Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8950–8954, September 1995
Genetics

A general genetic approach in *Escherichia coli* for determining the mechanism(s) of action of tumoricidal agents: Application to DMP 840, a tumoricidal agent

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Communicated by John R. Roth, The University of Utah, Salt Lake City, UT, April 13, 1995 (received for review December 19, 1994)

ABSTRACT **We describe here a simple and easily manipulatable *Escherichia coli*-based genetic system that permits us to identify bacterial gene products that modulate the sensitivity of bacteria to tumoricidal agents, such as DMP 840, a bisnaphthalimide drug. To the extent that the action of these agents is conserved, these studies may expand our understanding of how the agents work in mammalian cells. The approach briefly is to use a library of *E. coli* genes that are overexpressed in a high copy number vector to select bacterial clones that are resistant to the cytotoxic effects of drugs. A *tolC* bacterial mutant is used to maximize permeability of cells to hydrophobic organic molecules. By using DMP 840 to model the system, we have identified two genes, designated *mdaA* and *mdaB*, that impart resistance to DMP 840 when they are expressed at elevated levels. *mdaB* maps to *E. coli* map coordinate 66, is located between the *parE* and *parC* genes, and encodes a protein of 22 kDa. *mdaA* maps to *E. coli* map coordinate 18, is located adjacent to the glutaredoxin (*grx*) gene, and encodes a protein of 24 kDa. Specific and regulatable overproduction of both of these proteins correlates with DMP 840 resistance. Overproduction of the MdaB protein also imparts resistance to two mammalian topoisomerase inhibitors, Adriamycin and etoposide. In contrast, overproduction of the MdaA protein produces resistance only to Adriamycin. Based on its drug-resistance properties and its location between genes that encode the two subunits of the bacterial topoisomerase IV, we suggest that *mdaB* acts by modulating topoisomerase IV activity. The location of the *mdaA* gene adjacent to *grx* suggests it acts by a drug detoxification mechanism.**

The utility of tumoricidal drugs is often limited by a lack of information about the targets of the drug in cells and about the relationship between the presumed drug–target interactions and the cytotoxic effect(s) of the drug. In the absence of such information, it often becomes difficult to modify the drug to maximize desirable interactions and minimize undesirable ones.

There are primarily two general approaches for determining how a drug acts in mammalian cells: a biochemical approach and a genetic approach. The biochemical approach is largely based on clever speculation regarding the cellular target(s) of drug action that takes advantage of the structure and properties of the drug or its analogs and often relies on information about variation in cellular responses to the drug as a function of alterations in presumed target levels. The difficulty with this approach is that (*i*) it is based on a rather "hit-or-miss" process that depends largely on the availability of the above information for any drug and (*ii*) only limited conclusions can be drawn from the final analysis. Thus, the identification of a protein that is inhibited by a particular drug still fails to address issues such as whether this is the only protein inhibited by the drug; if there are others, which one is inhibited most efficiently by the drug; and, perhaps most important, whether the observed inhibition is responsible for the effects of the drug in cells.

The genetic approach to understanding drug mechanism has the potential to be more informative than the biochemical approach and can certainly deal with issues relating drug targets to drug-induced cellular changes. Thus, the isolation and characterization of mammalian cell lines that are resistant to cytotoxic anticancer agents such as camptothecin, amsacrine, and etoposide (1–3) reveal that these cells contain reduced levels or resistant forms of topoisomerase I or topoisomerase II enzymes and that the resistance correlates with mutations in genes that encode these enzymes (3–5). The difficulty with this approach as a general scheme in mammalian cells stems from the fact that these cells represent a difficult genetic system to work with. Thus, even if a cell line that is resistant to a particular drug has been isolated, in the absence of further information about the gene and mutation involved, one is left with the tedious task of trying to localize the region of the genome responsible (6, 7).

Thus, we have begun to develop a bacterial system in which we can rapidly analyze drug resistance in a well-developed easily manipulatable genetic system. We have chosen the bisnaphthalimide drug DMP 840 (8) for this initial study because it is a potent antitumor agent, because its presumed cellular target, DNA, is conserved, and because its mechanism of action remains unclear. *In vitro*, the drug intercalates into DNA with a preference for G+C-rich regions (9). In cultured mammalian cells, the drug preferentially fractionates and photocrosslinks to DNA (10) and appears to generate nicks but not double-strand breaks or covalent topoisomerase–DNA complexes (9). Finally, DMP 840-resistance patterns in various mammalian cell lines that are resistant to known topoisomerase I and II inhibitors have not indicated a clear correlation with any of these inhibitors (9).

We describe here the use of a multicoverage library of *Escherichia coli* genes to ask whether the overproduction of any one gene product can confer resistance to DMP 840. We have identified two *E. coli* genes that have this property and by simple genetic manipulation have isolated, mapped, sequenced, and regulated the expression of those genes.* These results confirm the utility of our approach.

