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Analysis of Clustered Genes Encoding Both Early and Late Steps in Daunomycin Biosynthesis by *Streptomyces* sp. Strain C5

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We recently described the isolation and sequence analysis of the daunomycin polyketide synthase biosynthesis genes of *Streptomyces* sp. strain C5 (J. Ye, M. L. Dickens, R. Plater, Y. Li, J. Lawrence, and W. R. Strohl, J. Bacteriol. 176:6270–6280, 1994). Contiguous to the daunomycin polyketide synthase biosynthesis gene region in *Streptomyces* sp. strain C5 are four additional genes involved in daunomycin biosynthesis, two of the products of which show similarity to different types of methyltransferases. The *dauC* gene, encoding aklanonic acid methyltransferase (AAMT), complements *dauC*-blocked mutants of *Streptomyces* sp. strain C5, restores in vitro AAMT activities to the mutant strains, and confers in vitro AAMT activity on *Streptomyces lividans*. Partial purification through gel filtration, followed by photoaffinity labeling of enriched AAMT with *S*-adenosyl-L-[^{3}H-*methyl*]methionine, indicates that AAMT is a homodimer with an M_r of ca. 48,000 (subunit M_r of ca. 24,000), which corresponds with the size of the deduced gene product. The *dauD* gene, encoding aklanonic acid methyl ester cyclase, is divergently arranged with respect to *dauC*. Immediately downstream and apparently translationally coupled with *dauD* is the *dauK* gene, encoding carminomycin 4-*O*-methyltransferase. The *dauK* gene confers in vitro carminomycin 4-*O*-methyltransferase activity on *S. lividans* and is nearly identical to a similar gene isolated from *Streptomyces peucetius* and characterized. Directly downstream of *dauK* lies a gene encoding a deduced protein that is similar to the methyl esterases.

Daunomycin (daunorubicin; Fig. 1B) and adriamycin (doxorubicin) are commercially important anthracycline antitumor agents. Recent data have indicated a putative pathway for daunomycin biosynthesis in *Streptomyces* sp. strain C5 and *Streptomyces peucetius* (4, 5, 8, 9, 33), a pathway in which two methyltransferase reactions have been proposed (8, 9, 33). Aklanonic acid methyltransferase (AAMT) catalyzes the methyl esterification of aklanonic acid (Fig. 1A) (8). Aklanonic acid methyl ester (AAME) cyclase catalyzes the formation of aklaviketone from AAME, and in wild-type anthracycline producers, aklaviketone is reduced to form aklavinone (8). We have generated mutants specifically blocked in AAMT activity (*dauC* mutants) and AAME cyclase activity (*dauD*) (4, 5).

Carminomycin 4-*O*-methyltransferase (CMT) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the 4-*O*-position of carminomycin and 13-dihydrocarminomycin (Fig. 1B) to form daunomycin and 13-dihydrodaunomycin, respectively (9, 10). CMT has been purified to near homogeneity and is an apparent homotetramer with an M_r of ca. 161,000 (10). A gene from *S. peucetius* ATCC 29050 encoding CMT has been isolated and sequenced (25). In this article, we show the isolation and sequence analyses of AAMT, AAME cyclase, and CMT of *Streptomyces* sp. strain C5. On the basis of a comparison of the sequence similarities and differences, the two methyltransferases apparently belong to two different subgroups. The AAME cyclase appears to be unusual with respect to both its activity and proteins in the databases. We also show the sequence of *dauP*, encoding an esterase-like function which we hypothesize also to be involved in daunomycin biosynthesis.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Streptomyces* sp. strain C5 and mutants derived from it have been described previously (4, 5). *Streptomyces lividans* TK24 (18) was obtained from D. A. Hopwood. *Streptomyces* strains normally were grown in YEME (18) supplemented with 20% sucrose. R2YE medium, also used for growth as well as for preparation of streptomycete protoplasts, was prepared as described by Hopwood et al. (18). Nitrate-defined-plus-yeast-extract (NDYE) medium, used for growth of *Streptomyces* sp. strain C5 and its mutants, has been described previously (9). Strains carrying streptomycete plasmids pIJ486 (38), pIJ702 (18), and pWHM3 (36) or derivatives of them were grown and stored on plates containing 40 μg of thiostrepton per ml.

Escherichia coli JM83 was used to propagate plasmids for sequencing and restriction analyses. *E. coli* was grown in Luria-Bertani medium, and plasmids were introduced into *E. coli* by transformation done by standard procedures (26). For *E. coli* strains harboring plasmids, ampicillin was added at a final concentration of 100 μg/ml.

General genetic manipulations. The procedures for protoplast formation, transformation, and regeneration of protoplasts for *Streptomyces* sp. strain C5 have been described elsewhere (24). The procedures used for the preparation of *Streptomyces* plasmid and chromosomal DNA have been described by Hopwood et al. (18). The 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 are described by Maniatis et al. (26).

DNA sequencing. Both strands of the DNA were sequenced with Sequenase V2.0 (United States Biochemical Corp., Cleveland, Ohio), double-stranded templates, and sequence specific primers and by labeling with α-thio-^{35}S-dCTP (1,000 to 1,500 Ci/mmol; Dupont-New England Nuclear, Boston, Mass.) as recently described (41). 7-Deaza-dGTP was substituted for dGTP to reduce compressions.

DNA and deduced amino acid sequence analyses and database searches. DNA sequence data were analyzed with 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.) (11). Open reading frames were detected in the sequenced DNA with the FRAME (7) and CODON PREFERENCE (39) algorithms by IBM-PC programs written in our laboratory (21). The amino acid sequences of potential gene products were derived by FRAME and CODON PREFERENCE analyses and were compared with those in the databases with BLAST (1).

