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Biochemistry

A bacterial analog of the *mdr* gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin

(ABC proteins/antiporter/antibiotic resistance)

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ABSTRACT **Sequence analysis of the *drrAB* locus from *Streptomyces peucetius* (American Type Culture Collection 29050) reveals the presence of two genes, *drrA* and *drrB*, both of which are required for daunorubicin and doxorubicin (Adriamycin) resistance in the heterologous host *Streptomyces lividans*. The DrrA protein is similar to a large family of ATP-binding transport proteins, including the proteins encoded by the *mdr* genes from mammalian tumor cells, which confer resistance to daunorubicin, doxorubicin, and some other structurally unrelated chemotherapeutic agents. The DrrB protein shows no significant similarity to other known proteins but is probably very hydrophobic, suggesting that it is located in the bacterial membrane. These two proteins may act jointly to confer daunorubicin and doxorubicin resistance by an analog of the antiport mechanism established for mammalian tumor cells that contain amplified or overexpressed *mdr* genes. Transcriptional analysis of the *drrAB* region supports the presence of one transcript containing *drrA* and *drrB* and indicates that these genes are expressed only during antibiotic production.**

Daunorubicin (Dnr) and doxorubicin (Dxr) are clinically important antitumor agents used to treat many types of cancer (1). A factor limiting the effectiveness of Dnr and Dxr therapy is the development of resistance to these agents by tumor cells. One of the most important causes of resistance is the expression of one or more of a family of proteins (P-glycoproteins), encoded by various *mdr* genes (2). P-glycoproteins cause the export of a variety of chemotherapeutic agents, including Dnr and Dxr, by an ATP-dependent antiport mechanism. The protein itself has two almost identical ATP-binding domains and a large hydrophobic domain consisting of several transmembrane regions (3). Current evidence suggests that the ATP-binding domains are required for energizing transport and that the hydrophobic domain anchors P-glycoprotein in the cell membrane and operates, perhaps as a pore, in facilitating extrusion of various cytotoxic agents (2). Sequence analysis indicates that P-glycoproteins are similar to a large family of bacterial transport proteins (4), leading to the speculation that *mdr* genes and genes encoding bacterial transport proteins share a common ancestry (5).

Streptomyces peucetius (American Type Culture Collection 29050 and 27952) produce Dnr and Dxr and are resistant to these two drugs as shown by the existence of Dnr-sensitive mutants of the 27952 strain (6). To investigate the mechanism of bacterial Dnr and Dxr resistance, we have cloned a Dnr/Dxr resistance locus, *drrAB*, from the wild-type 29050 strain. The results of expression of *drrAB* in *Streptomyces lividans* and sequence analysis† suggest that *drrAB* encodes two translationally coupled proteins, both of which are required for Dnr and Dxr resistance in this host. The sequence of the predicted product of *drrA* is similar to the products of other transport and resistance genes, most notably the P-glycoproteins from mammalian tumor cells. The product of *drrB* shows no significant similarity to other known proteins but is very hydrophobic, suggesting that this protein is located in the cell membrane. This intriguing similarity to Mdr suggests that DrrA uses ATP hydrolysis to energize transport of Dnr and Dxr via the presumably membrane-bound DrrB protein.

Transcriptional analysis indicates that the drrAB mRNA contains only drrA and drrB and further suggests that *drrAB* expression may be regulated so that the genes are only transcribed during antibiotic production.

MATERIALS AND METHODS

Biochemicals and Chemicals. Apramycin (Ap) was obtained from Eli Lilly; Dxr and Dnr were obtained from Sigma or from Farmitalia Carlo Erba; thiostrepton was obtained from Squibb. Restriction enzymes and other molecular biology reagents were obtained from standard commercial sources.

Bacterial Strains and Plasmids. *S. lividans* 1326 was obtained from David Hopwood (John Innes Institute and Agricultural and Food Research Council Institute of Plant Science, Norwich, U.K.). *S. peucetius* 29050 was obtained from the American Type Culture Collection. pGEM7zf(+) was obtained from Promega. pKC505 was obtained from Richard Baltz (Eli Lilly).

Growth Conditions. *S. lividans* 1326 was grown on R2YE medium (7) for protoplast regeneration, ISP4 (Difco) for spore preparation, tryptic soy agar (Difco) for resistance determinations, and precultivation medium containing 10 mM $CaCl_2$ (8) for plasmid isolations. Ap and thiostrepton concentrations of 50 μg/ml were used for selection of *S. lividans* transformants and maintenance of plasmids.