MATERIALS AND METHODS

Bacterial Strains, Vectors, and Media. NS3678 is strain AB1157 *tolC* (λLP1). The *tolC* mutation is due to a Tn*10*tet^{r} insertion in that gene that was transduced into NS3678 from strain CS1562 by using phage P1 (11, 12). The λLP1 prophage (Andrew Wright, Tufts University, Medford, MA) contains a

Abbreviations: r (as a superscript), resistant; ORF, open reading frame.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U18655 for clone 18a and U18656 for clone 66a).

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lacI^q repressor gene. The *tolC*⁻ phenotype of NS3678 was initially confirmed and periodically checked by assessing sensitivity of cells to crystal violet (13). The arabinose promoter vector pBAD-18 is described in ref. 14. M9 medium contains 1× M9 minimal salts (15) supplemented with 0.3% Casamino acids, 1 mM $MgCl_2$, thiamine (10 μg/ml), and 0.4% glucose. Induction of the arabinose promotor in pBAD-18 constructs was assessed by adding 0.2% glucose or 0.2% arabinose to the medium. M9 agar is M9 medium with 1.2% (wt/vol) agar. Tumoricidal agent-containing medium was protected from light to avoid photodamage to the drug.

Tumoricidal Agents. DMP 840 was obtained from DuPont Merck. Adriamycin, etoposide, amsacrine, and camptothecin were purchased from Sigma. The 5 mM stock solutions of drugs in dimethyl sulfoxide were stored at −20°C.

Standard Biochemical Procedures. Plasmid DNA isolation, digestion with restriction enzymes (New England Biolabs), and agarose gel analyses were as described (15). Proteins in crude cell extracts were analyzed by SDS/PAGE. DNA fragments, after being cloned into M13 vectors, were sequenced by the method of Sanger *et al.* (16).

Preparation of the pUC19–*E. coli* Gene Library. High molecular weight genomic DNA isolated from cell line NS3678 was digested with 1 unit of *Sau*3AI (New England Biolabs) at 37°C for 15 min as described by Pierce *et al.* (17). The 2- to 10-kb DNA was isolated from an agarose gel and purified. Fragments were ligated to *Bam*HI-digested pUC19 DNA (New England Biolabs) at a 3-fold molar excess of fragments, and the ligated product was used to transform electrocompetent JM101 bacteria. Colonies were scored after overnight incubation at 37°C on L agar plates containing ampicillin (150 μg/ml), isopropyl β-D-thiogalactoside, and 5-bromo-4-chloro-3-indolyl β-D-galactoside. Seventy thousand colonies were pooled. This represents the pUC19–*E. coli* gene library.

Drug-Resistance Assay in NS3678. Exponentially growing cells (OD_{590} = 0.3) in L broth supplemented with ampicillin (50 μg/ml) were diluted in M9 medium and spread onto M9 agar plates containing either glucose or arabinose with or without the tumoricidal agent to be evaluated. Plates were incubated at 37°C and scored after 18, 24, and 48 h for colony number and colony size.

RESULTS

Using a Library of *E. coli* Genes to Isolate DMP 840-resistant (DMP 840ʳ) Clones of *E. coli*. *The two elements of the system: A* tolC *mutant host and an* E. coli *gene library.* The *E. coli* host strain used in these studies (NS3678) contains a Tn*10* insertion mutation in the *tolC* gene (*tolC*⁻). This gene encodes a component of the *E. coli* cell membrane and its absence correlates with increased permeability of bacteria to various organic molecules (11–13). Consistent with this observation are results indicating that *tolC*⁻ bacteria can be 50–100 times more sensitive to the cytotoxic effects of DMP 840 than are *tolC*⁺ bacteria (N. Henderson, personal communication). Indeed, the elevated DMP 840 sensitivity exhibited by the *tolC* mutant permits us to carry out experiments at drug concentrations comparable to those used in mammalian cells (9). A second, less critical, feature of strain NS3678 is the presence of a *lacI^q* gene, which permits us to regulate the *lac* operon promoter in the vector (pUC19) that was used to construct the *E. coli* gene library (see below).

To construct a library of *E. coli* genes, high molecular weight (>100 kb) *E. coli* DNA from cell line NS3678 (*tolC*⁻) was partially digested with *Sau*3AI and fragments of 2–10 kb were ligated with *Bam*HI-digested pUC19 vector DNA. The ligated DNA was transformed into JM101 and 7×10^4 transformants were isolated. More than two-thirds of these contain inserts based both on the fraction of white vs. blue colonies on ampicillin/agar plates with isopropyl β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside and on an analysis of restriction enzyme digests of plasmid DNAs isolated from >50 transformants. If an average insert size is 4 kb per clone and the *E. coli* genome is 4 megabases, then 5×10^4 insert-containing clones would correspond to an ≈40-fold coverage of the *E. coli* genome. Plasmid DNA was isolated from the JM101 library and used to construct a similar library in strain NS3678. All subsequent studies were carried out with this NS3678 library. Note, the use of NS3678 genomic DNA to construct the library precludes the isolation of resistant clones containing a functional *tolC* gene.