Enzyme analyses. Mycelial extracts were prepared from 500-ml cultures of *Streptomyces* sp. strain C5, mutants derived from it, and recombinant *S. lividans* grown in YEME (18) for 7 days at 30°C. The mycelia were harvested by centrifugation at 10,000 × *g* for 10 min (4°C) and washed once in the appropriate buffer for each assay (see below). Pelleted, washed mycelia were broken with a French pressure cell (American Instrument Co., Urbana, Ill.) at 15,000 lb/in^2. The crude

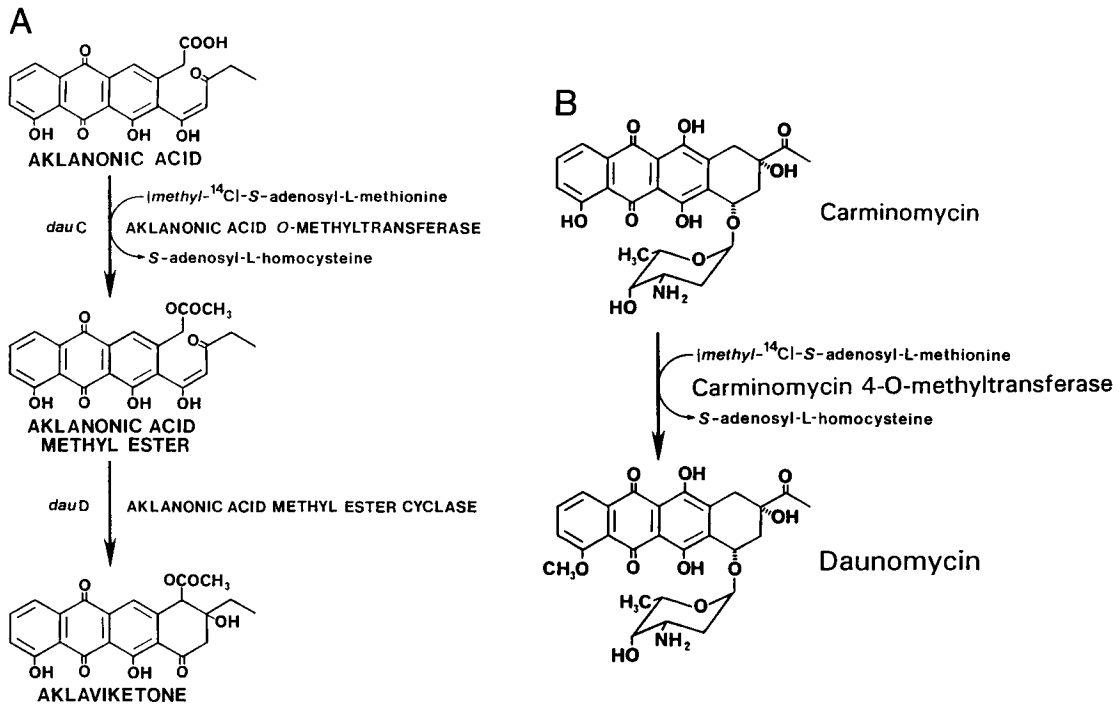

FIG. 1. Daunomycin biosynthesis reactions encoded by genes described in this work. (A) AdoMet-dependent methylation of aklanonic acid to aklanonic acid methyl ester coded for by *dauC*, followed by cyclization to form aklaviketone, coded for by *dauD*. (B) AdoMet-dependent methylation of carminomycin to form daunomycin, coded for by *dauK*.

mycelial extracts of unbroken mycelia and insoluble material were clarified by centrifugation at 15,000 × *g* for 30 min (4°C). The supernatants from these steps were used for enzyme assays.

AAMT was analyzed as described by Connors et al. (8), and the analysis was carried out with mycelial extracts prepared in 50 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 6.0). The assay used 25 nmol of aklanonic acid and 100 nmol of *S*-adenosyl-[*methyl*-^{3}H]-L-methionine (^{3}H-AdoMet; 5.5 μCi/μmol) as substrates in a reaction mixture of 0.25 ml. The final protein concentrations in these assay mixtures were 80 to 100 μg/ml. Preliminary experiments were run to optimize this assay with respect to pH, linearity with the protein and substrate concentrations, and linearity with time (12). The assay conditions above reflect those optimal conditions.

AAMT assays for the generation of the substrate for AAME cyclase assays were run with undiluted ^{3}H-AdoMet (83 Ci/mmol; 2,701 GBq/mmol). AAME assays were run with mycelial extracts of recombinant *S. lividans* strains prepared at pH 7.5 in 50 mM NaH_2PO_4-Na_2HPO_4.

CMT activity was analyzed as described by Connors and Strohl (10), and the analysis was carried out with mycelial extracts prepared in 50 mM Tris-HCl buffer (pH 8.0). The assay used 25 nmol of carminomycin and 100 nmol of ^{3}H-AdoMet (5.5 μCi/μmol) as substrates in a reaction mixture of 0.25 ml. The final protein concentrations of the extracts in these assay were 800 to 1,000 μg/ml. ^{3}H-AdoMet (83 Ci/mmol; 2,701 GBq/mmol) was obtained from Dupont-New England Nuclear.

Partial purification and photoaffinity labeling of AAMT. The product of the *dauC* gene, AAMT, was partially purified by fractionation through a precalibrated Pharmacia Superose-12 fast protein liquid chromatography column (HR 10/30) equilibrated with 50 mM NaH_2PO_4-Na_2HPO_4 (pH 6.0). Approximately 1.0 mg of protein was loaded onto the matrix. The flow rate of the buffer was 0.5 ml/min, and 1.0-ml fractions were collected for enzyme analysis.

