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Escherichia coli mazEF-Mediated Cell Death Is Triggered by Various Stressful Conditions

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***mazEF* is an *Escherichia coli* suicide module specific for a stable toxin and a labile antitoxin. Inhibiting *mazEF* expression appeared to activate the module to cause cell death. Here we show that several stressful conditions, including high temperatures, DNA damage, and oxidative stress, also induce *mazEF*-mediated cell death. We also show that this process takes place only during logarithmic growth and requires an intact *relA* gene.**

Programmed cell death (PCD) systems are generally considered to be characteristic of multicellular organisms (12). However, PCD has also been found in unicellular eukaryotes (4) and even in prokaryotes (19, 23, 28, 42). One version of these genetic systems is found on extrachromosomal elements like plasmids and phages (8, 12, 25, 45). They consist of a pair of genes, of which the downstream gene encodes a stable toxin and the upstream gene encodes a labile antitoxin. When the extrachromosomal element is present in the cell, the antitoxin antagonizes the toxin. When the extrachromosomal element is lost, however, the labile antitoxin is degraded, allowing the toxin to kill the cell, a process called postsegregational killing. For this reason, it is believed that the addiction modules participate in maintaining the extrachromosomal elements in the cell (8, 12, 25, 45). The chromosomes of many bacteria have been found to carry toxin-antitoxin systems that are homologous to these extrachromosomal addiction modules (3, 10, 11, 20, 29, 30, 31, 33).

The first chromosomal addiction module found to be regulatable and to function as a PCD system was *Escherichia coli mazEF* (3), located in the *relA* operon (31). The product of *mazF* (MazF) is a stable toxin that cleaves mRNA at a specific site(s) (11, 47). The mechanism of this cleavage has not yet been clarified, as contradictory results have been previously reported (11, 47). The product of *mazE* (MazE) is an anti-MazF labile protein that is degraded by the protease ClpAP (3). Thus, continuous production of MazE is required to prevent MazF-mediated death. In contrast to the results seen with the addiction modules that are triggered by the loss of the element (8, 12, 25, 45), death mediated by the chromosomally borne *mazEF* is triggered by several conditions that prevent *mazEF* expression. Initially, we found that the *mazEF* module is under the control of ppGpp (3, 13), the amino acid starvation signal molecule produced by the RelA protein (7). Overproduction of ppGpp leads to the inhibition of the expression of *mazEF* and, thereby, to cell death (3, 13). This inhibition also takes place in the presence of general inhibitors of transcription and/or translation such as the antibiotics rifampin, chloramphenicol, and spectinomycin (40) and through the inhibition of translation by the Doc protein of prophage P1 (21). In each case, the inhibition of gene expression leads to a relatively low level of the labile MazE that allows the stable MazF to kill the cell. Recently, we found that *mazEF*-mediated death is also triggered by thymine starvation (41), which is known to cause a unique form of DNA damage (2, 34).

Here we asked the following question: do other agents that cause DNA damage and do other kinds of stressful conditions also induce *mazEF*-dependent death? We examined the following stress conditions: high temperature (50°C), DNA damage (caused by UV irradiation and nalidixic acid and mitomycin C exposure), and oxidative stress (H_2O_2). Under each of these stressful conditions, we observed *mazEF*-dependent cell death. In addition, we observed no *mazEF*-mediated cell death during stationary growth; we found *mazEF*-mediated cell death only during logarithmic growth, and it seems to require the production of ppGpp.