Isolation and initial analyses of DMP 840ʳ clones. To isolate DMP 840ʳ clones, 10^7–10^8 cells derived from cultures of NS3678 containing the wild-type pUC19 plasmid or the library of pUC19–*E. coli* fragments were spread on M9/Casamino acids/minimal agar plates containing 1 μM DMP 840 (a concentration 3-fold higher than is needed to prevent colony growth) and the plates were incubated overnight at 37°C. Compared to the number of colonies on plates without the drug, 1×10^{-7} colonies were obtained for the pUC19 culture and 4×10^{-6} colonies were obtained for the culture with the pUC19–*E. coli* library. To confirm that the DMP 840ʳ phenotype was encoded by a member of the pUC19–*E. coli* library and was not due to a mutation in the bacterial genome, the plasmid DNA in several of the resistant clones was isolated and used to retransform NS3678. All of the transformants grew normally on minimal/Casamino acids/agar plates with 1 μM DMP 840. To exclude transformants that might act by decreasing permeability of the *tolC*⁻ cells, we tested each of the DMP 840ʳ transformants for their sensitivity to crystal violet by the standard colony-disk assay (13), and none demonstrated altered sensitivity.

Localization of Genes Responsible for DMP 840ʳ to Two Regions of the *E. coli* Genome. Thirty DMP 840ʳ clones containing members of the pUC19–*E. coli* library were chosen and plasmid DNAs were isolated from each clone. The insert DNAs from several of these plasmids were labeled with [α-³²P]dCTP by random priming, and the probes were hybridized to Biodyne filters (Bellco Glass, Vineland, NJ) (18) containing all 30 DNAs. The results indicated that the isolated clones fell largely into two classes, designated 18 and 66. The probes made from an individual of each class hybridized to all the members of that class but failed to cross-hybridize to any of the members of the other class. Based on differences in the restriction digestion patterns of the 30 plasmid DNAs, there were at least three isolates of each class of inserts in the group of 30. We chose to study further plasmids from each class with the smallest insert sizes: clone 18a contains an insert of 1.6 kb and clone 66a has an insert of 2.1 kb. Both of these clones show a similar pattern of DMP 840ʳ; namely, they are resistant to the drug at 2 μM but are sensitive to the drug at 5 μM. In contrast, clones containing a random member of the library are sensitive to DMP 840 at concentrations >0.3 μM.

Mapping and Sequencing Candidate *E. coli* Genes Responsible for the DMP 840ʳ Phenotype. The approach used to map and identify genes on the 66a and 18a clones was largely the same for both clones. (*i*) DNA sequence from both ends of each cloned insert was determined and that sequence was used to search the known *E. coli* database. (*ii*) If this failed to locate the insert DNA on the *E. coli* map, labeled insert DNA probes were used to hybridize to filters containing overlapping members of the Kohara cosmid library of *E. coli* DNA. (*iii*) The entire DNA sequence of both inserts was determined and possible open reading frames (ORFs) were noted.

By using 100–150 bp of DNA sequence derived from the ends of the insert in clone 66a, we found no similarity with any *E. coli* sequence in the GenBank or EMBL data bases by using the BLAST algorithm. In contrast, sequences from both ends of the insert in clone 18a revealed a 98% match to the C-terminal end of the *E. coli* glutaredoxin gene (*grx*) on one side and a

similar match to the sequence of the *E. coli rimK* gene on the other side (data not shown). The *E. coli grx–rimK* gene cluster is located at *E. coli* map coordinate 18 (19).

To map the insert in clone 66a, we hybridized a probe made from 66a insert DNA against members of the cloned Kohara library (20). The probe produced strong positive signals with only two overlapping cosmids, 17B2 and 6B12 (data not shown). The region of overlap shared by these two cosmids is located at *E. coli* map coordinate 66 and is thought to contain the *parE* and *parC* genes, genes that encode the two subunits of *E. coli* topoisomerase IV (Fig. 1). The sequences of *parE* and *parC* are known; both genes are transcribed in the same direction (21) and are separated by an unsequenced region of at least 6 kb. By using primer sets that are internal to the *parE* or *parC* genes, we could detect no amplification of the 66a DNA insert in a PCR, indicating that the fragment does not contain either gene (data not shown). Given that the ends of the 66a insert are in an unsequenced region, this suggests that this DNA is between *parE* and *parC* or between *parE* and the upstream *tolC* gene (the direction of *tolC* transcription is opposite that of *parE* and *parC*). The following two results indicate that the former is true. In PCRs containing genomic *E. coli* DNA, a primer from one end of the 66a insert (Fig. 1, primer 1) and primers from the C terminus of *parE* (Fig. 1, primer 2) or the N terminus of *tolC* (Fig. 1, primer 3), we generated PCR products of 650 bp and 4.5 kb, respectively. A second member of the 66 class of inserts (Fig. 1, clone 66b) has a fragment with one of its ends in the C-terminal portion of *parE* and the other end in unsequenced DNA. Since this fragment lacks the N-terminal portion of *parE* or *parC*, it presumably contains a DNA segment that extends from the C terminus of *parE* to a point within the unsequenced region between *parE* and *parC*.

