

## MazF-Mediated Cell Death in *Escherichia coli*: a Point of No Return

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***mazEF* is a stress-induced toxin-antitoxin module, located on the chromosome of *Escherichia coli*, that we have previously described to be responsible for programmed cell death in *E. coli*. *mazF* specifies a stable toxin, and *mazE* specifies a labile antitoxin. Recently, it was reported that inhibition of translation and cell growth by ectopic overexpression of the toxin MazF can be reversed by the action of the antitoxin MazE ectopically overexpressed at a later time. Based on these results, it was suggested that rather than inducing cell death, *mazF* induces a state of reversible bacteriostasis (K. Pederson, S. K. Christensen, and K. Gerdes, *Mol. Microbiol.* 45:501-510, 2002). Using a similar ectopic overexpression system, we show here that overexpression of MazE could reverse MazF lethality only over 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 sooner in minimal M9 medium than it did in the rich Luria-Bertani medium. We also describe a state in which the effect of MazF on translation could be separated from its effect on cell death: MazE overproduction could completely reverse the inhibitory effect of MazF on translation, while not affecting the bacteriocidal effect of MazF at all. Our results reported here support our view that the *mazEF* module mediates cell death and is part of a programmed cell death network.**

Toxin-antitoxin systems have been found on the chromosomes of many bacteria (1, 6, 10, 17–19, 21). In *Escherichia coli* such systems include *mazEF* (1, 18, 19), *chpBIK* (18), *relBE* (2, 7, 10), *yefM-yoeB* (4, 5, 11), and *dinJ-yafQ* (13). Each toxin-antitoxin system consists of a pair of genes, of which the downstream gene encodes a stable toxin and the upstream gene encodes a labile antitoxin. The first toxin-antitoxin system carried on a bacterial chromosome that was described as regulatable and responsible for programmed cell death was the *E. coli mazEF* module (1), located in the *relA* operon (19). The product of *mazF* (MazF) is a stable toxin that inhibits translation by cleaving mRNA at a specific site(s) (6, 26). In light of contradictory results from studies on this system, the mechanism of this cleavage is not yet well understood (6, 22, 26). The product of *mazE* (MazE) counteracts the action of MazF. Because MazE is a labile protein, degraded by the protease ClpAP (1), prevention of MazF-mediated death requires the continuous production of MazE. Thus, stressful conditions that prevent the expression of the chromosomally borne *mazEF* module should trigger cell death. Indeed, as predicted, we found several stressful conditions to cause *mazEF*-mediated cell death: (i) extreme amino acid starvation leading to the production of ppGpp (1, 9); (ii) inhibition of transcription and/or translation by antibiotics such as rifampin, chloramphenicol, and spectinomycin under specific growth conditions (25); (iii) inhibition of translation by the Doc protein of prophage P1 (15); (iv) DNA damage caused by thymine starvation (24) as well as by mitomycin C, nalidixic acid, and UV irradiation (14); and (v) oxidative stress (H<sub>2</sub>O<sub>2</sub>) (14).

Recently, it has been suggested by Pedersen et al. (23) that rather than inducing programmed cell death, chromosomal

toxin-antitoxin systems may induce a state of reversible bacteriostasis. They based their hypothesis on the results of their studies that included *mazEF* (*chpAIK*) and another chromosomal module, *relBE*, that is not homologous to *mazEF* (10). It was shown that ectopic overexpression of the toxins MazF (ChpAK) or RelE inhibits both translation and cell growth. In addition, both translation and cell growth resume if the cognate antitoxin, MazE (ChpAI) or RelB, is expressed at a later time (23). However, the experiments that show that the ectopic overexpression of the antitoxin MazE (or RelB) reverses MazF (or RelE) lethality were carried out over a short window of time, during only 5 hours after MazF (or RelE) induction (23).

