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The *Streptomyces peucetius drrC* Gene Encodes a UvrA-Like Protein Involved in Daunorubicin Resistance and Production

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The *drrC* gene, cloned from the daunorubicin (DNR)- and doxorubicin-producing strain of *Streptomyces peucetius* ATCC 29050, encodes a 764-amino-acid protein with a strong sequence similarity to the *Escherichia coli* and *Micrococcus luteus* UvrA proteins involved in excision repair of DNA. Expression of *drrC* was correlated with the timing of DNR production in the growth medium tested and was not dependent on the presence of DNR. Since introduction of *drrC* into *Streptomyces lividans* imparted a DNR resistance phenotype, this gene is believed to be a DNR resistance gene. The *drrC* gene could be disrupted in the non-DNR-producing *S. peucetius dnrJ* mutant but not in the wild-type strain, and the resulting *dnrJ drrC* double mutant was significantly more sensitive to DNR in efficiency-of-plating experiments. Expression of *drrC* in an *E. coli uvrA* strain conferred significant DNR resistance to this highly DNR-sensitive mutant. However, the DrrC protein did not complement the *uvrA* mutation to protect the mutant from the lethal effects of UV or mitomycin even though it enhanced the UV resistance of a *uvrA*$^+$ strain. We speculate that the DrrC protein mediates a novel type of DNR resistance, possibly different from the mechanism of DNR resistance governed by the *S. peucetius drrAB* genes, which are believed to encode a DNR antiporter.

Microorganisms require one or more self-resistance determinants to produce antibiotics, except in cases in which they are insensitive to the antibiotic's effect(s). The resistance genes usually are clustered with the structural (biosynthetic) and regulatory genes and encode proteins that either inactivate the antibiotic, facilitate its export, or modify the host to render it insensitive to the antibiotic (9). Multiple rather than single resistance mechanisms are often found; in this case, it is not known whether any one resistance mode is sufficient to ensure survival or antibiotic production. We address this question here.

Streptomyces peucetius, which produces the important antitumor drugs daunorubicin (DNR) (4, 10) and doxorubicin (DXR) (2), contains the *drrAB* (17) and *ric2* (8) resistance genes, which are assumed to provide self-resistance to these two antibiotics because they confer DNR and DXR resistance when introduced into *Streptomyces lividans*. The DrrA protein strongly resembles bacterial proteins that transport compounds by an ATP-dependent process (20), as well as the Mdr1 P glycoprotein responsible for DNR-DXR resistance of human cancer cells and known to act as an ATP-dependent transporter (19). DrrA thus is a candidate for a DNR-DXR binding and transport protein, whereas the hydrophobic DrrB protein could be responsible for binding DrrA to the bacterial membrane. The *ric2* locus, which unlike *drrAB* is not part of the cluster of DXR production genes (8), may also be important for self-resistance. A third gene, *drrC*, in the same cluster of genes as is *drrAB*, encodes the DrrC protein described here. *S. lividans drrC*$^+$ transformants display a DNR resistance phenotype similar to that of *drrAB* transformants (17). Moreover, *drrC* expression was coordinate with that of *drrAB* and the other DNR production genes (31). We show that even in the presence of the *drrAB* and *ric2* genes, *drrC* mutants were isolated only in a non-DNR-DXR-producing background and exhibited increased sensitivity to DNR, which suggests that *drrC* is vital to DNR-DXR production and self-resistance. The strong sequence similarity of DrrC to the *Escherichia coli* UvrA protein, a critical component of the UvrABC complex involved in excision repair of DNA damaged by the free radicals produced from the interaction of reduced, DNA-bound DNR or DXR with O_2 (14, 25), suggests that DrrC might be involved in a novel form of DNR-DXR resistance. Although expression of *drrC* in the *E. coli* UNC523 *uvrA* mutant (33) significantly increased its level of DNR resistance, the DrrC protein did not protect this mutant from the lethal effects of UV or mitomycin, classic factors that induce the excision repair process in *E. coli.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *E. coli* DH5α (40) and the pUC19 (46), M13mp19, and pGEM3Zf(−) plasmids (Promega, Madison, Wis.) were used for routine subcloning. The *E. coli* K802 *uvrA*$^+$ and UNC523 *uvrA* (33) strains were used to construct lysogens for expression of the *drrC* gene cloned in pET-17b and pET-26b (Novagen, Madison, Wis.). The other *E. coli* and *Streptomyces* strains, plasmids, and phages used in this study are listed in Table 1.

Biochemicals and chemicals. Thiostrepton was obtained from S. J. Lucania at Bristol-Myers Squibb (Princeton, N.J.), and DNR was obtained from Pharmacia S.p.a. (Milan, Italy). Apramycin was obtained from Eli Lilly and Company (Indianapolis, Ind.). Restriction enzymes and other molecular biology reagents were purchased from standard commercial sources.

Media and growth conditions. *E. coli* strains carrying plasmids were grown in Luria-Bertani (LB) medium (40) and selected with ampicillin (100 μg/ml). *S. peucetius* strains were grown on ISP4 medium (Difco Laboratories, Detroit, Mich.) with 50 ml of Difco yeast extract (10%, wt/vol) per liter for sporulation and on ISP4 medium alone to determine the fraction surviving after UV and/or antibiotic treatment. R2YE liquid medium (21) was used for the preparation of protoplasts, for the isolation of chromosomal and plasmid DNAs from *S. peucetius* strains, and in primer extension experiments. R2YE and GPS (11) liquid media were used for the determination of catechol dioxygenase activities in cell extracts of *S. peucetius* transformants. R2YE agar medium (21) was used for infection of *S. peucetius* with ϕC31 derivatives. *S. lividans* TK24 (22) and TK24(ϕC31) were grown on R2YE medium without sucrose for sporulation and

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TABLE 1. Bacterial strains, plasmids, and phages

