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

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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 *ftsQAZ* genes involved in cell division.

Mitomycin C (MMC), an antitumor agent isolated from *Streptomyces* cultures, is used in chemotherapy (30). It interacts with DNA by intercalation and adduct formation (37). These actions trigger the SOS response, the concerted induction of several DNA repair, and recombination activities controlled by the *lexA*-specified repressor in *Escherichia coli* (42). This interaction has been studied by in vitro and in vivo methods. The breadth of the SOS regulatory circuit has been approximated by screening a collection of *Escherichia coli* promoter-*lacZYA* gene fusions for those which displayed increased β-galactosidase activity in the presence of low levels of MMC (19). Similarly, two-dimensional electrophoretic separation of *E. coli* polypeptides induced by a DNA cleaving treatment has been catalogued (38). The expression of at least 29 genes is induced by DNA damage; 18 of these are regulated by the SOS response, while 11 are *lexA* independent (42).

Global regulatory circuits do not act in isolation (29). Rather, a stress treatment may induce many regulons, as has been observed by both two-dimensional protein separation methodology in studies of *Salmonella enterica* serovar Typhimurium (13) and gene fusion-based analyses of *E. coli* (7) after hydrogen peroxide treatment. The concerted action of all such induced regulons describes the responses to the stress caused by an individual chemical treatment. Such interactions may also be suggested by the analysis of pleiotropic mutants (20) resistant to DNA-damaging agents.

Despite the clinical, molecular biological, and historical importance of MMC-DNA interactions, the selection of MMC-resistant mutants has been limited to a single pseudoreversion study (24, 26). Further genetic studies might enhance our understanding of the cellular interactions with MMC. Demanding overexpression of a gene product is a classic means of overcoming chemical toxicity (20). Such selections were used to define the regulatory circuit controlling *his* operon expression in *Salmonella* serovar Typhimurium (32). This selection system was later exploited to select gene amplification events in bacteria (4). Similar selections with PALA, *N*-(phosphonacetyl)-L-aspartate, and methotrexate led to amplification of specific genes encoding enzymes targeted by the inhibitors in mammalian cells (34, 41). With the construction of yeast genomic DNA libraries in high-copy-number plasmids, the selection of inhibitor-resistant lines in yeast accelerated. Targets of inhibitor action were verified by using these methods (31), while the ability to define both target-specifying and unexpected resistance genes was uncovered (17). More recently, these methodologies were extended to a tumoricidal agent with an ill-defined mode of action using an *E. coli*-based multicopy plasmid library and a selection scheme (12). Such techniques have also been used to define the action of amino acid biosynthetic inhibitors (16; Z. Xue, D. R. Smulski, D. Delduco, S.-Y. Choi, M. H. Jia, and R. A. LaRossa, unpublished results; D. R. Smulski, L. X. Huang, T. K. Van Dyk, and R. A. LaRossa, unpublished results).

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 $P1_{vir}$ phage-mediated transduction with the donor strain, WX2, and the recipient strain, MG1655. Recombinants were selected using kanamycin (23). An electrotransformation method (33) was used to introduce plasmids into bacterial strains.

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype	Source or reference
E. coli		
MG1655	F^- λ^- *rph-1*	5
W3110	F^- λ^- *rph-1* IN(*rrnD-rrnE*)	5
RFM443	*rpsL200 galK2 lac*Δ*74*	22
WX2	Δ*lac-pro met pro zzz*::Tn*10* *sdiA*::Kan *thy supD* (r_K^- m_K^-)	43
DM800	F^- *metA28 lacY1* or *lacZ4 thi-1 xyl-5* or *xyl-7 galK2 tsx-6*	25
DM803	Same as DM800 but *lexA1*[a]	25
DPD2272	F^- λ^- *rph-1 sdiA*::Kan	This study[b]
DPD2668	RFM443(pUC19)	This study
DPD2669	RFM443(pDEW140)	This study
Plasmids		
pBR322	Cloning vector	33
pUC18	Cloning vector	33
pUC19	Cloning vector	33
pDEW133	pUC18 plus *gyrI*	This study
pDEW140	pUC19 plus *sdiA* (*Eco*RI)	This study
pDEW141	pUC19 plus *rob* (*Eco*RI)	This study

[a] *lexA1* [also *lexA*(Ind)] is a noninducible allele of *lexA*.
[b] P1(WX2) × MG1655 → Kan^r.

MIC of MMC. LB agar plates containing different MMC concentrations (10, 8, 5, 3, 1, 0.5, 0.1, 0.05, 0.01, and 0 μg/ml) were prepared. Strain RFM443 was streaked onto each plate, followed by incubation overnight at 37°C. The growth of RFM443 was checked by scoring for colony formation. The lowest concentration of MMC that inhibited colony formation of RFM443 was defined as the MIC of MMC. Strain RFM443 grew on all the plates with an MMC concentration of ≤1 μg/ml; growth was prevented by a concentration of ≥3 μg/ml. The MIC of MMC on plates for RFM443 was thus defined to be 3 μg/ml.

