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Functional Characterization and Transcriptional Analysis of a Gene Cluster Governing Early and Late Steps in Daunorubicin Biosynthesis in *Streptomyces peucetius*

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Sequence analysis of the *Streptomyces peucetius* daunorubicin biosynthetic gene cluster revealed a partial (*dnrQ*) and two complete (*dnrD* and *dnrP*) open reading frames flanking *dnrK*. Bioconversion experiments showed that DnrD converts aklanonic acid methylester to aklaviketone and that DnrC is a methyltransferase that converts aklanonic acid to aklanonic acid methylester. The deduced *dnrP* gene product, homologous to known esterases, may catalyze the conversion of 10-carbomethoxy-13-deoxycarminomycin to its 10-carboxy derivative. The *dnrKPQS* genes may be transcribed as a polycistronic mRNA.

Daunorubicin (DNR) and its C-14 hydroxylated derivative doxorubicin (DXR) are potent antitumor anthracycline antibiotics produced by *Streptomyces peucetius* and widely used in cancer chemotherapy. DNR biosynthesis is initiated by a type II polyketide synthase (8, 32) from one propionyl-coenzyme A and nine malonyl-coenzyme A precursor units (28). The early steps in DNR biosynthesis have been well characterized by the elucidation of the structures of anthracyclinone metabolites isolated from DNR fermentations (7), by the isolation and characterization of mutants blocked in DNR biosynthesis (1), and by enzyme assays (2). These steps (Fig. 1) result in the formation of ε-rhodomycinone (RHO), a predominant metabolite in *S. peucetius* fermentations (28). Daunosamine, the 2,3,6-trideoxy-L-amino sugar moiety of DNR, is synthesized separately and attached to RHO, resulting in the formation of 10-carbomethoxy-13-deoxycarminomycin (28). Even though the events that lead to the formation of carminomycin (CAR), DNR, and DXR from this glycoside are not understood completely, a hypothetical set of reactions has been proposed (3, 28).

To understand the regulation of antibiotic biosynthesis and to facilitate design of novel anthracyclines (11, 14), we have cloned and characterized several genes that code for enzymes involved in DXR biosynthesis (8, 19, 22) and for DNR-DXR resistance determinants (9, 29) and regulatory elements (17, 21, 29). Genes coding for the DNR polyketide synthase enzyme complex from *Streptomyces* sp. strain C5 (32) have also been cloned and sequenced. A DNA fragment that complements a mutation blocking an early step in DNR synthesis by *Streptomyces griseus* was recently shown by sequence analysis to

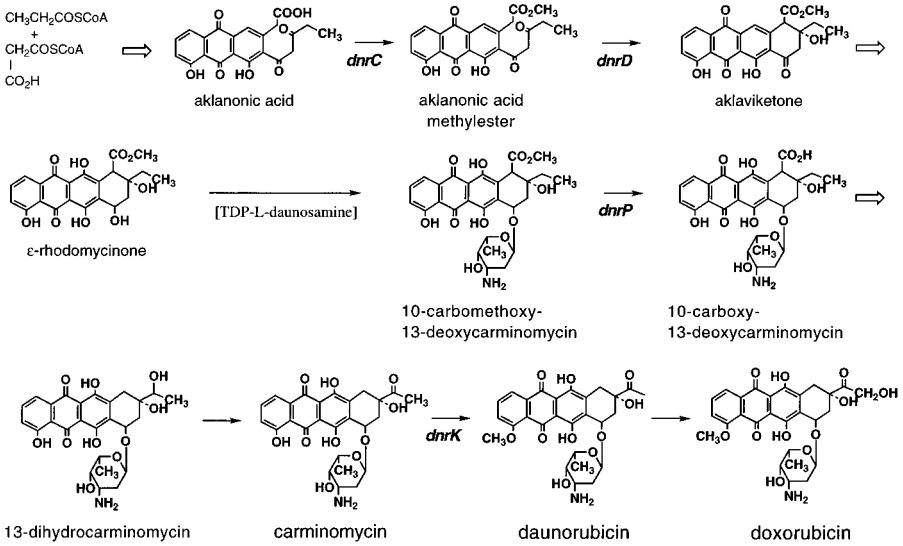

FIG. 1. DNR biosynthesis pathway. Only relevant intermediates and *dnr* genes discussed in the text are shown. Thick open arrows indicate that several enzymatic steps are necessary for the conversion. Thin arrows indicate a single enzyme-catalyzed reaction. 13-DeoxyCAR is the product of the decarboxylation of 10-carbomethoxy-13-deoxyCAR. 10-Carbomethoxy-13-deoxyCAR and its 10-carboxy derivative have been isolated from mutants of the DNR-producing *Streptomyces* sp. strain D788 (33).