The active fractions from gel filtration were analyzed by photoaffinity labeling of the enzyme with ^{3}H-AdoMet by procedures described by Yu (42). The reaction mixture for photoaffinity labeling in a 200-μl total volume included the following: the enzyme fraction, 20 μg; dithiothreitol, 1 μmol; ^{3}H-AdoMet, 130 pmol (11 μCi); and NaH_2PO_4-Na_2HPO_4 buffer (pH 6.0), 10 μmol. Aliquots (20 μl) of this reaction mixture were placed into the wells of noncoated, 96-well microtiter plates (Cellwells; Corning Glassware, Corning, N.Y.) on ice and irradiated with 1,200 mJ of UV light (254 nm) with the GS-Gene-Linker apparatus (Bio-Rad, Inc., Hercules, Calif.). The 20-μl aliquots were pooled and analyzed by both nondenaturing and denaturing polyacrylamide gel electrophoresis (PAGE) (23) and then by autoradiography. Resolving and stacking gels were 10 and 3% (wt/vol) acrylamide-bisacrylamide, respectively. When used, sodium dodecyl sulfate (SDS) was added at 0.1% (wt/vol). Proteins in the gels were silver stained essentially as described by Merrill et al. (27).

For autoradiography of the photoaffinity-labeled protein, the stained gels were treated with a 20-volume solution of Enlightning (Dupont-New England Nuclear) for 30 min. The treated gels were dried onto Whatman chromatography paper (3MM Chr; Whatman International, Ltd., Maidstone, United Kingdom) with a gel drier (model 1160 slab gel dryer; Hoefer, San Francisco, Calif.) and placed at −70°C with XR-100 X-ray film (Sterling Medical Film, Bio-World, Inc., Columbus, Ohio).

Detection of anthracyclines. Anthracyclines were extracted, isolated, and analyzed by methods described previously (5, 8–10).

Nucleotide sequence accession number. The 4,134-nucleotide (nt) DNA sequence described in this paper has been deposited at GenBank with the accession number L35154.

RESULTS AND DISCUSSION

Origin of DNA. We recently described the isolation of a ca. 30-kbp region of DNA from *Streptomyces* sp. strain C5 and sequence analysis of the putative daunomycin polyketide synthase (PKS) biosynthesis genes within an 8,089-nt region of that DNA (41). The genes described herein were found clustered directly downstream of the putative daunomycin PKS genes (Fig. 2). The localization of these daunomycin biosynthesis genes with the polyketide biosynthesis genes previously described (41) strongly reinforces the hypothesis that the PKS genes previously described encode daunomycin PKS.

Sequence analysis. A 4,134-nt DNA fragment was sequenced completely in both directions with a combination of subclones (Table 1) and universal forward and reverse primers and with sequence-specific primers. Only the sequence of 840 nt downstream of the *Eco*RI site (Fig. 2) containing the 5′ end of *dauC*, all of *dauD*, and the 5′ end of *dauK* is shown here (Fig. 3). By means of both the FRAME (7) and CODON PREFERENCE (39) analyses, four complete open reading frames were discovered in the complete 4,134-bp DNA frag-

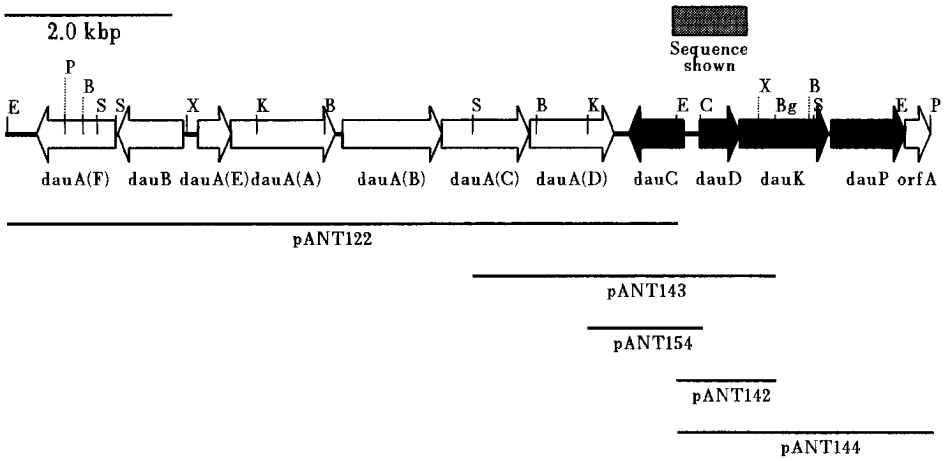

FIG. 2. Restriction map of part of the daunomycin biosynthesis gene cluster from *Streptomyces* sp. strain C5. The genes within the 4.13-kbp *Kpn*I-*Pst*I fragment described in this paper, including *dauC* (AAMT), *dauD* (AAME cyclase), *dauK* (carminomycin 4-*O*-methyltransferase), and *dauP* (uncharacterized putative esterase function), are indicated by solid arrows. The sequence of an 840-nt region within this fragment (denoted by the shaded box) is given in Fig. 3. The sequences and descriptions of *dauA*, *orfA* to *orfF*, and *dauB* have been described elsewhere (41). The inserts of plasmids pANT122, pANT142, pANT143, pANT144, and pANT154 are indicated. Abbreviations for restriction endonuclease sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I, S, *Sac*I; X, *Xho*I.

ment, one reading from right to left and three reading divergently from it (Fig. 2). The first open reading frame had a FRAME profile uncharacteristic of typical streptomycete open reading frames (12), but it was detected with CODON PREFERENCE. The G+C content of *dauC* was only 65.5 mol%, which is low for a streptomycete gene (7), whereas the G+C content of the entire 4,134-bp fragment, including both the genes and intergenic regions, was 69.3 mol%.

Function of DauC (AAMT). Plasmids pANT143 (Table 1; Fig. 2) and pANT154 (Table 1; Fig. 2) were used to transform *Streptomyces* sp. strains SC5-69, a *dauC* mutant, and SC5-147, a *dauC-dauE* double mutant, both of which accumulate aklanonic acid (4, 5). Both plasmids restored daunomycin production to strain SC5-69 and maggiemycin (the expected product of a *dauE* mutant [8]) production to SC5-147. The products of each experiment cochromatographed with authentic standards by thin-layer chromatography and high-pressure liquid chromatography (12) as described previously (5, 8–10). Moreover, plasmids containing the *dauC* gene conferred a strong increase in AAMT-specific activity in the *dauC Streptomyces* sp. strains SC5-69 and SC5-147 (Table 2).