Here, using a similar ectopic overexpression system of MazF and MazE, we found that as described previously (23), overexpression of MazE can resuscitate *E. coli* cells which overproduce MazF within a period of 6 h in liquid Luria-Bertani (LB) medium. When, however, we extended the period of MazF ectopic overproduction, the ability of MazE to resuscitate *E. coli* cells was drastically decreased. Moreover, the inability of MazE to reverse the bacteriocidal effect of MazF was even more dramatic when MazF was overexpressed in cells growing in liquid minimal M9 medium rather than in the rich LB medium. Thus, we found a “point of no return” which occurs sooner in minimal medium than in rich medium. In addition, we found that under the experimental conditions described previously (23), the effect of MazF on translation could be separated from its effect on cell death: MazE overproduction could completely reverse the inhibitory effect of MazF on translation, while not affecting the bacteriocidal effect of MazF at all. Our results reported here support our hypothesis that the *mazEF* module mediates a programmed cell death network in *E. coli*.

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### MATERIALS AND METHODS

**Materials and media.** [<sup>35</sup>S]methionine (1,175 Ci/mmol) was obtained from NEN (Boston, Mass.). Ampicillin was obtained from Biochemie GmbH (Kundl,

Austria). Chloramphenicol, L-arabinose, and IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) were obtained from Sigma (St. Louis, Mo.). Bacteria were grown either in M9 liquid medium (20) with 0.5% glycerol and a mixture of amino acids (2 mg/ml each, without methionine) or in LB liquid medium (20). The media were supplemented with 100  $\mu$ g of ampicillin per ml and 50  $\mu$ g of chloramphenicol per ml (final concentrations). Cultures were spread on LB plates containing 100  $\mu$ g of ampicillin per ml, 50  $\mu$ g of chloramphenicol per ml, and 0.2% glucose with or without IPTG (2 mM).

**Bacterial strains and plasmids.** We used *E. coli* strain MC4100  $\Delta mazEF relA1 lacI^q::tet$ . We constructed this strain by using P1 phage to transduce *lacI^q* from MC4100 *relA1 lacI^q::tet* (kindly provided by Gad Glaser) into MC4100  $\Delta mazEF relA1$  (1).

**Construction of pBAD-*mazF*.** *mazF* was PCR amplified from strain MC4100 *relA1* (3). The PCR fragment was cut with HindIII and XbaI and ligated to HindIII-XbaI sites of pBAD33 carrying a chloramphenicol resistance gene (12) (kindly provided by Larry Snyder), downstream from the *araBAD* promoter.

**Construction of pQE- $\Delta his$ -*mazE*.** *mazE* was PCR amplified from strain MC4100 *relA1* (3). The PCR fragment was cut with HindIII and EcoRI and ligated to the HindIII-EcoRI sites of pQE30 (Qiagen) carrying an ampicillin resistance gene, downstream from the T5 promoter and the *lac* operator. Transformation of strain MC4100  $\Delta mazEF relA1 lacI^q$  with pQE- $\Delta his$ -*mazE* results in the repression of *mazE* expression; the addition of IPTG to this same strain results in the induction of *mazE* expression. Note that cutting out pQE30 at these restriction sites resulted in the removal of both the His<sub>6</sub> and the ribosome binding sites. Because of this, in addition to the *mazE* sequence, we included the ribosome binding site sequence in our PCR fragment to compensate for its loss from the digested plasmid.

**Assays for studies on the effect of MazE overproduction on the viability of *E. coli* cells that overproduce MazF over different periods.** *E. coli* strain MC4100  $\Delta mazEF relA1 lacI^q$  was cotransformed with pQE- $\Delta his$ -*mazE*, carrying *mazE* (without a His tag), and pBAD-*mazF*, carrying *mazF*. The transformed culture was grown in either LB or M9 medium at 37°C to mid-logarithmic phase (optical density at 600 nm [OD<sub>600</sub>], 0.5). At time zero, *mazF* expression was induced by the addition of 0.2% arabinose to the culture. The induced culture was incubated at 37°C without shaking. At various time points, samples were withdrawn and spread on LB-ampicillin-chloramphenicol plates containing either 0.2% glucose (to repress *mazF* expression) or 0.2% glucose and 2 mM IPTG (to induce *mazE* expression).