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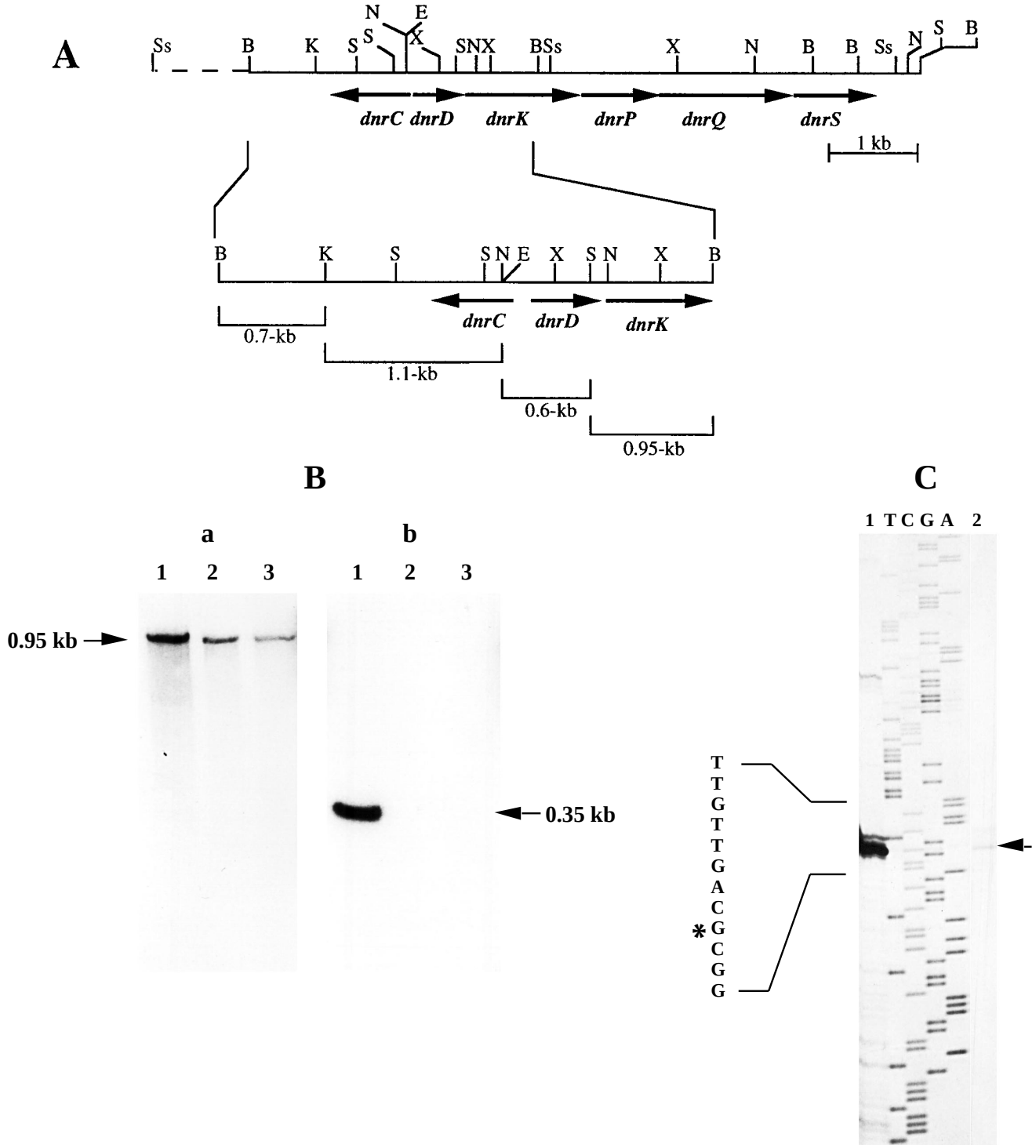