S. lividans TK24 was transformed with the *dauC*-containing plasmids pANT143 and pANT154 as well as with the control vectors pIJ702 and pWHM3. *S. lividans* transformed with plasmids containing an intact *dauC* gene had approximately 13-fold more AAMT activity than the strain transformed with control vectors (Table 2). By means of procedures identical to those previously described (8), the *dauC*-specific, ^{3}H-methyl-radiolabeled product was confirmed by thin-layer chromatography and autoradiography to comigrate with aklanonic acid methyl ester (12).

The *dauC* gene product was purified 6.5-fold from extracts of *S. lividans*(pANT154) by a single gel filtration step through Superose-12 (Fig. 4A). The active fraction yielded a protein with an approximate M_r of 45,000 (Fig. 4B), which is just slightly less than twice the size of the deduced protein predicted from the amino acid sequence (M_r, 24,319) and SDS-PAGE (Fig. 5A), indicating that AAMT is a homodimer.

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source or reference
pUC19	2.686 kbp; Amp^r; *E. coli* plasmid	J. N. Reeve
pIJ486	6.2 kbp; derivative of pIJ101; HC, Thio^r	D. Hopwood (38)
pIJ702	5.686 kbp; derivative of pIJ101; HC, Thio^r, Mel$^+$	D. Hopwood (18)
pWHM3	7.2 kbp; shuttle vector derivative of pIJ486 and pUC19; HC, Thio^r, Amp^r	36
pANT114	5.34 kbp; pUC19 with 2.65-kbp *Eco*RI fragment subcloned from phage P3 containing *dauK*	41
pANT117	4.19 kbp; 1.5-kbp *Bam*HI-*Eco*RI fragment from pANT114 subcloned into pUC19	This work
pANT121	10.9 kbp; pUC19 containing 8.1-kbp *Eco*RI fragment with daunomycin PKS genes and 3′ end of *dauC*	41
pANT122	14.3 kbp; pIJ486 containing 8.1 kbp *Eco*RI fragment from pANT121	41
pANT140	8.89 kbp; 6.2-kbp *Bgl*II fragment from phage P3 (41) subcloned into *Bam*HI site of pUC19	This work
pANT141	5.59 kbp; 1.4-kbp *Bam*HI-*Pst*I fragment from pANT140 subcloned into equivalent sites of pANT117	This work
pANT142	6.64 kbp; 1.3-kbp *Sst*I-*Bgl*II DNA fragment from pANT114 subcloned into pIJ702 containing *dauT*	This work
pANT143	9.1 kbp; 2.4-kbp *Eco*RI-*Sst*I DNA fragment from pANT122 subcloned into pANT142	This work
pANT144	7.94 kbp; 1.8-kbp *Pst*I-*Bgl*II fragment from pANT141 subcloned into equivalent sites in pANT142	This work
pANT149	4.09 kbp; 1.4-kbp *Cla*I-*Kpn*I fragment from pANT143 subcloned into *Acc*I-*Kpn*I sites of pUC19	This work
pANT154	8.6 kbp; 1.4-kbp *Hin*dIII-*Sst*I fragment from pANT149 subcloned into pWHM3	This work

[a] Abbreviations: HC, high-copy-number plasmid; Thio^r, thiostrepton resistance; Amp^r, ampicillin resistance; Mel$^+$, production of melanin. Unless otherwise stated, plasmids are streptomycete plasmids.

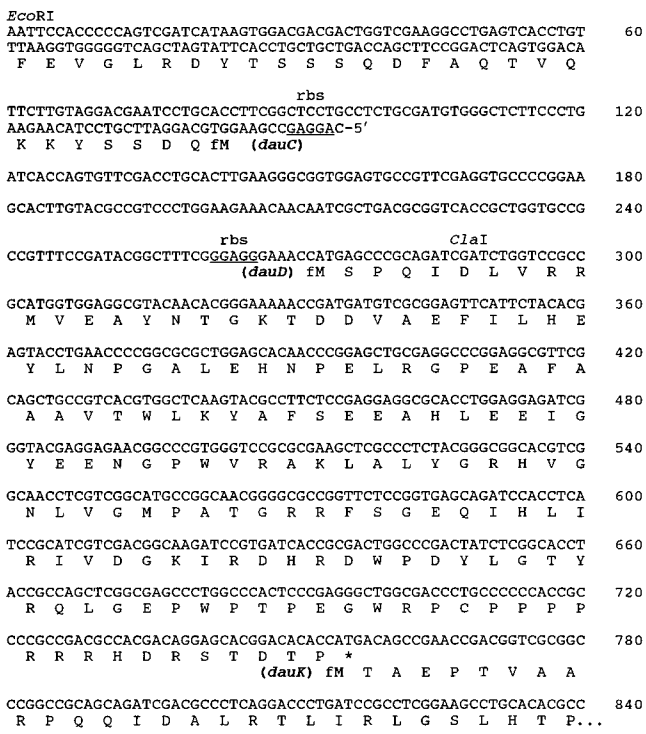

FIG. 3. Nucleotide sequence of an 840-bp DNA fragment reading downstream from the *Eco*RI site and containing the 5′ end of *dauC*, all of *dauD*, and the 5′ end of *dauK*. For the partial gene reading right to left, the second strand reading in the opposite direction has also been included. Where the double-stranded sequence is given, the top strand reads from 5′ to 3′. The deduced amino acid sequences of the proposed translation products are also given below the nucleotide sequence. The numbers at the right indicate nucleotide positions with respect to the *Eco*RI site. Potential ribosome binding sites are underlined and indicated by rbs. The *dauD* stop codon is indicated by *.

