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Characterization of a Mitomycin-Binding Drug Resistance Mechanism from the Producing Organism, *Streptomyces lavendulae*

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In an effort to characterize the diversity of mechanisms involved in cellular self-protection against the antitumor antibiotic mitomycin C (MC), DNA fragments from the producing organism (*Streptomyces lavendulae*) were introduced into *Streptomyces lividans* and transformants were selected for resistance to the drug. Subcloning of a 4.0-kb *Bcl*I fragment revealed the presence of an MC resistance determinant, *mrd*. Nucleotide sequence analysis identified an open reading frame consisting of 130 amino acids with a predicted molecular weight of 14,364. Transcriptional analysis revealed that *mrd* is expressed constitutively, with increased transcription in the presence of MC. Expression of *mrd* in *Escherichia coli* resulted in the synthesis of a soluble protein with an M_r of 14,400 that conferred high-level cellular resistance to MC and a series of structurally related natural products. Purified MRD was shown to function as a drug-binding protein that provides protection against cross-linking of DNA by preventing reductive activation of MC.

Streptomyces species are gram-positive soil bacteria known for their ability to produce a wide range of biologically active metabolites. In addition, many resistance genes have been cloned from these bacteria. Mechanisms of cellular self-protection include drug inactivation, target site modification, reduction of intracellular concentration via efflux, and drug binding (8). The presence of multiple modes of self-protection toward a single antibiotic is well documented (9), often with one or more resistance determinants located adjacent to the corresponding biosynthetic genes.

Mitomycin C (MC) was identified in 1956 as an antibiotic produced by *Streptomyces lavendulae* (18) and subsequently established as an important antitumor agent (19, 21). MC functions as a prodrug and requires enzymatic or chemical reduction to become a highly reactive alkylating agent (19, 40). The intracellular activation of MC is specified by endogenous flavoreductases (34) and proceeds by single electron reduction to the MC semiquinone radical. The relatively long-lived semiquinone species either rearranges to an alkylating intermediate (by further reduction) or transfers an electron to molecular oxygen to generate superoxide (32). Therefore, the ability of MC to inhibit bacterial and mammalian cell growth involves the combined action of DNA alkylation and the formation of reactive oxygen species. Other naturally occurring compounds within this class include bleomycin (37), enediynes (10), and the more recently discovered dihydrobenzoxazines (25, 42).

Recently, a locus, *mcr*, that confers high-level MC resistance in *S. lavendulae* has been reported (2, 3). The resistance gene, *mcrA*, encodes a flavoenzyme (MCRA) that reoxidizes reductively activated MC (23). In another example involving a DNA damaging agent, bleomycin self-resistance has been determined by drug modification (Bat) and binding (BLMA) proteins in *Streptomyces verticillus* (38). Beyond these examples, little is known about bacterial resistance to the growing class of bioreductively activated DNA alkylating and cleaving agents.

In this study we examine the function of a second gene (*mrd*) cloned from *S. lavendulae* that confers MC resistance when introduced into both *Streptomyces lividans* and *Escherichia coli*. Isolation and purification of the resistance protein, MRD, have revealed that it prevents reductive activation through specific binding of the prodrug.

MATERIALS AND METHODS

Strains and culture conditions. *E. coli* DH5αF′ (33) used as a host for generation of double-stranded plasmid and single-stranded M13 DNA (for sequencing) was grown at 37°C on Luria-Bertani and 2×YT media (33), respectively. *E. coli* BL21(DE3), used as a host for protein expression, was grown at 37°C in NZCYM medium (33). *S. lividans* 1326 was grown on R2YE (20) plates at 30°C for general use. *S. lividans* transformation was carried out by standard procedures (20), and transformants were selected on R2YE plates containing 50 μg of thiostrepton per ml.

DNA preparation and amplification. *S. lavendulae* genomic DNA was isolated by the lysozyme–2× Kirby mix method described by Hopwood et al. (20). General DNA manipulation and single-stranded DNA isolation were done as described previously (2). Oligonucleotides for PCR, primer extension, and sequencing were obtained from Gibco BRL (Gaithersburg, Md.). PCR amplifications were carried out by using a thermal cycler (Hybaid Ltd., Teddington, United Kingdom).

Cloning and sequencing of *mrd*. An *S. lavendulae* B619 DNA library was constructed in the high-copy-number *Streptomyces* vector pIJ702 (20) and used to transform *S. lividans* 1326 as previously described (2). Selection for resistance resulted in isolation of pDHS3001, which contains a 4.0-kb *Bcl*I DNA insert. From plasmid pDHS3001, restriction fragments of the insert DNA were isolated, and the ends were filled with the large fragment of DNA polymerase I. The blunt-ended fragments were then cloned into the *Bgl*II site of vector pIJ702, which was similarly blunt ended. The constructs were then transformed into *S. lividans* 1326 by the polyethylene glycol-mediated protocol described previously (20). Thiostrepton-resistant transformants were transferred to R2YE agar plates containing 25 μg of MC per ml, and pDHS7000 was isolated by its ability to confer drug resistance.

The 1.1-kb *Not*I-*Pvu*I fragment from pDHS3001 was treated with the large fragment of DNA polymerase I and cloned into the *Hin*cII site of pUC119. A set of nested deletion clones was generated with exonuclease III (Erase-A-Base kit;

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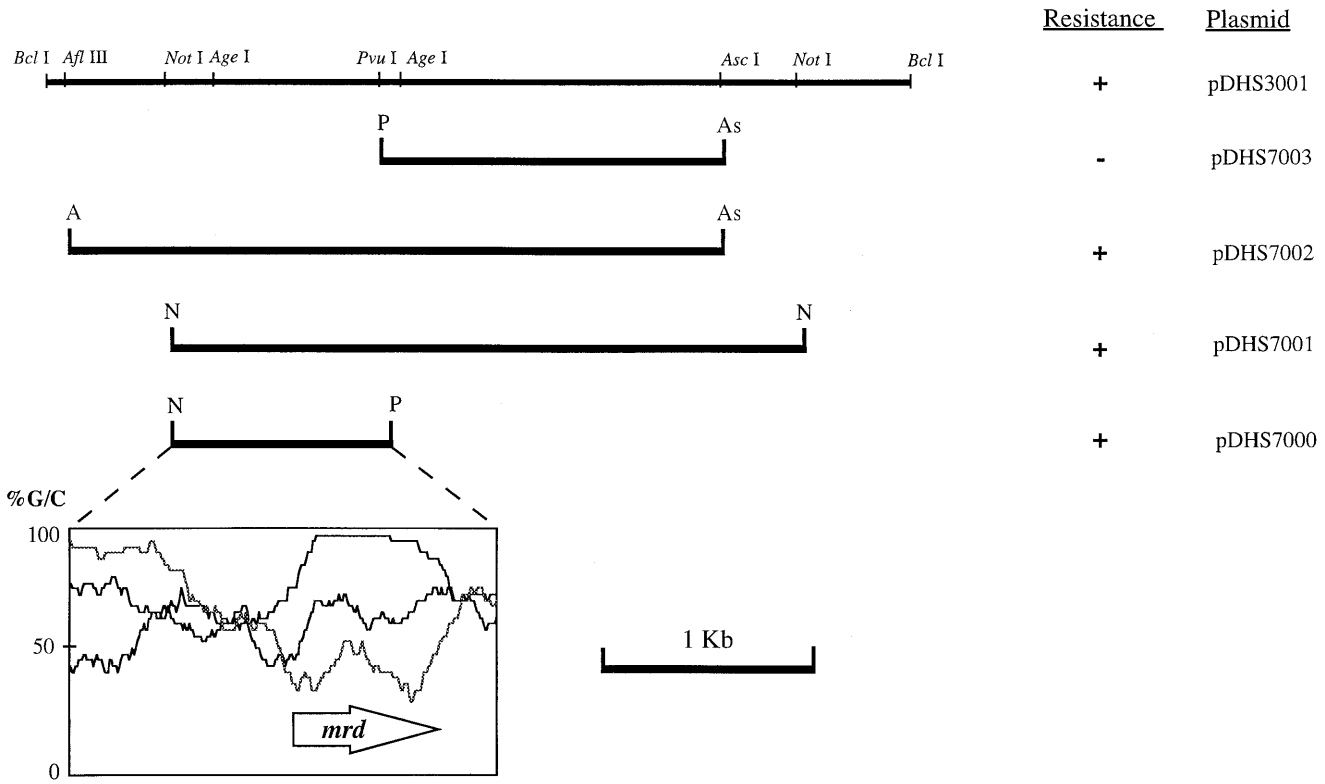

