Induction of *Escherichia coli* Chromosomal *mazEF* by Stressful Conditions Causes an Irreversible Loss of Viability

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***mazEF* is a stress-induced toxin-antitoxin module located on the chromosomes of many bacteria. Here we induced *Escherichia coli* chromosomal *mazEF* by various stressful conditions. We found an irreversible loss of viability, which is the basic characteristic of cell death. These results further support our previous conclusion that *E. coli mazEF* mediation of cell death is not a passive process, but an active and genetically “programmed” death response.**

Toxin-antitoxin systems have been found on the chromosomes of many bacteria (15, 16, 30, 32). Each system consists of a pair of genes that specify for two components: a stable toxin and an unstable antitoxin that interferes with the action of the toxin (for reviews, see references 14, 17, 19, and 22). In *Escherichia coli*, there are several such pairs of genes, including *mazEF* (2, 27, 28), *chpBIK* (26), *relBE* (4, 11, 20), *yefM-yoeB* (8, 9, 21), and *dinJ-yafQ* (22). Among them, *E. coli mazEF* was the first to be discovered (28) and has been described as being regulatable and responsible for programmed cell death in bacteria (2). *mazF* specifies for a stable toxin, MazF, while *mazE* specifies for a labile antitoxin, MazE, which is degraded in vivo by the ATP-dependent ClpPA serine protease (2). The toxin MazF was clearly shown to inhibit translation by cleaving mRNAs at specific sites (10, 31, 39). Based on both in vivo and in vitro studies, it has been shown that MazF is a sequence-specific endoribonuclease that preferentially cleaves single-stranded mRNAs at ACA sequences (39, 40). MazE counteracts the action of MazF. Because MazE is a labile protein, preventing MazF-mediated action requires the continuous production of MazE. We have found that the chromosomally borne *mazEF* is a stress-induced death-mediating module (2, 23, 24, 36, 37). Several stressful conditions that prevent the expression of the chromosomally borne *mazEF*, and thereby MazE synthesis, are triggering cell death. These include extreme amino acid starvation leading to the production of ppGpp (2, 18); the inhibition of transcription and/or translation by antibiotics, such as rifampin, chloramphenicol, and spectinomycin, under specific growth conditions (36); the inhibition of translation by the Doc protein of prophage P1 (24); DNA damage caused by thymine starvation (37) as well as by mitomycin C, nalidixic acid, and UV irradiation (23); and oxidative stress (H₂O₂) (23). Most of the antibiotics and stressful conditions that were used in these studies are well known for inducing bacterial cell death (1, 12, 35, 38). Our experiments clearly show that they cause bacterial cell death through the *mazEF* module.

It was reported by Pedersen and colleagues (33) that the

toxic effect obtained by an ectopic overproduction of MazF can be reversed by the action of the antitoxin MazE being ectopically overexpressed at a later time. Based on these results, Pedersen and colleagues have suggested that, rather than inducing cell death, *mazF* induces a state of reversible bacteriostasis (33). However, in a more recent report (3) where a similar ectopic overexpression system was used, we have shown that the overexpression of MazE could reverse MazF lethality for only a short window of time. The size of that window depended on the nature of the medium in which MazF was overexpressed. Thus, we found a “point of no return,” which occurred in minimal M9 medium much sooner than it did in rich Luria-Bertani (LB) medium.

Even so, it should be emphasized that in such an ectopic overexpression system previously used by others (33) and our laboratory (3), cells are flooded with toxic MazF that drastically affects bacterial pathways and networks. Therefore, these experiments may no longer reflect the actual physiological conditions under which a toxin-antitoxin system mediates cell death. Since the point of no return is the basic functional characteristic for cell death, here we tested this characteristic under conditions that are designed to mimic physiological ones. The *mazEF* module was located on the *E. coli* chromosome as a single copy in its natural context, and *mazEF* was induced by various stressful conditions. Under all of the stressful conditions applied, we found an irreversible loss of viability. Ectopic overexpression of MazE can reverse the lethal action of the chromosomal-directed MazF for only a short period of time.

We used the following *E. coli* strains: MC4100 *relA1* (6), MC410 *relA*⁺, and its $\Delta mazEF::kanR$ derivative (18). We also used *E. coli* strain K38 and its $\Delta mazEF$ derivative (23). For the overproduction of MazE, we used plasmid pQE- $\Delta his-mazE$, which is a derivative of the ampicillin resistance pQE30 (QIAGEN) carrying the *mazE* gene under the *lac* promoter (3). The bacteria were grown in liquid M9 minimal medium with 1% glucose and a mixture of amino acids (10 μ g/ml each) (29) and plated on rich LB agar plates as described previously (23). IPTG (isopropyl- β -D-thiogalactopyranoside), nalidixic acid, mitomycin C, trimethoprim, rifampin, chloramphenicol, spectinomycin, and ampicillin were obtained from Sigma (St. Louis, MO).