Identification of MMC-resistant clones. Libraries were previously prepared from *E. coli* strain W3110 genomic DNA partially digested with *Sau*3AI to ~4-kbp fragments (16). For each round of multicopy titration, 0.2 ng of the pBR322-based or 0.3 ng of the pUC18-based library was electrotransformed into RFM443. The colonies underwent single colony purification on the same medium. Plasmids were isolated from 1.5-ml overnight cultures of the single colonies in LB medium supplemented with ampicillin (150 μg/ml) using the Qiagen 96-well Turbo Plasmid Prep kit (Qiagen, Inc., Valencia, Calif.). DNA sequence data from both ends of each insert were obtained. The M13/pUC sequencing primer (−40) and the M13/pUC reverse sequencing primer (−48) were used in sequencing the pUC18-based inserts (33). The primers used in sequencing the pBR322-based insertions were 5′-GCC ACT ATC GAC TAC GCG-3′ and 5′-CGA TAT AGG CGC CAG CAA C-3′. BLASTn (3) searches identified the chromosomal segments harbored on each plasmid.

Subcloning of *gyrI, sdiA*, and *rob*. Primers were designed for PCR amplification of *gyrI, sdiA*, and *rob*, each with the flanking intergenic regions containing the corresponding promoter (Table 2). PCR amplification was carried out using the PCR AmpliTaq kit (Roche, Palo Alto, Calif.). The *Eco*RI-*Bam*HI double-digested PCR product of the *gyrI* region was subcloned into the *Eco*RI-*Bam*HI site of pUC18, and the resultant plasmid was designated pDEW133. The PCR products of the *sdiA* region and the *rob* region were each inserted into the polylinker site of pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen Co., Carlsbad, Calif.), and both inserts were subsequently placed into the *Eco*RI site of pUC19. The resultant plasmids were named pDEW140 and pDEW141, respectively. Their structures were confirmed by sequencing the insert junctions using the M13/pUC sequencing primer (−40) and the M13/pUC reverse sequencing primer (−48). pDEW133, pDEW140, and pDEW141 were electrotransformed into strains RFM443, DM800, and DM803 for further characterization.

Zone of inhibition assays. These were performed using the method modified (21) from that of Stephens et al. (36). Briefly, the test strains were grown overnight in LB medium supplemented with 150 μg of ampicillin per ml. Then, 0.1-ml portions of each culture and 2.5 ml of melted LB soft agar with 150 μg of of ampicillin per ml were mixed and poured over a 30-ml LB agar plate appended with 150 μg of ampicillin per ml. After the top layer was solidified, a sterile filter disc (7 mm in diameter) containing the desired amount of a chemical was placed at the center of the plate. For MMC, 15 or 30 μg was used; for nalidixic acid, 75 μg was used. The diameters of the inhibition zones were measured after overnight incubation at 37°C.

Microscopic examination of cultures. Both overnight and early-exponential-phase aliquots of a control strain, DPD2668, and one having an *sdiA* multicopy plasmid, DPD2669, were examined by confocal laser scanning microscopy. Portions (1 ml) of cultures, grown at 37°C in LB medium, were stained with 1 μl of SYTO13, a cell-permeant green fluorescent nucleic acid stain (5 mM solution in dimethyl sulfoxide, Molecular Probes, Eugene, Oreg.). Samples (ca. 1 μl) were spotted onto microscope slides and dried for 20 min by placement on a surface heated to 60°C. They were rehydrated in a drop of a glycerol-based mounting medium, Citifluor (Ted Pella, Inc., Redding, Calif.), designed to reduce photobleaching, and a coverslip was placed over the sample prior to examination by confocal laser scanning microscopy. Images were archived on a personal computer.

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 10^8 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 coinherited 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

TABLE 2. Primers used in genetic constructions

Primer	Sequence (5′-3′)
gyrI Bam-left	CGGGATCCCGCCGCGCACCAGACTAACAT
gyrI Eco-right	GGAATTCCGTCGGAACGGAACGCATCTGGTA
sdiAf	TGGCACGCAGGACAGAA
sdiAd	TAACAAATCAGCATAACTCAT
rob1	ACGACGGATCGGAATCAGCA
rob2	TAGTTCGTCACGGTAA