Strain(s), plasmid(s), or phage	Genotype and/or description	Source or reference(s)
Strains		
S. peucetius		
29050	Wild type	ATCC[a]
WMH1524	*dnrJ*	31
WMH1621	*dnrV*::*aphII*	36
WMH1629	*dnrZ*::*aphII*	35
H6125	*dnrS*	37
WMH1642 and WMH1643	*tsr aphII*; obtained after infection with phWHM263	This study
WMH1644 and WMH1645	*dnrJ drrC tsr aphII*; obtained after infection with phWHM263	This study
S. lividans TK24	SLP^- $SLP2^-$ *str*	22
E. coli		
WMH1646	*E. coli* UNC523 *uvrA* (λDE3) lysogen carrying pET-17b	This study
WMH1647	*E. coli* UNC523 *uvrA* (λDE3) lysogen carrying pWHM270	This study
WMH1648	*E. coli* K802 $uvrA^+$ (λDE3) lysogen carrying pET-26b	This study
WMH1649	*E. coli* K802 $uvrA^+$ (λDE3) lysogen carrying pWHM271	This study
Plasmids		
pFDNeo-S	pUC18 containing the *aphII* gene	12
pIJ2840	SCP2-derived plasmid carrying *tsr* and promoterless *xylE* genes	7
pWHM601	*E. coli-Streptomyces* low-copy-number shuttle vector carrying *aacIV* apramycin resistance gene	17
pWHM339	34-kb *S. peucetius* chromosomal DNA cloned in the KC505 cosmid	38
pWHM264	3.6-kb *Sac*I-*Sph*I fragment containing *drrC* cloned from pWHM339 into pGEM-3Zf(−)	This study
pWHM265	Internal 1.2-kb *Nru*I-*Bam*HI fragment of *drrC* cloned from pWHM264 into pFDNeo-S between the *Sma*I and *Bam*HI sites	This study
pWHM266	3.6-kb *Hin*dIII-*Eco*RI fragment containing *drrC* cloned from pWHM264 into pWHM601	This study
pWHM267	3.6-kb *Hin*dIII-*Eco*RI fragment containing *drrC* cloned from pWHM264 into pWHM601 in the opposite orientation	This study
pWHM268 and pWHM269	0.74-kb *Hin*dIII-*Bam*HI fragment containing the *drrC* promoter region cloned into pIJ2840 in the same orientation as the *xylE* gene (pWHM268) or in the opposite orientation (pWHM269)	This study
pWHM270	pET-17b with 2.6-kb *drrC* segment cloned between *Nde*I and *Xho*I sites	This study
pWHM271	pET-26b with 2.6-kb *drrC* segment cloned between *Nde*I and *Hin*dIII sites	This study
pWHM272	*drrC* coding region cloned in M13mp19	This study
pWHM908	*dnrJ* gene cloned in pWHM601	31
Phages		
φC31	Wild type; c^+ $attP^+$	28, 30
KC515	c^+ *attP*::*tsr*::*vph*	21
φC31R8	c^+ $attP^+$::*tsr*	6
phWHM263	c^+ *attP*::2.3-kb *Pst*I-*Sst*I fragment containing the Δ*drrC* and *aphII* genes cloned from pWHM265 into KC515	This study

[a] ATCC, American Type Culture Collection, Rockville, Md.

at 30°C in R2YE liquid medium for preparation of protoplasts. φC31 and its derivatives were propagated as described by Hopwood et al. (21) or in a liquid medium containing, per liter, Nutrient Broth (Difco), 8 g; NaCl, 9 g; and the following nutrients, which were added after autoclaving: 1 M $MgSO_4 \cdot 7\ H_2O$, 10 ml; 0.8 M $Ca(NO_3)_2$, 10 ml; and a 50% (wt/vol) solution of glucose, 20 ml.

Isolation and in vitro manipulation of DNA. Plasmid DNA was isolated from bacterial cells with the Bio 101, Inc. (Vista, Calif.), RPM kit, and φC31 phage DNA was isolated with the Qiagen (Chatsworth, Calif.) lambda kit. *S. peucetius* chromosomal and plasmid DNAs were isolated by the protocol of Hopwood et al. (21). Restriction endonuclease digestions and ligations were performed according to standard techniques (40). DNA fragments for labelling and subcloning were isolated using the Qiaex (Qiagen) gel extraction kit. The conditions for phage DNA transfection were as described by Hopwood et al. (21). Transformation of *S. peucetius* was done as described previously (38, 45) with protoplasts prepared from 1-day-old mycelium growing in R2YE at 30°C and screening of cell wall regenerants for antibiotic resistance with apramycin (15 μg/ml) or thiostrepton (25 μg/ml) added to the R2YE medium.

DNA sequencing. DNA fragments subcloned in M13 vectors were sequenced according to previous methods (17, 31) with the U.S. Biochemicals Sequenase version 2.0 sequencing kit according to the manufacturer's suggestions. 7-Deaza-dGTP was used instead of dGTP to avoid compressions.

Southern blot analysis. *Streptomyces* chromosomal DNA was digested with restriction enzymes for 4 h, electrophoresed in a 0.8% agarose gel overnight, and blotted to Hybond N membranes (Amersham, Arlington Heights, Ill.) by capillary transfer (40). Labelling, hybridization, and detection were carried out with the Genius 1 nonradioactive DNA labelling kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions under standard conditions (40).

Identification of the apparent transcriptional start site of the *drrC* gene. Primer extension experiments were carried out by previously described methods (17, 31) using 10 μg of RNA isolated from cells of *S. peucetius* 29050 and H6125 grown in R2YE medium at the times given in the legend to Fig. 4, 10 pmol of one of the two primers shown in Fig. 2B, and 200 U of RNA Superscript (Gibco/BRL Life Technologies, Cleveland, Ohio). Primer a (see Fig. 2B) was complementary to the RNA produced from positions −1 to −30 upstream of the predicted *drrC* translation start site, and primer b was complementary to that produced from positions +21 to +50 downstream of it.

Construction of *drrC*::*xylE* fusions for expression in *Streptomyces* species. A 0.74-kb *Nru*I fragment that extends from −500 nucleotides (nt) upstream to +240 nt downstream of the *drrC* start codon (see Fig. 2B) was cloned in both orientations into the *Hin*cII site of pGEM-3Zf(−), and then a *Hin*dIII-*Bam*HI fragment containing the *drrC* promoter was cloned from each of these plasmids into pIJ2840 (7) upstream of a promoterless *xylE* gene encoding catechol dioxygenase to give pWHM268, in which the *drrC* promoter is in the same orientation