FIG. 1. Restriction map of clone pDHS3001 and localization of the gene for low-level MC resistance. In the column labeled resistance whether the subclone is capable of conferring resistance to 25 μg of MC per ml is indicated. The enlarged box shows the frame analysis (6) of the nucleotide sequence (described in Materials and Methods). A, *Afl*III; As, *Asc*I; N, *Not*I; P, *Pvu*I.

Promega, Madison, Wis.), and both strands of the insert DNA were sequenced by the dideoxy chain termination method by using the double-stranded DNA Cycle Sequencing System (Gibco BRL), double-stranded templates, and [α-^{32}P]dCTP (Amersham, Arlington Heights, Ill.). Dimethyl sulfoxide (10%) was added to the reaction mixture to reduce compressions. Sequence data were analyzed by using the Geneworks (Intelligenetics, Mountain View, Calif.) software package. Deduced amino acid sequences were compared to those in the available protein databases by using the BLAST program of the Genetics Computer Group (Madison, Wis.) version 8.0 software.

RNA preparation. *S. lividans* 1326 containing pDHS3001 or pIJ702 was grown in YEME medium (20) supplemented with 5 mM $MgCl_2$–0.5% glycine–thiostrepton (5 μg/ml). For MC-induced cultures, 5 μg of drug per ml was added to the culture 30 min prior to RNA extraction. Total RNA was extracted from cultures after 72 h by the modified Kirby method (20) and resuspended in diethyl pyrocarbonate (DEPC)-treated deionized water. Contaminating DNA was removed by digestion with RNase-free DNase (Gibco BRL), and the concentration of RNA was determined spectrophotometrically.

Northern (RNA) blot analysis. RNA samples (40 μg of total RNA) were electrophoresed on a 1.2% agarose–formaldehyde denaturing gel in morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS, 5 mM sodium acetate, 2 mM EDTA [pH 7.0]) (4). After the gel was washed twice for 5 min (each) in DEPC-treated deionized water, RNA was transferred overnight with DEPC-treated 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (33) to a Hybond N nylon membrane (Amersham). The nucleic acids were UV cross-linked to the membrane. The probe used for hybridization was prepared by labeling of the 0.8-kb *Age*I-*Pvu*I fragment of pDHS3001 by using the Radprime random primers kit (Gibco BRL) and [α-^{32}P]dCTP. After prehybridization at 65°C for 15 min, hybridization was carried out overnight at 65°C in 0.5 M sodium phosphate (pH 7.2)–7% sodium dodecyl sulfate (SDS)–1 mM EDTA–1% nonfat dry milk (blocking agent)–probe at a concentration of 20 ng/ml. The blot was washed twice with 40 mM sodium phosphate (pH 7.2)–5% SDS–1 mM EDTA–0.5% nonfat dry milk at 65°C for 10 min and then once with 20 mM sodium phosphate (pH 7.2)–1% SDS–1 mM EDTA at 65°C for 30 min.

Primer extension analysis. Ten micrograms of total RNA plus 10^4 to 10^5 cpm of ^{32}P-end-labeled primer (5′-TTCCGGTAGAACTCCAGCGA-3′) was denatured at 95°C for 10 min and annealed at 60°C for 18 h. The reverse transcription reactions were carried out with the Primer Extension System kit (Promega). The DNA sequence ladder was generated with the same primer used in the extension reaction mixtures.

Construction and use of *mrd* expression plasmid. For the construction of the *E. coli* expression plasmid, *Nde*I and *Hin*dIII sites were introduced at the translational start codon and downstream of the translational stop codon of the *mrd* gene, respectively. The primers used for PCR were 5′-GGGAATTCCATATG TCAGCAAGGATTTCCC-3′ and 5′-GGGAATTCAAGCTTATAGCGCGGG AAGCCG-3′. The PCR was carried out with 2.5 U of *Taq* polymerase–0.4 μg of each primer–1 μg of pDHS7012 DNA as the template–10 mM (each) dATP, dGTP, dCTP, and dTTP–1.5 mM $MgCl_2$–10 μl of 10× Promega PCR buffer in a total volume of 100 μl. Amplification was achieved with 30 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 1 min, and extension at 70°C for 2 min.

The 0.71-kb PCR product was recovered by 1.0% agarose gel electrophoresis, digested with *Eco*RI-*Hin*dIII, and ligated into the T7 expression plasmid pT7SC (7), which had been similarly cut with *Eco*RI-*Hin*dIII, to give pDHS7006. pDHS7006 was introduced by transformation into *E. coli* BL21(DE3) to result in strain PJS100. Expression of the cloned gene was achieved when the culture was grown at 37°C in the presence of 100 μg of ampicillin per ml and the addition of isopropylthiogalactopyranoside (IPTG) to a final concentration of 1 mM (33). Proteins were analyzed by SDS–14% polyacrylamide gel electrophoresis (PAGE).

To analyze resistance conferred by the expression of *mrd* in *E. coli*, 10 μl of strain PJS100 was spread on LB agar medium containing 100 μg of ampicillin per ml, IPTG to a final concentration of 0.5 mM, and the antibiotic being analyzed. The cultures were grown overnight at 37°C, and the CFU were quantified.