We used *E. coli* strains MC4100 *relA1* and MC4100 *relA*$^+$ and their Δ*mazEF* derivatives, all of which have been used by members of our group (3, 13, 21, 40, 41). We also used strain K38 (39), which we are currently studying in our laboratory. Its Δ*mazEF* derivative was constructed here by P1 transduction of the kanamycin resistance gene from MC4100*mazEF*::*kanR*. For the overproduction of MazF we used plasmid pQE30*mazF*, which is a derivative of the ampicillin resistance-encoding plasmid pQE30 (Qiagen) carrying the *mazF* gene under the control of the *ptac* promoter. This plasmid was cotransformed together with the plasmid pREP4, which harbors a *lacI*^{q} gene (Qiagen) that enables the regulation of *ptac-mazF*. The bacteria were grown in liquid M9 minimal medium (40) and plated on rich Luria-Bertani (LB) agar medium (32) which we prepared with the following ingredients: 0.8% Bacto Tryptone, 0.5% yeast extract (both obtained from Difco, Sparks, Md.), 0.5% NaCl (Frutarom, Haifa, Israel), and 1.5% agar (Hispanagar, Burgos, Spain). Nalidixic acid, mitomycin C, rifampin, chloramphenicol, and ampicillin were obtained from Sigma (St. Louis, Mo.). H_2O_2 was obtained from Merck (Armstadt, Germany).

We studied the effects of various stressful conditions on cell

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viability by diluting (1/100) an overnight culture in M9 medium and growing the cells with shaking (150 rpm) in the same medium at 37°C until they reached logarithmic growth (optical density at 600 nm, 0.4 to 0.6) or, when indicated, for 18 h (optical density at 600 nm, about 2.0) to stationary phase. When the cells reached the stage of either logarithmic or stationary growth, they were incubated at 37°C for 10 min without shaking. The various stressful conditions were induced, and we then plated the cells on LB agar plates. We incubated the plates at 37°C overnight and then determined the ratio of the CFU of treated cells versus untreated cells.

High temperatures induced *mazEF*-mediated cell death. In previous work (37, 38), it was shown that *E. coli* cells are sensitive to short exposure (10 min) to temperatures higher than 48°C. Here we compared the sensitivities to high temperatures of the wild-type strains MC4100*relA*$^+$ and K38 to those of their Δ*mazEF* derivatives. In contrast to the wild-type cells, most of the Δ*mazEF* cells survived exposure to high temperatures between 48 and 50°C (MC4100*relA*$^+$) or at 49°C (K38). These Δ*mazEF* cells perished only at temperatures above 52°C (MC4100*relA*$^+$) or 49°C (Fig. 1).

Damage to DNA-induced *mazEF*-mediated cell death. In previous work (41), Sat et al. reported that the well-known phenomenon of thymineless death (2) is a result of the activation of the suicide module *mazEF*. As Sat et al. suggested then, thymine starvation might activate *mazEF*-mediated death by causing unique DNA damage (2, 34) that leads to the inhibition of the *mazEF* P_2 promoter activity (41). Here we asked the following question: do other types of DNA damage also trigger *mazEF*-mediated cell death? We chose to examine three modes for generating DNA damage: those of UV irradiation and of the two chemical agents nalidixic acid and mitomycin C. UV irradiation mainly causes the generation of cyclobutane pyrimidine dimers in template DNA during replication (6, 16). Nalidixic acid inhibits the topoisomerase gyrase (9), and mitomycin C induces DNA damage by causing DNA cross-links (5, 24).