Here we asked whether the induction of *mazEF*-mediated cell death can be reversed by a later overproduction of MazE. This was performed as follows: *E. coli* strains MC4100 *relA*⁺/

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pQE- Δhis -*mazE* and MC4100 *relA*⁺ $\Delta mazEF$ were grown to mid-logarithmic phase (optical density at 600 nm was 0.6, corresponding to CFU of $5 \times 10^8 \pm 2 \times 10^8$ cells/ml) in M9 medium. The cells were incubated for 10 min at 37°C. Stressful conditions were induced for the definitive time period required for chromosomal *mazEF* induction. The agents causing stressful conditions were removed by centrifugation and washing of the cells in preheated M9 medium. *mazE* was induced by the addition of IPTG (1 mM) for 1 h at different times after the removal of the stressful conditions. CFUs were determined by plating on LB plates that were incubated at 37°C for 12 h. The survivor ratio (percentage of control) was determined by comparing the number of treated cells to the number of untreated cells (before *mazEF* induction by each of the stressful conditions).

mazEF-mediated cell death was triggered in *E. coli* cells by three different groups of stressful conditions that were previously shown by us to induce *mazEF*-mediated cell death (2, 18, 23, 36). Group 1 included three different ways of inhibiting *mazEF* transcription (Fig. 1): the use of (i) the transcriptional inhibitor rifampin (Fig. 1A), which is known to inhibit the initiation of RNA synthesis by its interaction with the β subunit of RNA polymerase (12); (ii) serine hydroxamate (Fig. 1B), which induces the production of ppGpp (7) and thereby prevents transcription of *mazEF* (2, 18) (here, a strain carrying a *relA1* mutation which is therefore defective in the synthesis of ppGpp was used as a control; it verifies that *mazEF*-mediated cell death triggered by serine hydroxamate is ppGpp dependent [18]); and (iii) a high temperature (50°C) (Fig. 1C). At this temperature, the normal transcription factor σ^{70} becomes inactivated; σ^{70} is replaced by the periplasmic σ^E (34, 35) which should not initiate the transcription of the *mazEF* gene (23). We observed similar kinetics in the ability of MazE to reverse the loss of viability by each of the applied conditions (Fig. 1). The similarity is reflected both in the pattern of the gradual decrease in the ability of MazE to reverse the loss of viability and in the level of reversion in each time point (compare Fig. 1A, B, and C).

The stressful conditions we used for group 2 included the translational inhibitors spectinomycin (Fig. 2A) and chloramphenicol (Fig. 2B), affecting the machinery of translational elongation (12). We found that the kinetics of the ability of MazE to reverse the loss of viability were different for groups 1 and 2 (compare Fig. 1 and Fig. 2). For group 2, a very efficient reversion by MazE was observed during a longer period of time (120 to 150 min) than that for group 1 (30 to 60 min). Later, in contrast to the gradual decrease in the viability of group 1 (Fig. 1), in group 2 the reversion ability of MazE declines rapidly (Fig. 2).

In the stressful conditions for group 3, we used several agents causing DNA damage: nalidixic acid (Fig. 3A), an inhibitor of the topoisomerase gyrase (13); trimethoprim (Fig. 3B), which induces thymine starvation (1); and mitomycin C (Fig. 3C), which generates DNA cross-links (5). Here also, the overproduction of MazE could reverse the loss of viability over a short period of time. However, nonidentical kinetics were obtained by the different DNA damage-causing agents used (Fig. 3). Note that we induced MazE overproduction by the addition of IPTG for 1 h to the liquid medium. This duration of IPTG induction is optimal and sufficient for the reversion effect as well as for achievement of the most delayed point of

FIG. 1. A point of no return after the induction of the *E. coli* chromosomal-*mazEF*-mediated cell death by inhibition of translation (group 1). Bacterial cultures of *E. coli* strains MC4100 *relA*⁺/pQE- Δhis -*mazE*, MC4100 *relA1* (the strain was used for the induction of stressful conditions with serine hydroxamate), and MC4100 *relA*⁺ $\Delta mazEF$ were grown to mid-logarithmic phase. (A) Rifampin (20 μ g/ml) was added, the cells were incubated at 37°C for 10 min, and then rifampin was removed. (B) Serine hydroxamate (0.1 mg/ml) was added, the cells were incubated at 37°C for 1 h and then serine hydroxamate was removed. (C) Cells were incubated for 10 min in 50°C without shaking. For the rest of the experiment, see the text. The results describe the average of three independent experiments that were carried out in triplicate. Error bars indicate standard deviations. WT, wild type.