S. peucetius 29050 was grown in APM medium [consisting of yeast extract at 8 g/liter, malt extract at 20 g/liter, NaCl at 2 g/liter, Mops at 15 g/liter, 4 ml of antifoam B (Sigma), 1 ml of 10% (wt/vol) $MgSO_4$, 1 ml of 1% $FeSO_4$, 0.1 ml of 10% (wt/vol) $ZnSO_4$, 880 ml of tap water, 120 ml of a 50% (wt/vol) glucose solution added after autoclaving] for RNA isolation. Spores were inoculated into 5 ml of seed medium [consisting of yeast extract at 4 g/liter, malt extract at 10 g/liter, NaCl at 2 g/liter, $MgSO_4$ at 0.1 g/liter, and Mops sodium salt at 15 g/liter plus 10 ml of trace elements (7), 940 ml of tap water,

Abbreviations: Ap, apramycin; Dnr, daunorubicin; Dxr, doxorubicin; nt, nucleotide; ORF, open reading frame; RBS, ribosome binding site.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M73758).

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and 50 ml of a 50% (wt/vol) glucose solution added after autoclaving] and grown at 30°C in a 30-ml test tube for 20–25 hr. The 5-ml culture was inoculated into a 250-ml baffled flask containing 25 ml of APM medium and grown for an additional 20–60 hr.

Analysis of Drug Resistance. Clones of the *drrAB* region were tested in *S. lividans* initially using gradient plates (9). Further testing was done by either replica-plating Ap-resistant colonies to tryptic soy agar plates (Difco) containing Ap at 50 μg/ml and of Dnr at 5 μg/ml or by directly overlaying *S. lividans* on R2YE medium regeneration plates 20–24 hr after transformation with 2 ml of sterile, double-distilled water containing sufficient Dxr and Ap to give final concentrations of 100 and 50 μg/ml, respectively. Efficiency of plating analysis was done by plating spores of *S. lividans* transformants on tryptic soy agar plates containing Ap at 50 μg/ml and different concentrations of Dnr or Dxr.

DNA and RNA Isolation and Manipulation. DNA isolation was done by slightly modifying standard methods (7, 10–13). Exonuclease III deletions were made by using the Promega Erase-a-Base kit, according to the manufacturer's protocol. The shortened inserts were cleaved from the original plasmid by restriction enzyme digestion and then ligated into another plasmid. DNA fragments were purified from gels by using NA45 DEAE membrane (Schleicher & Schuell) according to the manufacturer's protocol, but the DNA was eluted into a 2.5 M ammonium acetate buffer and precipitated a single time with isopropanol. RNA was isolated essentially by the method of Fisher and Wray (14), except that cells were lysed by grinding with a mortar and pestle in the presence of liquid nitrogen and then adding lysis buffer.

DNA Sequencing. M13 clones were sequenced by the dideoxynucleotide chain-termination method by using Sequenase 2.0 or *Taq* DNA polymerase, according to the manufacturer's protocol, but the termination reactions were run at 5–10°C above standard temperatures. 7-Deaza-dGTP was used for dGTP to reduce the number of sequencing artifacts.

Northern (RNA) Hybridization. RNA samples were run in glyoxal dimethyl sulfoxide gels and blotted to a solid support (Hybond-N; Amersham) by capillary transfer using an alkaline elution buffer (13). Probes were prepared by nick-translating gel-purified DNA fragments (13). Prehybridizations and hybridizations were done at 45°C, as described by the manufacturer of Zeta-Probe membranes (Bio-Rad), but the dextran sulfate was omitted, and the final washing temperature was 75°C.