Amino acid sequence analysis of MRD. Strain PJS100 was grown as described above with incubation continuing for 6 h after induction with IPTG. Cells were harvested from 1 ml of the culture and lysed in gel loading buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Total cellular proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane by electroblotting (30) with a Bio-Rad semi-dry blotting apparatus. The membrane was stained with Coomassie brilliant blue R, and the protein band corresponding to MRD was removed and submitted to the University of Minnesota Microchemical Facility for N-terminal sequencing.

Purification of MRD. Strain PJS100 was grown overnight and used as the inoculum for fresh medium. After inoculation and growth for 2 h, IPTG was added to a final concentration of 1 mM and incubation was continued at 37°C for an additional 6 h. The harvested cells were sonicated in 50 mM Tris (pH 7.2), and insoluble cellular debris was removed by centrifugation. Total soluble protein was precipitated with ammonium sulfate, with fractions (60 to 80%) containing

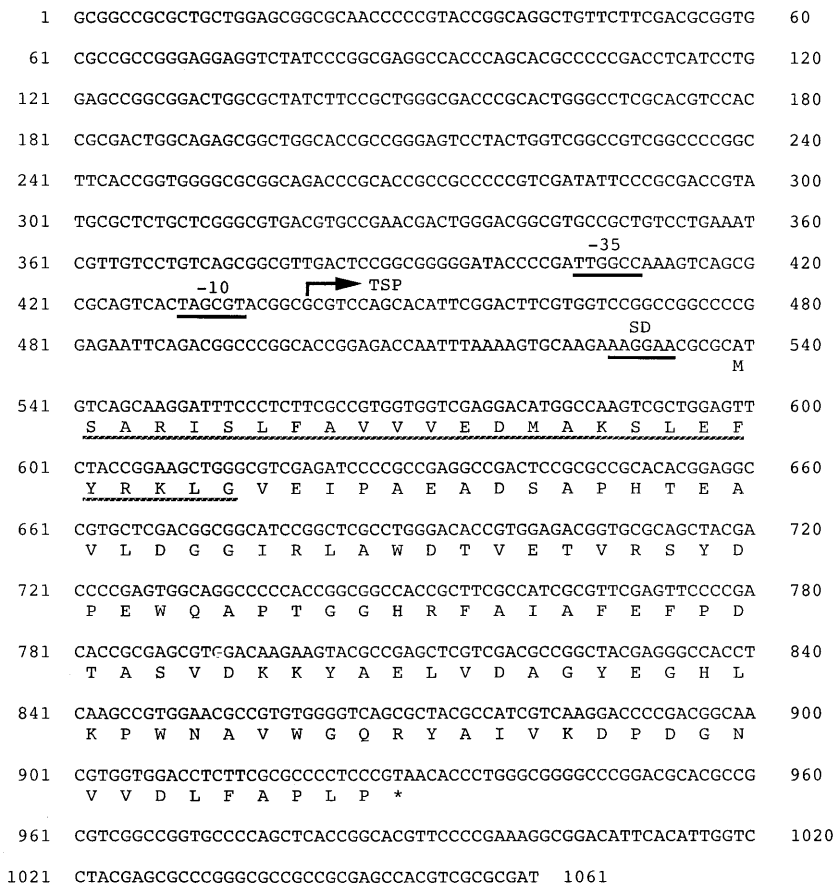

FIG. 2. Nucleotide sequence of the 1.06-kb insert of pDHS7000. The deduced amino acid sequence of *mrd* is indicated below the DNA sequence by single letter designations. The predicted promoter sequences and ribosome binding sites are indicated by solid underlining. The N-terminal amino acid sequence determined for MRD is marked with a dashed underline. The putative transcription start point (TSP) is denoted with an arrow.

MRD being dialyzed against 25 mM Tris (pH 7.2) and loaded onto a Macro-prep 50Q (Bio-Rad) anion-exchange column. Proteins were eluted with a linear gradient of 0 to 1.0 M NaCl in 25 mM Tris (pH 7.2). Fractions containing MRD were pooled, concentrated by ammonium sulfate precipitation, and loaded onto a Sephacryl-200 column. The desired protein was eluted from the column with 25 mM Tris (pH 7.2) buffer.

DNA protection assay. This assay was developed to demonstrate the ability of MRD to protect DNA from MC alkylation under conditions that reductively activate the drug. Reaction mixtures contained various concentrations of MRD plus 33.5 μM MC; 33 μM NADH; 0.4 μM E_3 (CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase), which is a protein containing both flavin adenine dinucleotide (FAD) and [2Fe-2S] center cofactors (31); and 11 μg of DNA in 20 mM Tris-HCl (pH 7.5) (100 μl, total volume). Each reaction mixture was made anaerobic by cycling all reactants except E_3 between vacuum and oxygen-scrubbed argon over a period of 10 min in airtight glassware. The concentrated E_3 was then added under argon pressure with a Hamilton gastight syringe, and the reaction mixture was lightly vortexed, incubated for 15 min, and quenched by being flushed with air. The gel sample (~1 μg of DNA) was denatured by incubation with 0.2 N NaOH for 5 to 10 min prior to the addition of the loading buffer and processing of the gel (0.8% agarose in 1× Tris-borate-EDTA with 1 mg of ethidium bromide per ml at 120 V for 45 min). The DNA used was CsCl-purified pIJ702 (20) from *S. lividans.*

Percentage of DNA protection and MC recovery as a function of [MCRA]/[MC] ratio. Densitometric analysis of the gel was performed with National Institutes of Health Image 1.6 (National Center for Supercomputing Applications, University of Illinois). The percent protection was calculated by dividing the total area of the top and bottom bands by the area of the bottom band. MC remaining in each reaction mixture after the incubation period was quantified by removing a 40-μl aliquot and diluting the aliquot with 100 μl of 20 mM Tris-HCl (pH 7.5). Protein and DNA were separated from the sample by using a Microcon 10 concentrator (Amicon, Beverly, Mass.). The filtrate was directly loaded onto an Econosil C_{18} column (4.6 mm by 250 mm, 10-μm diameter) fitted to a Hewlett-Packard 1090 high-pressure liquid chromatography (HPLC) instrument and separated by using the following elution profile: 0 to 30 min, 6 to 18% CH_3CN (buffer B) in 0.02 M NH_4 acetate (buffer A) (pH 5.2); 30 to 31 min, 18 to 100% buffer B; 31 to 41 min, 100% buffer B; and 41 to 42 min, 100% buffer B; and a final wash for 10 to 15 min with buffer A. The flow rate was 1 ml/min, with detection at 217 and 363 nm. The MC peak from each injection was quantified by manual integration, and the percent MC recovered was calculated based on control injections of the same quantity of MC originally placed in the reaction mixtures.

RESULTS

Cloning of *mrd* gene. The *mrd* locus was cloned from *S. lavendulae* B619 genomic DNA on a 4.0-kb *Bcl*I fragment with the high-copy-number vector pIJ702 (20) (Fig. 1). The resulting recombinant plasmid, pDHS3001, was subcloned further to a 1.06-kb *Not*I-*Pvu*I fragment to generate pDHS7000. When *S. lividans* contains either pDHS3001 or pDHS7000, both of which harbor the *mrd* gene, resistance is conferred to levels of approximately 25 μg of MC per ml, as opposed to *S. lividans* alone, which is unable to grow at concentrations of MC greater than 10 μg per ml.