**Assays for studies on the effect of MazE overproduction during growth in liquid medium on the ability of MazF-overproducing *E. coli* cells to form colonies and to synthesize proteins.** *E. coli* strain MC4100  $\Delta mazEF relA1 lacI^q$ , carrying pQE- $\Delta his$ -*mazE* and pBAD-*mazF*, was grown in M9 medium at 37°C to mid-logarithmic phase as described above. The culture was diluted 1:10 in the same medium, and *mazF* expression was induced by the addition of arabinose (0.2%). The induced culture was incubated at 37°C without shaking. At various time points, samples were withdrawn and split into two portions. To one, only 0.2% glucose was added, and to the other, both 0.2% glucose and 2 mM IPTG were added. The samples were further incubated at 37°C. At various times before and after the addition of glucose, we determined the levels of protein synthesis, viability (CFU), and OD<sub>600</sub>. To determine CFU, samples were spread on LB-ampicillin-chloramphenicol plates containing 0.2% glucose. Protein synthesis was determined by measuring the incorporation of [<sup>35</sup>S]methionine (10  $\mu$ Ci/ml) into a trichloroacetic acid (TCA)-insoluble fraction (final concentration of 5%). The TCA solutions contained 50  $\mu$ g of nonradioactive methionine per ml. The precipitates were incubated on ice for 30 min, filtered on Schleicher & Schuell (Dassel, Germany) filters, and washed with TCA (5%). The filters were allowed to dry, and the TCA-insoluble counts per minute were determined by using a scintillation counter (BETAmatic I/II; Kontron). The amount of radioactivity per OD<sub>600</sub> unit was determined. For any given time point, the relative percentage of protein synthesis was calculated compared to that at time zero.

## RESULTS

**Overproduced MazE did not resuscitate *E. coli* cells that had overproduced MazF over a prolonged period.** Here we carried out an experiment similar to the one described previously by Pedersen et al. (23). We used *E. coli* strain MC4100  $\Delta mazEF relA1 lacI^q$  cotransformed with plasmids pQE- $\Delta his$ -*mazE*, regulated by the T5 promoter and the *lac* operator, and pBAD-*mazF*, regulated by the *araBAD* promoter. The cells were grown in LB medium to mid-logarithmic phase. *mazF*

expression was then induced by the addition of arabinose. At various times after *mazF* induction, samples were withdrawn and spread on LB plates containing either only glucose (to repress *mazF* expression) or glucose and IPTG (to induce *mazE* expression).

Our results confirmed those of Pedersen et al. (23) and showed that the overproduction of MazE could resuscitate *E. coli* cells overproducing MazF during a period of 6 h (Fig. 1A). We found, however, that extending the period of *mazF* induction led to a dramatic decrease in the ability of MazE to resuscitate the cells. By 8 h after the induction of *mazF* expression, only about 20% of the cells survived, and by 24 h, survival was reduced to only 8% (Fig. 1A).

Unlike Pedersen et al. (23), we also examined the ability of MazE to reverse the toxic effect of MazF by growing the transformed cells in M9 medium rather than LB medium. In M9 medium, the ability of MazE to reverse the effect of MazF on cell survival was reduced even more significantly. As soon as 2 h after the induction of ectopic MazF overexpression, we observed only about 50% survival (Fig. 1B). By 6 h, survival was reduced to 10%, and by 24 h after *mazF* induction, survival was only 0.3% (Fig. 1B).

**There is a state in which MazE overproduction can reverse the inhibitory effect of MazF on translation but not its effect on colony formation.** Here, we again based our studies on the protocol used by Pedersen et al. (23), in which they studied the ability of MazE to reverse the effects of MazF on translation. However, we considered two new aspects. First, instead of studying the effect of MazE only after one specific period of MazF overproduction (3 h) (23), we explored the effect of MazE on cells in which MazF was previously overproduced for various periods (1 to 6 h). Second, we studied not only the ability of MazE to reverse the inhibitory effect of MazF on translation but also its ability to resuscitate cells from the same samples. We chose not to study the effect of MazF overproduction on replication, because the results of recent work by Christensen et al. (6) revealed that the effect of MazF on replication is secondary to its effect on translation.

