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In Vivo Titration of Mitomycin C Action by Four Escherichia coli Genomic Regions on Multicopy Plasmids

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Received 6 October 2000/Accepted 3 January 2001

Mitomycin C (MMC), a DNA-damaging agent, is a potent inducer of the bacterial SOS response; surprisingly, it has not been used to select resistant mutants from wild-type Escherichia coli. MMC resistance is caused by the presence of any of four distinct E. coli genes (mdfA, gyrI, rob, and sdiA) on high-copy-number vectors. mdfA encodes a membrane efflux pump whose overexpression results in broad-spectrum chemical resistance. The gyrI (also called sbmC) gene product inhibits DNA gyrase activity in vitro, while the rob protein appears to function in transcriptional activation of efflux pumps. SdiA is a transcriptional activator of rbsQ4Z genes involved in cell division.

In this study, the response to an MMC challenge was illuminated by the isolation of inhibitor-resistant mutants due to the presence of E. coli genomic fragments in multicopy plasmids. The sequencing of insert-vector junctions defines genes that confer inhibitor resistance when present in high copy. The application of such technology to MMC action is described here.

MATERIALS AND METHODS

Strains and media. The E. coli strains used in this study are all K-12 derivatives (Table 1). The strains were grown in Luria-Bertani (LB) medium. Ampicillin (at 100 or 150 μg/ml) or kanamycin (at 25 μg/ml) was added to the medium when necessary. The standard growth temperature was 37°C. Liquid cultures were aerated by rotary shaking at 250 rpm.

Strain construction. DPD2272 was constructed by P1vir phage-mediated transduction with the donor strain, WX2, and the recipient strain, MG1655. Recombinants were selected using kanamycin (23). An electrottransformation method (33) was used to introduce plasmids into bacterial strains.
TABLE 1: Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>E. coli MG1655</td>
<td>F-</td>
<td>pUC18 Cloning vector</td>
</tr>
<tr>
<td>W3110</td>
<td>F-</td>
<td>pUC19 Cloning vector</td>
</tr>
<tr>
<td>RFM443</td>
<td>F-</td>
<td>pUC18 Cloning vector</td>
</tr>
<tr>
<td>DM800</td>
<td>F-</td>
<td>pUC18 plus gyrI</td>
</tr>
<tr>
<td>DM803</td>
<td>Same as DM800 but lexA*</td>
<td></td>
</tr>
<tr>
<td>DPD2272</td>
<td>F-</td>
<td>This study*</td>
</tr>
<tr>
<td>DPD2668</td>
<td>F-</td>
<td>This study</td>
</tr>
<tr>
<td>DPD2669</td>
<td>F-</td>
<td>This study</td>
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</table>

Plasmids

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>Cloning vector</td>
<td>33</td>
</tr>
<tr>
<td>pUC18</td>
<td>Cloning vector</td>
<td>33</td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector</td>
<td>33</td>
</tr>
<tr>
<td>pDEW133</td>
<td>pUC18 plus gyrI</td>
<td>This study*</td>
</tr>
<tr>
<td>pDEW140</td>
<td>pUC18 plus sdiA (EcoRI)</td>
<td>This study</td>
</tr>
<tr>
<td>pDEW141</td>
<td>pUC18 plus rob (EcoRI)</td>
<td>This study</td>
</tr>
</tbody>
</table>

*lexA1 [also lexA(Ind)] is a noninducible allele of lexA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
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</thead>
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<tr>
<td>gyrI Bam-left</td>
<td>CCGGATCCTCGGGCCGCACCCAGACTAACAT</td>
</tr>
<tr>
<td>gyrI Eco-right</td>
<td>GGAATTCGCCGACCGGACGCTGTA</td>
</tr>
<tr>
<td>sdiA</td>
<td>TGGCACGCAGGACAGAA</td>
</tr>
<tr>
<td>sdiAd</td>
<td>TAACAAATCGGTAATCAGC</td>
</tr>
<tr>
<td>rob1</td>
<td>ACAGCGACGACGACGACGACGAC</td>
</tr>
<tr>
<td>rob2</td>
<td>TAGTCCGACGCA</td>
</tr>
</tbody>
</table>