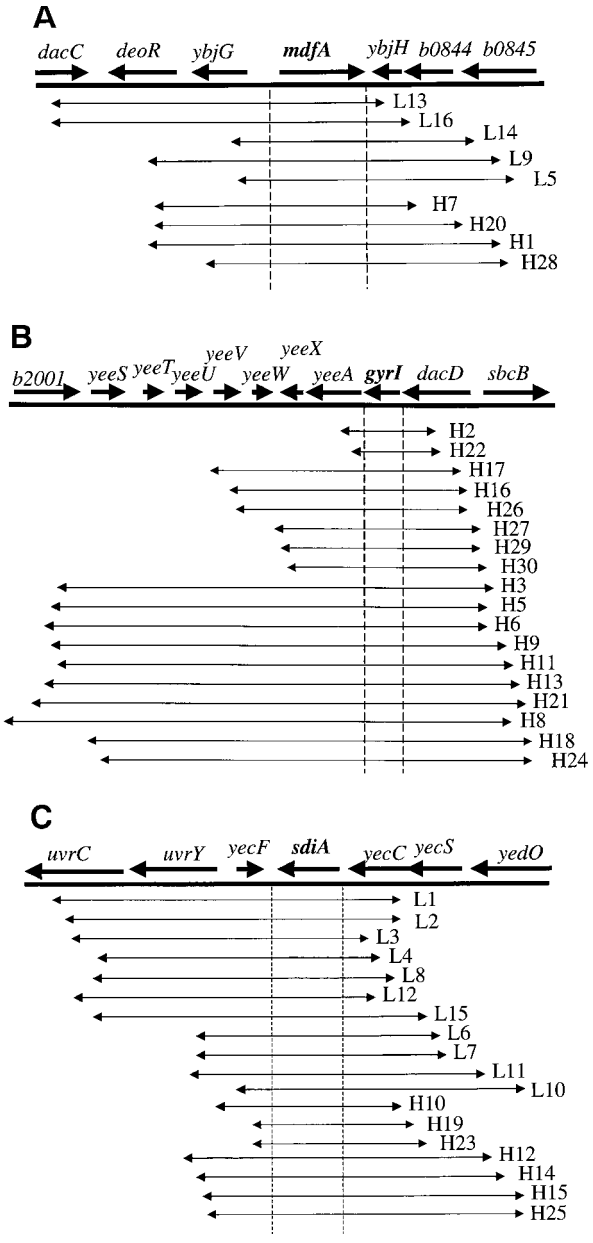

FIG. 1. Physical maps of *E. coli* genomic inserts conferring mitomycin resistance. "L" and "H" are plasmid names (e.g., L13 and H7), with "L" referring to pBR322-based plasmids and "H" referring to pUC18-based plasmids. (A) Region from min 9 in *E. coli*, where nine MMC-resistant clones were clustered (GenBank accession no. AE000186). (B) Region from min 44, where 18 MMC-resistant clones were clustered (GenBank accession no. AE000292). (C) Region from min 43, where 18 MMC-resistant clones were clustered (GenBank accession no. AE000284).

(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 multicopy 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.

TABLE 3. Zone of inhibition assays[a]

Plasmid	Inhibition zone diam (mm) with:	
	MMC (15 μg)	Nalidixic acid (75 μg)
pUC19	22	23
pDEW140 (*sdiA*)	16	16
pDEW141 (*rob*)	19	18
pUC18	19	19
pDEW133 (*gyrI*)	12	<7

[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.

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 multicopy 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 multicopy *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 4. *gyrI*-mediated multicopy resistance is *lexA* dependent

Strain	Inhibition zone diam (mm) with:	
	MMC (30 μg)	Nalidixic acid (75 μg)
DM800/pUC18	17	19
DM803/pUC18	22	22
DM800/pDEW133 (*gyrI*)	12	19
DM803/pDEW133 (*gyrI*)	22	22

TABLE 5. Modulation of *sdiA* multicopy resistance

Strain	Inhibition zone diam (mm) with:	
	MMC (15 μg)	Nalidixic acid (75 μg)
DM800/pUC19	20	20
DM803/pUC19	27	25
DM800/pDEW140 (*sdiA*)	<7	8
DM803/pDEW140 (*sdiA*)	16	13

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 *ftsQAZ*, 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* in 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

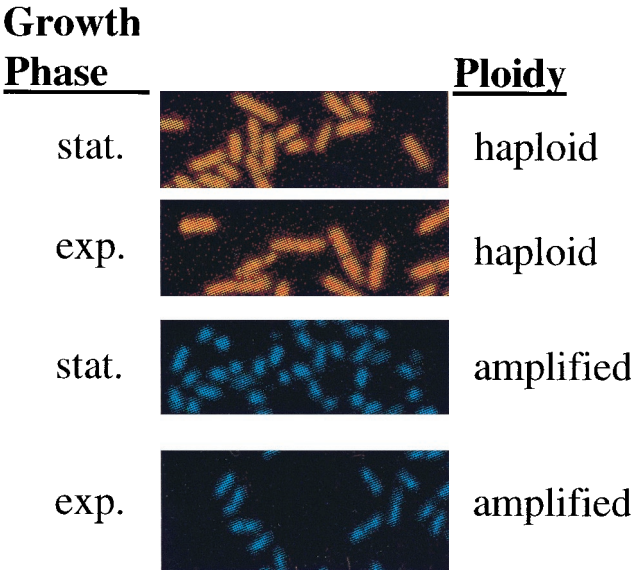

FIG. 2. Micrographs of *E. coli* cells. Each frame is enlarged to the same extent. The top two panels capture images of cells with a haploid *sdiA* (DPD2668) content, while the bottom two panels are of an isogenic strain containing multiple copies of *sdiA* (DPD2669). The first and third frames from 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 *ftsQAZ*. Expression of *ftsQAZ* is controlled by at least two regulators, RpoS and SdiA (35). Eliminating SdiA function does not prevent *ftsQAZ* expression, while amplification of *sdiA* results in overproduction of *ftsQAZ* 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.

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