Fractions 13 and 14 from the Superose-12 chromatography step containing AAMT activity were photoaffinity labeled with ^{3}H-AdoMet and separated by nondenaturing PAGE (Fig. 5B and C). A single protein from fraction 14 (Fig. 5B), which contained the bulk of AAMT activity, was strongly photoaffinity labeled with ^{3}H-AdoMet (Fig. 5C). Fraction 13, which contained only a lower level of AAMT activity, yielded a small amount of ^{3}H-AdoMet-labeled protein. Photoaffinity labeling of the protein, followed by SDS-PAGE, resulted in the ^{3}H-AdoMet labeling of a single polypeptide species with an M_r of 24,000 (the band marked with an arrow in Fig. 5A); unfortunately, SDS-PAGE substantially lowered the level of label remaining associated with the protein, making the band barely visible on the film even after 10 days of autoradiography (12).

In a control experiment, similar analysis of *S. lividans* (pIJ702) extracts yielded no protein fraction with AAMT activity (12). The ^{3}H-AdoMet-binding product of the *tsr* gene (23S rRNA methylase [35]) present on pIJ702 (a positive control for the photoaffinity-labeling experiment) was observed on the SDS-PAGE gel at a calculated M_r of 26,000 (12), which compares with the predicted M_r of 28,901 for Tsr (6).

Taken together, the complementation of *dauC* mutants, expression of *dauC* in *S. lividans*, partial purification of DauC from *S. lividans* by the AAMT assay and photoaffinity labeling of the enriched product, and similarities with members of a group of small methyltransferases (see Table 3 and later discussion) show that the *dauC* gene encodes AAMT, the first major modification step in the pathway.

Function of DauD (AAME cyclase). The deduced product of *dauD* has an apparent M_r of 18,495 and has little sequence identity with any known protein in the databases (12). DauD was found, however, to restore maggiemycin production (the product accumulated by *dauE* strains) to SC5-141, a previously isolated *dauD dauE* double mutant that accumulates AAME (4). Similarly, when introduced into *S. lividans* TK24, *dauD* conferred substantial AAME cyclase activity on the heterologous host (Fig. 6). AAME cyclase activity was observed at both pH 7.5 (Fig. 6) and pH 6.0 (12), suggesting that it has a broad optimal pH range for activity. Thus, both complementation

TABLE 2. Specific activities of AAMT and CMT in streptomycete strains

Strain	Relevant genotype	Plasmid[a]	Assay[b]	Specific activity[c]
Streptomyces sp. strain C5	Parental	None	AAMT	668 ± 11
Streptomyces sp. strain SC5-69	*dauC*	None	AAMT	28 ± 6
Streptomyces sp. strain SC5-69	*dauC*	pWHM3	AAMT	180 ± 41
Streptomyces sp. strain SC5-69	*dauC*	pANT142	AAMT	198 ± 11
Streptomyces sp. strain SC5-69	*dauC*	pANT143	AAMT	2,271 ± 282
Streptomyces sp. strain SC5-69	*dauC*	pANT154	AAMT	2,590 ± 110
Streptomyces sp. strain SC5-147	*dauC dauE*	None	AAMT	47 ± 7
Streptomyces sp. strain SC5-147	*dauC dauE*	pWHM3	AAMT	375 ± 24
Streptomyces sp. strain SC5-147	*dauC dauE*	pANT154	AAMT	1,239 ± 104
S. lividans TK24	*dauC* minus	None	AAMT	55 ± 18
S. lividans TK24	*dauC* minus	pWHM3	AAMT	21 ± 3
S. lividans TK24	*dauC* minus	pIJ702	AAMT	152 ± 33
S. lividans TK24	*dauC* minus	pANT142	AAMT	165 ± 7
S. lividans TK24	*dauC* minus	pANT143	AAMT	2,079 ± 160
S. lividans TK24	*dauC* minus	pANT154	AAMT	2,194 ± 209
Streptomyces sp. strain C5	Parental	None	CMT	0 ± 4
Streptomyces sp. strain C5	Parental	pANT144	CMT	0 ± 13
S. lividans TK24	*dauK* minus	None	CMT	2 ± 1
S. lividans TK24	*dauK* minus	pIJ702	CMT	1 ± 3
S. lividans TK24	*dauK* minus	pANT144	CMT	23 ± 1

[a] Plasmid construction is described in Table 1, and inserts of *Streptomyces* sp. strain C5 DNA in the plasmids can be seen easily in Fig. 2.
[b] The AAMT and CMT assays were carried out at pH 6.0 and 7.5, respectively, as described in Materials and Methods.
[c] The values given are mean picomoles per minute per milligram of protein ± the standard deviation of at least six replicate assays.