RESULTS

sdiA, gyrI, rob, and mdfA in multicopy conferred resistance to MMC upon E. coli. MMC-resistant clones were selected on LB agar plates with 6 μg of MMC per ml (twice the MIC determined in this study) and 150 μg of ampicillin per ml. Resistant colonies appeared after 1 day of incubation at 37°C. E. coli genomic DNA libraries in pUC18 and pBR322 were separately transformed into strain W3110. Thirty MMC-resistant isolates were found among approximately 10^9 ampicillin-resistant colonies obtained from the pUC18 library. The plating of approximately 10^9 ampicillin-resistant colonies obtained from the pBR322 library yielded 16 MMC-resistant clones. Plasmids were isolated from each of these 46 lines and used to retransform strain RFM443 selecting for ampicillin resistance. In each backcross, MMC resistance was co-inherited with ampicillin resistance, indicating that the MMC resistance determinants were plasmid-borne. The ends of these 46 inserts conferring MMC resistance were sequenced to identify the regions of the E. coli chromosome harbored within each multicopy plasmid. Inserts were derived from four distinct chromosomal loci.

Four pUC18-derived plasmids and five pBR322-derived plasmids contained a region of the genome that mapped to min 19 (Fig. 1A). The only gene present in all nine plasmids was mdfA, suggesting that mdfA in multiple copies conferred MMC resistance. Eighteen other pUC18-derived plasmids clustered at a second locus, the gyrI (also known as sbmC) region of the chromosome at min 44 (Fig. 1B). gyrI was the single gene in common among the 18 inserts. Another cluster of 7 pUC18-derived plasmids and 11 pBR322-derived plasmids shared a single common gene, sdiA from min 43 (Fig. 1C). The final pUC18-derived plasmid conferring MMC resistance contained three intact E. coli genes, i.e., rob, creA, and creB (map not shown), in the vicinity of min 100. To confirm these assignments of resistance determinants, the gyrI, sdiA, and rob genes with just their flanking intergenic regions were individually inserted into either pUC18 or pUC19.
(see Materials and Methods). The resultant plasmids, when introduced by transformation into strain RFM443, were tested for their abilities to alter the strains’ responses to MMC and nalidixic acid, an inhibitor of DNA gyrase that causes strand scissions through interference with the gyrase ligation reaction (42). The results (Table 3) demonstrated that each of these three genes in multicy copy conferred resistance to both MMC and nalidixic acid, since strains bearing these genes in high copy displayed smaller zones of inhibition than strains containing control plasmids.

An sdiA null mutant did not show hypersensitivity to MMC. Inhibition zone assays were performed on the isogenic sdiA+ and sdiA strains MG1655 and DPD2272. Obvious differences in the zones of inhibition caused by exposure to MMC or nalidixic acid were not observed. Both strains showed 19-mm-diameter zones of inhibition with 15 μg of MMC or 75 μg of nalidixic acid.

Modulation of multicy copy resistance to DNA-damaging agents by lexA(Ind). Strain DM800 (lexA+) and strain DM803 [lexA(Ind)], each individually transformed with a set of plasmids (pUC18, pUC19, pDEW133 [gyrI], pDEW140 [sdiA], and pDEW141 [rob]), were tested for MMC and nalidixic acid sensitivities (Tables 4 and 5). The lexA(Ind) product is resistant to proteolysis by activated RecA and thus prevents induction of the SOS response (42). As expected, the lexA(Ind) mutants displayed larger zones of inhibition than those of the isogenic lexA+ strain. A multicy copy gyrI plasmid (pDEW133) in the lexA(Ind) background did not confer significant resistance to either MMC or nalidixic acid. Thus, the SOS response was needed for the gyrI-associated phenotype. In contrast, the presence of sdiA in high copy (pDEW140) conferred resistance in both the lexA+ and lexA(Ind) backgrounds. Thus, the phenotype conferred by sdiA amplification did not rely upon the SOS response. rob in high copy (pDEW141) did not confer significant resistance in the DM800/DM803 background used for testing lexA(Ind) dependence (data not shown).