The sequences of the entire 66a and 18a inserts were determined. Portions of the sequences are shown in Fig. 2. The 2128-bp sequence of 66a contains two nearly completely overlapping ORFs (Fig. 2*A*) that could encode polypeptides of 22 kDa. ORF-1 is transcribed in the same direction as is *parE* and *parC* but appears to lack any obviously recognizable translation start signal. In contrast, ORF-2 is transcribed in the opposite direction from *parE–parC* and has an appropriately positioned upstream Shine–Dalgarno ribosome binding site. The 1569-bp sequence of the 18a insert reveals that it contains, in addition to the entire *grx* gene, an ORF that could encode a polypeptide of 24 kDa (Fig. 2*B*). When the amino acid sequence of either of the two 22-kDa 66a ORFs or the 24-kDa 18a ORF was used to search the GenBank or European Molecular Biology Laboratory data bases with the BLAST algorithm, no region of significant homology was detected.

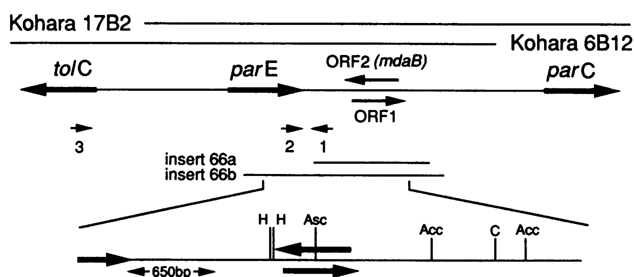

FIG. 1. Map of the region of *E. coli* genome at coordinate 66 containing the cloned 66a fragment. The top two lines indicate the overlap of the two Kohara cosmid DNAs that hybridize to 66a insert DNA. The next line down depicts the location and direction of transcription of the *tolC*, *parE*, *parC*, and clone 66a ORF-1 and ORF-2 (*mdaB*) genes. Below this line are the three primers used in PCRs to better localize the 66a insert DNA. The region covered by that insert DNA and the region of the insert in a related clone, 66b, are shown in the next two lines. The line at the bottom shows a restriction map of the region. H, *Hpa* I; Asc, *Asc* I; Acc, *Acc* I; C, *Cla* I.

The 66a 22-kDa ORF-2 and the 18a 24-kDa ORF Are Responsible for the DMP 840 Resistance of Clones 66a and 18a. *Mutational analysis.* For the 66a insert, digestion and fill-in of the unique *Cla* I site or deletion of 473 bp between the two *Acc* I sites in this insert had no effect on DMP 840 resistance. Both of these mutations are located outside of the two ORFs in this insert (Fig. 1). In contrast, mutations that would be expected to inactivate both ORFs—cleavage and fill-in of a unique *Asc* I site creating a frameshift mutation in either ORF or deletion of DNA between the upstream *Hpa* I site and the *Asc* I site (Figs. 1 and 2*A*)—destroyed the ability of the 66a clone to confer resistance to DMP 840. For the insert in the 18a clone, an 84-bp deletion of DNA between *Bgl* II sites (positions 118 and 202) in the *grx* gene (sequence not shown) did not affect the ability of the clone to confer DMP 840 resistance. However, a deletion that extends from the N-terminal *Bgl* II site in *grx* to the *Bgl* II site in the 24-kDa ORF (Fig. 2*B*) did destroy this property of the clone.

Arabinose-regulated expression of the 22-kDa 66a ORF-2 and the 24-kDa 18a ORF confers DMP 840 resistance. To determine which of the two overlapping 66a ORFs is responsible for the resistance to DMP 840, the ORF-1 and ORF-2 structural genes along with 25 or 20 bp of upstream sequence and 2 or 4 bp of downstream sequence, respectively, were cloned in the sense orientation downstream of the P_{BAD} arabinose promoter in the pBAD-18 vector (14). This promoter is induced by adding 0.2% arabinose to the culture medium and is repressible by glucose. While the original pUC19-66a clone was resistant to DMP 840 regardless of the sugar added to the culture medium, the P_{BAD}–ORF-1 and P_{BAD}–ORF-2 clones behaved differently (Table 1). Thus isolates with the P_{BAD}–ORF-1 plasmid failed to express resistance regardless of which sugar was used. However, the isolates with the P_{BAD}–ORF-2 plasmid were resistant to the drug in the presence of arabinose, but sensitive to it in the presence of glucose. We conclude that ORF-2 expression is sufficient to confer resistance to DMP 840 and will, henceforth, refer to this gene as *mdaB* (modulator of drug activity B).