S1 Mapping and Primer Extension. Standard hybridization and digestion conditions were used for S1 mapping (13). Single-stranded, uniformly labeled hybridization probes were generated by a labeling reaction with an M13mp18 clone extending from nucleotide (nt) 295 to the *Bam*HI site at nt 583 of *drrA*, with the universal primer and Sequenase 2.0, as described by Sharrocks and Hornby (15), except that after digestion with a restriction enzyme that cleaved at the 5′ end of the labeled strand, the complementary strand was removed by exonuclease III digestion, and the probes were purified on a Sephadex G-50 column (13). Primer-extension reactions were done as described by Stein *et al.* (16), except the dithiothreitol concentration was 10 mM and dGTP, dATP, and dTTP were added to a final concentration of 1 mM, followed by the addition of unlabeled dCTP to 1 mM after 30-min incubation. The oligonucleotide 5′-TGGCCCGT-GTCGGCTGCGTGTTCAC-3′, extending from 20 nt downstream of the probable translational start codon for *drrA*, was used to initiate the primer-extension reaction.

RESULTS

Localization of the *drrAB* Region. Analysis of overlapping cosmid clones had previously suggested that a Dnr/Dxr resistance gene resided in a 7.5-kilobase (kb) region of the *dnr* gene cluster (6). Testing of subclones of these cosmids (obtained from K. Stutzman-Engwall and S. L. Otten, University of Wisconsin, Madison) further localized the resistance gene to a 5.6-kb fragment on the basis of the Dnr resistance conferred to *S. lividans* by pWHM603 (Fig. 1). Difficulties were encountered in further subcloning work with pIJ702 (17), pIJ943 (7), and pWHM3 (18) because selection for thiostrepton resistance greatly increased Dnr resistance in *S. lividans* by some unknown mechanism. To circumvent this problem, pWHM601, which contains an Ap resistance gene and a convenient polylinker, was constructed from the low-copy *Escherichia coli–Streptomyces* shuttle vector pKC505 (19) and the *E. coli* plasmid pGEM-7zf(+). Analysis of the properties of subclones made in pWHM601 showed that the smallest deletion that still conferred Dnr/Dxr resistance to *S. lividans* was the 2-kb DNA fragment in pWHM618 (Fig. 1). Both ORFs present in this region are required for Dnr or Dxr resistance because in-frame deletions of parts of *drrA* (pWHM620) or *drrB* (pWHM624) abolished the ability to confer Dnr/Dxr resistance to *S. lividans* (Fig. 1).

Comparison of Dxr and Dnr Resistance Levels. The level of Dxr and Dnr resistance conferred to *S. lividans* by the *drrAB* locus varied significantly (Table 1). Although *S. lividans* is itself not markedly more sensitive to Dnr than to Dxr, *drrAB* apparently confers significantly higher levels of resistance to Dxr than to Dnr in *S. lividans* transformants. We speculate that one reason for the lower level of resistance in pWHM618

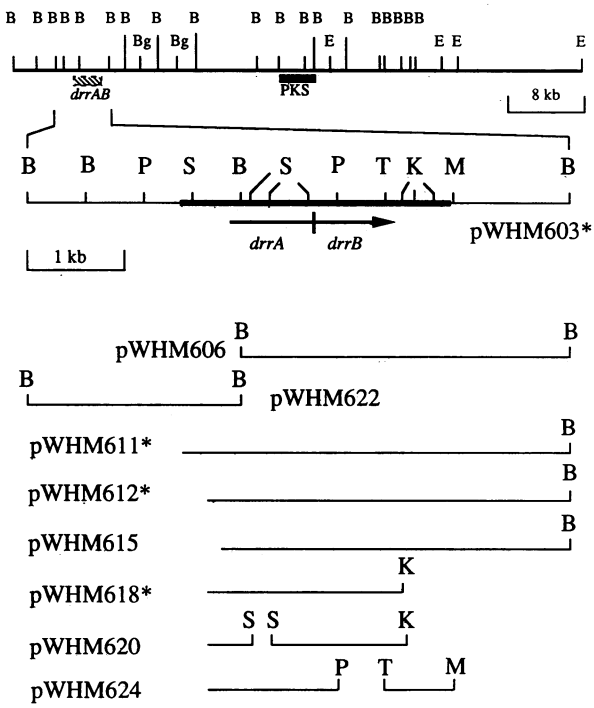

FIG. 1. Restriction map and subclones from the region of the *dnr* gene cluster containing the *drrAB* locus. At top is a map of region IV of the *S. peucetius* 29050 chromosome, which contains most or all of the Dnr biosynthetic genes (6). PKS, polyketide synthase genes. The detailed map below is of a 5.6-kb region, contained on the far-left side of cosmid pWHM338 (6). The thickened dark line indicates the extent of the sequenced region, and the arrow below the line indicates the open reading frames (ORFs) and direction of transcription. Constructs with an asterisk conferred Dnr/Dxr resistance to *S. lividans*. All inserts are in the same orientation in plasmid pWHM601, except pWHM606, which has the insert in the opposite orientation, and pWHM622, which has the insert in the same orientation in a modified version of pWHM601 lacking ≈200 base pairs (bp) of the pGEM-7zf(+) DNA. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; K, *Kpn* I; P, *Pst* I; M, *Mlu* I; S, *Sph* I; T, *Sst* I.