Microscopic examination of cultures harboring an sdiA plasmid. In the early exponential phase the DPD2668 (control) culture displayed a typical rod-shaped morphology, and in stationary phase the culture was composed of rods that might be slightly shorter than cells in the exponential phase of growth. In contrast, the stationary-phase culture of DPD2669 (harboring sdiA in high copy) was dominated by rounded cells that transformed into rods upon reaching logarithmic growth after subculturing. These rods were significantly shorter than those

### Table 3. Zone of inhibition assaysa

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Inhibition zone diam (mm) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMC (15 μg)</td>
</tr>
<tr>
<td>pUC19</td>
<td>22</td>
</tr>
<tr>
<td>pDEW140 (sdiA)</td>
<td>16</td>
</tr>
<tr>
<td>pDEW141 (rob)</td>
<td>19</td>
</tr>
<tr>
<td>pUC18</td>
<td>19</td>
</tr>
<tr>
<td>pDEW133 (gyrI)</td>
<td>12</td>
</tr>
</tbody>
</table>

a RFM443, harboring various plasmids, was challenged with either 15 μg of MMC or 75 μg of nalidixic acid. The diameter of each resultant inhibition zone is presented.

### Table 4. gyrI-mediated multicy copy resistance is lexA dependent

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inhibition zone diam (mm) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMC (30 μg)</td>
</tr>
<tr>
<td>DM800/pUC18</td>
<td>17</td>
</tr>
<tr>
<td>DM803/pUC18</td>
<td>22</td>
</tr>
<tr>
<td>DM800/pDEW133 (gyrI)</td>
<td>12</td>
</tr>
<tr>
<td>DM803/pDEW133 (gyrI)</td>
<td>22</td>
</tr>
</tbody>
</table>
observed in the early-exponential-phase control culture. Representative fields of equal magnification are shown in Fig. 2.

In contrast, exponential- and stationary-phase E. coli cultures harboring either mdfA or rob on a multicopy plasmid displayed a morphology indistinguishable from that of the control cultures of DPD2668. The gyrI-containing plasmid, however, was associated with an intermediate phenotype having a rounded morphology in the stationary phase but a normal shape in the exponential phase.

**DISCUSSION**

Random E. coli genomic DNA fragments on a medium-copy-number vector, pBR322, and a high-copy-number vector, pUC18, were screened in an E. coli K-12 strain for variants resistant to MMC, a DNA-damaging agent. The clones obtained were clustered at four chromosomal regions. Only one gene in each region was responsible for the MMC resistance phenotype; these genes are mdfA, sdiA, rob, and gyrI.

Since mdfA is a multidrug resistance locus (15), the demonstration that resistance was conferred by minimal plasmids expressing only one E. coli gene was restricted to the analysis of rob, sdiA, and gyrI. One chromosomal region, defined by a single plasmid, contained rob and creBC. Since rob is involved in drug resistance (45), while creBC functions in carbon metabolism (10), rob was subcloned and shown to confer resistance to MMC when present at a high copy number.

The finding that these four genes confer resistance, though unanticipated, can be incorporated into a plausible model. The gyrI product inhibits DNA gyrase activity in vitro (28), while the sdiA product activates the transcription of fisQAZ, genes involved in septum formation at an early stage of cell division (43). SdiA also activates the expression of several other genes (1, 44), including uvrY and uvrC, which are involved in the protection of the cells from UV irradiation, and the acrA, acrB, acrD, acrE, and acrF genes, whose products are responsible for acridine efflux. The rob product also appears to function in transcriptional activation of efflux pump genes, including the acrAB operon (9, 27, 45). AcrAB connects to form an export channel with the tolC-encoded outer membrane porin (2).

Since tolC mutants are hypersensitive to MMC (14), we propose that MMC is exported from cells by the action of the efflux pumps, while DNA gyrase activity facilitates the intercalation of MMC into the chromosome. Thus, amplification of gyrI and mdfA may prevent MMC from interacting with DNA, its macromolecular target. Amplification of gyrI has previously been shown to protect cells from the action of microcin B17, a DNA-cleaving agent (6). This compound causes double-stranded DNA breaks in vivo and in vitro only in concert with DNA gyrase (39). Perhaps, rob overexpression also acts to enhance efflux, while elevated levels of the sdiA product might overcome cell division arrest imposed by DNA damage. Thus, exhaustive selection of multicopy resistance, in conjunction with previous knowledge of MMC action, has allowed us to define the integrated response to this chemical insult.