FIG. 2. (A) A restriction map of the region of the *S. peucetius* genome that contains genes governing late steps in DNR biosynthesis. The map of the 3.35-kb *Bam*HI DNA fragment containing the apparent start site of the *dnrDKPQS* transcript with the four DNA fragments used in low-resolution S1 analyses is shown in the lower portion. The location and the direction of transcription of *dnrC*, *dnrD*, and *dnrK* are indicated by arrows. The *dnrK* gene extends beyond the *Bam*HI site at the right end. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; N, *Nco*I; K, *Kpn*I; S, *Sph*I; Ss, *Sst*I; and X, *Xho*I. (B) S1 nuclease protection analysis of the *dnrDKPQS* transcript in *S. peucetius*. (a) Low-resolution analysis of mRNA isolated from 24-, 48-, and 72-h cultures with the 0.95-kb *Bam*HI-*Sph*I DNA fragment (lanes 1 to 3, respectively). mRNA (40 μg), isolated according to a previously published protocol (9), was hybridized overnight with the DNA fragment at 55°C and digested with 150 U of S1 nuclease under standard conditions (23), and the reaction mixture was resolved on a 4% denaturing polyacrylamide gel and blot transferred to a Hybond N membrane. The membrane was probed with the ^{32}P-labelled 0.95-kb *Sph*I-*Bam*HI DNA fragment. (b) Low-resolution analysis of mRNA from a 24-h culture with the 0.6-kb *Eco*RI-*Sph*I, 1.1-kb *Eco*RI-*Kpn*I, and 0.7-kb *Bam*HI-*Kpn*I DNA fragments (lanes 1 to 3, respectively). The membrane was probed with the ^{32}P-labelled 3.35-kb *Bam*HI DNA fragment (shown in panel A). The locations of the protected fragments are indicated by arrows. (C) Determination of the apparent transcriptional start point of the *dnrDKPQS* mRNA. A 24-mer oligodeoxynucleotide primer (5′-GTTGTATGCCTCCACCATGCGGCG-3′), complementary to a sequence 24 nucleotides downstream of the predicted translation start codon of *dnrD*, was annealed to the M13mp18 single-stranded template containing a 0.35-kb *Eco*RI-*Xho*I DNA fragment, and a labelling reaction was performed with the Sequenase enzyme to obtain single-stranded uniformly labelled probe according to the method of

contain several genes that are implicated in daunosamine biosynthesis (16).

In the present investigation we have cloned and tentatively characterized a gene that codes for a late-acting enzyme in DNR biosynthesis and two genes coding for early pathway enzymes that convert aklanonic acid to aklaviketone.

Cloning, sequencing, and analysis of the *dnrDP* genes. Since antibiotic biosynthesis genes are commonly clustered, the DNA on both sides of the previously reported *dnrK* gene (19), present at the left end of a 5.8-kb *Sph*I fragment (Fig. 2A), was analyzed in order to identify other DNR biosynthesis genes. DNA fragments subcloned in M13 vectors (31) were sequenced according to the previously published strategy (19). Two complete and one partial open reading frame, *dnrD*, *dnrP*, and *dnrQ* (Fig. 3), respectively, were identified by CODON PREFERENCE analysis (5) through the high G+C content characteristic of the third position of codons of *Streptomyces* genes. *dnrD* is located on the right side, and *dnrPQ* are located on the left side, of *dnrK*. Sequence analysis of the remaining portion of the 5.8-kb *Sph*I fragment has been completed, and it contains the rest of the *dnrQ* gene and *dnrS*, both of which have been identified as daunosamine biosynthesis genes (20). The *dnrP* gene is 897 nucleotides long and is presumed to code for a protein of 298 amino acids with a molecular weight of 32,228. Comparison of the deduced protein sequence with the protein and gene sequence data banks by FASTA and TFASTA analyses (5) indicated that DnrP shows 50 and 53% similarity, respectively, to the poly(3-hydroxyalkanoate) depolymerase from *Pseudomonas oleovorans* (13) and to esterase V from *Pseudomonas* sp. strain KWI-56 (25). *dnrD* is 438 nucleotides long and is presumed to code for a protein of 145 amino acids with a molecular weight of 16,715. The deduced protein sequence of DnrD did not show significant similarity to available protein sequences in the data banks by FASTA and TFASTA analyses (5), except for its close homolog DauD from another DNR producer, *Streptomyces* sp. strain C5 (6).