DNA sequence analysis of *mrd* gene. Sequence analysis of the 1.06-kb insert of pDHS7000 revealed one complete open reading frame (Fig. 1) by using the Geneworks software and calculations based on the frame analysis (6). The *mrd* open reading frame was predicted to begin with the ATG codon at position 539 and end with the TAA codon at position 929 (Fig. 2), resulting in a 130-amino-acid protein with a predicted molecular weight of 14,364. A potential Shine-Dalgarno sequence (36) was found 5 bp upstream (AAGGAA) from the ATG

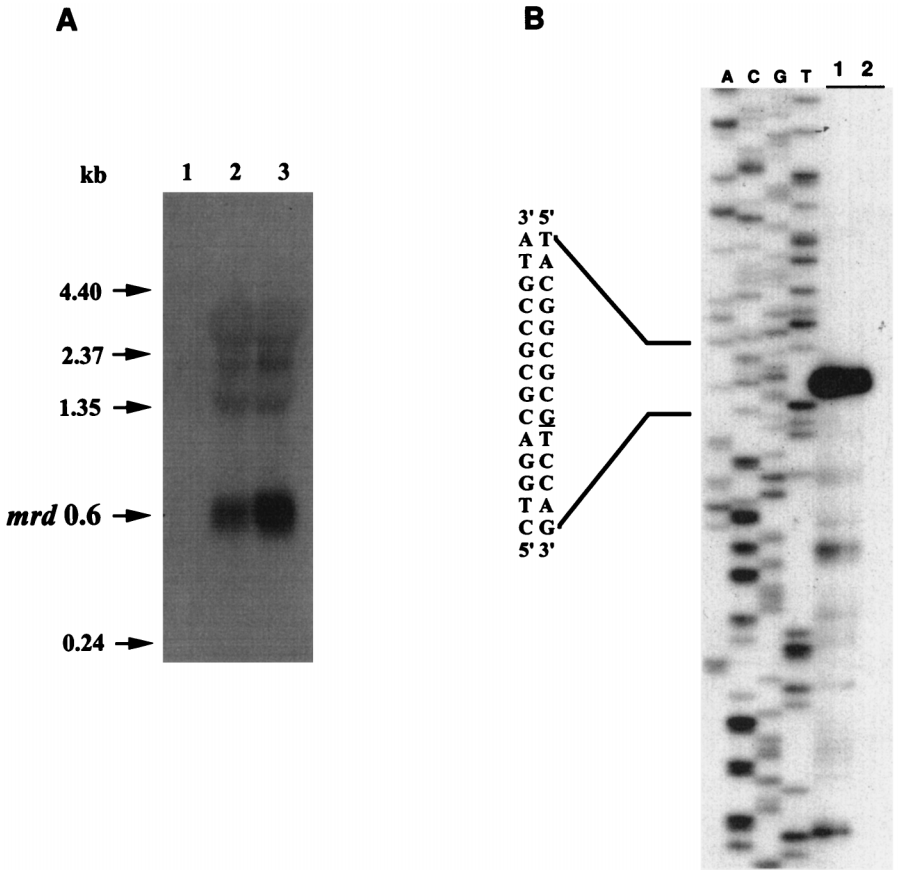

FIG. 3. Northern blot and primer extension analysis of *mrd* expression. (A) Total RNA was isolated from 72-h cultures of *S. lividans* containing either vector (pIJ702) alone (lane 1) or pDHS3001 (lanes 2 and 3). Blotting was done with 40 μg of RNA isolated from a 72-h culture 30 min after the addition of 5 μg of MC per ml (lane 3) or no addition (lane 2). (B) Identification of *mrd* transcription start point by primer extension. The sequence ladder was generated with the same oligonucleotide (described in Materials and Methods) utilized for the primer extension experiments. Primer extensions were carried out with 10 μg of total RNA isolated from a 72-h culture of *S. lividans*/pDHS3001 30 min after the addition of 5 μg of MC per ml (lane 1) or no addition (lane 2). The underlined G residue represents the transcription start point.

initiation codon (Fig. 2). Searching available protein databases with the deduced MRD amino acid sequence failed to reveal significant similarities.

Transcriptional analysis of *mrd*. To define further the gene structure and regulation of *mrd*, transcriptional analysis was performed by Northern hybridization and primer extension. With the 0.8-kb *Age*I fragment of pDHS3001 (Fig. 1) as a probe, a single transcript of approximately 0.6 kb was detected (Fig. 3A). Significantly, *mrd* appears to be constitutively expressed, with increased levels (about 2.5-fold as determined by densitometry) of the specific mRNA detected when cells are grown in the presence of MC. Identical results were obtained with total RNA isolated from *S. lividans*/pDHS3001 and with wild-type *S. lavendulae* (data not shown).

Primer extension analysis was performed to map the 5′ end of the *mrd* mRNA. A single mRNA 5′ end was located 95 bp upstream of the AUG start codon (Fig. 3B). In accord with the Northern hybridization results, the transcript level was greater (approximately 2.5-fold) when the cells had been grown in the presence of MC. Examination of the DNA sequence upstream from the *mrd* start codon in the region of the mapped 5′ end at −95 revealed a potential *Streptomyces* promoter (36) with 4 of 6 bases conserved in both the −10 and −35 hexamers, which are separated by a 19-nucleotide spacer region.

Expression of the *mrd* gene in *E. coli* confers resistance to MC and structurally related natural products. Interestingly, direct cloning of the 1.06-kb *Not*I-*Pvu*I fragment from pDHS3001 into pUC119 (pDHS7012) and introduction into *E. coli* DH5α did not afford protection against the drug (see Discussion). *mrd* was amplified by PCR and cloned into the protein expression vector pT7SC to give pDHS7006 (Table 1). Introduction of pDHS7006 into *E. coli* BL21(DE3) (strain PJS100) and induction with IPTG (see Materials and Methods) resulted in the high-level synthesis of a protein with an apparent molecular weight of 14,400 (Fig. 4A). Significantly, IPTG-induced cultures of PJS100 exhibited resistance to MC at drug concentrations 30-fold greater than those for *E. coli* BL21(DE3) containing vector alone (Table 2).

To establish if MRD-mediated resistance involved a direct response against mitomycins or functioned indirectly to cellular stress (14), the sensitivity of PJS100 to several classes of antibiotics was assessed. In addition to providing resistance to analogs of MC, MRD conferred resistance to the dihydrobenzoxazine FR900482, an antitumor antibiotic that is structurally and mechanistically (25, 41) related to the mitomycins (Table 3). The results establish that MRD reacts against a structurally restricted class of DNA alkylating agents that require reductive activation. Notably, MRD did not provide cellular protection against the known redox cycling agent menadione (Table 3).