In our experiments, *E. coli* strains MC4100*relA*$^+$ and K38 and their Δ*mazEF* derivatives were exposed to DNA-damaging conditions (nalidixic acid [Fig. 2A] or mitomycin C [Fig. 2B] treatment or UV irradiation [Fig. 2C] and the percentages of survivors were determined. The survival of the wild-type strains MC4100*relA*$^+$ and K38 was affected by short exposures to nalidixic acid at concentrations of 1.0 mg/ml and higher (Fig. 2Aa) and to mitomycin C at concentrations of 0.05 μg/ml and higher (Fig. 2Ba). In contrast, the survival of the Δ*mazEF* derivative of MC4100*relA*$^+$ was not affected by nalidixic acid at concentrations of 2.0 mg/ml and lower (Fig. 2Aa) or by mitomycin C at concentrations of 1.5 μg/ml and lower (Fig. 2Ba). A similar pattern was observed in strain K38 (Fig. 2Ab and Bb). Furthermore, its Δ*mazEF* derivative seems to be resistant to even higher concentrations of nalidixic acid and mitomycin C than MC4100*relA*$^+$ (Fig. 2Aa versus 2Ab and Fig. 2Ba versus 2Bb). In addition, UV irradiation also affected the survival of the Δ*mazEF* derivatives less than it affected their corresponding wild-type strains. Only 2 s of irradiation (25 J/m^2) was required to reduce the survival of both strains MC4100 *relA*$^+$ and K38, while more than 8 s of irradiation (25 J/m^2) was required to affect the survival of their Δ*mazEF* derivatives (Fig. 2C). Thus, we conclude that the *mazEF* system is involved in the process of death caused by DNA damage.

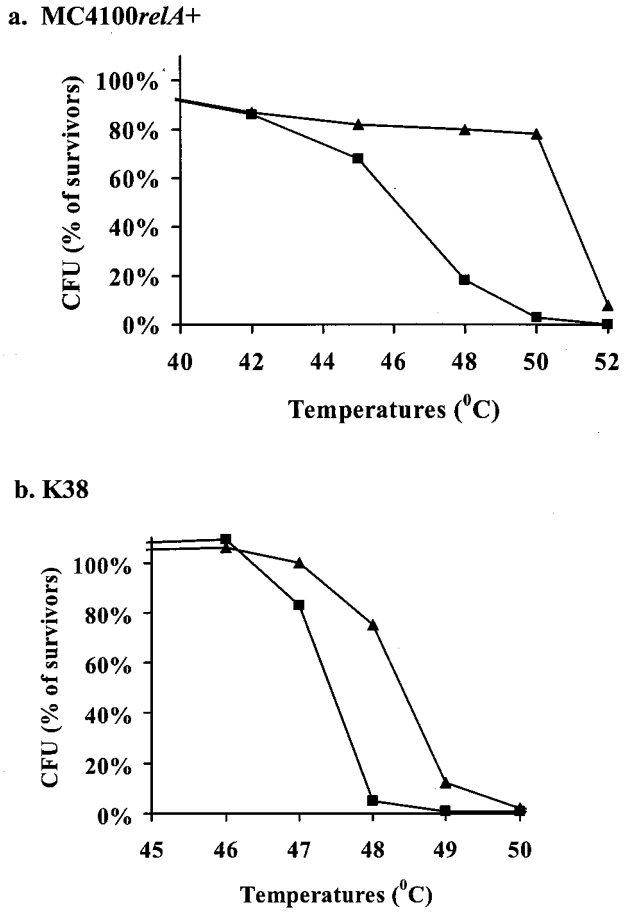

FIG. 1. The effect of *mazEF* on bacterial viability after exposure to high temperatures. *E. coli* wild-type strains (■) and their Δ*mazEF* derivatives (▲) were incubated for 10 min at various temperatures. Cell viability was determined by CFU counting on LB plates incubated at 37°C overnight. The survivor ratio was determined by comparing the number of CFU of treated cells to that of untreated cells. The results represent one experiment out of three independent similar experiments. (a) *E. coli* MC4100 *relA*$^+$; (b) *E. coli* K38.

Oxidative stress induced *mazEF*-mediated cell death. An increased level of reactive oxygen causes oxidative stress that leads to damage to all cellular components (43). To induce oxidative stress, we treated *E. coli* cells with hydrogen peroxide (H_2O_2) (Fig. 3). The wild-type strain MC4100 *relA*$^+$ died after a short exposure to H_2O_2 at concentrations of 5 mM and higher; however, most of the cells of its Δ*mazEF* derivative survived at concentrations of H_2O_2 up to 50 mM (Fig. 3). A similar pattern was observed in strain K38, although this strain seems to be slightly less sensitive to H_2O_2 than MC4100*relA*$^+$. Thus, *mazEF*-mediated cell death was also involved in death caused by oxidative stress in the presence of H_2O_2.

