JOURNAL OF BACTERIOLOGY, June 1996, p. 3389–3395 Vol. 178, No. 11
0021-9193/96/$04.00+0

Isolation and Characterization of a Gene from *Streptomyces* sp. Strain C5 That Confers the Ability To Convert Daunomycin to Doxorubicin on *Streptomyces lividans* TK24

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Received 5 February 1996/Accepted 15 March 1996

DNA sequence analysis of a region of the *Streptomyces* sp. strain C5 daunomycin biosynthesis gene cluster, located between the daunomycin polyketide biosynthesis gene cluster and a *dnrI* (transcriptional activator) homolog, revealed the presence of a gene encoding a P-450-like enzyme with a deduced M_r of 46,096. Expression of this gene, named herein *doxA*, in *Streptomyces lividans* TK24 resulted in in vivo bioconversion of daunomycin to doxorubicin. DoxA showed specificity for only daunomycin and 13-dihydrodaunomycin, both of which were converted to doxorubicin. Daunomycinone (daunomycin aglycone), carminomycin, 13-dihydrocarminomycin, idarubicin, and aklavin were not apparent substrates for DoxA. In vector controls or in vectors in which *doxA* was poorly expressed, *S. lividans* catalyzed the reduction of daunomycin and other 13-oxo-anthracyclines and -anthracyclinones to their 13-dihydro homologs.

Daunomycin (daunorubicin) and doxorubicin (14-hydroxydaunomycin; adriamycin) (Fig. 1) are clinically important anthracycline chemotherapeutic agents. Daunomycin is used primarily to treat adult myelogenous leukemia, whereas doxorubicin is widely used to treat a variety of neoplasias (2), making it the more valuable of these antitumor drugs (36). Daunomycin was discovered in the early 1960s (9, 15) by research groups at Rhône-Poulenc and Farmitalia. Subsequently, several streptomycete strains have been shown to produce daunomycin (31, 36). *Streptomyces peucetius*, a daunomycin-producing strain, was later mutagenized to produce doxorubicin (3). To our knowledge, this mutant, *Streptomyces peucetius* subsp. *caesius* (3), is the only organism capable of producing doxorubicin that has been described.

Streptomyces sp. strain C5 produces daunomycin and baumycins (23, 24). In the 10 years that we have studied daunomycin biosynthesis by this strain (4–7, 13, 14, 31, 32, 37), we have never observed it to produce doxorubicin. Nevertheless, we have been intrigued that *Streptomyces peucetius*, a daunomycin-producing organism, was mutated to form doxorubicin, suggesting that other daunomycin-producing strains may have the genetic capability to produce doxorubicin. Here we describe the isolation and characterization of a gene from the *Streptomyces* sp. strain C5 daunomycin biosynthesis gene cluster (Fig. 2) encoding a cytochrome P-450 that confers on *Streptomyces lividans* the ability to hydroxylate daunomycin at C-14 to form doxorubicin.

Bacterial strains, plasmids, media, and genetic manipulations. *Streptomyces* sp. strain C5 has been described previously (5, 24). Mutations in the *Streptomyces* sp. strain C5 genes that block daunomycin biosynthesis, *dauA74* (blocked in polyketide synthase formation), *dauCE147* (blocked in formation of aklanonic acid methyl ester), *dauE24* (blocked in aklavinone formation from aklaviketone), and *dauH54* (accumulates ε-rhodomycinone), have also been described (4). *Streptomyces* sp. strain C5 and mutants of it were grown in NDYE medium as described previously (6). *S. lividans* TK24 (17) was obtained from D. A. Hopwood and was grown in YEME medium (17) supplemented with 20% sucrose. *Escherichia coli* JM83, transformed with plasmids by standard procedures (21), was grown in Luria-Bertani broth containing 100 μg of ampicillin per ml. Procedures for routine molecular techniques, protoplast transformation, and preparation of *Streptomyces* plasmid and chromosomal DNA have been described previously (17). The plasmids used or constructed in this work are described in Table 1. DNA sequencing and sequence analyses were carried out as previously described (13, 37).

Analysis of open reading frames. We recently isolated ca. 30 kbp of DNA from *Streptomyces* sp. strain C5 within which genes encoding daunomycin polyketide synthase (32, 37) and other daunomycin biosynthesis genes (13, 14, 32) were found. The genes described in this report are located ca. 10 kbp from the daunomycin polyketide synthase genes (Fig. 2).