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Bacterial strains		
S. lavendulae		
B619	MC^+ Mc^r	Abbott Laboratories
NRRL 2564	MC^+ Mc^r	American Type Culture Collection
S. lividans 1326	MC^- Mc^s	20
E. coli		
DH5αF′	F′ ϕ80 d*lac*Z ΔM15	Gibco BRL
DH5α	F^- *rec*A f80 d*lac*Z ΔM15	Gibco BRL
BL21(DE3)	F^- *ompT hsdS gal dcm* (DE3)	Novagen
PJS100	BL21(DE3) containing pDHS7006	This work
Plasmids		
pUC119	High-copy-number *E. coli* vector; Amp^r	38
pIJ702	*tsr mel*	20
pT7SC	Protein expression vector; Amp^r	6
pDHS3001[b]	pIJ702 with 4.1-kb *Bcl*I DNA insert from *S. lavendulae* B619; contains *mrd* locus	This work
pDHS7000[b]	pIJ702 with 1.02-kb *Not*I-*Pvu*I subclone from pDHS3001	This work
pDHS7001[b]	pIJ702 with 2.7-kb *Not*I subclone from pDHS3001	This work
pDHS7002[b]	pIJ702 with 3.2-kb *Afl*III-*Asc*I subclone from pDHS3001	This work
pDHS7003	pIJ702 with 1.6-kb *Pvu*I-*Asc*I subclone from pDHS3001	This work
pDHS7006[c]	pT7SC with 0.7-kb insert containing *mrd*	This work
pDHS7012	pUC119 with 1.02-kb *Not*I-*Pvu*I subclone from pDHS3001	This work

[a] Mc^s, MC sensitive; Mc^r, MC resistant; MC^+, MC production; *tsr*, gene encoding thiostrepton resistance; *mel*, gene encoding melanin production.
[b] Confers resistance to 25 μg of MC per ml of medium in *S. lividans.*
[c] Confers resistance to 30 μg of MC per ml of medium in *E. coli.*

MRD protects DNA against MC-mediated cross-linking. Since the amino acid sequence of MRD failed to show significant similarity to known proteins, biochemical characterization of the purified protein was initiated. We were able to recover MRD from the cytosolic fraction of whole-cell lysates of strain PJS100 (Fig. 4A). Based on the relatively low pI (4.37) and small size (14.4 kDa) of MRD (determined by SDS-PAGE), anion-exchange and size exclusion chromatographic steps, respectively, were sufficient to purify the protein (Fig. 4B). The molecular weight of MRD determined by gel filtration was 17,000, indicating that it functions as a monomer.

MC cytotoxicity occurs when reductively activated drug covalently binds complementary strands of DNA (22, 39). The presence of MC-DNA cross-links can be detected by a number of techniques, including standard agarose gel electrophoresis. We exploited the ability of the flavoreductase E_3 (31) (see Materials and Methods) to transfer electrons from NADH to MC in the design of an in vitro assay (23) to measure MRD activity. Various concentrations of MRD were incubated with constant concentrations of all other reaction components (MC, NADH, E_3, and DNA). In these experiments MRD protected DNA from MC-mediated cross-linking (Fig. 5A). At a 1:1 stoichiometric ratio with MC, MRD inhibited the formation of cross-links over 90%, as shown by the level of single-stranded DNA after denaturation (Fig. 5A, lane 2). Without MRD in the reaction mixture the DNA was almost completely resistant to denaturation (Fig. 5A, lane 6). As a control, bovine serum albumin (BSA), at a concentration comparable to that of MRD (33.5 μM), did not inhibit the formation of cross-links (Fig. 5A, lane 7).

MRD reversibly binds MC. Structural modification of antibiotics is a common mechanism of cellular self-protection (8, 9). To determine if MC was chemically modified by MRD, aliquots of the DNA protection assay reaction mixtures were filtered to remove contaminating components (protein, DNA, and MC-DNA adducts), and the filtrate was analyzed by HPLC. Since the levels of MC recovered were surprisingly low (Fig. 5B), we were compelled to examine the potential interaction between MRD and MC. Various concentrations of MRD were incubated with MC in the presence and absence of reducing agent followed by ultrafiltration. The results revealed that as the concentration of MRD increased, decreasing amounts of MC were recovered (Fig. 6). However, when the reaction mixtures were boiled, near complete recovery of MC was achieved. Importantly, there was no apparent structural modification of the recovered drug, as deduced by HPLC, spectrophotometric analysis, and biological activity (data not shown). As a control, BSA, at concentrations comparable to those of MRD, did not appreciably retard the recovery of MC (Fig. 6). Equilibrium ultrafiltration (35) showed specific binding of MC to MRD with a mean affinity of approximately 10^4 M^{-1} compared to a value of approximately 10^2 M^{-1} for BSA. Interestingly, MRD-mediated binding of MC was found to be dependent on the presence of a reducing agent such as NADH or dithiothreitol (Fig. 6). A similar cofactor requirement has been previously described for the albicidin resistance protein from *Alcaligenes denitrificans* (5).

DISCUSSION

S. lavendulae contains at least two genetic loci for resistance to MC, *mcr* and *mrd*. Previously, cloning and analysis of *mcr* and its product (MCRA) were shown to involve the oxidation of activated MC (2, 3, 23). In contrast, MRD appears to confer resistance by binding MC as a complex that prevents drug activation. The formation of MC-DNA adducts is highly efficient and selective for guanine nucleosides in the specific sequence 5′-CpG-3′ (27, 28). On the basis of an average G+C