The induction of *mazEF*-mediated cell death by stress conditions is carried out at the logarithmic growth stage and requires an intact *relA* gene. All of the previous experiments of Aizenman et al. and Sat et al. (3, 40, 41) and all of the experiments described above showing that various stressful condi-

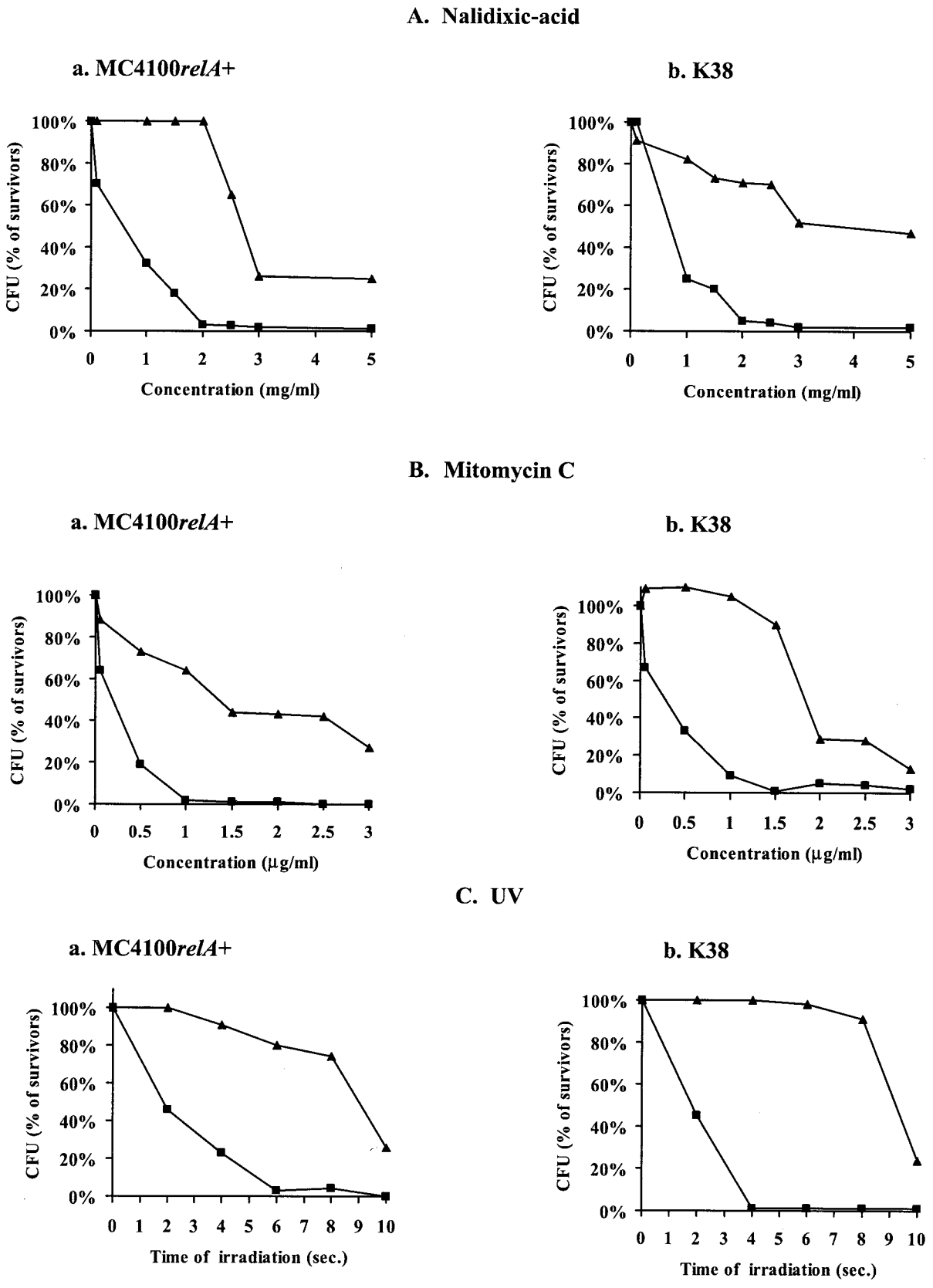

FIG. 2. The effect of *mazEF* on bacterial viability after exposure to agents causing DNA damage. *E. coli* MC4100 *relA*$^+$ (a) and K38 (b) and their Δ*mazEF* derivatives were treated for 10 min at 37°C under the following DNA-damaging conditions: nalidixic acid exposure (A), mitomycin C exposure (B), and UV irradiation (C). The cells were washed with saline and diluted in LB medium. For UV irradiation, a series of decimal dilutions were carried out and 10-μl drops were plated on LB plates, dried, and UV irradiated (25 J/m^2). In all cases, the