Figure 3 shows the DNA sequence spanning the region from the 3′ end of *orf1*, a putative ketoreductase (14), to the 5′ end of *dauI* (37). The two complete open reading frames found within this sequence, *orfA* and *doxA*, encode deduced proteins with M_rs of 28,808 and 46,096, respectively. The deduced product of *orfA* has 36% amino acid identity to *Streptomyces griseus* OrfD, a deduced protein of unknown function encoded by a gene linked with a putative threonine/serine kinase gene (34).

The second open reading frame, *doxA*, overlaps *orfA* by one nucleotide in a gene arrangement commonly found in streptomycete polyketide synthase genes (37). *doxA* encodes a protein that has 29 to 32% amino acid identity with deduced cytochrome P-450 enzymes from *Streptomyces griseus* ($P\text{-}450_{SOY}$) (33), *Streptomyces antibioticus* (OleP; putative hydroxylase in oleandomycin biosynthesis) (29), *Saccharopolyspora erythraea* (P-450-107B1) (1), and *Streptomyces* sp. strain SA-COO (ChoP) (18). Both the highly conserved heme pocket (FGDG PHYCIG) and oxygen-binding (AGHDT) domains, characteristic of cytochrome P-450s (28, 33), are present in DoxA (Fig. 3). We have not found a ferredoxin or NADPH:ferredoxin oxidoreductase gene within the ca. 25 kbp of the *Streptomyces* sp. strain C5 daunomycin biosynthesis gene cluster that we have sequenced, which includes DNA more than 6 kbp in either direction from *doxA* (13, 14, 32, 37). When genes for

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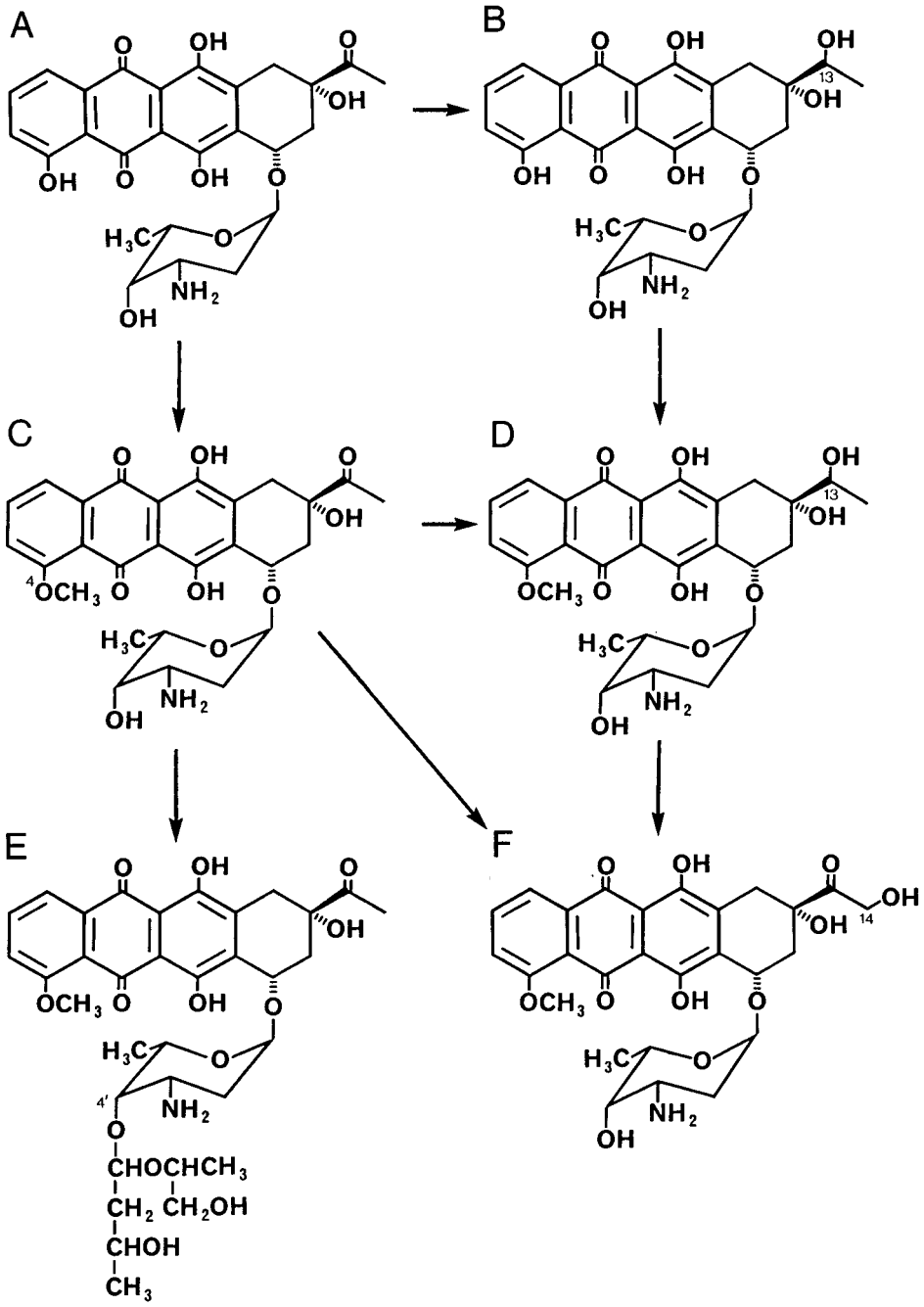