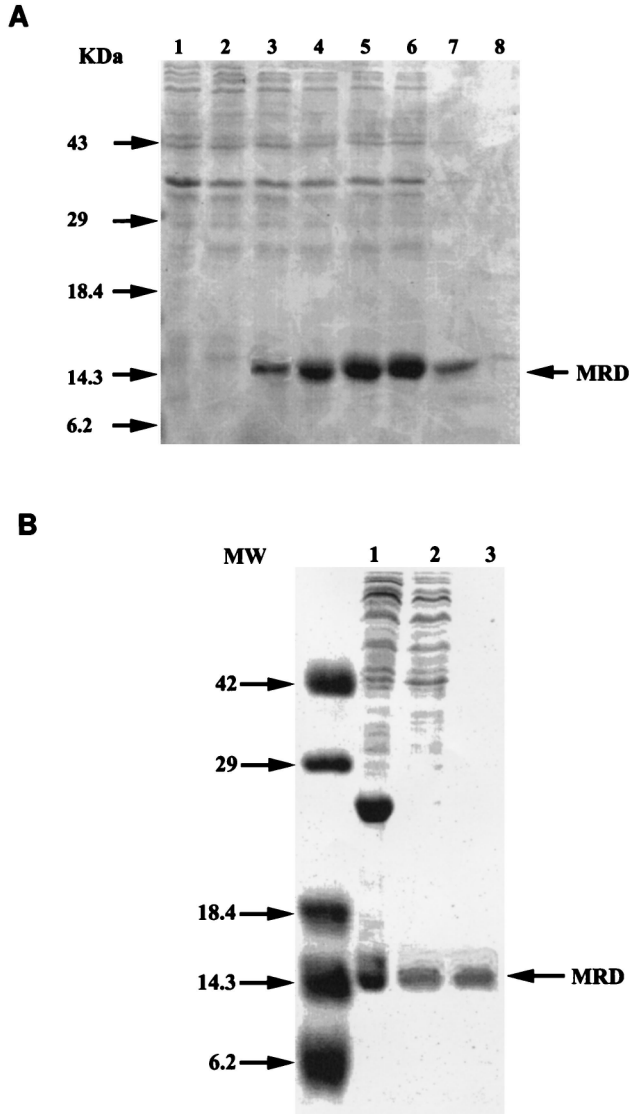

FIG. 4. Time course of MRD expression and protein purification. (A) Total cell lysates were analyzed by SDS–14% PAGE. Lanes: 1, pT7SC vector control; 2, strain PJS100 at 0 h; 3 to 6, strain PJSS100 1 to 4 h after addition of IPTG, respectively; 7, PJS100 soluble fraction; 8, PJS100 insoluble fraction. Positions of molecular mass markers are shown on the left. (B) Purification of MRD. Lanes: 1, 60 to 80% ammonium sulfate precipitate; 2, anion-exchange column; 3, gel filtration column. MRD is denoted with an arrow. Molecular weights (MW) (in thousands) are indicated on the left.

content of 70%, there may be as many as 10^6 target sites within the genome of a typical streptomycete. Considering that a single cross-link is sufficient to cause cell death (22), the protection afforded by multiple resistance determinants appears crucial for *S. lavendulae*. Indeed, *S. lavendulae* mutants lacking *mrd* show considerably (approximately 10-fold) higher levels of sensitivity to MC (34a).

We have shown that the *mrd* gene is expressed constitutively with increased levels of expression when cells are challenged with the drug. Conceivably, as antibiotic production increases, the ability to increase the level of gene expression affords greater concentrations of MRD. We found that the *mrd* gene was not expressed from its native promoter in *E. coli*. Examination of the −10 and −35 hexamers of the *mrd* promoter revealed that they appear to fit into a class of *Streptomyces* promoters that show similarity to the consensus sequence for *E. coli* promoters recognized by $E\sigma^{70}$ (36). However, the inability to successfully express *mrd* with the native promoter is likely attributable to the nonoptimal spacing of the −10 and −35 hexamers.

TABLE 2. Resistance of *E. coli* strains to various concentrations of MC

Concn of MC (μg/ml)	CFU/ml		
	BL21(DE3)	BL21(DE3)/pT7SC	PJS100
0.0	$>10^7$	$>10^7$	$>10^7$
0.01	$>10^7$	$>10^7$	$>10^7$
0.1	9.8×10^3	7.3×10^3	$>10^7$
1.0	0.0	0.0	3.3×10^6
5.0	0.0	0.0	2.7×10^6
10.0	0.0	0.0	6.1×10^5
20.0	0.0	0.0	2.5×10^5
30.0	0.0	0.0	5.0×10^2

E. coli is able to survive harmful changes in the environment by elevating the levels of specific cellular proteins encoded within stress regulons (29). Responses have been observed for disturbances such as oxidative stress (12), heat shock (43), and treatment with DNA-damaging agents (11). Since MRD was unable to protect *E. coli* from the lethal effect of menadione (Table 3), a compound known to produce O_2 radicals in *E. coli* (17), it is unlikely that the protein facilitates the repair of DNA damage caused by O_2 radicals, either directly or through the transcriptional activation of oxidative stress genes or DNA repair systems. Additionally, MRD failed to protect *E. coli* challenged with chloramphenicol, tetracycline, or nalidixic acid (Table 3), compounds that have been associated with multiple antibiotic resistance mediated by the activation of stress regulons *sox*RS and *mar*RAB (14, 16). Significantly, cellular resistance provided by MRD is specific for the mitomycins and/or very closely related compounds.

The ability to overproduce a functional (and soluble) form of MRD in *E. coli* greatly facilitated our investigation of this unique protein. The results of the DNA protection assay (Fig. 5A) show that the purified protein retains its protective activity in vitro. Indeed, when the concentration of the protein was not sufficient, DNA was cross-linked by reductively activated MC (Fig. 5A, lanes 3 to 5). The use of *Streptomyces*-derived plasmid DNA (G+C content of 70%) in the protection assay provides an enormous number of potential cross-linking sites. Therefore, masking of the target site through protein-DNA interactions is an unlikely mode of protection against attack by acti-

TABLE 3. Resistance of *E. coli* to antibiotics other than MC

Antibiotic (concn)[a]	CFU/ml	
	BL21(DE3)/pT7SC	PJS100
Tetracycline	0.0	0.0
Chloramphenicol	0.0	0.0
Nalidixic acid	0.0	0.0
Menadione (4 mM)	0.0	0.0
Mitomycin A	0.0	$>10^7$
Mitomycin B	0.0	$>10^7$
Mitomycin D	0.0	$>10^7$
Porfiromycin	0.0	$>10^7$
FR-900482 (10 μg/ml)	1.0×10^4	$>10^7$
FR-900482 (50 μg/ml)	0.0	$>10^7$

[a] Concentration of 1.0 μg/ml unless otherwise specified.