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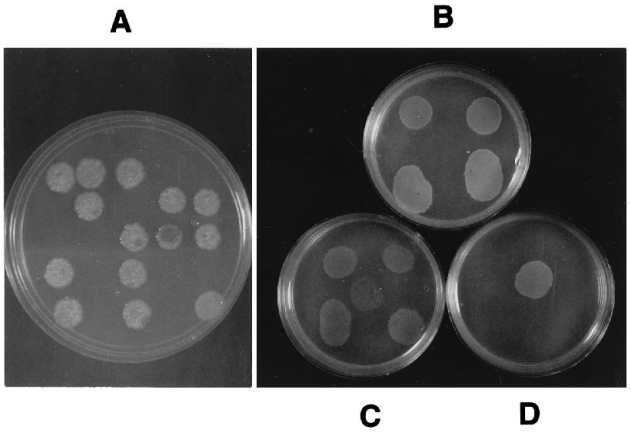

FIG. 1. Screening of recombinant phages carrying the *aphII* gene by the plate spot test method described in Materials and Methods. (A) Areas of resistant growth from single *aphII*-carrying transfectant plaques on plates containing spores of *S. lividans* TK24 and an *S. lividans* TK24(ϕC31) lysogen plus neomycin in soft agar overlays. (B to D) Phenotypes of strains bearing phWHM263. Four drops of the phWHM263 stock as well as a drop of KC515 stock (center) were put on plates containing spores of *S. lividans* TK24 and an *S. lividans* TK24(ϕC31) lysogen plus neomycin (B), thiostrepton (C), or viomycin (D) in soft agar overlays. KC515 carries the *tsr* and *vph* genes conferring thiostrepton and viomycin resistance.

as *xylE*, and pWHM269, in which it has the opposite orientation. In the latter case, the promoter of a putative gene that is transcribed divergently from *drrC* should control *xylE* expression. Each plasmid was introduced into *S. peucetius* by transformation, and the catechol dioxygenase activities of cell extracts of the resulting strains grown in R2YE or GPS medium were determined by established methods (24).

Construction of the phWHM263 phage carrying an internal segment of the *drrC* gene. The internal 1.2-kb *Nru*I-*Bam*HI fragment of the *drrC* gene (see Fig. 2B) was cloned between the unique *Sma*I and *Bam*HI sites of the plasmid pFDNeo-S (12) to create pWHM265. From this plasmid, a 2.3-kb *Sac*I-*Pst*I fragment containing the *drrC* segment and the *aphII* resistance gene was cloned into the *Pst*I and *Sac*I sites of KC515, resulting in the loss of the 0.7-kb *Sac*I-*Pst*I fragment that contains a significant part of the *vph* gene in the phage (21). Protoplasts of *S. lividans* TK24 were transfected with the ligated mixture, and plaques were observed after overnight incubation. The recombinant phWHM263 phage containing the *drrC* segment joined to the *aphII* gene was isolated with the help of the following spot test method developed in this study. Single plaques were transferred to 0.2 ml of Nutrient Broth, and 10 μl from each sample was pipetted as spots onto plates overlaid with soft R2YE agar containing a mixture of *S. lividans* TK24 and TK24(ϕC31) lysogen spores. After 17 h of incubation at 30°C the plates were overlaid with soft agar containing neomycin. Infection with phages carrying the *aphII* gene resulted in spots of neomycin-resistant growth after another overnight incubation at 30°C (Fig. 1A). The recombinant phages were purified through a second round of the spot test to confirm the presence of the *aphII* and *tsr* resistance genes. High-titer phage stocks were then prepared by standard methods (21) and tested for the presence of the *tsr*, *vph*, and *aphII* resistance genes by the spot test (Fig. 1). In this way phWHM263 was characterized as containing the *aphII* and *tsr* resistance genes (Fig. 1B and C), and the presence of the cloned DNA was confirmed by restriction endonuclease digestion analysis.

Screening of recombinant phage by the spot test method described above was carried out by selection for neomycin resistance with neomycin (10 μg/ml) added to R2YE growth medium. The neomycin (*aphII*), viomycin (*vph*), and thiostrepton (*tsr*) resistance markers were scored in the presence of neomycin (10 μg/ml), viomycin (200 μg/ml), and thiostrepton (30 μg/ml) in R2YE. Selection for integration of ϕC31 phage which had *attP* deleted was carried out on minimal medium (21) with neomycin (10 μg/ml) or thiostrepton (30 μg/ml).

Disruption of the *drrC* gene in *S. peucetius*. The 29050 wild-type and WMH1524 *dnrJ* strains were chosen as recipients of phWHM263 in attempts to insertionally inactivate *drrC*. The two strains were infected with phWHM263 on R2YE agar plates (5×10^7 spores per ml and between 1×10^8 and 2×10^8 phage) to allow phage multiplication and host sporulation. After sporulation the infected cultures were replica plated onto minimal medium (21) containing neomycin. Primary neomycin-resistant clones were isolated, and their phenotypes were redetermined after a second round of single-colony isolation. Clones resistant to neomycin and thiostrepton were obtained from both strains.

Expression of *drrC* in *E. coli* strains. To express *drrC* in *E. coli*, an *Nde*I site was introduced at the ATG immediately upstream of the predicted translational