FIG. 1. Conversion of carminomycin (A) to 13-DHC (B) or daunomycin (C), which can be converted to 13-DHD (D), baumycins A1 and A2 (19, 23) (E) or doxorubicin (F). Evidence for reactions A→C, B→D, and A→C→D was shown previously (7). Reaction C→E is presumed because of the products formed by the strain (23), and evidence for reactions C→F and D→F is given in the text. (B, D, and F) Pertinent carbons for reactions described in the text are numbered in the structures.

other antibiotic biosynthesis-related P-450s have been identified (e.g., *eryF* [35] and *eryK* [30]), ferredoxin and NADPH: ferredoxin oxidoreductase genes also are not clustered with the P-450 genes. On the other hand, in *Streptomyces griseus* and *Streptomyces griseolus*, the genes encoding P-450 enzymes putatively involved in catabolic reactions, $P450_{SOY}$ and $P450_{SU1}$/$P450_{SU2}$, respectively, are linked with genes encoding the ferredoxin specific for their activities (25, 33).

Construction of *doxA* expression plasmid. A 1.67-kbp *Sal*I-*Sst*I fragment from pANT235 containing the 3′ end of *doxA* and the 5′ end of *dauI* was purified from an agarose gel and ligated into pUC19 to make pANT185 (Table 1). Plasmid pANT235 was used as the template in PCRs for the generation of the 5′ end of *doxA* containing a strong upstream ribosome binding site and appropriate restriction sites at both ends. The primers synthesized were 5′-GACATGCATGCGGAGGGGT GCCTC-3′ for the 5′ end (containing an *Sph*I site) and 5′-GACGCAGCTCCGGAACGGGG-3′ for the 3′ end (carrying a *Bsp*EI site). The amplified DNA fragment was digested with *Sph*I and *Bsp*EI to generate a 285-bp fragment that was ligated