As in our experiments described above, we used *E. coli* strain MC4100  $\Delta mazEF relA1 lacI^q$  cotransformed with plasmids pQE- $\Delta his$ -*mazE* and pBAD-*mazF*. The cells were grown in M9 minimal medium to mid-logarithmic phase, at which time the cultures were diluted in the same medium and 0.2% arabinose was added to induce *mazF* expression. At 1, 4, and 6 h after the induction of *mazF* expression, samples were withdrawn and split into two portions. To one portion, only glucose was added (to repress *mazF* expression), and to the other portion, glucose and IPTG were added (to induce *mazE* expression). The samples were further incubated, and the abilities to synthesize proteins (Fig. 2Aa to Ac) and to form colonies (Fig. 2Ba to Bc) were determined 1 and 3 h later (for details, see also Materials and Methods).

We found that at every point from 1 to 6 hours after the induction of the overexpression of MazF, in the absence of MazE, the rate of translation was drastically reduced. During this period, the rate of translation was only 5 to 16% of that of untreated cells (Fig. 2A). This inhibitory effect of MazF on translation was completely reversed by overproduction of MazE. As soon as 1 h after the induction of MazE expression, the rate of translation was restored to 100% regardless of the

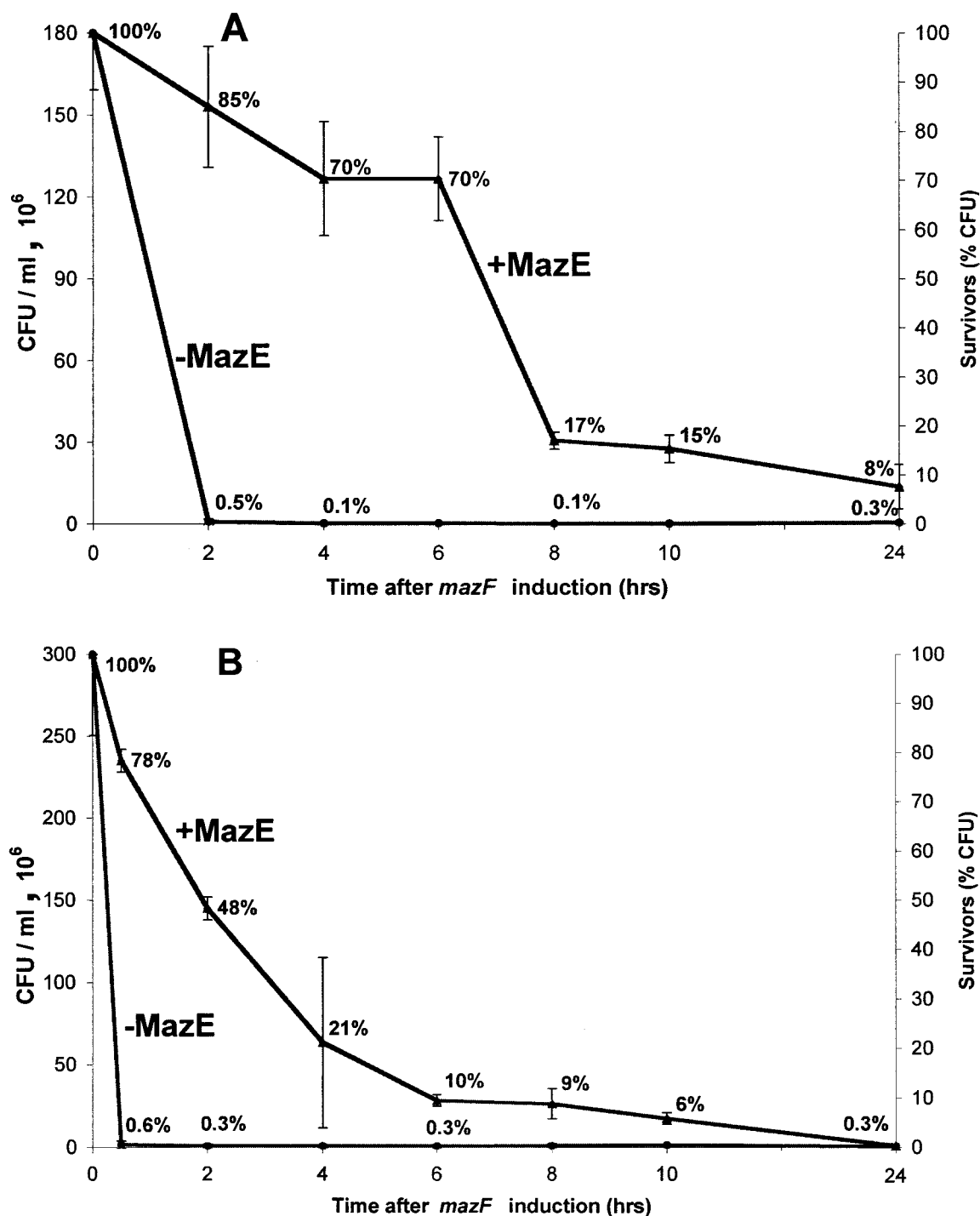


FIG. 1. Ability of *E. coli* cells that had been ectopically overexpressing MazF in liquid medium to form colonies when ectopically overexpressing MazE on plates. *E. coli* strain MC4100  $\Delta mazEF relA1 lacI^S$  was cotransformed with pQE- $\Delta his-mazE$ , carrying *mazE* (without a His tag), and pBAD-*mazF*, carrying *mazF*. The cultures were grown in LB medium (A) or M9 minimal medium with 0.5% glycerol (B) at 37°C to mid-logarithmic phase (OD<sub>600</sub>, 0.5). At time zero, 0.2% arabinose was added to the cultures to induce *mazF* expression. To determine CFU, samples were withdrawn at various time points and spread on LB plates containing 100 µg of ampicillin per ml, 50 µg of chloramphenicol per ml, and either 0.2% glucose (to represses *mazF* expression) or 0.2% glucose and 2 mM IPTG (to induce *mazE* expression). Induced culture spread on plates with IPTG, ▲; induced culture spread on plates without IPTG, ●. The percentage of survivors (indicated next to the plotted measurements) was calculated by comparing the CFU of the *mazF*-induced culture to that of the uninduced culture at time zero. Error bars indicate standard deviations.