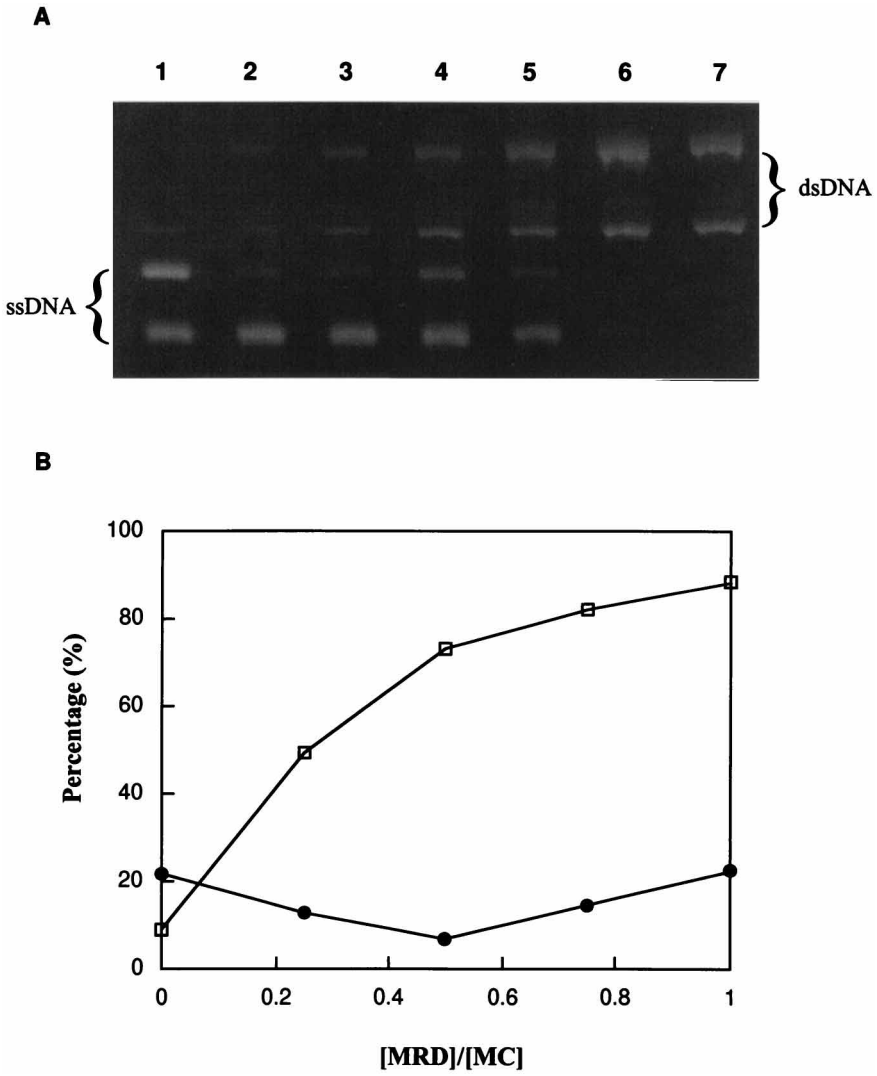

FIG. 5. Anaerobic MRD-mediated DNA protection assay. (A) The gel depicts the results of a series of anaerobic DNA protection reactions (see Materials and Methods). Lanes: 1, DNA denaturation control; 2, 33.5 μM MRD; 3, 25.1 μM MRD; 4, 16.7 μM MRD; 5, 8.3 μM MRD; 6, no MRD; 7, 35 μM BSA. The bands labeled ssDNA are the linear (upper band in lane 1) and circular (lower band in lane 1) strands of the denatured plasmid DNA, while the dsDNA bands are the open circular (upper band in lane 7) and covalently closed circular (lower band in lane 7) forms of the native plasmid DNA (24). (B) Densitometric analysis of DNA protection gel image (percent protection from DNA cross-linking [□]) and HPLC analysis of each reaction mixture (percent recovery [●] of MC).

vated MC. Interestingly, it appears that nearly full protection from DNA cross-linking is attained when MRD is present with MC at a 1:1 ratio. Considering the native state of MRD to be a monomer, this suggests that one molecule of MRD interacts with one molecule of MC. Although equimolar binding of the drug and MRD appears to be an inefficient mechanism of resistance, it is possible that MRD is recycled as part of a drug transport complex (1, 15).

In contrast to the low recovery of MC in the presence of MRD, drug recovery is high when MCRA is present in the reaction mixtures (23). Previously, it has been observed that unreduced MC does not appreciably bind to DNA (26). Consequently, failure to recover sufficient amounts of MC from the DNA protection reaction mixtures (Fig. 5B) suggested that MRD was responsible for drug sequestration. Recovery of unmodified MC after the protein was boiled indicates that the resistance protein binds MC to form a complex that prevents DNA alkylation. Drug binding as a mechanism of cellular self-protection has been observed for bleomycin (13, 38) and albicidin (5). The possibility exists that MRD provides resistance to MC and FR900482 through a redox-mediated pathway similar to that of MCRA. However, spectrophotometric and inductively coupled atomic plasma spectrometry analysis of the purified protein (34a) failed to indicate the presence of any cofactors, such as FAD or metals, that could facilitate electron transfer.

In summary, the present work provides evidence that MRD binds to unreduced MC (and other mitosanes produced by *S. lavendulae*), thus shielding the molecule from in vivo reductive activation catalyzed by intracellular flavoreductases (34). The benefit of preventing reduction of the drug includes the inhibition of the precovalent recognition of DNA (26) by the reactive quinone methide and the suppression of oxygen radical formation due to redox cycling of the MC semiquinone radical (32). Continued biochemical characterization of the MC-MRD complex may provide further insight into the mechanism of this important class of antitumor compounds. To date, it appears that MC resistance in *S. lavendulae* does not

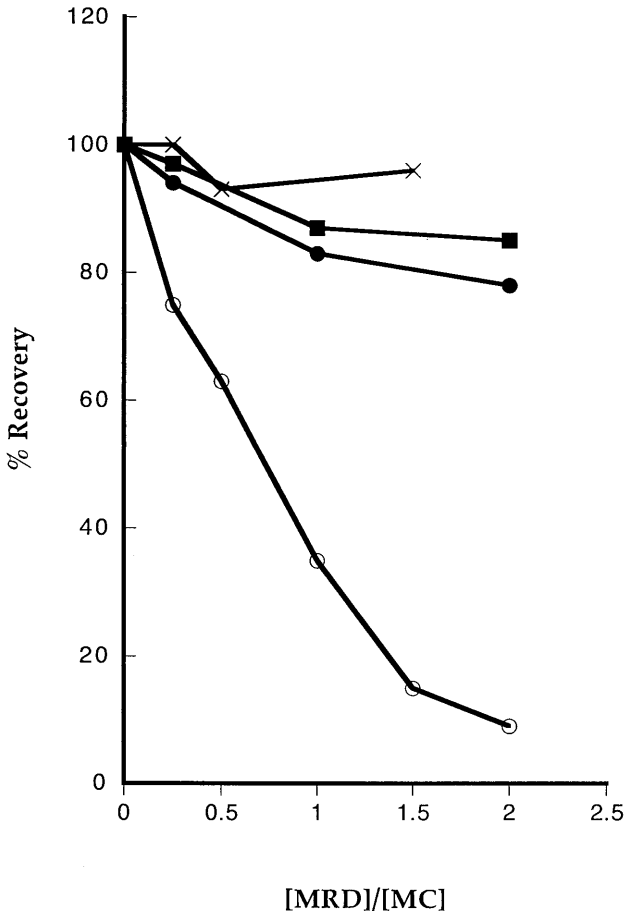

FIG. 6. Percent recovery of MC after incubation with MRD and ultrafiltration. MC recovery was quantified by HPLC from filtrates of reaction mixtures lacking NADH (■) or containing NADH (○) and after the reaction mixtures were boiled (●). As a control, BSA was substituted for MRD in the reaction mixtures (×).

include the use of enzymes that covalently modify the antibiotic. Rather, reversal (MCRA) and prevention (MRD) of reductive bioactivation have evolved as effective modes of cellular self-protection.