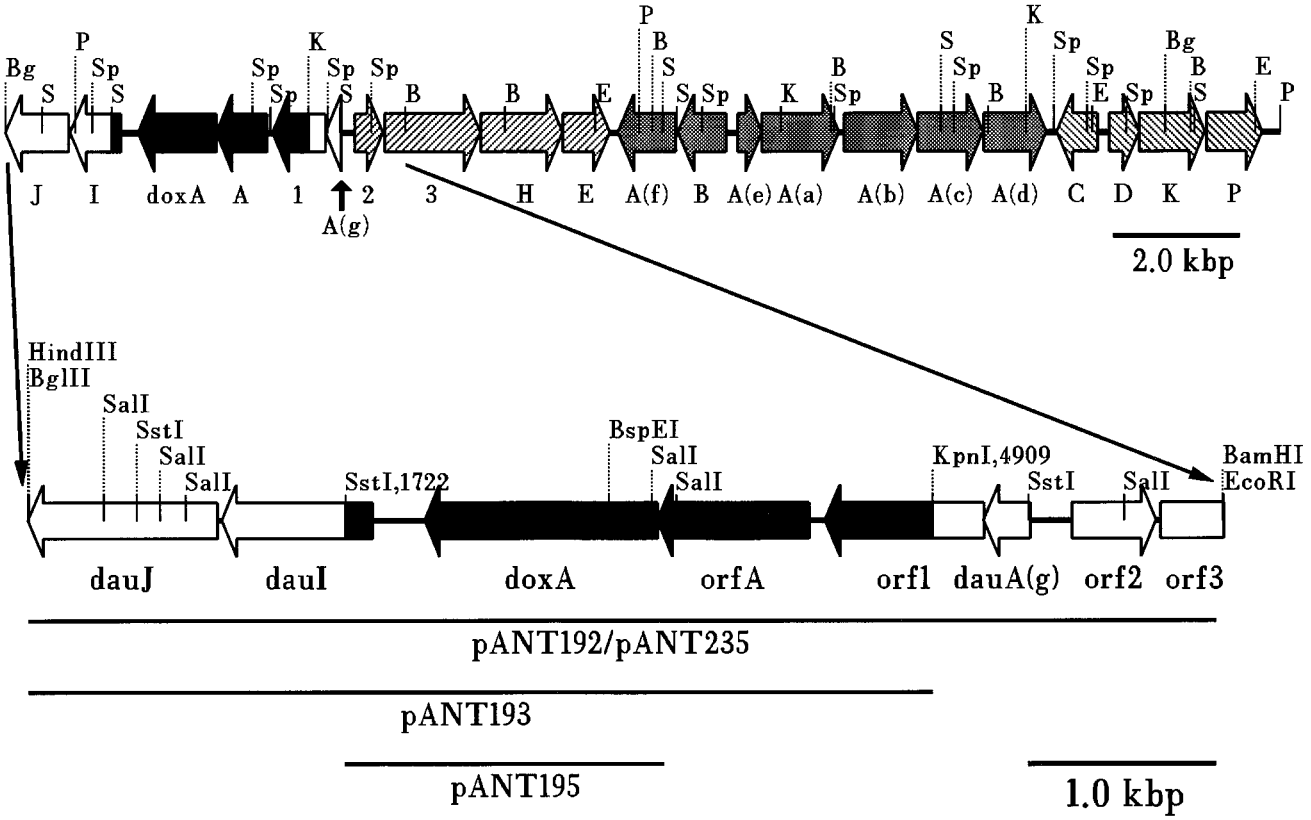

FIG. 2. Restriction map of daunomycin biosynthesis gene cluster from *Streptomyces* sp. strain C5, generated from complete sequence data (13, 14, 32, 37), with a more detailed restriction map of pANT235. The genes in the top diagram indicated by the right-slanting arrows, shaded arrows, and left-slanting arrows are described in references 14, 37, and 13, respectively. The DNA sequence of the 3,196-bp *Sst*I-*Kpn*I fragment shown in black is given in Fig. 3. The sequences and deduced products of *doxA* (daunomycin C-14 hydroxylase) and *orfA* (unknown function) are described in the text. The DNA contained within plasmid inserts described in Table 1 is shown at the bottom. Abbreviations for restriction endonuclease sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; S, *Sst*I; Sp, *Sph*I.

into similarly digested pANT185 to generate pANT186. The nucleotide sequence of the PCR product was confirmed by sequencing, and the 1.72-kbp *Sph*I-*Sst*I insert from pANT186, containing the complete *doxA* gene and a strong ribosome binding site (GGAGGT), was ligated into pANT849, a streptomycete expression vector constructed in our laboratory (10) from pANT42 (20), to form pANT195 (Table 1), which was introduced into *Streptomyces lividans* TK24 by protoplast transformation. In this construct, *doxA* expression was driven by the SnpR-activated *snpA* promoter, a very strongly expressed promoter in pANT849 (10).

Bioconversion of daunomycin to doxorubicin by recombinant *Streptomyces lividans* TK24. For the bioconversion studies, recombinant *Streptomyces lividans* strains were grown in 50 ml

TABLE 1. Bacterial plasmids used and constructed in this study

Plasmid	Relevant characteristics[a]	Source or reference
pUC19	2.686 kbp; Amp^r; *E. coli* plasmid	J. N. Reeve
pANT185	4.35 kbp; pUC19 containing a 1.67-kbp *Sal*I-*Sst*I construct of *doxA* from pANT235 in which the 5′ 27 nucleotides of *doxA* are absent	This work
pANT186	4.39 kbp; pANT185 in which the *Sph*I-*Bsp*EI fragment was replaced with a PCR-generated 285-bp *Sph*I-*Bsp*EI fragment that regenerated the 5′ end of *doxA* as well as a strong ribosome binding site and appropriate restriction sites	This work
pANT192	11.84 kbp; 6.52-kbp *Hin*dIII-*Eco*RI fragment from pANT235 (including the entire *Bgl*II-*Bam*HI fragment shown in Fig. 2) in pANT849	This work
pANT193	10.28 kbp; 4.95-kbp *Kpn*I-*Hin*dIII fragment from pANT235 in pANT849	This work
pANT195	7.04 kbp; pANT849 containing the 1.72-kbp *Sph*I-*Sst*I fragment insert containing intact *doxA* from pANT186	This work
pANT235	9.2 kbp; pUC19 containing a 6.48-kbp *Bam*HI-*Bgl*II DNA fragment from *Streptomyces* sp. strain C5 carrying the *dnrRI*-hybridizing region	37
pANT849	5.34 kbp; expression vector derived from pANT42 (20) by removal of a 1.95-kbp *Kpn*I fragment and a 1.42-kbp *Sph*I-*Mlu*I fragment and insertion of a 42-bp polylinker into the resulting *Sph*I-*Mlu*I sites; HC, Thi^r	10

[a] Abbreviations: HC, high-copy-number plasmid; Thi^r, thiostrepton resistance; Amp^r, ampicillin resistance.