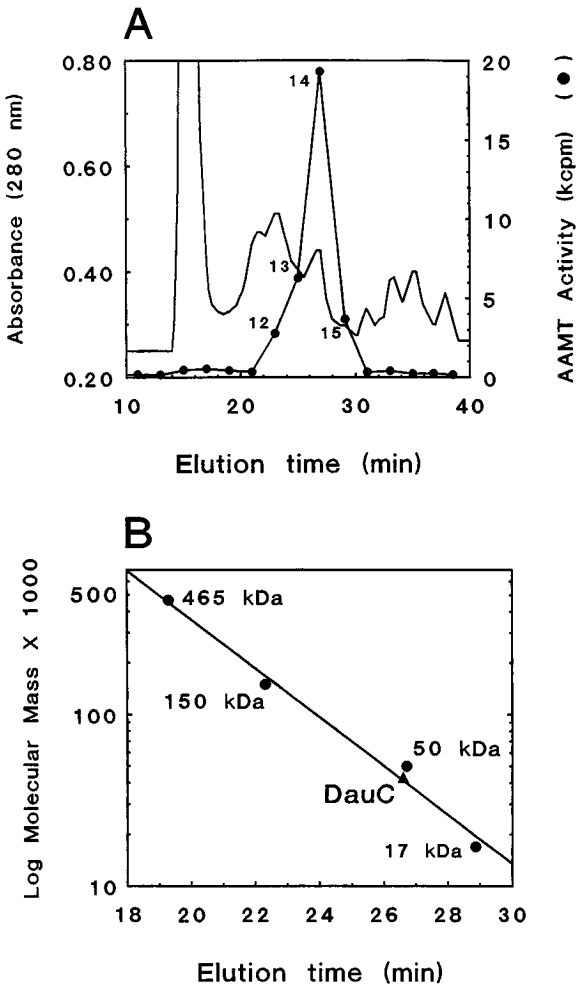

FIG. 4. Determination of approximate apparent M_r of AAMT by Superose-12 fractionation and SDS-PAGE. (A) Superose-12 fractionation of AAMT activity in clarified mycelial extracts from cultures of *S. lividans*(pANT154). The AAMT activity came out in four fractions at about 26.8 min of elution time. Fractions 12 to 15 (of which fraction 14 contained the greatest activity) are noted on the figure. No AAMT activity was eluted from Superose-12 in extracts of *S. lividans*(pIJ702) (12). (B) Calculation of approximate apparent M_r of *Streptomyces* sp. strain C5 AAMT from extracts of *S. lividans*(pANT154). The standards (in kilodaltons) are *E. coli* β-galactosidase (465), sheep immunoglobulin G (150), the Fab fragment of sheep immunoglobulin G (50), and horse skeletal muscle myoglobin (17). The elution of AAMT is indicated by the solid triangle.

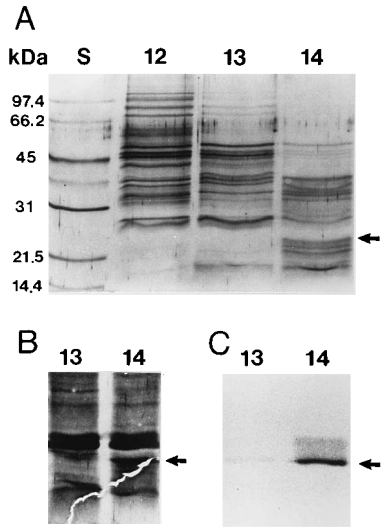

FIG. 5. PAGE and photoaffinity labeling of fractions from Superose-12 chromatography (Fig. 4) containing the highest AAMT activity. (A) SDS-PAGE of fractions 12, 13, and 14 as labeled. Fraction 14 contained the bulk of the AAMT activity. The arrow indicates the probable AAMT on the basis of the control lanes of *S. lividans*(pIJ702) (not shown). Lane S gives size standards in kilodaltons: rabbit muscle phosphorylase *b* (97.4), bovine serum albumin (66.2), hen egg white ovalbumin (45), bovine carbonic anhydrase (31), soybean trypsin inhibitor (21.5), and hen egg white lysozyme (14.4). The proteins in the gel were strained with silver (27). (B) Nondenaturing PAGE of proteins from fractions 13 and 14 as labeled, stained with silver. The arrow indicates AAMT. (C) ^{3}H-AdoMet-photoaffinity-labeled nondenatured proteins in fractions 13 and 14 as shown. The only protein that is cross-linked with ^{3}H-AdoMet corresponds to the protein specifically enriched in this fraction, which also corresponds to the strongest AAMT activity.

and heterologous expression data support the contention that *dauD* encodes AAME cyclase.

Function of DauK (CMT). Plasmids containing intact *Streptomyces* sp. strain C5 *dauK* conferred CMT activity on *S. lividans* TK24 (Table 2). By means of procedures identical to those previously described (9, 10), the *dauK*-specific [^{3}H] methyl-radiolabeled product was confirmed by thin-layer chromatography and autoradiography to comigrate with daunomycin (12). Interestingly, extra copies of *dauK* in *Streptomyces* sp. strain C5 did not significantly increase CMT specific activity in that strain (Table 2), presumably because the putative enzyme inhibitor, previously observed during purification of CMT from *Streptomyces* sp. strain C5 (10), was not completely titrated out by the additional enzyme.

The deduced product of *Streptomyces* sp. strain C5 *dauK* was 95% identical to *S. peucetius dnrK*, which has been shown to encode CMT (25), and has strong regions of similarity with DnrK and other methyltransferases (Table 3). The predicted M_r for DauK is 38,713. We previously purified the *Streptomyces* sp. strain C5 CMT to near homogeneity and found that the nondenatured enzyme had a calculated M_r of ca. 161,000. In conjunction with our previous data (10), the current data indicate that CMT is a homotetramer with an M_r of 154,852.

Speculated function of DauP. The *dauP* gene is found immediately downstream of *dauK* (Fig. 2). The deduced product of *dauP* has an M_r of 32,427. DauP has a 56% amino acid sequence identity with RdmC (28) (Fig. 7), a deduced protein from *Streptomyces purpurascens* that was recently shown, along with the products of at least three other genes, to be involved in the conversion of aclacinomycin and aklavin to 10-demethoxy-10-hydroxy derivatives of those compounds (28, 29). DauP also has a 24 to 26% sequence identity with *Pseudomonas* carboxymethylesterase Est5 (32) and *Acinetobacter calcoaceticus* CatD (β-ketoadipate enol-lactone hydrolase [17]) (Fig. 7). DauP contains the typical conserved serine hydrolase-active site, GXSXG, at amino acids 100 to 104 (Fig. 7) as well as a conserved histidine residue at position 275, conserved with the other deduced proteins analyzed, that was suggested by Shimada et al. (32) to be involved in the catalytic triad required for the charge-relay mechanism common to esterases (32). The four proteins also contain four conserved aspartate residues that could also be involved in the catalytic triad (Fig. 7).