Table 1. Efficiency of plating of *S. lividans* clones on tryptic soy agar containing Dnr or Dxr

Antibiotic μg/ml	Clone, no. of cfu (%)		
	pWHM601	pWHM612	pWHM618
Ap (50)	70	125	558
Ap (50)/Dxr (5)	0 (0)	134 (107)	427 (77)
Ap (50)/Dxr (10)	0 (0)	96 (77)	111 (20)
Ap (50)/Dnr (5)	0 (0)	20 (16)	0 (0)
Ap (50)/Dnr (10)	0 (0)	0 (0)	0 (0)

Efficiency of plating was determined by measuring the number of colony-forming units (cfu).

compared with pWHM612 transformants is reduced mRNA stability of the shorter transcript from pWHM618. The insert in pWHM618 ends 10 nt downstream of the probable translational stop codon for *drrB* and upstream of an imperfect inverted repeat that might be important for transcriptional termination or mRNA stability, as shown recently for the *E. coli crp* gene (20).

Sequence of the *drrAB* Locus. Phenotypic and sequence analysis of plasmids made from exonuclease deletions (Fig. 1) suggested that a promoter was likely to be found in the region between the 5′ ends of pWHM612 and pWHM615 because clone pWHM612 (extending from nt 295) but not pWHM615 (extending from nt 428) conferred resistance to Dnr and Dxr in *S. lividans*. CODON PREFERENCE analysis (21) indicates that *drrA* begins downstream of the 5′ end of pWHM615 (both of these plasmids contain inserts oriented in the same direction and inserted at the same site). This result was confirmed by the results of transcript mapping, which identified the apparent transcriptional start site at nt 399 (Table 2). Further analysis of the *drrAB* region showed that it contains two genes, *drrA* and *drrB*, both of which are required for Dnr/Dxr resistance in *S. lividans* (Fig. 1). These genes are likely to encode proteins the translation of which is coupled because the TGA stop codon for *drrA* at nt 1461 overlaps the most likely ATG start codon for *drrB* at nt 1460 (5′-ATGA-3′). The formation of an ≈80-kDa, rather than 106-kDa, *malE* (27) fusion protein upon expression of a *malE*::*drrAB* fusion in *E. coli* DH5α confirmed that *drrAB* contains two ORFs and not one long one (unpublished data). There are two possible translational start points for *drrA*: One is at nt 471 (GTG), preceded by a ribosome binding site (RBS) (GGGGG), which shows a high degree of complementarity to the 3′ end of *S. lividans* 16S rRNA (5′-ACCUCCUUUCU-$_{OH}$3′) (22); another one is the GTG codon located at nt 540, which is also preceded by a potential RBS (GGACG). It is unlikely that *drrA* starts downstream of this latter GTG codon because a conserved nucleotide-binding site (28) begins at nt 591, and there are no in-frame translational start codons between these two regions. We favor the GTG codon at nt 471 as the translational start site for DrrA, because, by CODON PREFERENCE analysis (21), the third position G+C bias becomes typical for *Streptomyces* codons at or near that point. The probable translational start for *drrB* is an ATG codon at nt 1460, preceded by a RBS (AGGAG), and its translational stop is the TGA at nt 2309. Another ORF begins downstream of the *drrB* gene at nt 2603 (GTG), preceded by a potential RBS (GGAG). An additional ORF that would be transcribed in the opposite direction relative to *drrA* begins at nt 237 (GTG), preceded by a RBS (GGAGG). This may be the beginning of the aklavinone C-11 hydroxylase gene because *S. lividans* transformants containing a plasmid with the same insert as in pWHM622 (Fig. 1) could bioconvert aklavinone to ε-rhodomycinone, a step in Dnr biosynthesis catalyzed by a putative hydroxylase (ref. 29, A. Colombo, personal communication). Several imperfect inverted repeats are located downstream of the TGA stop codon for *drrB*, which may function as transcription terminators, as has been demonstrated for similar sequences in *Streptomyces* (30).