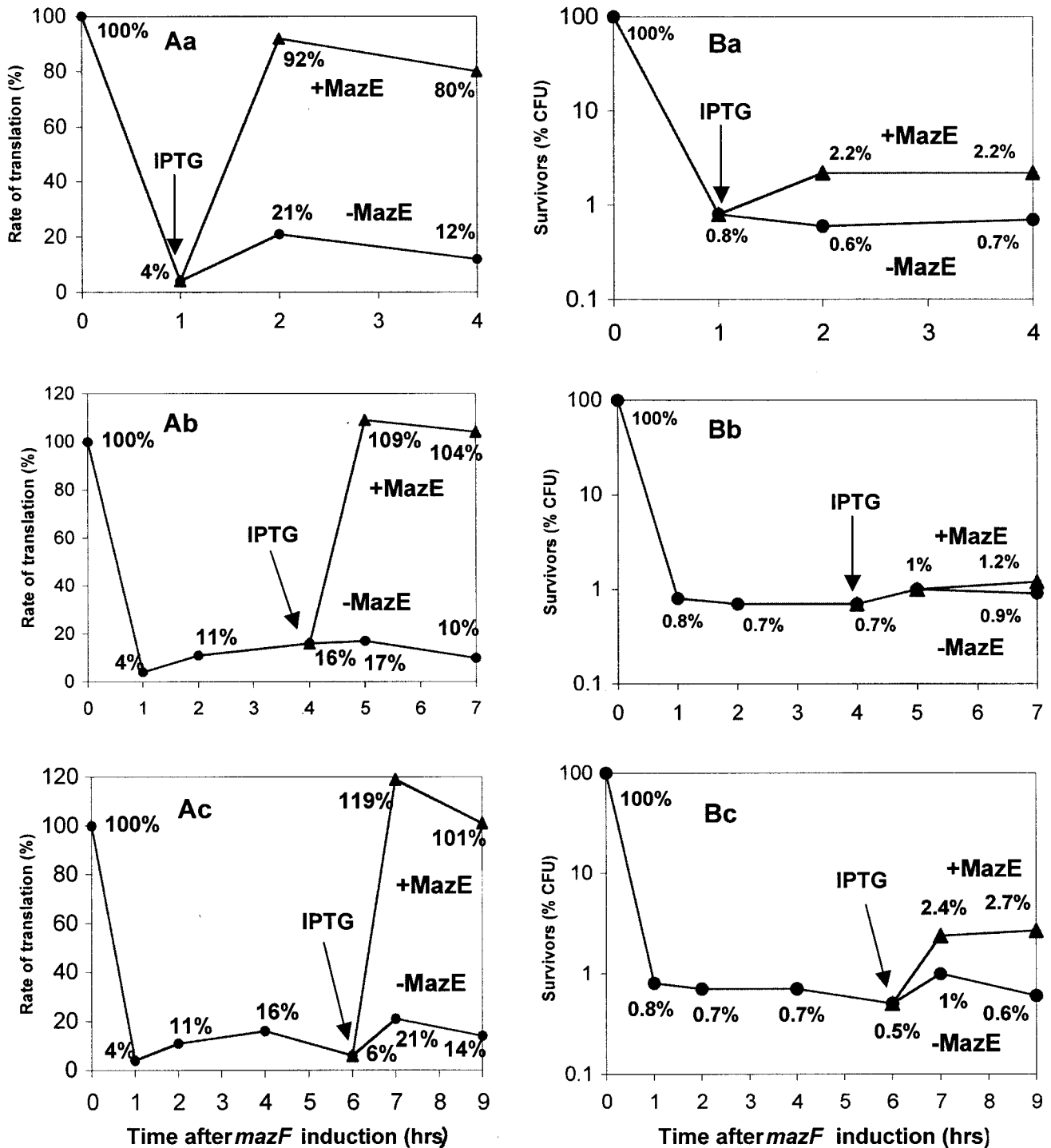


FIG. 2. Effect of MazE overproduction during growth in liquid medium on the ability of MazF-overproducing *E. coli* cells to synthesize proteins and to form colonies. *E. coli* strain MC4100  $\Delta mazEF$  *relA1* *lacI*<sup>q</sup> was cotransformed as described in the legend to Fig. 1. The cotransformed cells were grown, induced, labeled, and plated as described in Materials and Methods. Aa to Ac, Rate of translation. Ba to Bc, Percentage of survivors. To induce *mazE* expression, IPTG was added to the bacterial culture at 1 h (Aa and Ba), 4 h (Ab and Bb), and 6 h (Ac and Bc) after *mazF* induction at time zero. The effects of the ectopic overexpression of MazE were measured at 1 and 3 h after the induction of *mazE* expression. *mazE*-induced culture,  $\blacktriangle$ ; uninduced culture,  $\bullet$ . The relative percentage of protein synthesis and viability (indicated next to the plotted measurements) was calculated compared to that at time zero. The values shown are from one out of three similar experiments.

amount of time after the induction of MazF overproduction (Fig. 2Aa to Ac). However, in spite of the ability of MazE to reverse the inhibitory effect of MazF on translation, it could not reverse the bacteriocidal effect of MazF (Fig. 2Ba to Bc).