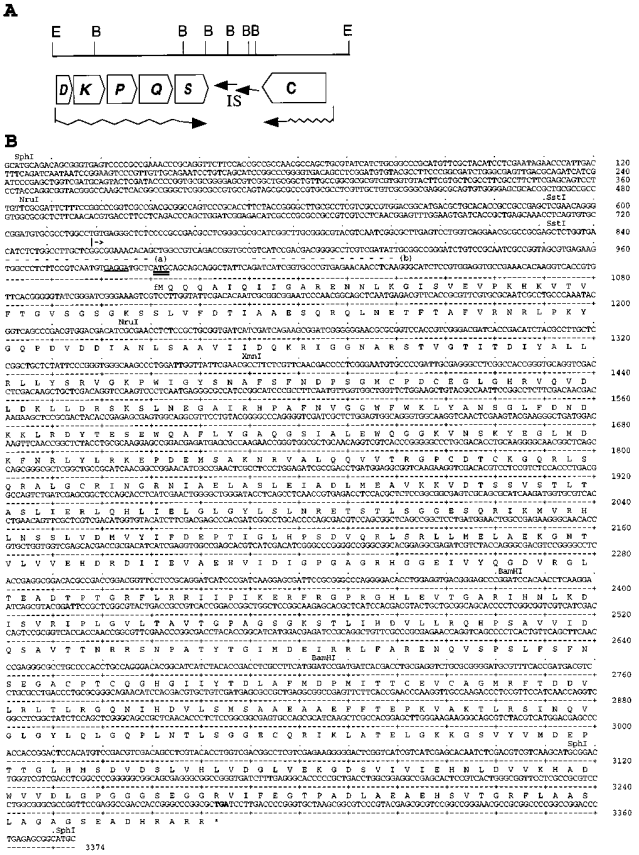

FIG. 2. (A) Organization of a portion of the cluster of DXR biosynthesis genes that contains *drrC*. IS, putative insertion element (37). Wavy arrows, mapped transcripts (32). (B) DNA and deduced protein sequences of the *drrC* gene. Restriction sites of interest are shown above their hexameric recognition sequence. The proposed translational start and stop sites of *drrC* are doubly underlined and in bold type, respectively, and the putative ribosome binding site is underlined. The stop codon is indicated by an asterisk. The apparent transcriptional start site at nt 858 is indicated by an arrow. The locations of the two primers, a and b, used in primer extension experiments (Fig. 4) are indicated by dashed lines above the complementary DNA sequence between nt 960 and 1080.

start codon shown in Fig. 2B and the second codon (CTC) was changed to CTG (in bold type in the first of the two sequences that follow), the preferred codon in *E. coli*, by PCR methods using the following two primers: 5′-GCACGGATCC ATATG**CTG**ATGCAGCAGCAGGCTATTCAG-3′ and 5′-GCAGGAATTCG CGATGTCGTCCACGTCGG-3′ (the latter primer contains the *Nru*I site at nt 1225 in Fig. 2B). Each primer (0.5 μg) was incubated with 30 ng of pWHM272, which contains the *drrC* coding region cloned in M13mp19, in a solution containing 10 mM Tris-HCl (pH 9), 1.25 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 100 mg of bovine serum albumin, 60 μM (each) dCTP and dGTP, and 40 μM (each) dATP and dTTP in a total volume of 99.5 μl. After being overlaid with mineral oil, the reaction mixture was incubated at 100°C for 5 min and then cooled to 70°C, and 2.5 U of *Taq* polymerase (Promega) was added. The PCR was carried out in a model 480 DNA thermal cycler (Perkin-Elmer Cetus). Amplification was achieved with 25 cycles of denaturation at 97°C for 50 s followed by annealing and extension at 70°C for 2.5 min. The resulting 250-bp fragment was recovered from a 1.5% agarose gel after electrophoresis and digested with *Nde*I and *Nru*I. The *drrC* gene was reassembled by three-piece ligation of this 0.25-kb *Nde*I-*Nru*I fragment, the 2.1-kb *Nru*I-*Hin*dIII fragment from pWHM272 containing the remainder of the *drrC* coding region, and the *Nde*I-*Hin*dIII fragment of pET-26b (Novagen) to produce pWHM271. The resulting *drrC* gene was transferred as a 2.4-kb *Nde*I-*Xho*I fragment into pET-17b between its *Nde*I and *Xho*I sites to produce pWHM270. A 1.4-kb *Xba*I-*Bam*HI fragment containing the entire region made by the PCR was subcloned from pWHM271 into pUC19 between the *Xba*I and *Bam*HI sites. The DNA sequence of this region was verified by sequence analysis according to the methods described above.

E. coli UNC523 *uvrA* was lysogenized with the λDE3 phage (Novagen) according to the Novagen protocol, and then the pET-17b vector and pWHM270

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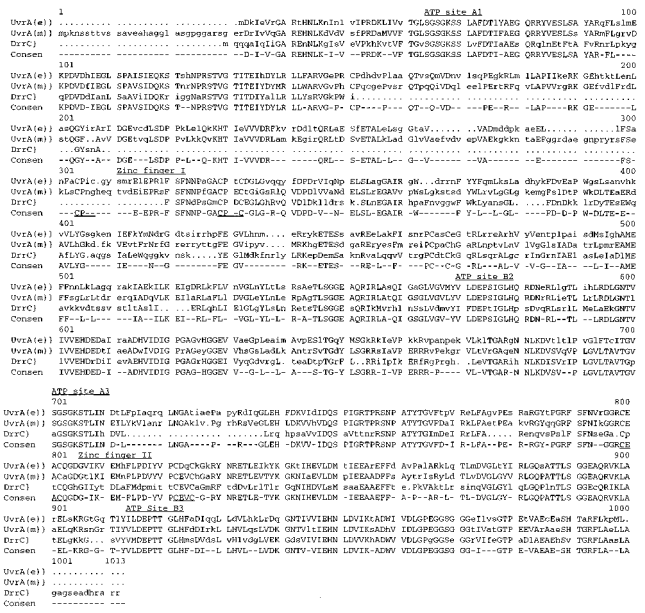

FIG. 3. PILEUP analysis (13) of DrrC and UvrA proteins from *E. coli* [UvrA(e)] and *M. luteus* [UvrA(m)]. The two zinc finger (CXXC at each end is underlined) and four ATP binding sites are indicated for each protein. Capital letters indicate amino acids in consensus sequence. Consen, consensus.

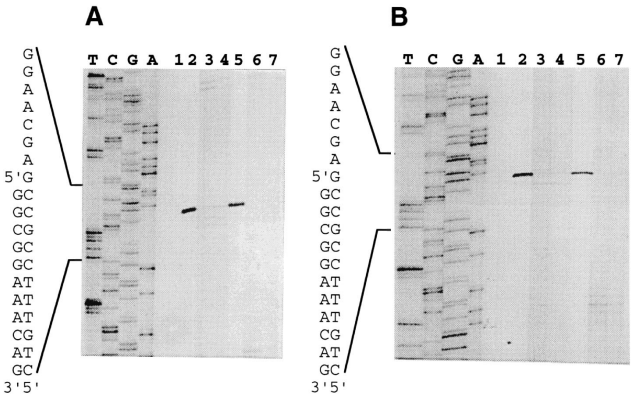

FIG. 4. Mapping of the apparent 5′ end of the *drrC* mRNA by primer extension analyses using RNA isolated at 24 (lanes 2 and 5), 48 (lanes 3 and 6), and 72 (lanes 4 and 7) h from cultures grown in R2YE medium. (A and B) Data obtained with primers a and b identified in Materials and Methods, respectively. Lanes T, C, G, and A represent the DNA sequencing ladder. Lanes 1, yeast tRNA control; lanes 2 to 4, *S. peucetius* 29050; lanes 5 to 7, *S. peucetius* H6125. The apparent transcriptional start points (G) are shown at the positions complementary to the DNA sequence.

were introduced by transformation into separate *uvrA* strains, resulting in *E. coli* WMH1646 and WMH1647 (Table 1). These two strains were grown in LB medium with kanamycin (20 μg/ml), thiostrepton (20 μg/ml), and apramycin (100 μg/ml) at 37°C. At an optical density at 600 nm of 0.6, 0.4 mM isopropyl-thiogalactopyranoside (IPTG) was added and the cells were collected after 1 h. The cells were then suspended in sample buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% [wt/vol] sodium dodecyl sulfate [SDS], 0.1% [wt/vol] bromophenol blue, 10% [vol/vol] glycerol) and heated in boiling water for 3 min, and then the supernatant fractions were run on an SDS–7.5% polyacrylamide gel electrophoresis (PAGE) gel to observe the proteins produced in each sample (data not shown).