Analysis of the functions of *dnrC* and *dnrD*. The functions of the adjacently located and divergently transcribed *dnrD* and *dnrC* (previously named *ORF5* [8]) genes were tested on the basis of the ability of their gene products to bioconvert intermediates in DNR biosynthesis. *Streptomyces lividans* TK24 (12) transformants containing pWHM918 (carrying *dnrC*) and the *dnrI*::*aphII* strain (WMH1445) containing pWHM917 (carrying *dnrD* [the constitutive *ermE** promoter that regulates *dnrD* expression is not affected by the *dnrI*::*aphII* mutation]) were able to bioconvert aklanonic acid to aklanonic acid methylester (AAME) and AAME to aklaviketone (Fig. 1), respectively, according to the results of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analyses (data not shown). The R_f values on TLC, retention time on HPLC, and the fluorescence of the products under acidic, basic, and neutral conditions were identical to those of authentic AAME and aklaviketone. To confirm the identity of the products of bioconversion, AAME and aklaviketone were purified by TLC from cultures bioconverting aklanonic acid and AAME and fed to the WMH1535 strain, a Δ*dpsB* mutant blocked in the first step of DNR biosynthesis (8). Most of the added AAME- and aklaviketone-like compounds were bioconverted to RHO by this strain (Fig. 4B), suggesting that the products of bioconversion of aklanonic acid and AAME by the appropriate strains are indeed AAME and aklaviketone, respectively. These deductions are fully consistent with the functions of the homologous *Streptomyces* sp. strain C5 *dauC* and *dauD* genes (6). The deduced sequences of the *dnrC* and *dnrD* gene products are 88.6 and 95.9% identical to those of the deduced products of the *dauC* and *dauD* genes, respectively. However, the *dnrD* gene product is shorter than the deduced *dauD* gene product by 16 amino acids. Recently, *dnrD* was overexpressed in *Escherichia coli*, and the estimated size of DnrD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was in reasonable agreement with the deduced size (18).

Analysis of the function of *dnrP*. Although we were unable to disrupt the chromosomal *dnrD* and *dnrP* genes, we were able to isolate mutants blocked in *dnrK* by gene disruption as follows. A 1.6-kb *Sst*I fragment containing the *aphII* neomycin-kanamycin resistance gene was cloned into the *Sst*I site of the *dnrK* gene. The DNA segment containing the mutated *dnrK* was then cloned into the integrating vector pDH5 (10) that does not have a *Streptomyces* origin of replication and used to replace the wild-type copy in the genome according to previously published protocols (21). Two mutants, WMH1536 and WMH1537, with the desired genotype were obtained; in each of these strains the wild-type copy of *dnrK* is replaced by the mutated copy, as confirmed by Southern analysis (data not shown). WMH1536 and WMH1537 accumulated RHO, and the mutation in the WMH1536 strain was complemented by pWHM907 containing the *dnrKPQS* genes but not by pWHM902 containing *dnrK* alone, suggesting that the introduction of *aphII* into *dnrK* had inactivated the *dnrKPQS* genes. The WMH1536 strain was able to bioconvert 13-deoxyCAR to CAR and DNR to DXR. This indicates that *dnrP* is either a daunosamine biosynthesis gene or one of the two genes necessary for the conversion of 10-carbomethoxy-13-deoxyCAR (Fig. 1) to 13-deoxyCAR (28). The similarity of DnrP to esterase V and poly(3-hydroxyalkanoate) depolymerase suggests that DnrP may remove the methyl group from 10-carbomethoxy-13-deoxyCAR to yield 10-carboxy-13-deoxyCAR, as has been predicted for the DauP enzyme in *Streptomyces* strain C5 (6).