## DISCUSSION

Pedersen et al. (23) have previously reported that the artificial overproduction of the toxin MazF (ChpAK) does not confer cell death. Instead, they suggest that this toxin induces bacteriostasis that can be fully reversed by overexpression of the antitoxin MazE (ChpAI). Here, we have confirmed their results by carrying out similar experiments with a similar ectopic overexpression system for MazF and MazE. We found that during the first 6 h of MazF overproduction in liquid LB medium, there is indeed almost a complete reversion of cell survival by a subsequent overproduction of MazE during overnight incubation on LB plates (Fig. 1A). However, when we extended the period of MazF overproduction to 8 h, the ability of MazE to resuscitate *E. coli* cells was drastically decreased. Furthermore, when MazF was overproduced in cells growing in the minimal M9 medium, the inability of MazE to reverse the bacteriocidal effect of MazF was even more dramatic (Fig. 1B). Thus, our results indicate that there is a point of no return and that in the minimal M9 medium this point occurs sooner than it does in the rich LB medium (compare Fig. 1A and B). By the time that the cells reach this point of no return, they have undergone so much MazF-induced damage that their death is unavoidable and they can no longer be resuscitated by the action of MazE. Since in M9 and LB media the abilities of MazE to revive cells damaged by MazF were different, we understand that this point of no return is variable and is a function of growth conditions.