UV irradiation of *E. coli* cells. The source of UV was a 15-W low-pressure mercury germicidal lamp. The cells to be irradiated were grown at 37°C to an optical density at 670 nm of 0.6, and IPTG (0.4 mM) was added. After 1 h the cells were diluted in SM buffer (40) to 1×10^8 to 2×10^8 cells per ml and exposed to various doses of UV at a 60-cm distance from the source.

Determination of antibiotic resistance. Strains were analyzed for DNR resistance by spreading spore suspensions on R2YE plates containing different concentrations of DNR and apramycin (50 μg/ml), if necessary. Antibiotic resistance was also determined after treatment of spore suspensions with antibiotics. The spore suspensions to be treated were diluted in SM buffer to 10^7 spores per ml, DNR (4 mg/ml in distilled water) was added at the concentrations indicated in the text, and the incubations were carried out for 1 h at 37°C with agitation. The spore suspensions were then diluted and plated onto ISP4 plates without or with neomycin (10 μg/ml) or apramycin (20 μg/ml), and growth was scored as CFU observed after 96 h at 30°C.

E. coli WMH1646 and WMH1647 were incubated in LB medium (40) supplemented with tetracycline (20 μg/ml), kanamycin (20 μg/ml), and apramycin (100 μg/ml), and the cultures were grown at 37°C and 260 rpm to an optical density at 600 nm of 0.85 and then diluted into the same liquid medium to 2×10^8 cells per ml. DNR (4-mg/ml stock) or mitomycin (1-mg/ml stock) was added at the concentrations indicated in the text, and then the cultures were incubated for 40 min at 37°C at 260 rpm. The cells were recovered by centrifugation, resuspended in SM buffer, and plated onto antibiotic-supplemented LB plates.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in EMBL and GenBank under accession number L76359.

TABLE 2. DNR resistance of *S. lividans* transformants

Plasmid introduced	*drrC* gene present	Growth with DNR at the following concn (μg/ml)[a]:					
		0	5	8	10	12	15
pWHM601	No	+	+	−	−	−	−
pWHM266	Yes	+	+	+	+	+	−
pWHM267	Yes	+	+	+	+	+	−
pWHM339	Yes	+	+	+	+	+	−

[a] Concentration in R2YE agar medium. + and − signify visible growth and no growth, respectively, of a lawn of cells from 10^7 spores after 4 days at 30°C.

RESULTS

Analysis of the DNA sequence of the *drrC* gene. The sequence of a 3.4-kb *Sph*I DNA segment downstream of the *dnrQS* genes and an intervening insertion element (37) (Fig. 2A) revealed one complete open reading frame (Fig. 2B) by CODON PREFERENCE analysis (13). The *drrC* open reading frame, most likely beginning and ending with ATG and TGA codons at nt 991 and 3285, respectively (Fig. 2B), is transcribed convergently with respect to *dnrS* and is predicted to encode a 764-amino-acid protein (M_r, 83,386 without the formylmethionyl) with a calculated pI of 6.8 and no significant hydrophobic character as determined by Kyte-Doolittle analysis (13). A database search by means of the TFASTA (13) and BLASTA (1) programs showed that the sequence of the putative DrrC protein resembles those of a large number of proteins involved in ATP-dependent metabolite transport. It also has especially strong overall similarities (75 and 77%, respectively, as determined by GAP [13] analysis) to the sequences of the *E. coli* (23) and *Micrococcus luteus* (44) UvrA proteins involved in excision repair. The zinc finger region essential for the DNA-binding property of UvrA and at least two of the sites for binding of ATP, from which UvrA derives its ability to scan DNA and complex with UvrB (41), can easily be seen, but the other zinc finger found in UvrA is absent in DrrC because the latter protein contains a 163-amino-acid gap in the corresponding region (Fig. 3).

Examination of the sequence of the 990 nt upstream of the *drrC* gene (Fig. 2B) did not reveal a clear open reading frame longer than about 200 nt by CODON PREFERENCE analysis in either direction.

Expression of the *drrC* gene in *S. lividans* and *S. peucetius*. When *S. lividans* contained pWHM266, which carries the *drrC* gene cloned in the low-copy-number vector pWHM601, DNR

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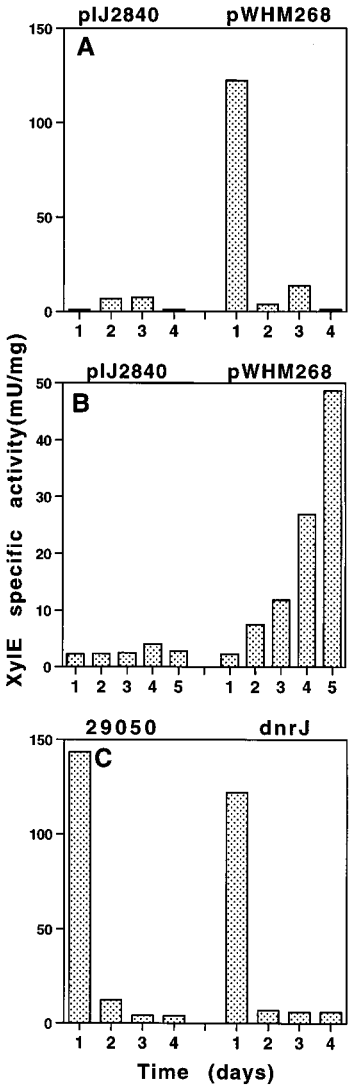

FIG. 5. Catechol dioxygenase activities in cell extracts of *S. peucetius* 29050 strains. (A and B) XylE specific activities (in milliunits per milligram of protein) (24) at 24, 48, 72, and 96 (or 120) h (1, 2, 3, and 4 [or 5] days) of *S. peucetius* 29050(pIJ2840) and *S. peucetius* 29050(pWHM268) grown in R2YE and GPS media, respectively. (C) XylE specific activities (in milliunits per milligram of protein) (24) at 24, 48, 72, and 96 h (1, 2, 3, and 4 days) of *S. peucetius* 29050 and *S. peucetius* WMH1524 *dnrJ*, each transformed with pWHM268 and grown in R2YE medium.

