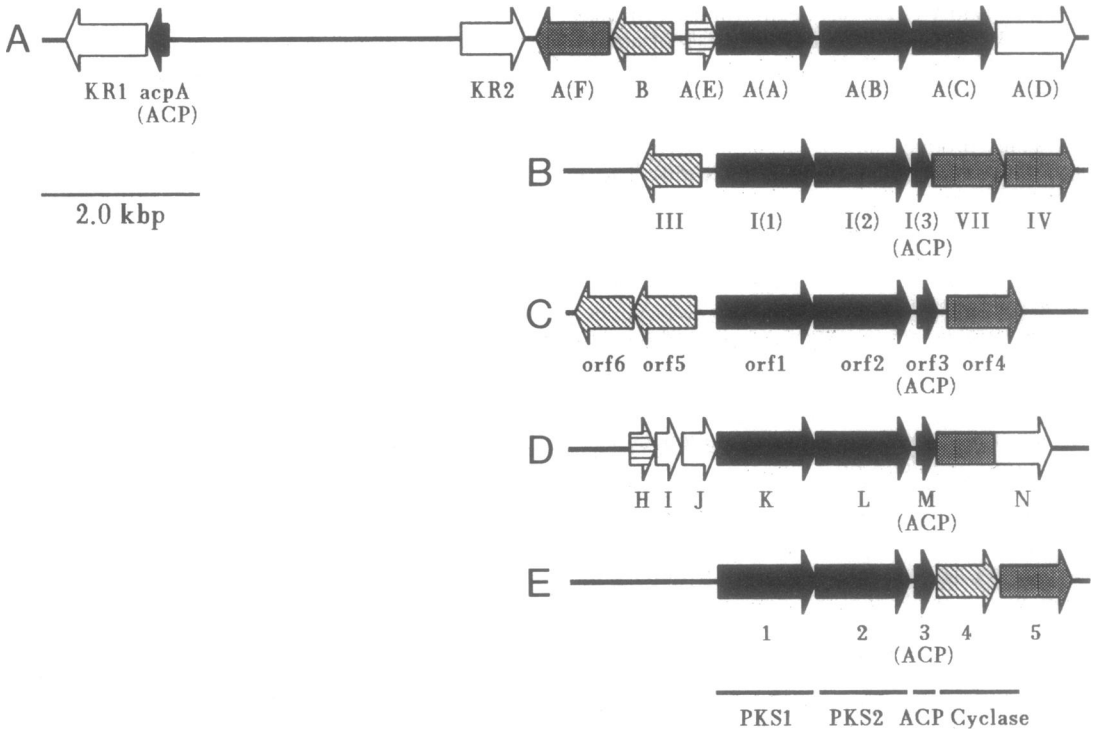

FIG. 7. Comparison of the gene structures of streptomycete type II PKSs. The genes encoding putative subunits of the PKSs (including the ACP genes) (solid arrows), and cyclase and/or dehydrase functions (shaded arrows), the PKR genes (diagonally hatched arrows), the genes of the tetracenomycin and daunomycin gene clusters that encode oxygenase-like functions (arrows with horizontal lines), and other genes of either unknown function or functions not included above (open arrows) are indicated. The positions of the genes encoding ACP for each cluster are designated. (A) *Streptomyces* sp. strain C5 daunomycin PKS gene region. Note that the gene encoding the putative daunomycin ACP (14) is located ca. 7 kbp to the left of the daunomycin PKS genes rather than directly downstream of the PKS subunit genes as found in the other type II PKS gene clusters (rows B through E). (B) *S. coelicolor* A3(2) actinorhodin PKS gene region, produced by *S. coelicolor* strain A3(2) (19). (C) *S. violaceoruber* Tü22 dihydrogranaticin PKS gene region (42). (D) *S. glaucescens* tetracenomycin C PKS gene cluster (7). (E) *S. cinnamonensis* Mon PKS gene locus of unknown function (2). The generalized conserved positions for the genes encoding PKS1, PKS2, ACP, and cyclases of type II PKS gene regions are shown by the lines at the bottom.