The only theoretical reaction in daunomycin biosynthesis that would include a hydrolysis reaction of this type, i.e., hydrolysis or deesterification, would be the removal of the methyl group added by aklanonic acid methyltransferase as part of the process of converting Type II anthracyclines such as aclacinomycin (i.e., those containing the carbomethoxyl group at C-10) to Type I anthracyclines such as daunomycin and doxorubicin

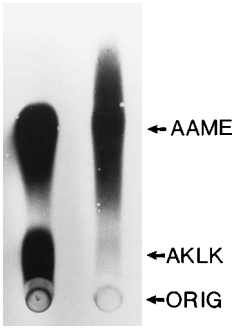

FIG. 6. Thin-layer chromatography and autoradiography of assay for AAME cyclase activity in recombinant *S. lividans* TK24 containing genes isolated from *Streptomyces* sp. strain C5. The solvent used was benzene-acetone-methanol (100:10:1). In the left lane, the extract from *S. lividans* TK24(pANT154) (*dauC*$^+$) was incubated at pH 6.0 with ^{3}H-AdoMet and aklanonic acid as described in Materials and Methods. The accumulated product (AAME) was extracted with chloroform-methanol (9:1), dried, and reconstituted in methanol as previously described (8) for use as a substrate in a second incubation with the extract from *S. lividans* TK24(pANT142) (*dauD*$^+$) prepared at pH 7.5. Part of the AAME accumulated in the AAMT reaction was converted by the second reaction to aklaviketone (AKLK). In the right lane, the extract from *S. lividans* TK24(pANT154) (*dauC*$^+$) was incubated at pH 6.0 with ^{3}H-AdoMet and aklanonic acid as described in Materials and Methods. The accumulated product was extracted as described above and used as a substrate for incubation with the extract from *S. lividans* TK24(pWHM3) (control) prepared at pH 7.5. None of the AAME accumulated in the first AAMT reaction was converted to AKLK in the absence of DauD. ORI, origin.

that lack the carbomethoxyl moiety at C-10 (14, 33). We have previously suggested that this reaction takes place after the glycosylation of ε-rhodomycinone (33), although no solid evidence for the order of this reaction in the pathway is yet available. As mentioned above, RdmC has been implicated in reactions which include deesterification of aklavin and aclacinomycin A (28, 29). This is consistent with, although clearly not proof for, *dauP* encoding anthracycline 15-methyl esterase.

Comparison of methyltransferase sequences. Upon analysis of the amino acid sequence of human erythrocyte D-aspartyl/L-isoaspartyl methyltransferase, Ingrosso et al. (19) suggested that a wide range of methyltransferases contain three conserved domains, which they labeled Regions I, II, and III (Table 3). Regions I and III coincide with the consensus regions observed by other investigators (37, 40); Region I also contains highly conserved AdoMet-binding sequences (19, 37, 40).

DauC was found to align with a group of deduced 22- to 28-kDa proteins (containing 203 to 254 amino acids), four of which are well characterized methyltransferases (Table 3). *E. coli* UbiG catalyzes AdoMet-dependent methylation of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone, the terminal step in the formation of ubiquinone (40), and *Rhodobacter sphaeroides* PmtA catalyzes the N-methylation of phosphatidylethanolamine (3). Catechol *O*-methyltransferase, the only methyltransferase which has been crystallized and studied by X-ray diffraction (37), fell into the same size group as DauC. Also belonging to this group is D-aspartyl/L-isoaspartyl methyltransferase, a protein methyltransferase (19). DauC has significant similarity with the product of the *bioC* gene, which encodes an uncharacterized step in the biotin biosynthesis pathway (30). DauC also has lower but still significant sequence similarities with the gene products of the germination factor *gerC2* from *Lactococcus lactis* (15), *E. coli ubiG* (40), and *R. sphaeroides pmtA* (3), particularly in regions of conserved domains (Table 3). The functions of *bioC* and *gerC2* are not yet known, although our analysis suggests that these genes may bind AdoMet in some manner (Table 3). The Genetics Computer Group PILE-UP and LINE-UP algorithms (11) were used to show that this group of methyltransferases clustered separately from the group of larger methyltransferases, i.e., those containing 332 to 393 amino acids, to which DauK belongs (12).

CMT is highly similar in size, overall amino acid sequence, and spacing of the three conserved domains to other methyltransferases involved in antibiotic biosynthesis pathways (Ta-

TABLE 3. Conserved domains in two size groups of methyltransferases and related proteins

Enzyme[a]	Length (amino acids)	Region I (sequence position)	Region II (sequence position)	Region III (sequence position)	Reference
Group I					
DauK	356	VLDVGGGKGGFA--AAIAR (183–199)	PLPRRADAIIL (241–251)	ALEPGGR-ILI (273–282)	This work
DnrK	356	VLDVGGGKGGFA--AAIAR (183–199)	PLPRKADAIIL (241–251)	ALEPGGR-ILI (273–282)	25
HIOMT	350	ICDLGGGSGALA--KACVS (182–198)	ALPE-ADLYIL (240–249)	ACRTGGG-ILV (271–280)	13
TcmN	332	IADLGGGDGWFL--AQILR (169–185)	PVPTGYDAYLF (228–238)	AIGDDDARLLI (260–270)	34
TcmO	333	FVDLGGARGNLA--AHLHR (167–183)	PLPR-ADVFIV (226–235)	ALTPGGA-VLV (257–266)	34
DmpM	339	VVDIGGADGSLLAAVLSAH (171–189)	RVPGGGDLYVL (229–239)	AMPAHAR-LLV (261–270)	22
CrtF	393	VMDVGGGTGAFL--RVVAK (232–248)	PIPQGADVITL (285–295)	ALPPGGR-LII (317–326)	2
Group II					
DauC	220	VLDIGCGRGACL-FPAAEK (48–65)	PA-RSFDLVMG (110–119)	ILDHGGR-IAF (139–148)	This work
UbiG	240	VLDVGCGGGILA--ESMAR (60–76)	KHAGQYDVVTC (118–128)	LVKPGGD-VFF (148–157)	40
PmtA	203	VLEVGVGTGLSL-PLYSHR (42–59)	PFDETFDTVVA (100–110)	VCRKGGE-VVI (130–139)	3
BioC	251	VLDAGCGPGWMS-RHWRER (46–63)	LATATFDLAWS (99–109)	VVRPKGV-VAF (129–138)	30
GerC2	252	ILDLCCGTGDWT-FDLSES (57–74)	FEKGSFDVVTI (118–128)	VLKPGGR-VVC (148–157)	15
AIPMT	226	ALDVGSGSGILT--ACFAR (81–97)	AEEAPYDAIHV (147–157)	QLKPGGR-LIL (171–180)	19
MycF	254	VLETGVWRGGAC---IFAR (81–96)	DDDHPMDAEMN (123–133)	RLSPGGF-AII (206–214)	20
MdmC	231	VLEIGTFTGYST--LCMAR (64–80)	DGALVFDADKA (134–144)	LVRPGGL-VAI (159–168)	16
COMT	221	VLELGAYCGYSA--VRMAR (62–78)	DM-VFLDHWKD (136–145)	LLEKCGLRKGT (153–163)	37
Conserved[b]		VLDoGuGuGooo AR	oD	oLRPGGX-ooo	