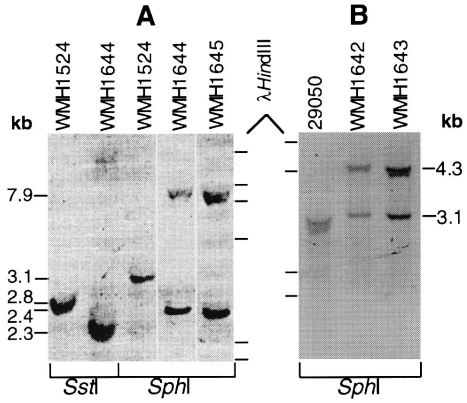

FIG. 6. Southern blot hybridization analysis of the genomic DNAs from wild-type *S. peucetius* 29050, WMH1524 *dnrJ*, WMH1642 and WMH1643 neomycin- and thiostrepton-resistant transductants, and WMH1644 and WMH1645 *dnrJ drrC* strains probed with the 1.2-kb *Sac*I-*Bam*HI fragment of pWHM265 that contains *drrC*. (A) WMH1524, WMH1644, and WMH1645 DNAs digested with *Sst*I or *Sph*I. (B) 29050, WMH1642, and WMH1643 DNAs digested with *Sph*I. The apparent doublet for 29050 DNA is an artifact, since only one band appeared in panel A and in all the Southern blot analyses of the 29050 DNA samples whose data are not shown.

resistance was increased (Table 2) to the level observed for *S. lividans*(pWHM339) transformants (38), in which *drrC* is contained within the 34-kb insert cloned in the KC505 cosmid, from which pWHM601 was derived (17). *S. lividans*(pWHM612) transformants carrying the *drrAB* genes cloned in pWHM601 exhibited a similar level of DNR resistance (17).

To permit analysis of the timing of *drrC* expression, the apparent transcriptional start point of the *drrC* gene in *S. peucetius* 29050 and H6125 was localized by primer extension experiments, as described in Materials and Methods, to a G 132 nt upstream of the predicted start of translation (Fig. 4). The transcript was detected in the 29050 strain after 24 h of growth in R2YE medium and then disappeared by 48 and 72 h. Similar results were obtained for the H6125 strain, which is more sensitive to DNR than the wild-type 29050 strain (38). Under these growth conditions, the 29050 strain produces only a low level of DNR, whereas the H6125 strain produces ε-rhodomycinone because it is blocked in DNR biosynthesis because of a mutation in the *dnrS* gene required for attachment of daunosamine to ε-rhodomycinone (37).

These results were confirmed by the behavior of the wild-type *S. peucetius* strain containing a *drrC*::*xylE* fusion, pWHM268, in which the apparent transcriptional start site of *drrC* lies approximately in the middle of the 0.74-kb *Nru*I fragment (Fig. 2B) that was cloned upstream of the promoterless *xylE* gene in pIJ2840 (see Materials and Methods). The catechol dioxygenase activities of extracts from cells grown in the R2YE medium showed that maximal XylE activity was present at 24 h (Fig. 5A), which is near the end of the exponential stage of growth, as determined by measurements of protein concentration at 12-h intervals (data not shown), and then declined to a low level. In GPS medium (11), in which cells produce much higher levels of DNR, maximal XylE activity was observed much later, i.e., after 120 h (Fig. 5B), coincident with the time of greatest DNR production in this medium (data not shown). Consequently, *drrC* expression is growth medium dependent.

Expression of *drrC* in the WMH1524 *dnrJ* mutant that is blocked in DNR biosynthesis and accumulates ε-rhodomycinone because of the effect of the *dnrJ* mutation on daunosamine biosynthesis (31) was also studied. As shown by the catechol dioxygenase activities of pWHM268 transformants grown in the R2YE medium (Fig. 5C), *drrC* was expressed in the *dnrJ* mutant in a manner similar to that of its expression in

TABLE 3. DNR sensitivities of spores of *S. peucetius* wild-type and *dnrJ* strains

Strain	Fraction of spores surviving after treatment with DNR at the following concn (μg/ml)[a]:	
	10	50
29050 (wild type)	$(5.3 \pm 0.3) \times 10^{-1}$	$(3.1 \pm 0.2) \times 10^{-1}$
WMH1524 *dnrJ*	$(4.2 \pm 0.7) \times 10^{-2}$	$(6.2 \pm 0.8) \times 10^{-4}$
WMH1644 *dnrJ drrC*	$(8.6 \pm 1.2) \times 10^{-3}$	$(4.3 \pm 1.2) \times 10^{-5}$

[a] Data are averages with standard errors. $n = 2$ for 29050 and $n = 4$ for the other two strains.

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TABLE 4. DNR sensitivities of spores of the *S. peucetius dnrJ* strain and its *dnrJ*$^+$ transformant

Strain	Fraction of spores surviving after treatment with DNR at the following concn (μg/ml)[a]:	
	50	100
WMH1524 *dnrJ*	$(4.8 \pm 1.1) \times 10^{-4}$	$(1.2 \pm 0.3) \times 10^{-4}$
WMH1524(pWHM908)	$(1.1 \pm 0.1) \times 10^{-1}$	$(7.2 \pm 0.7) \times 10^{-2}$

[a] Data are averages with standard errors ($n = 4$).

the wild-type 29050 strain. Thus, the DNR sensitivity of WMH1524 described below cannot be due to a lack of *drrC* expression.