FIG. 2. A point of no return after the induction of the *E. coli* chromosomal-*mazEF*-mediated cell death by inhibition of translation (group 2). Bacterial cultures of *E. coli* strains MC4100 *relA*⁺/pQE- Δhis -*mazE* and MC4100 *relA*⁺ $\Delta mazEF$ were grown as described in the legend of Fig. 1. Agents causing the inhibition of translation were added and then removed after incubation without shaking at 37°C for 10 min. (A) Spectinomycin (0.2 mg/ml). (B) Chloramphenicol (0.04 mg/ml). For the rest of the experiment, see the text. The results describe the average of three independent experiments that were carried out in triplicate. Error bars indicate standard deviations. WT, wild type.

no return; similar results were obtained when IPTG was added to the LB plates rather than to the liquid medium. In addition, in our experimental system, IPTG induction for at least 30 min was required for the reversion of MazF lethality by MazE overproduction. Therefore, it seems that the accumulation of a sufficient amount of MazE is necessary.

As found previously in the ectopic overexpression system (3), we also found a point of no return here when the chromosomal *mazEF* module was triggered by each of various stressful conditions applied. As shown in Fig. 1 to 3, MazE could reverse MazF lethality over only a short period of time. Because of reasons yet unknown, MazE could reverse *mazEF*-mediated cell death within a wider window of time when *mazEF* is triggered by the inhibition of translation (120 to 150 min) (Fig. 2) than that when *mazEF* is triggered by the inhibition of transcription (30 to 60 min) (Fig. 1) or by DNA damage (60 to 90 min) (Fig. 3). Later on, in all the stressful conditions studied (Fig. 1 to 3), there was a gradual or even

FIG. 3. A point of no return after the induction of the *E. coli* chromosomal-*mazEF*-mediated cell death by DNA damage (group 3). Bacterial cultures of *E. coli* strains MC4100 *relA*⁺/pQE- Δhis -*mazE* and MC4100 *relA*⁺ $\Delta mazEF$ were grown as described in the legend of Fig. 1. (A) Nalidixic acid (1 mg/ml) with incubation for 10 min. (B) Trimethoprim (2 μ g/ml) with incubation for 1 h. (C) Mitomycin C (0.25 μ g/ml) with incubation for 10 min. For the rest of the experiment, see the text. The results describe the average of three independent experiments that were carried out in triplicate. Error bars indicate standard deviations. WT, wild type.

drastic decrease in the ability of MazE to reverse MazF lethality. However, for all cases, MazE was not able to reverse MazF lethality at 210 min after MazF induction. Similar results were also obtained for another *E. coli* strain, K38 (data not shown).

In summary, the point of no return described previously (3) and here (Fig. 1 to 3) suggests the existence of a stage of commitment after which death is unavoidable. Based on our previous results (3) as well as the herein-described point of no return in *mazEF*-mediated cell death, we suggested a model (14) in which the endoribonucleolytic effect of MazF would be one of the initial steps in the programmed cell death pathway. In this model, this initial step can be still be reversed by the antagonistic effect of MazE on MazF. Further cleavage of mRNAs and tmRNA by MazF would be prevented by MazE, and the previously truncated mRNAs could be released from the ribosomes through the action of de novo-synthesized, un-cleaved tmRNA (for a review, see reference 25). However, we suggest that MazE cannot reverse the downstream events that were already initiated by MazF. Thus, if the process could not be stopped in time, eventual cell death would be unavoidable. How might the inhibition of translation by MazF induce such a downstream cascade leading to cell death? Currently, we can envisage two mechanisms that may act simultaneously: (i) some of the MazF-cleaved mRNAs are specifying for proteins required for cell survival and (ii) the action of MazF that cleaves mRNAs at specific sites (39, 40) could lead to the selective synthesis of proteins encoded by mRNAs resistant to its cleavage. These proteins may be involved in the death pathway. It is possible that the differences in the kinetics of the ability of MazE to reverse the loss of viability caused by members of groups 1, 2, and 3 (Fig. 1 to 3) reflect the involvement of different death pathways.

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