Both MMC and nalidixic acid are known to induce the SOS response in E. coli (38, 42). While the lexA+ strain, DM800, showed sensitivity to MMC and nalidixic acid, strain DM803 [lexA(Ind)], which is incapable of mounting the SOS response due to a noncleavable form of the LexA repressor, showed an increased sensitivity to both chemicals. In the lexA(Ind) background, the strains harboring pUC18 or the gyrI-containing pDEW133 both displayed inhibition zones of the same size when challenged with MMC. This indicates that gyrI from multicopy did not confer resistance in DM803. Thus, gyrI multicopy-mediated resistance to MMC was dependent upon lexA function; this gyrI function was thus defined to be a part of the SOS response; a result congruent with other studies of this gene (6). In contrast, multicopy sdiA conferred resistance to MMC or nalidixic acid in both lexA+ and lexA(Ind) backgrounds, suggesting that this phenotype was at least not directly related to the lexA and recA circuitry which defines the SOS response. It was not clear to us why gyrI amplification in strain DM800 did not confer resistance to nalidixic acid as it did to MMC. This, at least, reflects differences in the action of the two chemicals. That rob amplification did not confer resistance in a DM800 or DM803 strain background reinforces that MMC resistance is a complex trait influenced by several genetic factors.

While sdiA conferred resistance in multicopy, a null mutation in sdiA did not result in hypersensitivity to MMC. It has been observed that the strain with sdiA in high copy, DPD2669, forms rounder and shorter cells than control cells in both the

**Table 5. Modulation of sdiA multicopy resistance**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inhibition zone diam (µm) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMC (15 µg)</td>
</tr>
<tr>
<td>DM800/pUC19</td>
<td>20</td>
</tr>
<tr>
<td>DM803/pUC19</td>
<td>27</td>
</tr>
<tr>
<td>DM800/pDEW140 (sdiA)</td>
<td>&lt;7</td>
</tr>
<tr>
<td>DM803/pDEW140 (sdiA)</td>
<td>16</td>
</tr>
</tbody>
</table>

**Growth Phase**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>stat.</td>
<td>haploid</td>
</tr>
<tr>
<td>exp.</td>
<td>haploid</td>
</tr>
<tr>
<td>stat.</td>
<td>amplified</td>
</tr>
<tr>
<td>exp.</td>
<td>amplified</td>
</tr>
</tbody>
</table>

**FIG. 2.** Micrographs of E. coli cells. Each frame is enlarged to the same extent. The top two panels capture images of cells with a haploid sdiA (DPD2668) content, while the bottom two panels are of an isogenic strain containing multiple copies of sdiA (DPD2669). The first and third frames from the top are pictures of stationary (stat.)-phase cultures, while the second and fourth panels from the top are images of exponential (exp.)-phase cultures. Color differences in the images of the two strains are a result of computer processing.
exponential and the stationary growth phases, and the same strain forms slightly shorter cells in the stationary phase than those in the exponential phase. These observations agree with the knowledge that sdiA overexpression speeds up cell division (43), and the expression of sdiA is decreased 50 to 80% in mid- to late-exponential growth phase with the appearance of an extracellular factor in the growth medium that specifically downregulates sdiA expression (18). It is also known that sdiA null mutants did not have obviously different phenotypes in cell division or growth (18). This may be because sdiA encodes a transcriptional activator only partially responsible for expression of the cell division genes fitsQAZ. Expression of fitsQAZ is controlled by at least two regulators, RpoS and SdiA (35). Eliminating SdiA function does not prevent fitsQAZ expression, while amplification of sdiA results in overproduction of fitsQAZ transcripts (44). In contrast, amplification of gyrI encoding a protein that inhibits DNA supercoiling, increased resistance to MMC (this work), while a null mutation in gyrI resulted in a strain that is twofold more sensitive to MMC and another DNA-damaging agent, microcin B17 (6).

Biosensors that can detect genotoxic agents have been developed (8, 40), providing one means of categorizing different DNA-damaging agents. The genetic titration of inhibitor action, coupled with the completed E. coli genomic sequence (11) and the availability of high-throughput, automated sequencing facilities, has proven to be a very powerful technique for the characterization of one DNA-damaging agent, MMC. The broad application of this methodology to several DNA-damaging agents may be quite informative.

ACKNOWLEDGMENT

Tina Van Dyk’s provision of the pUC18-based and pBR322-based E. coli genomic DNA libraries was an instrumental starting point for this work. Prior work of Dana Smulski and David Elsemore on genetic characterization of one DNA-damaging agent, microcin, provided one means of categorizing different DNA-damaging agents.

REFERENCES