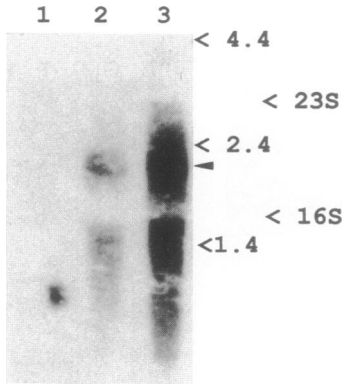

FIG. 2. Northern hybridization of *S. peucetius* 29050 DNA. Lanes: 1, 40 μg of *S. peucetius* RNA from a 20-hr culture; 2, 35 μg of *S. peucetius* RNA from a 40-hr culture; 3, ≈45 μg of *S. peucetius* RNA from a 60-hr culture. Numbers at right of gel indicate positions of size markers (kbp) from an RNA ladder (Bethesda Research Laboratories). Also indicated are locations of 23S and 16S RNA, which occupy all binding sites on the membrane and generate the clear areas. Arrowhead indicates position of the 2100-nt-long drrAB transcript. The blot was made as described and was probed with a nick-translated 1.1-kb DNA probe from the *Bam*HI site in *drrA* to the *Pst* I site in *drrB* (Fig. 1).

Identification of the Size and Precise Start Point of the *drrAB* Transcript. From the results of Northern hybridization experiments, the size of the *drrAB* transcript is ≈2100 nt (Fig. 2), which is consistent with both *drrA* and *drrB* being part of the same transcript and with transcription terminating near the inverted repeats at the 3′ end of *drrB*. Alternatively, the (fainter) hybridization to higher-molecular-weight RNA seen in Fig. 2 could be interpreted as indicating that the actual transcript is longer than 2100 nt and that the 2100-nt message is a stable decay product. Regardless, the results of Northern hybridization, coupled with the primer-extension and S1 mapping data, indicate that *drrA* and *drrB* are part of the same transcript. The apparent transcriptional start point was determined to be at nt 399 by primer-extension and S1 mapping (Fig. 3, Table 2). The corresponding −10 and −35 regions

Table 2. Comparison of *drrAB* promoter with other *Streptomyces* promoters

Promoter	Sequence*	Ref.
Streptomyces consensus	TTGaca −18 bp− tAGgat −6 bp−	22
drrAB-P	GAAGCCATCGCGGCCATGAAGTGTCCTCATTGGGGGCTACGGTACTCAAC	
choP-P	GGGGGAACGATCTCGTTGACAGCCTTCACATCGCCTCCATACGGTCATTTC	23
aml-P	GGGGGCGTCCGGAGGGTTGACCGGGCGTCGGGGCACTCGTACGGTCACGGCTG	24
aphD-P2	GTGCGGCTCACCGAGGACGGTCGGGCGCTGCTACGTTGGCTGAAC	25
orfl-P1	TCCGTGGACGGTGGTGGGGCCGAGGACCGCGGGATACGGTCGCCGC	26

*Sequences are aligned with the transcript start point at right. For the *aml* promoter (P) the most-5′ transcript start point was used. Underlining indicates sequence identity between the *drrAB* promoter region and the −10 and −35 regions of the other promoters.