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REFERENCES

1. **Ames, G. F. L.** 1986. Bacterial periplasmic transport systems: structure, mechanism, and evolution. Annu. Rev. Biochem. **55:**397–425.
2. **August, P. R., M. C. Flickinger, and D. H. Sherman.** 1994. Cloning and analysis of a locus (*mcr*) involved in mitomycin C resistance in *Streptomyces lavendulae*. J. Bacteriol. **176:**4448–4454.
3. **August, P. R., J. R. Rahn, M. C. Flickinger, and D. H. Sherman.** 1996. Inducible synthesis of the mitomycin C resistance gene product (MCRA) from *Streptomyces lavendulae*. Gene **175:**261–267.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
5. **Basnayake, W. V., and R. G. Birch.** 1995. A gene from *Alcaligenes denitrificans* that confers albicidin resistance by reversible antibiotic binding. Microbiology **141:**551–560.
6. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30:**157–166.
7. **Brown, W. C., and J. L. Campbell.** 1993. A new cloning vector and expression strategy for genes encoding proteins toxic to *Escherichia coli*. Gene **127:**99–103.
8. **Cundliffe, E.** 1989. How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. **43:**207–233.
9. **Cundliffe, E.** 1992. Self-protection mechanisms in antibiotic producers. Ciba Found. Symp. **171:**199–214.
10. **Dedon, P. C., and I. H. Goldberg.** 1992. Free-radical mechanisms involved in the formation of sequence-dependent bistranded DNA lesions by the antitumor antibiotics bleomycin, neocarzinostatin, and calicheamicin. Chem. Res. Toxicol. **5:**311–332.
11. **Demple, B.** 1987. Adaptive responses to genotoxic damage: bacterial strategies to prevent mutation and cell death. Bioessays **6:**157–160.
12. **Demple, B.** 1991. Regulation of bacterial oxidative stress genes. Annu. Rev. Genet. **25:**315–337.
13. **Gatignol, A., H. Durand, and G. Tiraby.** 1988. Bleomycin resistance conferred by a drug-binding protein. FEBS Lett. **230:**171–175.
14. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-encoded efflux of tetracycline. J. Bacteriol. **155:**531–540.
15. **Gilson, E., A. Genevieve, T. Schmidt, J. P. Claverys, R. Dudler, and M. Hofnung.** 1988. Evidence for high affinity binding-protein dependent transport systems in Gram-positive bacteria and in *Mycoplasma*. EMBO J. **7:**3971–3974.
16. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**6181–6185.
17. **Hassan, J. M., and I. Fridovich.** 1979. Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. Arch. Biochem. Biophys. **196:**385–395.
18. **Hata, T., Y. Sano, R. Sugawara, A. Matsumae, K. Kanamori, T. Shima, and T. Hoshi.** 1956. Mitomycin, a new antibiotic from *Streptomyces*. J. Antibiot. Ser. A **9:**141–146.
19. **Henderson, C. I.** 1993. Recent advances in the usage of mitomycin. Oncology **50:**1–84.
20. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. S. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Institute, Norwich, United Kingdom.
21. **Iyer, V. N., and W. Szybalski.** 1963. A molecular mechanism of mitomycin action: linkage of complementary DNA strands. Proc. Natl. Acad. Sci. USA **50:**355–362.
22. **Iyer, V. N., and W. Szybalski.** 1964. Mitomycin or porfiromycin: chemical mechanism of activation and cross-linking of DNA. Science **145:**55–56.
23. **Johnson, D. A., P. R. August, C. Shackleton, H. W. Liu, and D. H. Sherman.** Microbial resistance to mitomycins involves a redox relay mechanism. J. Am. Chem. Soc., in press.
24. **Kieser, T.** 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12:**19–36.
25. **Kiyoto, S., T. Shibata, M. Yamashita, T. Komori, M. Okuhara, H. Terano, M. Kohsaka, H. Aoki, and H. Imanaka.** 1987. A new antitumor antibiotic, FR900482. II. Production, isolation, characterization, and biological activity. J. Antibiot. **50:**594–599.
26. **Kumar, G. S., Q. He, D. Behr-Ventura, and M. Tomasz.** 1995. Binding of 2,7-diaminomitosene to DNA: model for the precovalent recognition of DNA by activated mitomycin C. Biochemistry **34:**2662–2671.
27. **Kumar, S., R. Lipman, and M. Tomasz.** 1992. Recognition of specific DNA sequences by mitomycin C for alkylation. Biochemistry **31:**1399–1407.
28. **Li, V., and H. Kohn.** 1991. Studies on the binding specificity for mitomycin C-DNA monoalkylation processes. J. Am. Chem. Soc. **113:**275–283.
29. **Mager, W. H., and A. De Kruijff.** 1995. Stress-induced transcriptional activation. Microbiol. Rev. **59:**506–531.
30. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:** 10035–10038.
31. **Miller, V. P., J. S. Thorson, O. Ploux, S. F. Lo, and H. W. Liu.** 1993. Cofactor characterization and mechanistic studies of CDP-6-deoxy-delta 3,4-glucoseen reductase: exploration into a novel enzymatic C-O bond cleavage event. Biochemistry **32:**11934–11942.
32. **Pritsos, C. A., and A. C. Sartorelli.** 1986. Generation of reactive oxygen radicals through bioactivation of mitomycin antibiotics. Cancer Res. **46:** 3528–3532.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a

laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Sartorelli, A. C., W. F. Hodnick, M. F. Belcourt, M. Tomasz, B. Haffty, J. J. Fischer, and S. Rockwell.** 1994. Mitomycin C: a prototype bioreductive agent. Oncol. Res. **6:**501–508.
34a. **Sheldon, P. J., and D. H. Sherman.** Unpublished data.
35. **Sophianopoulos, J. A., S. J. Durham, A. J. Sophianopoulos, H. L. Ragsdale, and W. P. Cropper.** 1978. Ultrafiltration is theoretically equivalent to equilibrium dialysis but much simpler to carry out. Arch. Biochem. Biophys. **187:**132–137.
36. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20:**961–974.
37. **Stubbe, J., and J. W. Kozarich.** 1987. Mechanisms of bleomycin-induced DNA degradation. Chem. Rev. **87:**1107–1136.
38. **Sugiyama, M., C. J. Thompson, T. Kumagai, K. Suzuki, R. Deblaere, R. Villarroel, and J. Davies.** 1994. Characterization by cloning of two genes from *Streptomyces verticillus* encoding resistance to bleomycin. Gene **151:**11–16.
39. **Tomasz, M.** 1995. Mitomycin C: small, fast and deadly (but very selective). Chem. Biol. **2:**575–579.
40. **Tomasz, M., A. K. Chawla, and R. Lipman.** 1988. Mechanism of monofunctional and bifunctional alkylation of DNA by mitomycin C. Biochemistry **27:**3182–3187.
41. **Woo, J., S. T. Sigurdsson, and P. B. Hopkins.** 1993. DNA interstrand cross-linking reactions of pyrrole-derived, bifunctional electrophiles: evidence for a common target site in DNA. J. Am. Chem. Soc. **115:**3407–3415.
42. **Woo, J., S. T. Sigurdsson, and P. B. Hopkins.** 1993. DNA interstrand cross-linking by reductively activated FR900482 and FR66979. J. Am. Chem. Soc. **115:**1199–1200.
43. **Yura, T., H. Nagai, and H. Mori.** 1993. Regulation of the heat-shock response in bacteria. Annu. Rev. Microbiol. **47:**321–350.