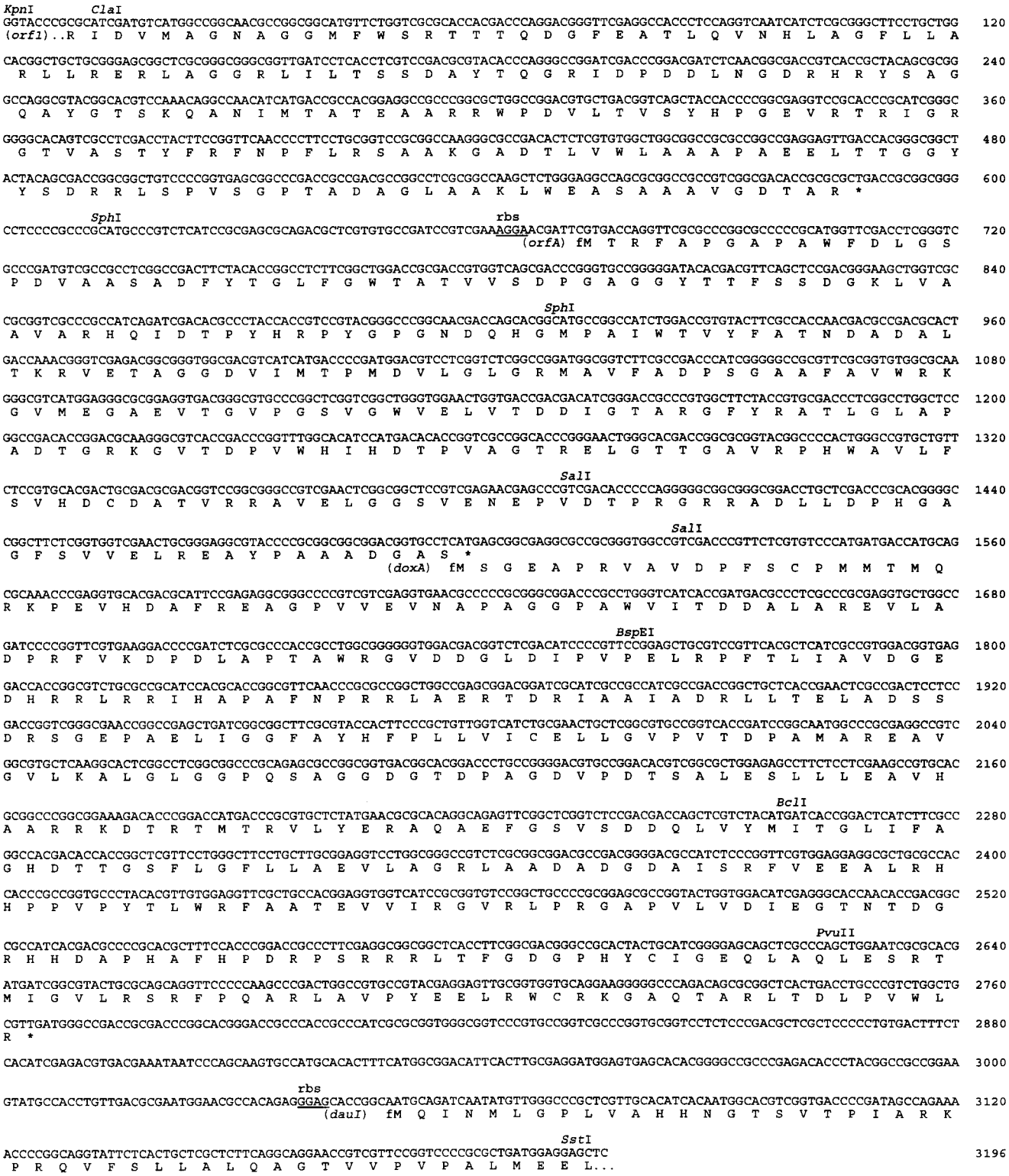

FIG. 3. Nucleotide sequence of the 3,196-bp DNA fragment from *Streptomyces* sp. strain C5 containing the daunomycin C-14 hydroxylase gene. The deduced amino acid sequence of the proposed translation product is given below the nucleotide sequence. The numbers on the right indicate nucleotide positions. Potential ribosome binding sites (rbs) are noted, as are significant restriction endonuclease sites.