To distinguish between the two possible functions of *dnrP*, several gene cassettes containing different combinations of the *dnrDKPQS* genes were made (Table 1) and introduced into the WMH1536 mutant and other strains. The transformants were analyzed after 96 h of growth in the GPS production medium (4) for the presence of anthracyclines. The WMH1536 transformants containing *dnrDQS* or *dnrQS* on the low-copy-number vector pWHM601 (9) accumulated two new metabolites in addition to RHO. The retention times of the new metabolites were identical to those of authentic 13-dihydroCAR and DNR by HPLC analysis (data not shown). To further characterize the compound similar to DNR, it was purified from a TLC plate and hydrolyzed with 0.1 N HCl for 60 min at 90°C. The resulting product and the hydrolysis product of authentic DNR

Sharrocks and Hornby (24). The labelled DNA was purified on a denaturing polyacrylamide gel (23) after digesting the labelling reaction mixture with an enzyme that cut the probe DNA at its 3′ end. The labelled DNA was used in high-resolution S1 nuclease protection analysis according to standard conditions of hybridization and digestion (23). Primer extension analysis was performed by the method of Stein et al. (26), with modifications described elsewhere (9). mRNA (40 μg) from a 24-h culture of *S. peucetius* was annealed to the 24-mer oligodeoxynucleotide and extended with murine leukemia virus reverse transcriptase. The product of the reaction was resolved on a denaturing 6% polyacrylamide gel. Lane 1 shows the product of the primer extension reaction. Lanes T, C, G, and A show the dideoxy sequencing ladder generated from the 0.35-kb *Eco*RI-*Xho*I DNA fragment with the same primer. The ∗G in the portion of the sequence shown indicates the transcriptional start site. Lane 2 shows the product of the high-resolution S1 nuclease protection analysis reaction. The arrow indicates the location of the protected fragment.

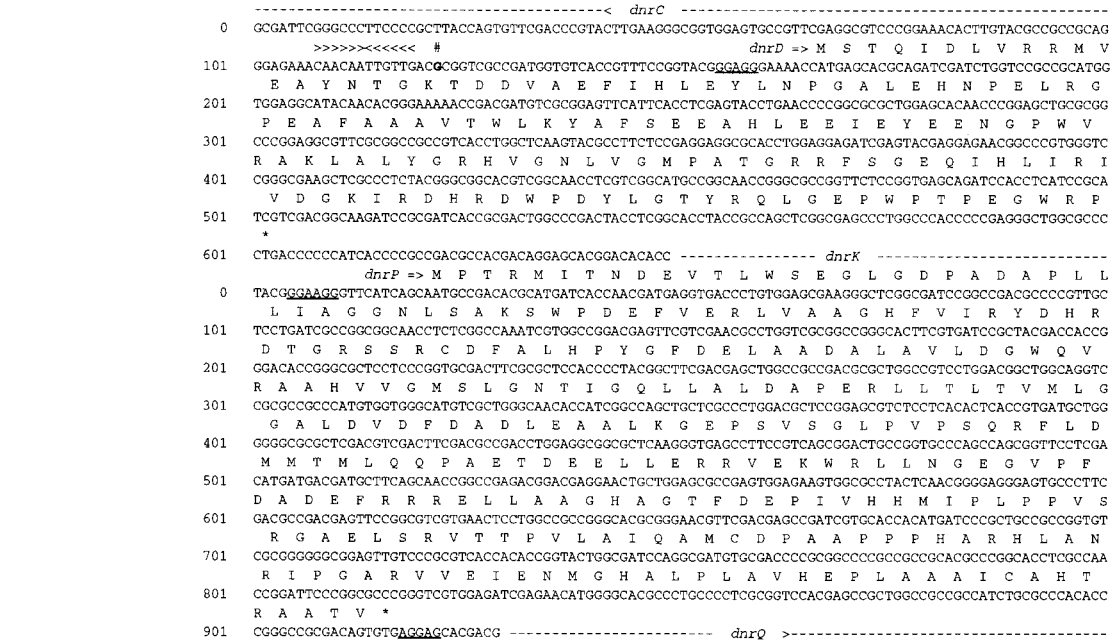

FIG. 3. The nucleotide sequence of the region containing *dnrD* and *dnrP*. The locations of the previously described DNA sequences containing the *dnrC* (*ORF5* [8]) and *dnrK* (19) genes are indicated by dashed lines. The predicted translational start codon of *dnrQ* is located at the left end of the dashed line shown after the sequence of *dnrP*. Putative ribosomal binding sites are underlined, an inverted repeat sequence is indicated by arrows above the sequence, and the apparent transcript start site is indicated (#) above the boldfaced G.

had identical retention times by HPLC analysis (Fig. 4A). This suggests that the DNR-like compound is glycosylated. Since *dnrQS* appear to be daunosamine biosynthesis genes (20) and since the lack of even a single daunosamine gene causes the mutant to accumulate RHO, we conclude that *dnrP* is not a daunosamine biosynthesis gene and that DnrP acts after the synthesis of the first glycosylated intermediate.