To show that the 24-kDa ORF in clone 18a is responsible for the drug-resistant phenotype of this clone, we cloned that gene along with 49 bp of upstream sequence (positions 642–1569 in the cloned 18a fragment; Fig. 2*B*) in both orientations downstream of the P_{BAD} promoter in pBAD-18. As expected, the isolate with the 24-kDa ORF gene in the sense orientation relative to P_{BAD} conferred resistance in the presence of arabinose but not in the presence of glucose (Table 1). The gene in the antisense orientation failed to confer DMP 840 resistance under any circumstance and the original 18a clone conferred resistance regardless of the sugar used. Henceforth, we will refer to the gene encoding the 24-kDa protein as *mdaA*.

Arabinose-regulated expression of polypeptides encoded by mdaB *and* mdaA. When proteins present in cell lines containing the original 66a or 18a clones were analyzed by PAGE, a prominent polypeptide band was observed in each case, neither of which was present in cells containing a control plasmid from the library (clone 6). The 66a polypeptide is 22 kDa (Fig. 3*A*, lanes 4 and 5) and the 18a polypeptide is 24 kDa (Fig. 3*B*, lanes 4 and 5). To determine whether the overproduction of these proteins correlates with the drug-resistant phenotype, we analyzed proteins produced in cells containing the various arabinose-promoter constructs. With constructs containing either of the 66a ORFs in the pBAD-18 vector, it is clear that only the construct containing ORF-2 produces a large amount of the 22-kDa polypeptide, and only in the presence of the inducer arabinose (Fig. 3*A*, lanes 6–9). With the pBAD-18 constructs containing the 24-kDa ORF, a prominent 24-kDa protein was detected only with the clone containing the gene in the sense orientation and only in the presence of arabinose (Fig. 3*B*, lanes 6–9). These results correlate exactly with DMP 840^r phenotypes of the P_{BAD} clones and strongly support our contention that overproduction of either the 22-kDa ORF-2

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A                                  ORF-1──►
         | Hpa 1                   M   L   A   I   F   F   S   I   A   G   N   I   R   H   F
444  TAGTTAACCAAAAATTTCCACAAGATGCTTGCGATATTCTTCAGTATAGCGGGGAACATCAGGCATT
      3'AATTGGTTTTTAAAGGTGTTCTACGAACGCTATAAGAAGTCATATCGCCCCTTGTAGTCCGTAA
         *   G   F   I   E   V   L   H   K   R   Y   E   E   T   Y   R   P   V   D   P   M

       Y   H   V   I   S   D   K   C   R   Q   R   F   H   T   E   E   L   V   C   F   M   E
511  TTTATCACGTCATTAGCGATAAATGTCGGCAGCGGTTCCATACCGAGGAATTGGTTTGCTTTATGGA
     AAATAGTGCAGTAATCGCTATTTACAGCCGTCGCCAAGGTATGGCTCCTTAACCAAACGAAATACCT
      K   I   V   D   N   A   I   F   T   P   L   P   E   M   G   L   F   Q   N   A   K   H

      R   Q   I   H   T   V   N   A   N   A   V   E   E   L   I   F   F   G   E   G   F   H
578  ACGGCAGATACACACCGTCAACGCCAACGCCGTGGAAGAACTGATCTTTTTCGGTGAAGGCTTCCAT
     TGCCGTCTATGTGTGGCAGTTGCGGTTGCGGCACCTTCTTGACTAGAAAAAGCCACTTCCGAAGGTA
     F   P   L   Y   V   G   D   V   G   V   G   H   F   F   Q   D   K   E   T   F   A   E   M

     W   C   V   P   G   Q   R   K   H   I   F   F   A   L   Y   Q   A   A   G   T   V   F   F
645  TGGTGCGTTCCAGGTCAGAGAAAGCATATATTTTTTGCCCTGTACCAGGCCGCCGGAACCGTATTTT
     ACCACGCAAGGTCCAGTCTCTTTCGTATATAAAAAACGGGACATGGTCCGGCGGCCTTGGCATAAAA
       P   A   N   W   T   L   S   L   M   Y   K   K   G   Q   V   L   G   G   S   G   Y   K

       R   R   I   F   A   G   T   T   I   A   G   I   Q   R   P   V   T   F   G   E   Y   I
712  TTCGACGGATCTTTGCGGGTACGACCATCGCTGGCATACAGCGTCCCGTGACCTTCGGTGAATACAT
     AAGCTGCCTAGAAACGCCCATGCTGGTAGCGACCGTATGTCGCAGGGCACTGGAAGCCACTTATGTA
      K   S   P   D   K   R   T   R   G   D   S   A   Y   L   T   G   H   G   E   T   F   V
                                             | Asc 1
      I   N   V   F   F   H   C   P   R   R   A   H   P   P   A   W   H   L   P   D   H   H
779  CATCAATGTATTTTTTCACTGTCCACGGCGCGCCCATCCACCAGCCTGGCATCTGCCAGATCACCAC
     GTAGTTACATAAAAAAGTGACAGGTGCCGCGCGGGTAGGTGGTCGGACCGTAGACGGTCTAGTGGTG
     D   D   I   Y   K   K   V   T   W   P   A   G   M   W   W   G   P   M   Q   W   I   V   V