of YEME medium plus 10 μg of thiostrepton per ml in a 250-ml Erlenmeyer flask containing a coiled spring (6). After incubation of the cultures at 30°C in a rotary shaker (250 rpm) for 48 h, 100 μg of filter-sterilized daunomycin in distilled water was added to each culture. The culture containing substrate was incubated for another 48 h, after which the whole culture broth was brought to a pH of 8.5 with 5 N NaOH and extracted with an equal volume of chloroform-methanol (9:1). The organic extract was dried, dissolved in methanol, and spotted onto 0.25-mm-diameter silica gel thin-layer chromatography plates (Whatman, Clifton, N.J.), which were developed with chloroform-methanol-acetic acid-water (80:20:16:6). The following compounds, detected by pigmentation and fluorescence under UV irradiation at 365 nm, had R_f values as indicated: carminomycin, 0.86; 13-dihydrocarminomycin (13-DHC), 0.70; daunomycin, 0.56; 13-dihydrodaunomycin (13-DHD), 0.39; and doxorubicin, 0.36. Anthracyclines also were separated, identified, and quantified by high-performance liquid chromatography (HPLC) (14) with a Waters C_{18} μBondapak reverse-phase column and a mobile phase of methanol-water (65:35) brought to pH 2.5 with 85% phosphoric acid. Bioconversion products were compared with authentic standards cochromatographed or run in parallel. HPLC retention times were 18.3 min for carminomycin, 15.8 min for 13-DHC, 13.3 min for daunomycin, 10.6 min for 13-DHD, and 8.4 min for doxorubicin.

The only theoretical monooxygenase-like (e.g., hydroxylation) steps in doxorubicin biosynthesis are (i) hydroxylation of C-11 of aklavinone to form ε-rhodomycinone, (ii) C-13 hydroxylation of 13-deoxycarminomycin to form 13-DHC, and (iii) hydroxylation of C-14 to convert daunomycin to doxorubicin (31, 32). The gene encoding C-11 hydroxylase (*dnrF*) has been previously shown to be located in a different part of the gene cluster in *Streptomyces peucetius* (16). In preliminary experiments, no apparent C-13 hydroxylase activity was observed to be conferred by the presence of pANT195 in *Streptomyces lividans* (12). Thus, we tested the ability of 48-h-old cultures of *Streptomyces lividans* TK24(pANT195) to hydroxylate daunomycin at C-14 to form doxorubicin. After 48 h of incubation, we observed a 100% conversion of daunomycin to doxorubicin by cultures of *Streptomyces lividans* TK24(pANT195), as determined by cochromatography with thin-layer chromatography and HPLC.

Control cultures of *Streptomyces lividans* TK24(pANT849), TK24(pIJ702), and TK24 (without a plasmid), grown, incubated with daunomycin, and analyzed as described above, converted 100% of the daunomycin to 13-DHD. Nonspecific reduction of daunomycin to 13-DHD has been observed previously (22, 26), suggesting that this reaction can be catalyzed by a common bacterial enzyme (or enzymes) of low substrate specificity.

Other *doxA* constructs were tested for their ability to confer daunomycin C-14 hydroxylation ability to *Streptomyces lividans.* *Streptomyces lividans* TK24(pANT192), containing the *dauA-orfg* gene (encoding acyl carrier protein) and its putative promoter, *orf1*, *orfA*, and *doxA*, converted approximately 25% of the daunomycin added to doxorubicin; the remaining 75% was converted to 13-DHD or not converted to any product. *Streptomyces lividans* TK24(pANT193), containing part of *orf1*, all of *orfA*, and *doxA* driven by the *snpA* promoter, converted 80% of the daunomycin to doxorubicin; 20% was converted to 13-DHD.