Disruption of the *drrC* gene by insert-directed phage integration. We attempted to inactivate *drrC* by homology-directed integration of an *attP* mutant temperate phage. Lampel and Strohl (27) have shown that *S. peucetius* is sensitive to infection by the *attP*$^+$ ϕC31 phage (28) and its *attP* derivative KC515 (21). KC515 can integrate into the chromosome only if it carries a cloned insert capable of undergoing homologous recombination (5). Use of the phage vector ensured that each transductant was the product of an independent recombination event. A *Pst*I-*Sac*I fragment containing an internal 1.2-kb *Nru*I-*Bam*HI segment of *drrC* (Fig. 2B) and the *aphII* gene from pWHM265 (Table 1) was cloned into the *Pst*I-*Sst*I sites of KC515 to produce the phWHM263 phage carrying the *tsr* and *aphII* genes, as described in Materials and Methods. This *attP* phage was expected to produce thiostrepton- and neomycin-resistant transductants in which *drrC* is disrupted by a single-crossover recombination event.

The 29050 wild-type and WMH1524 *dnrJ* strains were infected with phWHM263, and neomycin- and thiostrepton-resistant transductants were obtained from two sets of infection experiments with both strains; nine clones from the wild type and five clones from the *dnrJ* mutant were chosen for further analysis.

The hybridization pattern of *Sac*I- and *Sph*I-digested DNA from the five clones obtained from the *dnrJ* mutant confirmed that homologous integration had occurred at the *drrC* locus. Integration by a single crossover between the cloned fragment and the recipient chromosome within a 1.2-kb *Bam*HI-*Nru*I region of the *drrC* locus was expected to produce hybridizing *Sph*I fragments of 2.4 and 7.9 kb (instead of the 3.1-kb fragment generated from a nonrecombinant strain) and a 2.3-kb *Sac*I fragment (instead of a 2.8-kb band). The expected patterns were found for two representative strains, WMH1644 and WMH1645 (Fig. 6A); the three others isolated had identical hybridization patterns (data not shown).

Surprisingly, analysis of *Sph*I-digested DNA from the nine neomycin- and thiostrepton-resistant clones obtained from the wild-type strain showed that phWHM263 had integrated into another, unknown region of the chromosome without disrupting the *drrC* gene. As shown in Fig. 6B for the representative WMH1642 and WMH1643 strains, 3.1- and 4.3-kb hybridizing bands were identified with the same probe used for the *dnrJ drrC* mutants. Since the 3.1-kb hybridizing band is present in both the 29050 wild-type strain and the neomycin- and thiostrepton-resistant WMH1642 and WMH1643 strains, integration took place without disrupting the *drrC* locus. The 4.3-kb hybridizing band was the same size in the seven other clones from the wild-type strain analyzed (data not shown). This result strongly suggests that integration of phWHM263 occurred in the same region of the *S. peucetius* chromosome and involved the same place in the phage, somewhere between the cloned *drrC* fragment and the *cos* site at the right-hand end of phWHM263, because a 7.1-kb *Sph*I fragment would have resulted if recombination had taken place at another region in the phage. Since a clone with a disrupted *drrC* gene was not isolated for the wild-type strain but was consistently isolated in the *dnrJ* background, the elimination of *drrC* function may be deleterious in DNR-producing strains of *S. peucetius.*

The *S. peucetius drrC* mutant is less resistant to DNR. The strong similarity between the deduced products of the *drrC* and *uvrA* genes led us to the hypothesis that *drrC* could be involved in repairing DNA that had been damaged by the DNR-DXR-induced formation of hydroxyl radicals or in providing resistance to DNR by some other mechanism. To explore these possibilities, we studied the levels of resistance of spores of the parental *dnrJ* strain and the WMH1644 *dnrJ drrC* recombinant clone to UV irradiation and DNR. There was no difference in UV sensitivity between the wild-type and *dnrJ* strains. The *dnrJ drrC* mutant was more sensitive to UV than the *dnrJ* strain, but the difference was small, i.e., not more than two- to threefold (data not shown).

To study the comparative sensitivities of the *S. peucetius* wild-type strain and its *dnrJ* and *dnrJ drrC* mutants to DNR and other antibiotics, we determined the levels of resistance to DNR in efficiency-of-plating experiments with spore suspensions, as described in Materials and Methods, because we could not devise a satisfactory means to measure DNR resistance of the mycelial cells taken from the exponential or stationary stage of growth. The WMH1524 *dnrJ* strain was significantly more sensitive to DNR than the wild-type strain was, and the WMH1644 *dnrJ drrC* strain was more sensitive than the WMH1524 strain was (Table 3). (No difference in sensitivity between the wild-type strain and its WMH1642 neomycin- and thiostrepton-resistant transductant was observed [data not shown].) These results indicate that the *drrC* gene could function as a DNR resistance gene. We also found that the DNR resistance of the *dnrJ* strain was restored to a level near that of the wild-type strain when WMH1524 was transformed with pWHM908, a low-copy-number pWHM601-derived plasmid carrying the wild-type *dnrJ* gene (31) (Table 4). (No difference in DNR sensitivity between the *dnrJ* mutant strain and its pWHM601 transformant was observed [data not shown].) This result plus the fact that the *S. peucetius dnrV* and *dnrZ*

TABLE 5. DNR sensitivities of *E. coli* UNC523 *uvrA* (λDE3) lysogens with and without the *drrC* gene

Strain	Plasmid introduced	Fraction of organisms surviving after treatment with DNR at the following concn (μg/ml)[a]:		
		200	500	1,000[b]
WMH1646	pET-17b	$(2.4 \pm 0.5) \times 10^{-1}$	$(6.5 \pm 1.0) \times 10^{-2}$	$(3.7 \pm 1.0) \times 10^{-4}$
WMH1647	pWHM270 (*drrC*$^+$)	$(6.0 \pm 0.6) \times 10^{-1}$	$(3.7 \pm 0.9) \times 10^{-1}$	$(1.4 \pm 0.3) \times 10^{-2}$

[a] Data are averages with standard errors ($n = 4$).
[b] *E. coli* K802 *uvrA*$^+$ (λDE3) lysogens exhibited values of $(8.3 \pm 1.0) \times 10^{-2}$ and $(2.7 \pm 0.5) \times 10^{-2}$ with and without pWHM271, respectively, for this concentration of DNR.