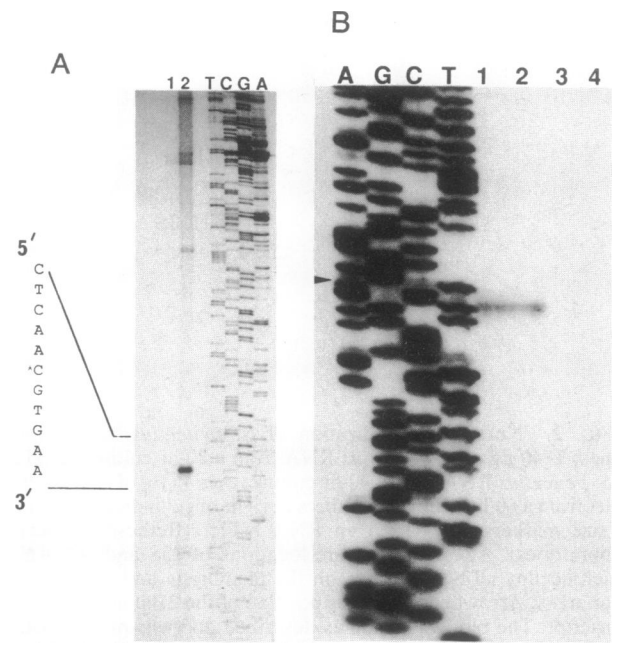

FIG. 3. Determination of the start point of *drrAB* transcription. (*A*) Primer-extension analysis of the 5′ end of *drrA*. Lanes: 1, primer plus 60 μg of RNA from 20-hr *S. peucetius* 29050 culture; 2, primer plus 50 μg of RNA from 60-hr *S. peucetius* culture. Lanes TCGA, dideoxynucleotide chain-termination sequencing reactions with the same primer. A portion of the sequence of the complementary strand is given with the apparent transcriptional start site indicated by an asterisk. The faint higher-molecular-weight bands may represent the products of low-frequency mispriming or low-abundance, longer mRNA species. (*B*) High-resolution S1 mapping of the 5′ end of the *drrA* gene. Lanes: 1, probe plus ≈60 μg of RNA from 60-hr *S. peucetius* 29050 culture; 2, probe plus 60 μg of RNA from 40-hr *S. peucetius* culture; 3, probe plus 120 μg of RNA from 20-hr *S. peucetius* culture; 4, probe plus 20 μg of tRNA. Lanes AGCT, size markers generated from dideoxynucleotide chain-termination sequencing reactions of the same region of DNA. The sequence is offset by 49 nt compared with the S1 lanes because the sequence lanes include the universal primer and a portion of the M13 polylinker. Arrowhead indicates size of the expected S1 protected fragment, based on the primer-extension results shown in *A*.

show some similarity to the *Streptomyces* vegetative promoter consensus sequence (22) and even greater similarity (especially in the −10 region) to 4 of 85 *Streptomyces* promoters analyzed (from a list provided by W. R. Strohl, Ohio State University) (Table 2). That at least some of the promoters most similar to *drrAB-P* belong to regulated genes [*aml* (24) and *aphD* (25)] is consistent with the absence of *drrAB* expression early in culture growth (Figs. 2 and 3) and suggests that *drrAB* transcription is regulated to coincide with antibiotic production. This hypothesis is further supported by thin-layer chromatograms of chloroform extracts from the broths of the 20-, 40-, and 60-hr cultures, which showed detectable Dnr and Dxr only at 40 and 60 hr (unpublished data).

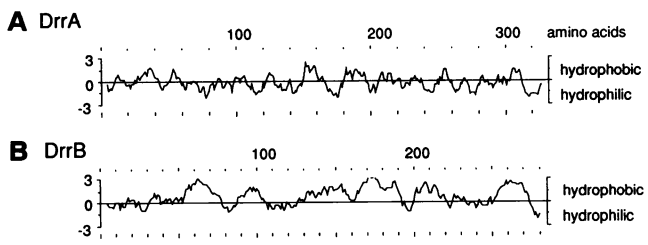

FIG. 4. Kyte–Doolittle (31) hydrophobicity analysis of the deduced products of *drrA* and *drrB* using the program PEPPLOT (21). (*A*) Hydrophobicity profile of DrrA. (*B*) Hydrophobicity profile of DrrB. The range 3 to −3 on the left-hand edge of each graph indicates relative hydrophobicity.

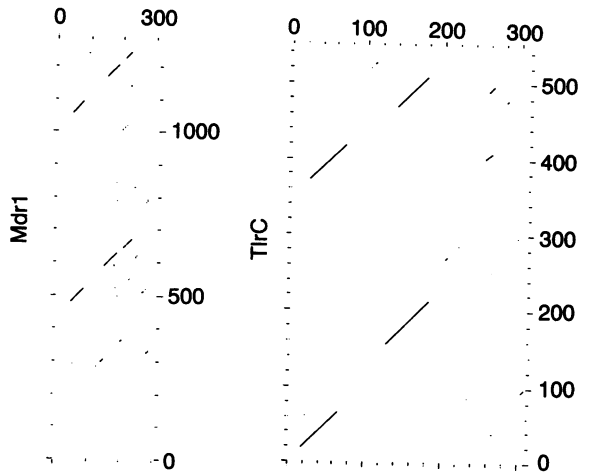

FIG. 5. Comparison of predicted DrrA protein sequence with two other proteins by using programs COMPARE and DOTPLOT (21). In each case the window was 40, the stringency was 20, and the DrrA protein sequence is plotted along the *x* axis. Numbers along axes refer to the number of amino acids from the N terminus of each protein.