A 42-bp in-frame insertion mutation at the *Sst*I site in the *dnrK* gene unexpectedly caused the mutant strain WMH1538 to accumulate RHO. In this case, however, the *dnrK* gene (introduced as pWHM902 [19]) restored DNR production, indicating that the mutation had inactivated only *dnrK*. The D-to-A mutation at position 184 (D184A) in the putative *S*-adenosylmethionine cofactor binding site (15) completely abolished DnrK activity when assayed in *E. coli*(pWHM919) transformants as described previously (19), but WMH1538 (pWHM919) transformants still accumulated RHO and not CAR. Since the lack of CAR accumulation is not due to rapid degradation of CAR (data not shown), the anomalous accumulation of RHO instead of CAR may reflect a second unknown function of DnrK not revealed by the effect of the D184A mutation (for instance, the ability of DnrK to act as both an enzyme and an essential component of a multienzyme complex involved in the conversion of RHO to DNR).

Transcript mapping. Since the *dnrK*::*aphII* mutation appeared to be polar on expression of the downstream *dnrPQS* genes, we sought to detect and determine the time of appearance of the putative polycistronic mRNA. A 0.95-kb *Bam*HI-*Sph*I DNA fragment containing the first half of *dnrK* and some upstream sequence (Fig. 2A) was cloned in M13 in the appropriate orientation and used in a low-resolution S1 nuclease protection experiment (Fig. 2B). This portion of the transcript was present in mRNA isolated from 24-, 48-, and 72-h cultures. HPLC analysis of culture extracts indicated the presence of DNR at these sampling times.

Since the whole 0.95-kb *Bam*HI-*Sph*I DNA fragment was protected, it was conceivable that *dnrD* and *dnrK* are cotranscribed. To identify the apparent start site of such a transcript, three DNA fragments, 0.6-kb *Eco*RI-*Sph*I, 1.1-kb *Eco*RI-*Kpn*I, and 0.7-kb *Bam*HI-*Kpn*I, from the 3.35-kb *Bam*HI DNA seg-