Pedersen et al. (23) also reported that ectopic overexpression of the toxin MazF inhibits translation, which can be resumed if the antitoxin MazE is expressed at a later time. Carrying out similar experiments, we confirmed their results. Here we even extended the period of MazF expression to 6 h. During this period, the inhibitory effect of MazF on translation was completely reversed after as little as 1 h of MazE overexpression (Fig. 2Aa to Ac). Although these cells were able to translate proteins, they were hardly able to form colonies (Fig. 2Ba to Bc). Thus, under these experimental conditions, the cells were in a state in which MazE overproduction could completely reverse the inhibitory effect of MazF on translation but could not reverse the effect of MazF on colony formation. In other words, the bacteriocidal effect of MazF and the effect of MazF on translation seem to be two separate functions that could be dissociated by the ectopic overexpression of MazE.

Our results reported here indicate on the one hand that in the bacteriocidal effect of MazF there is a point of no return (Fig. 1) and on the other hand that the effect of MazF on translation and its bacteriocidal effect (Fig. 2) may be separate functions. These results support our conclusions reported in our previous publications (8, 14, 15, 24, 25), in which we have suggested that *mazEF* is part of a programmed cell death network. In such a network, MazF would be seen as a mediator rather than as an executor. Other researchers have suggested that the inhibition of protein synthesis by MazF is due to its endoribonucleolytic effect on mRNAs (6, 22, 26) and tmRNA

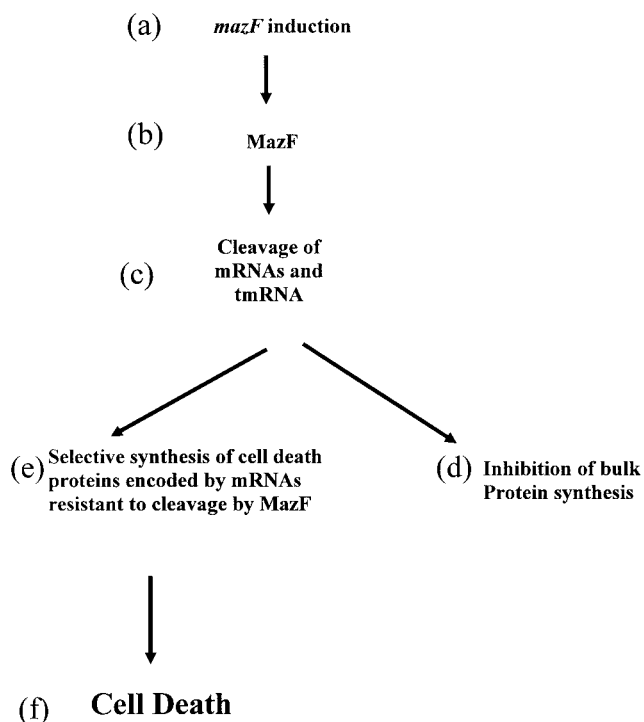


FIG. 3. A model for the *mazF*-induced death pathway. For further details, see Discussion.

(16). Based on our results reported here, we have built a model (Fig. 3) in which this action of MazF (Fig. 3, step c) would be one of the initial steps in the programmed cell death pathway. In our model, this initial step can still be reversed by the antagonistic effect of MazE over 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 (16). However, we suggest that MazE cannot reverse the downstream cascade already initiated by MazF (Fig. 3, steps e and f); thus, if the process is not stopped in time, eventually cell death would be unavoidable. How might the inhibition of translation by MazF induce such a downstream cascade leading to cell death? This might be caused by the unique mechanism through which MazF cleaves mRNAs at specific sites (6, 22, 26). In this way, the action of MazF could lead to the selective synthesis of proteins encoded by mRNAs that are resistant to the cleavage by MazF (Fig. 3, step e). We believe that these proteins may be a part of an elaborate pathway to cell death. The described model (Fig. 3) may be unique to the *mazEF* system; however, other *E. coli* chromosomal toxin-antitoxin systems may act in a similar way.

Based on their experimental results, Pedersen et al. (23) concluded that the toxin MazF does not mediate cell death but only induces bacteriostasis that can be reversed by the antitoxin MazE. Our results reported here contradict their conclusion. We suggest that although intermediary stages in the *mazF*-mediated cascade may be reversible, the process as a whole leads to cell death.

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