Characteristics of the Putative DrrAB Proteins. With the translational start and stop codons described above, *drrA* would encode a protein containing 330 amino acids with a molecular mass of 35,668 Da and a pI of 5.1. Kyte–Doolittle analysis (31) of this putative protein, using the program PEPPLOT (21), suggests it is predominantly hydrophilic (Fig. 4*A*). Similarly, the protein encoded by *drrB* would contain 283 amino acids with a molecular mass of 30,614 Da and a pI of 7.5. Kyte–Doolittle analysis of this deduced protein (Fig. 4*B*) indicates that the DrrB protein would be extremely hydrophobic and, therefore, likely to be bound to or embedded in the cell membrane.

Using the TFASTA and FASTA programs (21), many proteins were identified as similar to the DrrA protein; most notably, the products of several *mdr* genes from eukaryotic tumor cells (32) (Fig. 5). In addition, other genes coding for members of the large ABC family of ATP-binding transport proteins, such as *hisP* from *E. coli* (ref. 33; data not shown) and the *tlrC* tylosin-resistance gene from *Streptomyces fradiae* (ref. 34; Fig. 5) were found to be similar. In contrast, the deduced product of *drrB* was not significantly similar to proteins in the European Molecular Biology Laboratory or GenBank data bases as of February 1, 1991.

DISCUSSION

We have reported the cloning, sequencing, and transcriptional analysis of the *drrAB* locus from *S. peucetius* 29050. Deletion analysis of *drrAB* in *S. lividans*, the *drrAB* sequence data, and the size of a MalE::DrrA fusion protein suggest that two translationally coupled proteins are required for the resistance phenotype in *S. lividans* and presumably in *S. peucetius*. This is an example of an antibiotic resistance gene that encodes two structural proteins, both of which are required for resistance in a heterologous host.

Two types of transport-based antibiotic resistance mechanisms have so far been found in prokaryotic organisms. (*i*) The first is exemplified by the TetA resistance proteins in *E. coli*, where resistance is conferred by a single large integral membrane protein that probably uses proton-motive force to energize transport of antibiotics (35). (*ii*) The second type is exemplified by the *drrAB* and *tlrC* (34) gene products, in

which two or more separate domains or proteins are required for resistance, and the energy for transport of the antibiotics is likely to come directly from ATP hydrolysis. The chemical structure of the antibiotic being transported does not seem to determine the transport mechanism because tetracycline resistance-like proteins are involved in transporting a variety of antibiotics ranging from the cyclopentanoid methylenomycin (36) to the polycyclic aromatic antibiotics tetracycline (37) and tetracenomycin C (unpublished data). The Mdr protein family also transports a broad range of chemically dissimilar antibiotics (2).

The putative protein encoded by *drrA* is an example of a prokaryotic protein that both shows sequence similarity to the product of the *mdr* genes and is probably also involved in transport of the same substrates as the Mdr proteins, namely Dnr and Dxr. This similarity suggests that the *mdr* genes may have originated from the same ancestral gene as *drrAB*, as has been suggested for some other antibiotic resistance genes (38). The sequence (and presumably mechanistic) similarity also suggests that *drrAB* may provide a useful genetic system for unraveling some of the mechanistic complexity associated with the very large (≈1300 amino acids) P-glycoproteins (2). Current evidence indicates that the Mdr family of proteins transport drugs through a membrane-spanning protein (or domain) and that the energy for this active-transport process comes from ATP hydrolysis, which occurs on a separate hydrophilic protein (or protein domain) (2). By analogy, the DrrA protein could energize transport of the antibiotics through a membrane pore made from the DrrB protein. Which protein is actually involved in binding the two antibiotics and whether other proteins, such as those produced from the other Dnr-resistance genes identified in *S. peucetius* (ref. 6; A. Colombo, personal communication), are required for transport of these antibiotics out of *S. peucetius* has yet to be determined.

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