     I   S   P   E   K   V   L   Y   F   R   F   D   I   V   V   A   V   G   A   N   D   A   D
846  ATCAGCCCAGAGAAAGTTTTGTACTTCCGCTTTGACATCGTAGTCGCTGTCGGCGCGAACGATGCGG
     TAGTCGGGTCTCTTTCAAAACATGAAGGCGAAACTGTAGCATCAGCGACAGCCGCGCTTGCTACGCC
       D   A   W   L   F   N   Q   V   E   A   K   V   D   Y   D   S   D   A   R   V   I   R

       I   M   P   K   V   A   Q   C   A   I   R   D   F   G   Q   G   V   V   Q   L   T   I
913  ACATCATGCCCAAGGTCGCGCAGTGTGCCATCCGCGACTTCGGTCAGGGTGTCGTTCAGTTGACCAT
     TGTAGTACGGGTTCCAGCGCGTCACACGGTAGGCGCTGAAGCCAGTCCCACAGCAAGTCAACTGGTA
      V   D   H   G   L   D   R   L   T   G   D   A   V   E   T   L   T   D   N   L   Q   G

      G   V   G   E   F   F   R   A   V   D   N   Q   D   V   A   H   F   L   T   S   R   *
980  TGGAGTGGGCGAATTTTTTCGCGCCGTTGATAATCAGGATGTTGCTCATTTTTTAACCTCAAGGTGA
     ACCTCACCCGCTTAAAAAAGCGCGGCAACTATTAGTCCTACAACGAGTA  5'
     N   S   H   A   F   K   K   A   G   N   I   I   L   I   N   S   M
                                                       ◄──────
                                                          ORF-2
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B                                       ◄──glutaredoxin
221   AGTAAGGGCAACCCGAACGACCAAAAATAACGGTTTGCATTATTTCTC.........//........
      mdaA
      ──►
      M   T   P   T   I   E   L   I   C   G   H   R   S   I   R   H   F   T   D   E   P   I
690   AATGACGCCAACCATTGAACTTATTTGTGGCCATCGCTCCATTCGCCATTTCACTGATGAACCCATT

      S   E   A   Q   R   E   A   I   I   N   S   A   R   A   T   S   S   S   S   F   L   Q   C
757   TCCGAAGCGCAGCGTGAGGCGATTATTAACAGCGCCCGTGCGACGTCCAGTTCCAGTTTTTGCAGT

        S   S   I   I   R   I   T   D   K   A   L   R   E   E   L   V   T   L   T   G   G   Q
824   GCAGTAGCATTATTCGCATTACCGACAAAGCGTTACGTGAAGAACTGGTGACGCTGACCGGCGGGCA
                                                                    Bgl II |
       K   H   V   A   Q   A   A   E   F   W   V   F   C   A   D   F   N   R   H   L   Q   I
891   AAAACACGTAGCGCAAGCGGCGGAGTTCTGGGTGTTCTGTGCCGACTTTAACCGCCATTTACAGATC

      C   P   D   A   Q   L   G   L   A   E   Q   L   L   L   G   V   V   D   T   A   M   M   A
958   TGTCCGGATGCTCAGCTCGGCCTGGCGGAACAACTGTTGCTCGGTGTCGTTGATACGGCAATGATGG

        Q   N   A   L   I   A   A   E   S   L   G   L   G   G   V   Y   I   G   G   L   R   N
1025  CGCAGAATGCATTAATCGCAGCGGAATCGCTGGGATTGGGCGGGGTATATATCGGCGGCCTGCGCAA

       N   I   E   A   V   T   K   L   L   K   L   P   Q   H   V   L   P   L   F   G   L   C
1092  TAATATTGAAGCGGTGACGAAACTGCTTAAATTACCGCAGCATGTTCTGCCGCTGTTTGGGCTGTGC

      L   G   W   P   A   D   N   P   D   L   K   P   R   L   P   A   S   I   L   V   H   E   N
1159  CTTGGCTGGCCTGCGGATAATCCGGATCTTAAGCCGCGTTTACCGGCCTCCATTTTGGTGCATGAAA

        S   Y   Q   P   L   D   K   G   A   L   A   Q   Y   D   E   Q   L   A   E   Y   Y   L
1226  ACAGCTATCAACCGCTGGATAAAGGCGCACTGGCGCAGTATGACGAGCAACTGGCGGAATATTACCT