[a] The abbreviations for the methyltransferases are those used in the references cited except for human erythrocyte D-aspartyl/L-isoaspartyl protein methyltransferase, which is here designated AIPMT, and *L. lactis* GerC2, which was identified in reference 15 as a homolog of the *Bacillus subtilis gerC2* gene product.

[b] Designations: u, small, neutral amino acid; o, hydrophobic amino acid; X, any amino acid.

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DauP      MPTRMI TKDEVTLWSE GIGDPADAPL LL--IAGGNL SARSWPDEFV ERLAAAGHFV IRYDHRDTGR SSRYDFALHP   74
RdmC      MSERIV PSGDVELWSD DFGDPADPAL LL--VMGGNL SALGWPDEFA RRLADGGLHV IRYDHRDTGR STTRDFAAHP   74
Est5         MIF HNGNVNL-SY DVAGHGECIF FI----AGTA SDKSMWDGLR QELS-GKYRT VAFDNRDSGE STICD---QP   64
CatD   MPVFHFKDTL TAQDVALNYA TFGQADRPAL IFSNSLGTNL SMWQQQIAYF Q----DKYFV ICYDTRGHGA SST---PVGP   73
Cons   ....m..ri. t.gdV.Lws. dfgdpadpal ll....ggnl Sa.swpdef. qrla.g.yfv iryDhRdtGr S.t.d.a.hP

DauP   YGFDELATDA LAVLDAWQVR AAHVVGMSLG NTIGQLLALD APERLLTLTV MLGGALDVDF DADLEAA-LK GEPSVSGLPV  153
RdmC   YGFGELAADA VAVLDGWGVD RAHVVGLSMG ATITQVIALD HHDRLSSLTM LLGGGLDIDF DANIERV-MR GEPTLDGLPG  153
Est5   YTMLDLAKDA LSVMDAEGLQ KAHIVGHSLG GMIAQELAIL APDRVSTLSL -------VNT ASRIDNY-MR ---SVIELAR  133
CatD   YRIDQLGTDV IALLDHLQIP QATFCGISMG GLTGQWLAIH FPERFNQVIV ANTAAKIGEA QAWQARAQLV REQGLTPIAQ  153
Cons   YgfdeLatDa lavlDaw.v. .AhvvG.S.G gtigQ.lA.d ap.Rlstltv .lggaldvdf da.iera..r geps..gl..

DauP   PSRRFLDMMM LLQQPAGTDE ELLERRVEKW RLLNGEGVPF DSDEFRRREL LAAGHAGT-F DEPIVHHMIP QPPVSRGAEL  232
RdmC   PQQPFLDALA LMNQPAEGRA AEVAKRVSKW RILSGTGVPF DDAEYARWEE RAIDHAGGVL AEPYAHYSLT LPPPSRAAEL  233
Est5   DWSKTITDQR LLNRS----- ---------L YFLALGSKAL GSDIFNQVVD FASGSQSQPR EALIRQWEID L-TVDTTDRL  198
CatD   TAATRWFTPG FIEDSPEIVE KL-------- ---------- -------SHD LAQGSA---- -EGYASCCEA LAEADVRPQL  203
Cons   p...fld... llnq.ae..e .l.......w r.l.g.gvpf dsdef.r.ed lA.g.ag... .ep.ah..i. lppv.r.aeL

DauP   ARITTPVLAI QAMCDPAAPP PHARHLADRI PGARVVEIEN MGHALPLAVH EPLAAAICAH TRAATV*                 298
RdmC   REVTVPTLVI QAEHDPIAPA PHGKHLAGLI PTARLAEIPG MGHALPSSVH GPLAEVILAH TRSAA*                 298
Est5   SLINAKTHVI WASEDKIVTK DQQKMLVNGI SGAKFTCIEE SGHFPMIEAP EEFIRVLSGF IDKS*                  262
CatD   QRISIPVLVI AGAQDPVTTV ADGQFLCEHI VHSTL.EVLE ASHISNVEQP QAFNHAVEAV MKRFN*                 267
Cons   .rit.p.lvI qa..Dpia.. phgkhLa..I pgarl.eiee mgHalp.ev. ep.a..i.ah tr.a..
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FIG. 7. LINE-UP analysis of the PILE-UP comparison (11) of the deduced amino acid sequences of *Streptomyces* sp. strain DauP with CatD (17), Est5 (32), and RdmC (28). The highly conserved putative active site GXSXG residues (where X is any residue) are enclosed within a box. The conserved histidine residue suggested by Shimada et al. (32) to be involved in the catalytic triad is designated with bullets. The translation stop is indicated with an asterisk. Dashes indicate gaps generated by PILE-UP. The consensus sequence shown was determined by LINE-UP. Parameters used: gap weight, 3.0; gap length weight, 0.1.

ble 3). These methyltransferases also constitute a discreet cluster as observed by PILE-UP analysis (12). This group of larger methyltransferases appears to be more highly conserved in all three regions than the members of the group of smaller methyltransferases (Table 3). Our analysis (Table 3) suggests that DauC and DauK are members of two different subgroups of methyltransferases, each of which shares characteristics such as size and approximate position of conserved regions within the amino acid sequences. These groups are not meant to be inclusive; it is well known that other clustered groups of methyltransferases exist. For example, bacterial DNA cytosine methyltransferases form a separate cluster of methyltransferases (31).

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