cells were plated on LB medium for CFU counting and the survivor ratio was determined by comparing the number of treated cells to the number of untreated cells as described in the legend to Fig. 1. The results represent one experiment out of three independent similar experiments. ■, wild-type strains; ▲, Δ*mazEF* derivatives of the wild-type strains.

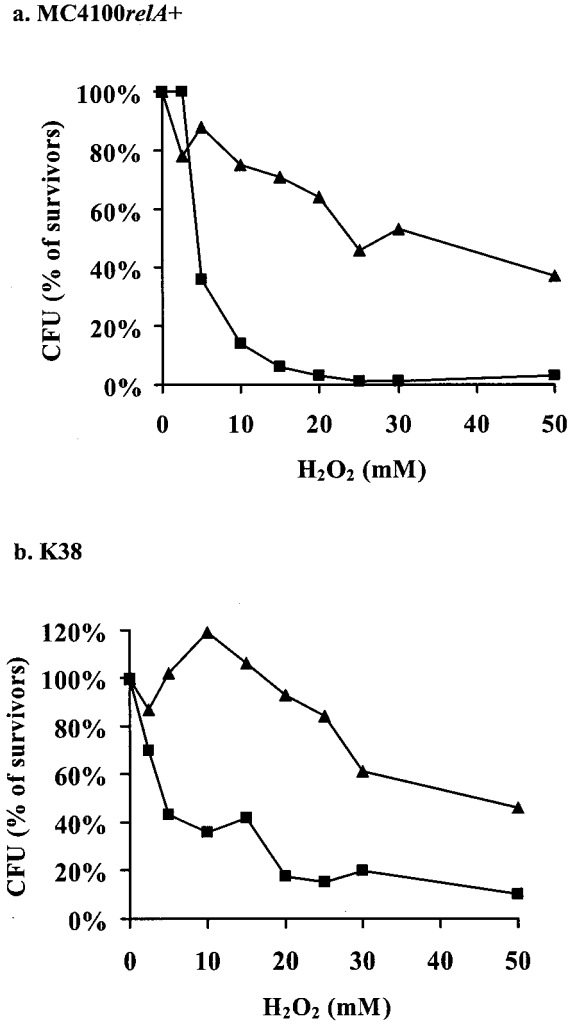

FIG. 3. The effect of *mazEF* on bacterial viability after exposure to oxidative stress. *E. coli* strains MC4100*relA*$^+$ (a) and K38 (b) and their Δ*mazEF* derivatives were incubated in the presence of various concentrations of H_2O_2 at 37°C for 10 min. Cell viability and survivor ratios were determined as described in the legend to Fig. 1. The results represent one experiment out of three independent similar experiments. ■, wild-type strains; ▲, Δ*mazEF* derivatives of the wild-type strains.