       T   R   G   S   N   N   R   R   D   T   W   S   D   H   I   R   R   T   I   I   K   E
1293  CACCCGTGGCAGCAATAATCGCCGGGATACCTGGAGCGATCATATCCGCCGAACAATCATTAAAGAA

      S   R   P   F   I   L   D   Y   L   H   K   Q   G   W   A   T   R   *
1360  AGCCGCCCATTTATTCTGGATTATTTGCACAAACAGGGTTGGGCGACGCGCTAAAACCGCCACGTCG
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FIG. 2. (*A*) Nucleotide sequence of 66a ORF-1 and ORF-2. The sequence of both strands was determined. The amino acid sequence of these ORFs is shown above and below the nucleotide sequences of the two strands. (*B*) Nucleotide sequence of the 18a insert. The amino acid sequence of the 24-kDa ORF is shown above the DNA sequence. The direction of *grx* transcription and the location of the *grx* start codon (the end of the arrow) are also shown.

protein at *E. coli* map position 66 or the 24-kDa ORF protein at *E. coli* map coordinate 18 is responsible for the bacteria's resistance to DMP 840.

***mdaA*- and *mdaB*-Mediated Resistance to Other Topoisomerase-Inhibiting Tumoricidal Agents.** Because *mdaB* is located in a region of the *E. coli* map that contains the genes for the two subunits of topoisomerase IV, the bacterial homolog of the mammalian topoisomerase II (21), we decided to investigate the effects of overexpressing either the *mdaB* or *mdaA* genes on the sensitivity of bacteria to various known inhibitors of the mammalian topoisomerases I and II. The results are summarized in Table 2 and indicate that *mdaB* confers resistance to Adriamycin and etoposide and *mdaA* confers resistance only to Adriamycin.

DISCUSSION

Resistance of cells to a variety of tumoricidal agents has been most extensively studied in mammalian cells, where several different mechanisms have been proposed. Resistance has been associated with overexpression of the P180 glycoprotein that mediates the active efflux of toxic hydrophobic molecules from cells (22). Indeed, a similar membrane pump responsible for the efflux of hydrophobic cations from *E. coli* has been identified (23). Another mechanism of drug resistance involves the overproduction of agents that effect the metabolism and

Table 1. Growth of $tolC^-$ bacteria containing various multicopy plasmids in the presence of DMP 840

Plasmid used	Growth	
	+ glucose	+ arabinose
pUC19-insert 6	−	−
pUC19-66a	+	+
P_{BAD}-66a ORF-2	−	+
P_{BAD}-66a ORF-1	−	−
pUC19-18a	+	+
P_{BAD}-18a ORF sense	−	+
P_{BAD}-18a ORF antisense	−	−

Colony formation was assessed on minimal agar plates with 1 μM DMP 840 and either glucose (0.6%) or arabinose (0.4% glucose/0.2% arabinose). +, 1000 normal-sized colonies (>1.2 mm in diameter); −, no colonies after 24 h of incubation at 37°C.

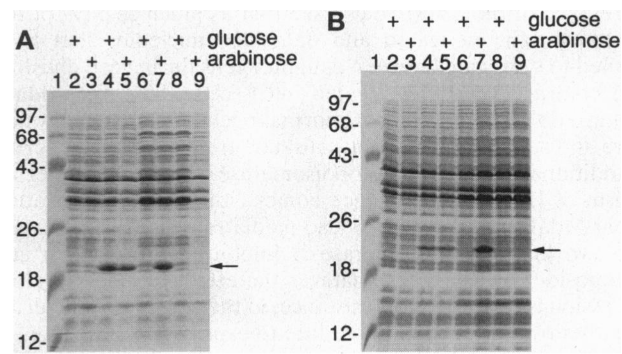

FIG. 3. (*A*) Arabinose-regulated expression of the 22-kDa ORF-2 (MdaB) protein. SDS/PAGE analyses of various whole cell extracts stained with Coomassie blue. Lane 1 contains protein size markers (identified in kDa). Lanes: 2, 4, 6, and 8, clones grown in the presence of glucose; 3, 5, 7, and 9, clones grown in the presence of arabinose; 2 and 3, clone 6, a random member of the pUC19–*E. coli* library; 4 and 5, clone 66a; 6 and 7, P_{BAD} ORF-2 clones; 8 and 9, P_{BAD} ORF-1 clones. The arrow indicates the position of the 22-kDa ORF-2 protein. (*B*) Arabinose-regulated expression of the 24-kDa ORF (MdaA) protein. An identical SDS/PAGE analysis of whole cell extracts as in *A* with lane 1 as in *A*. Lanes: 2, 4, 6, and 8, clones grown in glucose; 3, 5, 7, and 9, clones grown in arabinose; 2 and 3, control clone 6; 4 and 5, clone 18a; 6 and 7, 24-kDa P_{BAD} ORF clones in the sense orientation; 8 and 9, 24-kDa P_{BAD} ORF clones in the antisense orientation. The arrow indicates the position of the 24-kDa Mda-18 protein.