(Fig. 2), was inserted in pIJ486 to make pANT164. The DNA in pANT164 complemented the *S. coelicolor* B41 (*actIII*; PKR) and *S. coelicolor* B40 (*actVII*; cyclase/dehydrase) mutants to produce actinorhodin and *S. galilaeus* ATCC 31671 (PKR-minus [5, 50]) to produce aklavinone and aclacinomycin A (Table 3). The production of actinorhodin by *S. coelicolor* B41(pANT164) was not stable, as evidenced by the loss of the blue phenotype upon transfer of the blue transformants. Plasmid pANT164 did not complement the mutation in *Streptomyces* sp. strain SC5-74 (*dauA74*), indicating that the mutation, which was complemented by the 8.1-kbp *Eco*RI fragment, is within one of the *dauA orfA* to *orfE* genes.

It is not surprising that the putative daunomycin biosynthesis PKR and cyclase genes of *Streptomyces* sp. strain C5 heterologously complemented the analogous mutants of *S. coelicolor* blocked in actinorhodin production. We previously found that pANT43, containing the *actI-orf1* and *actI-orf2* genes (19), caused *S. galilaeus* ATCC 31133 to produce aloesaponarin II (5). Since the *actI-orf123*, *actIII*, *actVII*, and *actIV* genes (and the absence of *actVI*) are required for aloesaponarin II biosynthesis (5, 44), we deduced that *S. galilaeus* ATCC 31133 provided the enzymatic functions of ActI-Orf3 (ACP), ActIII, ActVII, and ActIV (5, 44). Thus, aloesaponarin II production by *S. galilaeus* 31133(pANT43) was the result of *S. coelicolor* and *S. galilaeus* gene products operating interfunctionally (5, 44).

Identity of genes sequenced. Several unsuccessful attempts were made to disrupt the putative daunomycin PKS genes of *Streptomyces* sp. strain C5. Moreover, transformation of *S. lividans* TK24 with pANT122 did not result in the formation of a daunomycin-like precursor. Thus, we have not proven unequivocally that these genes encode daunomycin biosynthesis. Nevertheless, considering that the genes shown herein are clustered with both the *dnrK* and *dnrJ* homologs, genes known from published reports to encode daunomycin biosynthesis reactions (31, 45), that they complement *dauA* mutants, and that they represent the only *actI* homologs in *Streptomyces* sp. strain C5, it is likely that they encode daunomycin polyketide biosynthesis.

Comparison of type II PKS gene organization. The structure of the *Streptomyces* sp. strain C5 putative daunomycin PKS gene region is shown in Fig. 7 in comparison with other type II PKS gene regions. The putative daunomycin PKS gene cluster contains several homologs to genes found in the other type II PKS gene clusters, e.g., *dauA-orfA* (*actI-orf1* homolog), *dauA-orfB* (*actI-orf2* homolog), and *dauB* (PKR; *actIII* homolog). The *dauB* gene is oriented in the same position relative to *dauA-orfA* as are the PKR genes in actinorhodin and granaticin (*actIII* and *gra-orf5*, respectively [21, 42]) PKS gene clusters. The putative daunomycin PKS locus also contains substantial deviations from the structures of previously described type II PKS loci, the most significant of which are the presence of *dauA-orfC* and *dauA-orfD* directly downstream of *dauA-orfB* and the absence of a gene encoding ACP directly 3′ of *dauA-orfB*. A gene encoding ACP has been found in the *Streptomyces* sp. strain C5 daunomycin biosynthesis gene cluster approximately 7 kbp to the left of *dauA-orfA* on the restriction map shown in Fig. 2 (14).

The *dauA-orfC* gene product lacks the condensing enzyme active site associated with the proteins with which it is most closely related (49), so it may have a mostly structural role. The product of *dauA-orfD*, also a likely member of the daunomycin PKS complex on the basis of the gene structure shown, is an acyltransferase homolog that we speculate to be propionyl-CoA:acyl-carrier-protein acyltransferase. Whether this protein is a subunit or a dissociable soluble protein of the daunomycin PKS complex is as yet unknown.

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