tions induced *mazEF*-mediated death were carried out during logarithmic growth (Fig. 1 to 3). Here, we found that cells in the stationary growth phase survived these stressful conditions (Fig. 4A). These conditions included high temperature (50°C) (Fig. 4Ab), UV irradiation (Fig. 4Ac), and the addition of rifampin (Fig. 4Ad). During stationary phase, moreover, even the induction of the toxin MazF by IPTG (isopropyl-β-D-thiogalactopyranoside) was not lethal (Fig. 4Ae). Thus, we found that *mazEF*-mediated death was activated only during logarithmic growth. We do not yet understand why MazF appears to be inactive during stationary growth. However, this inactivity may contribute to the well-known resistance of cells to stressful conditions during stationary growth (22).

All of the previous experiments in which Sat et al. used rifampin or chloramphenicol (40) and our experiments described above (Fig. 1 to 3) were done using bacterial strains that carried an intact *relA* gene. To test the involvement of the *relA* gene, we compared the effects of various stressful conditions which we found to induce *mazEF*-mediated death on MC4100*relA*$^+$ and on MC4100*relA1* strains during logarithmic growth (Fig. 4B). The *relA1* mutation consists of an amino-terminal IS*2* insertion between codons 85 and 86. This mutant allele produces only residual amounts of ppGpp (7). We treated each strain for 10 min under the following stressful conditions: 50°C (Fig. 4Ba), UV irradiation (Fig. 4Bb), nalidixic acid exposure (Fig. 4Bc), H_2O_2 exposure (Fig. 4Bd), rifampin exposure (Fig. 4Be), or chloramphenicol exposure (Fig. 4Bf). In contrast to the *relA*$^+$ cells, which do not survive these conditions of treatment, the *relA1* cells did survive (Fig. 4B). Interestingly, overproduction of MazF kills both *relA*$^+$ and *relA1* cells (Fig. 4Bg). Thus, it seems that ppGpp is involved in triggering the *mazEF* death rather than affecting the toxic action of MazF.

Loss of viability of *E. coli* cells due to high temperatures (37, 38), oxidative stress (43), and DNA damage by UV irradiation (6), nalidixic acid exposure (9), and mitomycin C exposure (24) was previously described. Here we have shown that *mazEF* is involved in cell death under stressful conditions (Fig. 1 to 3). In other words, it appears that under these conditions the cells die because of the activation of an internal death machinery. It should be noted that we observed *mazEF*-mediated death only in a window of mildly stressful conditions. Within this window of conditions, the Δ*mazEF* derivatives were relatively resistant to the stresses (Fig. 1 to 3). However, we found that under extreme stressful conditions the Δ*mazEF* cells also died (Fig. 1 to 3). It is still not clear whether the Δ*mazEF* cells died because of the induction of some other toxin-antitoxin systems or through the inactivation of some essential component.

As mentioned above, members of our group have previously shown that *mazEF*-mediated death can be triggered by various stressful conditions that inhibit the expression of *mazEF*. These conditions include the inhibition of transcription by the overproduction of ppGpp (3, 13) and by the presence of antibiotics that inhibit transcription and/or translation (rifampin, chloramphenicol, and spectinomycin) (40). The inhibition of *mazEF* expression may also be involved in the induction of *mazEF*-dependent death at high temperatures (Fig. 1). At such stressful temperatures, σ^{70}, the normal transcription factor, becomes inactivated; σ^{70} is replaced by periplasmic σ^E (37, 38). Judging on the basis of investigations of the promoter recognition sites of σ^E (46), it should not initiate the transcription of the *mazEF* genes. Recently, Sat et al. also showed that DNA damage due to thymine starvation prevents *mazEF* transcription (41). Whether the herein-described *mazEF*-mediated death induced by UV, nalidixic acid, mitomycin C, and H_2O_2 also inhibits *mazEF* expression remains to be determined.