Table 2. Drug-resistance patterns of plasmid-containing bacterial strains that overexpress either the MdaB or the MdaA protein

Plasmid used	Drug resistance			
	No drug	DMP 840	Adriamycin	Etoposide
pUC19-insert 6	+	−	−	−
pUC19-66a	+	+	+	+
P_{BAD}-66a ORF-2 (*mdaB*)	+	+	+	+
*p*UC19-18a	+	+	+	−

Resistance of plasmid-containing NS3678 bacteria to various drugs was measured. The drugs were used at the concentrations that just kill cells with pUC19-insert 6 control plasmid: DMP 840 (0.5 μM), Adriamycin (0.7 μM), and etoposide (0.8 μM). + and − are as in Table 1. Amsacrine and camptothecin could not be tested here due to the resistance of *tolC*$^-$ cells to relatively high concentrations (5 μM) of these two drugs.

inactivation of the drug. Included in this class of resistance are mutants that exhibit elevated levels of redox agents, such as thioredoxin and glutathione (24, 25). Finally, a class of resistance has been characterized that appears to be associated with drugs that damage DNA. Resistance to agents such as cisplatin and ditercalinium has been attributed to increased cellular DNA repair capacity (25, 26) and resistance to topoisomerase inhibitors is frequently associated with either a reduction in the particular topoisomerase target or specific topoisomerase mutations that interfere with the interaction of the enzyme and the inhibitor (2–5).

How do the *mdaA* and *mdaB* genes mediate DMP 840 resistance? The most likely possibilities derive from information about the location of *mdaA* and *mdaB* on the *E. coli* map. Thus *mdaB* is located between two genes that encode the subunits of the mammalian topoisomerase II analog, *E. coli* topoisomerase IV. Accordingly, it is tempting to speculate that DMP 840 resistance mediated by the overproduction of the 22-kDa MdaB protein is due to a modulation of topoisomerase IV activity, which is primarily associated with chromosome segregation in *E. coli* (21). Consistent with that hypothesis are preliminary results in which *tolC*$^-$ bacteria treated with DMP 840 exhibit a segregation defect. Thus, by 4′,6-diamidino-2′-phenylindole dihydrochloride staining of cellular DNA in the presence of the drug, we can see that as much as 50% of the cells contain elongated and deformed nucleoids that have failed to segregate between daughter cells during cell division. In contrast, DMP 840-treated *tolC*$^-$ cells that overproduce MdaB do not contain this abnormal nucleoid morphology and are indistinguishable from cells not treated with the drug. Additional support for a topoisomerase IV-mediated mechanism of DMP 840 resistance comes from the demonstration that MdaB overproduction also mediates resistance in *E. coli* to two known topoisomerase II inhibitors, Adriamycin and etoposide. In any case, whatever the effect of *mdaB* overexpression is on DMP 840 resistance, in the absence of the drug, neither overexpression nor failure to express this gene (e.g., in *mdaB* gene knockouts) affects cell growth (data not shown).

The *mdaA* gene is located on the *E. coli* map adjacent to a gene for glutaredoxin, a prime candidate for a drug detoxification activity. Consequently, it is tempting to speculate that *mdaA* also encodes such an activity. This conclusion is supported by recent results that indicate that DMP 840-treated cells containing the *mdaA* plasmid overproduce a drug metabolite barely detectable by HPLC in cells without the plasmid (data not shown). Moreover, *mdaA* and *mdaB* probably act by different mechanisms, since both genes afford resistance to Adriamycin, but only the latter provides resistance to etoposide.

A variety of experiments suggest that MdaA and MdaB do not act by increasing either a cellular efflux pump or cellular DNA repair capacity. We have evaluated the efflux pump hypothesis by measuring the uptake of ^{14}C-labeled DMP 840 into cells directly, by taking advantage of the ability of the drug to be photocrosslinked to DNA when exposed to intense light of >360 nm (10). Our results showed little difference in the level of *in vivo* crosslinking of the drug to DNA as a function of *mdaA* or *mdaB* expression (data not shown). Moreover, these two gene products appear to afford resistance to the lethal effects of various topoisomerase II inhibitors but not the effects of crystal violet, kanamycin, or tetracycline. These results also indicate that neither *mdaB* nor *mdaA* overexpression alters the permeability properties of the *tolC*$^-$ host. With regard to the DNA repair hypothesis, we see no effect of *mdaA* or *mdaB* arabinose-mediated gene induction on the sensitivity of cells to UV treatment. Moreover, *uvrA* and *recA* mutations do not affect the level of resistance to DMP 840 when either of the *mda* genes is overexpressed.

We thank Dr. K. Abremski for data base searches, S. Stack and J. Bunville for DNA sequencing, and N. Henderson and Drs. G. Cohen, N. Shepherd, R. Grafstrom, M. Kirschenbaum, and J. L. Gross for helpful discussions.

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