Analysis of bioconversion products. For mass spectrometry (MS) analysis, 500 μg of daunomycin was incubated for 48 h with 48-h-old cultures of *Streptomyces lividans*(pANT195) grown in YEME medium. The bioconversion product was extracted, dried, resuspended in 100 μl of methanol, spotted onto a 250-μm (thickness) layer (Aldrich, Milwaukee, Wis.) 20 by 20 cm glass-backed thin-layer chromatography plate, and separated from contaminants by chromatography for 2 h with a mobile phase of chloroform-methanol-acetic acid-water (80:20:16:6). The silica gel containing the product was scraped from the plate with a spatula and extracted three times each with 1.5 ml of methanol. The methanol extracts were combined, filtered through a 0.2-μm-pore-size Nylon Acrodisc 13 filter, and then dried in air. The dried product was resuspended in 500 μl of chloroform-methanol (9:1) and back-extracted with an equal volume of water (brought to pH 10.0 with Na_2CO_3), and the organic phase from this extraction procedure was removed and dried. The dried sample was resuspended in 500 μl of methanol, from which 50 μl was removed for HPLC and thin-layer chromatography analyses, and the remainder was redried for MS analysis. MS analysis was carried out with a SCIEX API III+ triple quadrupole mass spectrometer fitted with an atmospheric pressure chemical ionization source operating in the positive ion mode.

The product of daunomycin bioconversion by *Streptomyces lividans* TK24(pANT195) had an M+1 of 543.90, the same as that obtained with authentic doxorubicin. MS/MS analysis of the parent 543.90 peak revealed major fragmentation species of 396.80, 378.90, 361.00, and 130.20, identical to those observed after MS/MS analysis of the 543.65 M+1 parent peak of authentic doxorubicin. The product of daunomycin bioconversion by cultures of *Streptomyces lividans* TK24(pANT849) had an M+1 of 529.85 and MS/MS fragmentation species of 382.80, 364.60, 320.75, and 129.95, the same as those obtained with authentic 13-DHD.

Substrate specificity of DoxA. For substrate specificity studies, 48-h-old cultures of *Streptomyces lividans* TK24(pANT195) or TK24(pANT849) (control), grown in YEME medium containing 10 μg of thiostrepton per ml, were incubated for an additional 48 h in the same medium with an anthracycline substrate. The products were extracted and analyzed as described previously. Strain TK24(pANT195) converted >90% of 13-DHD to doxorubicin within 48 h. In control cultures, strain TK24(pANT849) did not convert 13-DHD to any observable product. These results suggest that two reactions, oxidation of the hydroxy to a keto group at C-13 and oxidation of the methyl to hydroxyl at C-14, occur as a result of the presence of DoxA in *Streptomyces lividans*; it is not probable that DoxA catalyzes both reactions; however, the mechanism for this bioconversion is unknown. The bioconversion of 13-DHD to doxorubicin also has been observed for a non-anthracycline-producing mutant of *Streptomyces peucetius* subsp. *caesius* (26). Cultures of TK24(pANT195) did not hydroxylate daunomycinone, carminomycin, 13-DHC, idarubicin (4-demethoxydaunomycin), or aklavin, indicating an apparently strict substrate specificity of DoxA for daunomycin and 13-DHD.

The fact that 13-DHD, a shunt product of daunomycin biosynthesis (7, 8), was not converted to 13-dihydrodoxorubicin suggests that formation of 13-dihydrodoxorubicin in vivo by *Streptomyces peucetius* and other doxorubicin-producing strains occurs by reduction of the 13-oxo group of doxorubicin rather than by C-14 hydroxylation of 13-DHD. Only trace levels of 13-dihydrodoxorubicin were observed in cultures of *Streptomyces lividans* TK24(pANT195) incubated with either daunomycin or 13-DHD, indicating that doxorubicin was probably not a good substrate for *Streptomyces lividans* TK24 C-13 ketoreductase activity.

Protection of anthracyclines by DoxA from reduction by *Streptomyces lividans* C-13 ketoreductase. Since cultures of *Streptomyces lividans* TK24(pANT849) converted daunomycin to

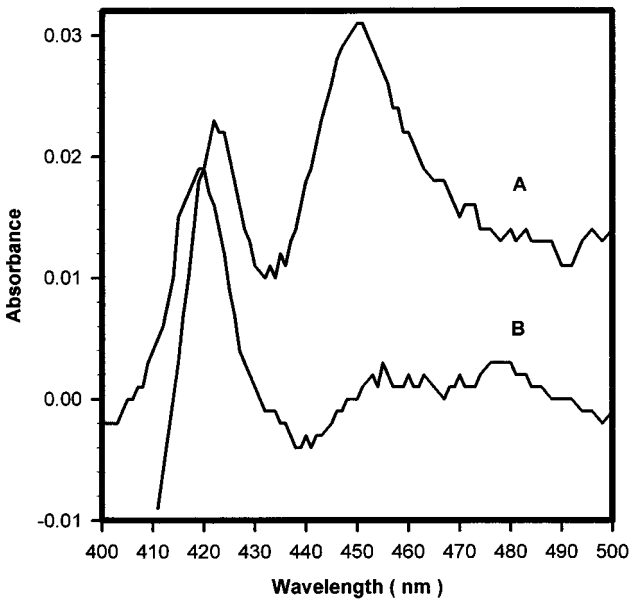

FIG. 4. Dithionite reduced-plus-CO minus reduced difference spectra of extracts prepared from cultures of *Streptomyces lividans* TK24(pANT195) (A) and TK24(pANT849) (B). Note the DoxA-specific peak at 450 nm in curve A.