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TABLE 6. UV sensitivities of *E. coli* K802 *uvrA*$^+$ (λDE3) lysogens with and without the *drrC* gene

Strain	Plasmid introduced	Fraction of organisms surviving after UV irradiation for the following period (s)[a]:		
		30	45	60
WMH1648	pET-26b	$(2.5 \pm 0.4) \times 10^{-1}$	$(8.5 \pm 2.2) \times 10^{-2}$	$(1.4 \pm 0.4) \times 10^{-2}$
WMH1649	pWHM271 (*drrC*$^+$)	$(7.7 \pm 0.8) \times 10^{-1}$	$(4.2 \pm 0.3) \times 10^{-1}$	$(2.2 \pm 0.5) \times 10^{-1}$

[a] Data are averages with standard errors ($n = 4$). *E. coli* UNC523 *uvrA* (λDE3) lysogens exhibited values of $(2.4 \pm 0.7) \times 10^{-2}$ and $(2.2 \pm 0.3) \times 10^{-2}$ with and without pWHM270, respectively, after 5 s of irradiation.

strains, two other non-DNR-producing mutants also unable to convert ε-rhodomycinone to glycosylated intermediates of DNR biosynthesis (35), were more sensitive to DNR than the wild-type strain was when tested in a similar manner (data not shown) suggests that DNR resistance may be induced by DNR, DXR, or their glycosylated precursors.

Expression of the *drrC* gene in *E. coli* increases DNR resistance but does not complement a *uvrA* mutation. Since *uvrA*$^+$ *E. coli* K802(λDE3) lysogens were quite resistant to DNR, even though introduction of the *drrC* gene increased the resistance somewhat (Table 5), we capitalized on the reported high-level DXR sensitivities of *E. coli uvrA* strains (25) to determine whether the DrrC protein could impart DNR resistance to *E. coli*. The *drrC* gene was cloned in the T7-based expression vector pET-17b as pWHM270 and introduced into an *E. coli* UNC523 *uvrA* (33) λDE3 lysogen to produce the WMH1647 strain. A protein with the mobility expected for DrrC in SDS-PAGE analysis was produced in WHM1647 upon induction with IPTG (see Materials and Methods), and WMH1647 was 3- to 30-fold more resistant to DNR than the isogenic WMH1646 strain containing the pET-17b vector only was (Table 5). DNR resistance was determined without IPTG induction of *drrC* expression because of the difficulty of maintaining a known and consistent population of induced cells during DNR treatment (some degree of T7-controlled gene transcription usually occurs in λDE3 lysogens [34]). Interestingly, DrrC considerably enhanced the resistance of the wild-type *uvrA*$^+$ *E. coli* K802 λDE3 lysogen to the lethal effect of UV (Table 6, the WMH1649 strain, which carried *drrC* cloned in pET-26b as pWHM271). There was no difference in UV sensitivity between the *E. coli* UNC523 *uvrA* λDE3 lysogens with and without *drrC* (Table 6). There was also no difference in mitomycin sensitivity between these strains (data not shown).

DISCUSSION

Self-resistance is an important requirement for antibiotic-producing microorganisms that are sensitive to the antibiotic produced. The frequent occurrence of more than a single resistance gene within or outside the cluster of structural and regulatory genes in producing organisms attests to this fact (9). For example, tylosin resistance in *Streptomyces fradiae* involves both target site modification (47, 48) and apparent export mechanisms (39, 42) mediated by the *tlrAD* and *tlrBC* genes, respectively. Resistance to actinorhodin (15) and tetracenomycin (18), in contrast, appears to involve a single transport-mediated mechanism, which in the case of actinorhodin has been shown to be essential for antibiotic export (15).

The *drrC* gene appears to be a bona fide DNR resistance gene because it conferred significant DNR resistance to both *S. lividans* (Table 2) and *E. coli* (Table 5). Furthermore, disruption of *drrC* in the *dnrJ* mutant clearly resulted in an increased DNR sensitivity (Table 3), even though the *dnrJ drrC* mutant still retained a low level of DNR resistance. In fact, it was possible to introduce pWHM908 containing the wild-type *dnrJ* gene into the *dnrJ drrC* mutant and reisolate it from the WMH1644(pWHM908) transformants (29), perhaps because pWHM908 restores only a low level of DNR production to the *dnrJ* mutant (31). Nonetheless, the results of the infection experiments with phWHM263 suggest that *drrC* may be important for DNR production, since the loss of its function was disfavored unless *S. peucetius* was unable to make DNR or its antibiotically active precursors. The *drrAB* genes (17) or the *ric2* locus (8) thus are apparently not able to compensate for loss of *drrC* function.

Why does *S. peucetius* seem to require multiple DNR resistance mechanisms? It is plausible that in addition to drug export mediated by the *drrAB* genes, the organism needs a way to minimize the amount of DNR and DXR bound to genomic DNA to avoid interference with gene expression or free radical damage resulting from reduction of the bound drug, as in *E. coli* (14, 25). We speculate that DrrC could inhibit or destabilize the binding of DNR and DXR to genomic DNA because of its similarity to bacterial UvrA proteins (Fig. 3) that are DNA-binding proteins (16, 43). Yet we cannot exclude a DNR-DXR export function for DrrC, since its sequence also resembles those of proteins known to facilitate metabolite transport by an ATP-dependent process. DrrC may bind DNR (and DXR) specifically, because it did not also confer mitomycin resistance to the *E. coli uvrA* mutant. (Mitomycin resistance involves a novel type of drug modification in the producing strain, *Streptomyces lavendulae* [3].) Since DrrC was unable to protect the *E. coli uvrA* mutant from the lethality of UV or mitomycin, classic factors that induce the UvrABC excision repair system in *E. coli* (16), it is unlikely that DrrC facilitates the repair of DNA damage resulting from the free-radical-promoting effects of DNR and DXR. However, DrrC clearly has a UV-protective effect on *E. coli* (Table 6). One possibility is that the higher-level UV resistance of the *uvrA*$^+$ lysogens could be due to a stimulating effect of DrrC on the (A)BC excinuclease mechanism, through an enhancement of UvrA dimerization or $(UvrA)_2(UvrB)_1$ complexation, critical initial steps in the mechanism (43).

A further unresolved issue is why the *dnrJ*, *dnrV*, and *dnrZ* non-DNR-producing mutants are more sensitive to DNR than the wild-type strain is if exogenous DNR is able to induce *drrAB* and/or *drrC* expression equally in all of these strains. Perhaps the true inducer is a glycosylated precursor of DNR also not synthesized in the mutants. This idea is consistent with the report (26) that expression of the *tlrA* tylosin resistance gene, which was induced in *S. fradiae* by all the glycosylated tylosin precursors tested, was induced only by the monosaccharide and not the disaccharide precursors in *Streptomyces albus* carrying the *tlrA* gene.

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