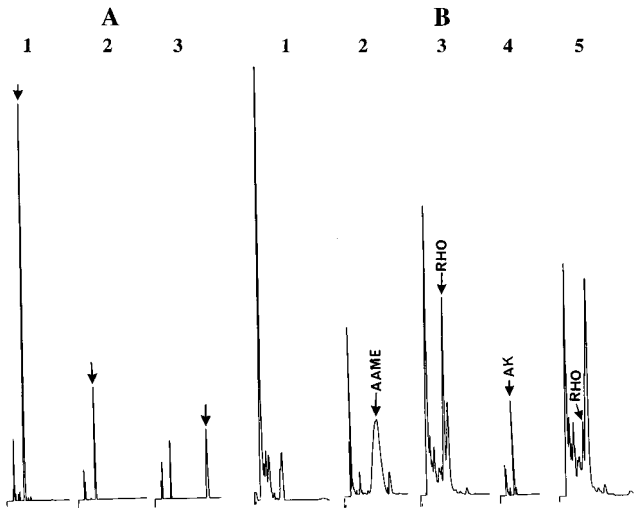

FIG. 4. HPLC analysis of extracts of *Streptomyces* cultures. The solvent system used was 60% methanol in water (pH adjusted to 2.0 with phosphoric acid), and the flow rate was 3 ml/min. (A) Analysis of the products of acid hydrolysis of anthracycline metabolites isolated from the WMH1536(pWHM916) and the wild-type *S. peucetius* strains. (1) Hydrolyzed DNR; (2) hydrolyzed DNR-like compound; and (3) unhydrolyzed DNR-like compound. Arrows indicate the locations of the appropriate anthracyclines. (B) Analysis of the products of bioconversion of AAME and aklaviketone by the WMH1535 strain. (1) Extract of WMH1535 culture without any supplementation; (2) AAME purified from *S. lividans* transformants bioconverting aklanonic acid; (3) extract of WMH1535 cultures bioconverting the purified AAME; (4) aklaviketone purified from WMH1445 transformants bioconverting AAME; and (5) extract of WMH1535 cultures bioconverting the purified aklaviketone. AK, aklaviketone.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Reference or source
Strains		
S. peucetius WMH1536	*aphII* gene inserted into the *Sst*I site of the *dnrK* gene	This study
S. peucetius WMH1538	42-bp linker inserted into the *Sst*I site of the *dnrK* gene	This study
Plasmids		
pWHM3	High-copy-number *E. coli* and *Streptomyces* shuttle vector	30
pWHM601	Low-copy-number *E. coli* and *Streptomyces* shuttle vector	9
pWHM907	5.8-kb *Sph*I fragment containing *dnrKPQS* cloned into pWHM601	This study
pWHM912	3.25-kb *Sac*II fragment containing *dnrQS* cloned into pWHM601	This study
pWHM913	0.83-kb DNA fragment containing *dnrD* cloned into pWHM3, using a synthetic linker	This study
pWHM916[a]	3.92-kb DNA fragment containing *dnrDQS* cloned into pWHM601	This study
pWHM917	Insert in pWHM916 cloned under the control of *ermE** promoter in pWHM601	This study
pWHM918	1.42-kb *Xho*I-*Kpn*I fragment containing *dnrC*, cloned into *Sal*I-*Kpn*I sites of pWHM3	This study
pWHM919	pWHM907 (19) containing a mutated *dnrK* gene (created by the method used in reference 21) that produces the D184A DnrK protein.	This study

[a] Cassettes containing *dnrDQS* were made with the help of a synthetic linker containing the *Nco*I, *Sac*II, *Xho*I, and *Sst*I sites (5′-CATGGTCTGACCGCGGCTC GAGCT-3′ and 5′-CAGACTGGCGCCGAGC-3′). A 0.83-kb *Eco*RI-*Nco*I fragment containing *dnrD* was cloned into the *Eco*RI and *Sst*I sites of pWHM3 with the help of the linker having *Nco*I and *Sst*I sticky ends, to obtain pWHM913. The *dnrQS* genes were cloned downstream of *dnrD* as follows. The 3.07-kb *Sac*II-*Stu*I segment containing *dnrQS* was cloned downstream of *dnrD* (excised from pWHM913 as an *Eco*RI-*Sac*II fragment) in pWHM3 at its *Eco*RI and *Bam*HI sites (after blunt ending the *Bam*HI site), to obtain pWHM915. Then the *dnrDQS* genes from pWHM915 were cloned into pWHM601 as an *Eco*RI-*Hin*dIII fragment, to obtain pWHM916.

ment upstream of *dnrK* (Fig. 2A) were cloned in M13 and used in low-resolution S1 nuclease protection experiments. A 0.35-kb fragment was protected when the 0.6-kb *Eco*RI-*Sph*I DNA fragment was used, suggesting that the *dnrK* transcript might start upstream of *dnrD* (Fig. 2B). However, this analysis does not unambiguously rule out the possibility of two different transcripts, one coding for DnrD and the second one coding for DnrK (and possibly DnrPQS, as suggested by the results of the *dnrK*::*aphII* complementation experiments described above). A 24-mer oligodeoxynucleotide corresponding to a sequence close to the likely translation initiation site of *dnrD* was synthesized and used in high-resolution S1 nuclease protection and primer extension analysis experiments. The results (Fig. 2C) show that an apparent mRNA 5′ end is located at a G 44 nucleotides upstream of the predicted translational start codon of *dnrD*. The fainter bands above and below the strong signal in the lane containing the products of the primer extension reaction (Fig. 2C) may represent low-frequency mispriming or low-abundance transcripts. Comparison of the sequence immediately upstream of the start site of the transcript coding for DnrD with those of a large number of *Streptomyces* promoter sequences (27) did not yield any consensus sequences typical of *Streptomyces* promoter regions.

Nucleotide sequence accession number. The DNA sequence data described in this paper have been deposited at EMBL and GenBank with accession number L40425.

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