13-DHD, in cultures of *Streptomyces lividans* TK24(pANT195) host C-13 ketoreductase activity and recombinant DoxA C-14 hydroxylase activity must compete for daunomycin. From the results observed, overexpressed DoxA must be able to outcompete the host C-13 ketoreductase for substrate. Similarly, incubation of strain TK24(pANT849) cultures with carminomycin, idarubicin, daunomycinone, or carminomycinone resulted in 100% bioconversion of these compounds to their 13-dihydro derivatives (12), indicating that *Streptomyces lividans* TK24 C-13 ketoreductase has a broad substrate specificity for these anthracyclines and anthracyclinones.

Incubation of TK24(pANT195) cultures, containing DoxA, with carminomycin or idarubicin, however, resulted in >85% recovery of these 13-oxo substrates (the remaining ca. 10 to 15% of each was converted to the 13-dihydro derivative). In the absence of additional data, we interpret these results to suggest that DoxA protects these substrates from *Streptomyces lividans* TK24 C-13 ketoreductase by an unknown mechanism. The anthracyclinones daunomycinone and carminomycinone, on the other hand, were converted to their 13-hydroxy homologs by TK24(pANT195), indicating that they were not protected by DoxA (12).

Spectral analysis of cytochrome P-450. Recombinant *Streptomyces lividans* TK24 strains were grown in YEME medium plus thiostrepton (10 μg/ml) for 48 h at 30°C, harvested, washed by centrifugation, and then broken in sodium phosphate buffer (100 mM, pH 7.5) in a French pressure cell at 15,000 lb/in^2. The extracts were clarified by pelleting cell debris and unbroken mycelia at 10,000 × *g* for 30 min (4°C), after which the supernatant was analyzed by visible spectrometry. Reference and experimental samples were reduced by a few grains of sodium dithionite. Experimental samples were bubbled with CO for 1 min prior to analysis. Spectra were obtained with a Beckman model DU-64 single-beam spectrophotometer and reduced-plus-CO minus reduced difference spectra were obtained by electronic subtraction.

Reduced-plus-CO minus reduced difference spectra of extracts of *Streptomyces lividans* TK24(pANT195) yielded a peak at 450 nm, characteristic of P450s (27), not observed in extracts of the control culture, *Streptomyces lividans* TK24(pANT849) (Fig. 4). Moreover, a polypeptide with an M_r of ca. 42,000, close to the predicted size of DoxA, was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in crude mycelial extracts of TK24(pANT195) that was not present in extracts of TK24(pANT849) (12). Despite repeated attempts, however, we have not yet observed in vitro DoxA activity in TK24(pANT195).

Daunomycin bioconversion to doxorubicin by *Streptomyces* sp. strain C5. Cultures of *Streptomyces* sp. strain C5 and C5 mutants with alterations in the *dauA74*, *dauCE147*, *dauE24*, and *dauH54* genes that were grown for 48 h in NDYE and incubated for 48 h with 100 μg of exogenously added daunomycin converted ca. 10% of the substrate to doxorubicin. The remainder (ca. 90%) of the daunomycin was converted to compounds that were hydrolyzable to daunomycin with oxalic acid (i.e., baumycins A1 and A2) (19, 24). These results indicate that *Streptomyces* sp. strain C5 has the genetic and enzymatic capability to produce doxorubicin but that other reactions, e.g., formation of baumycins A1 and A2, outcompete native DoxA, which is probably produced at very low levels. These experiments suggest that a double mutant of *Streptomyces* sp. strain C5 deficient in baumycin production and upregulated for DoxA activity should produce doxorubicin.

Nucleotide sequence accession number. The DNA sequence described in this paper has been deposited in GenBank under the accession no. U50973.

We are deeply indebted to Chuck DeSanti for pANT849, to Kevin M. Downard for assistance with the MS and MS/MS analyses, and to Nigel Priestley for discussion of the results. We also thank J. Lunel of Rhône-Poulenc, Inc., for authentic samples of carminomycin and 13-dihydrocarminomycin, and Adria, Inc., and the Frederick Cancer Research Center for additional authentic anthracycline and anthracyclinone samples.

This work was supported by the National Science Foundation (grant DMB-94-05730).

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