To the best of our knowledge, this report is the first to suggest that ppGpp has a role in *E. coli* cell death caused by various stressful conditions (Fig. 4B). These results are in addition to previous results showing that artificial overproduction of ppGpp can by itself cause *mazEF*-dependent death, which seems to be due to the inhibition of the transcription of *mazEF* (3, 13). Furthermore, we have shown here that the combined action of stress conditions together with ppGpp affects only the induction of *mazEF* and not the MazF toxicity (Fig. 4Bg). More particularly, since death by MazF action does not require

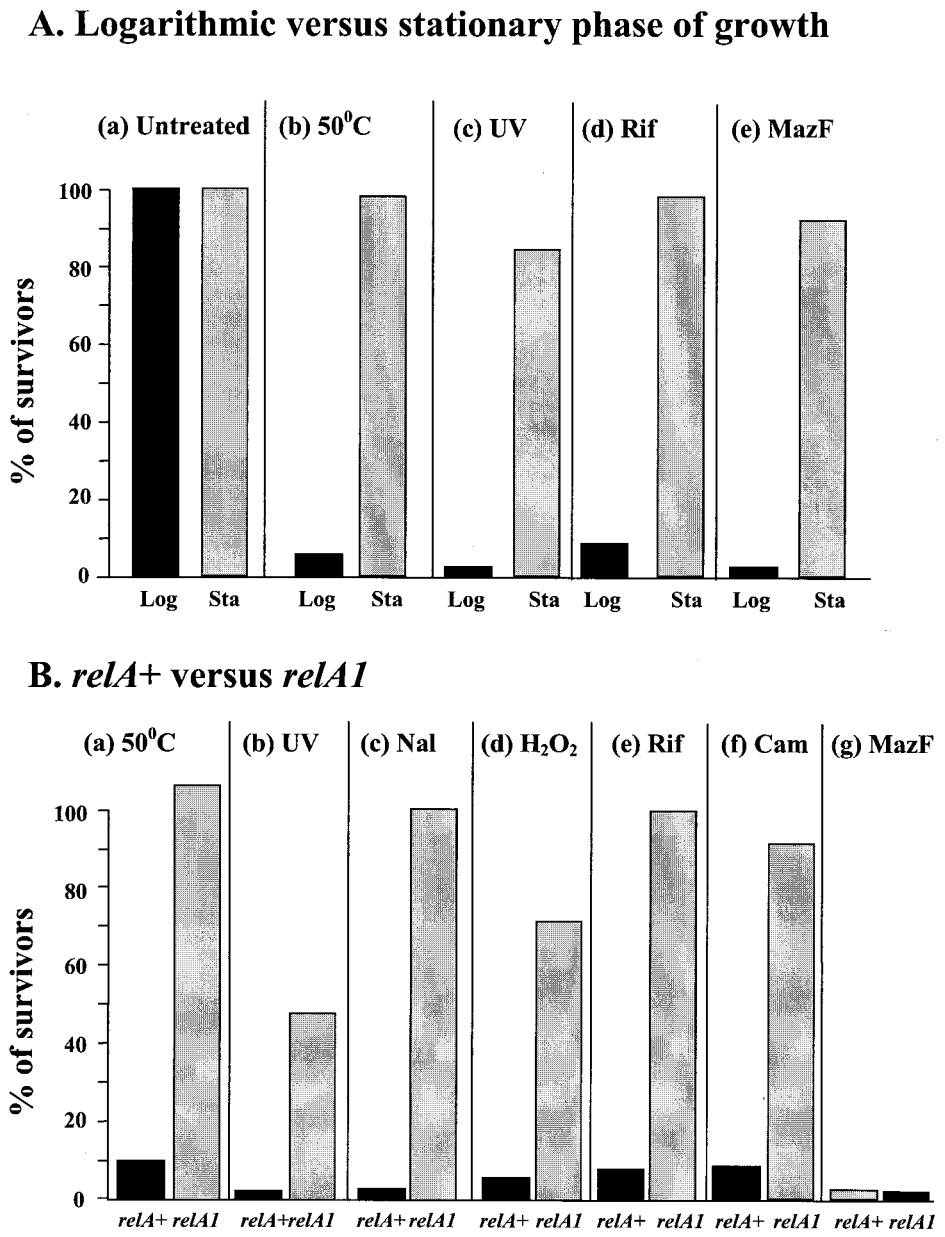

FIG. 4. The effects of the growth stage (A) and ppGpp (B) on the induction of *mazEF*-mediated cell death. (A) Logarithmic (Log) and stationary (Sta) *E. coli* MC4100 *relA*$^+$ cells were either left untreated (a) or submitted for 10 min to high temperature (50°C) (b), UV irradiation (c), rifampin exposure (20 μg/ml) (d), or the overproduction of MazF (e). (B) Logarithmic *E. coli* MC4100*relA*$^+$ and MC4100*relA1* cells were submitted for 10 min to high temperature (50°C) (a), UV irradiation (b), nalidixic acid (Nal) exposure (2 mg/ml) (c), H_2O_2 exposure (15 mM) (d), rifampin (Rif) exposure (20 μm/ml) (e), chloramphenicol (Cam) exposure (50 μg/ml) (f), or the overproduction of MazF (g). Cell viability and survivor ratios were determined as described in the legend to Fig. 1. The results represent one experiment out of three independent similar experiments.

the presence of ppGpp (Fig. 4Bg) we have concluded that the combined action of stressful conditions together with ppGpp only affects the induction of *mazEF* and not the toxicity of MazF itself.

Many gram-positive and gram-negative bacteria carry genes that are homologous to *mazEF* (14, 33). It is interesting that in several gram-positive bacteria, including *Staphylococcus aureus*, *Bacillus subtilis* (33), and *Bacillus anthracis*, the *mazEF* homologues are located immediately upstream of the *sigB* gene (27, 33). This gene encodes σ^B, which is a general stress-response component (26). Moreover, during heat stress the *mazEF* homologues are cotranscribed with *sigB*, at least in *S. aureus* (18). Thus, it seems that the link between stressful conditions and the *mazEF* system is not limited to *E. coli*. Moreover, even in eukaryotes various stressful conditions, including DNA damage, nitric oxide exposure, and heat shock, have been found to trigger apoptosis (35, 44).

Recently, it has been suggested that the chromosomal toxin-

antitoxin system *mazEF* and its nonhomologous chromosomal module *relBE* (10) are not involved in cell death but rather induce a state of reversible bacteriostasis (36). This view is based on experiments showing that ectopic overexpression of the toxin MazF or RelE inhibits translation and cell growth, which can be resumed if the cognate antitoxin is expressed at a later time (36). However these experiments were carried out within a small window of time, during only the 5 h after MazF expression. Using the same system, we found that overexpressing MazE after a prolonged overexpression of MazF did not lead to the reversal of cell death. Thus, it seems that there is a point of no return (15; S. Amitai and H. Engelberg-Kulka, unpublished results). Even so, it should be emphasized that ectopic overexpression drastically affects bacterial pathways and networks, so that the conditions in the cell no longer reflect the actual physiological conditions under which toxin-antitoxin systems mediate cell death. The experiments described here and previously (3, 13, 21, 40, 41) were done when the *mazE* module was located on the *E. coli* chromosome as a single copy and in its natural context, which seems to be more appropriate than ectopic overexpression for studying bacterial pathways and physiological networks. These experiments clearly show that *E. coli mazEF*-mediated cell death is triggered by various stressful conditions. It may be that under stressful conditions, *mazEF* has some additional regulatory function(s), as suggested previously (10, 17); however, the present report and other previous reports (3, 13, 21, 40, 41) relate only to *mazEF*-mediated cell death, i.e., loss of viability.

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