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Postsegregational Killing Mediated by the P1 Phage "Addiction Module" *phd-doc* Requires the *Escherichia coli* Programmed Cell Death System *mazEF*

RONEN HAZAN, BOAZ SAT, MYRIAM RECHES, AND HANNA ENGELBERG-KULKA*

Department of Molecular Biology, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

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"Addiction modules" consist of two genes; the product of the second is long lived and toxic, while the product of the first is short lived and antagonizes the lethal action of the toxin. The extrachromosomal addiction module *phd-doc*, located on the P1 prophage, is responsible for the postsegregational killing effect (death of plasmid-free cells). The *Escherichia coli* chromosomal addiction module analogue, *mazEF*, is responsible for the induction of programmed cell death. Here we show that the postsegregational killing mediated by the P1 *phd-doc* module depends on the presence of the *E. coli mazEF* system. In addition, we demonstrate that under conditions of postsegregational killing, mediated by *phd-doc*, protein synthesis of *E. coli* is inhibited. Based on our findings, we suggest the existence of a coupling between the *phd-doc* and *mazEF* systems.

In *Escherichia coli* cultures, programmed cell death is mediated through "addiction modules" consisting of two genes; the product of the second gene is long lived and toxic, whereas the product of the first is short lived and antagonizes the lethal action of the toxin. Until recently, such genetic systems of bacterial programmed cell death have been found mainly in a number of *E. coli* extrachromosomal elements (for reviews, see references 5, 7, 8, and 15), where they are responsible for what is called the postsegregational killing effect; they are responsible for the death of plasmid-free cells. When bacteria lose the extrachromosomal elements, the cured cells are selectively killed because the unstable antitoxin is degraded faster than is the more stable toxin. One of the best-studied systems belonging to this category is the *phd-doc* module of plasmid prophage P1 (9, 10). This module consists of an operon the organization of which is similar to that of the operons of other addiction modules: the antitoxic gene, *phd* (prevents host death), precedes the toxic gene, *doc* (death on curing). Doc acts as a cell toxin to which the short-lived Phd protein is an antidote. Phd is degraded by the serine protease ClpPX (10). Like that of other previously described addiction modules, the expression of *phd-doc* is also subjected to an autoregulatory circuit (11, 12). The cellular target of Doc is not yet known. However, based on recent studies it is assumed to be a step in protein synthesis (7; M. Yarmolinsky, personal communication).

Members of our group have reported on the *E. coli mazEF* system, the first known regulatable prokaryotic chromosomal addiction module (1). It consists of two genes, *mazE* and *mazF*, located downstream from the *relA* gene in the *rel* operon (13). Through our work, we have found that *mazEF* has all the properties required for an addiction module. MazF is toxic and long lived, while MazE is antitoxic and short lived and is degraded by the ClpPA serine protease. MazE and MazF are coexpressed, and they interact. In addition, the *mazEF* system has a unique property: its expression is regulated by guanosine-3′5′-bispyrophosphate (ppGpp), which is synthesized by the RelA protein under conditions of amino acid starvation (3). Moreover, overproduction of ppGpp induces *mazEF*-mediated cell death (1, 6). These properties suggest that the *mazEF* addiction module may be responsible for programmed cell death in starving cultures of *E. coli* (1). In an accompanying report (14a), we are showing that cell death mediated by the *E. coli mazEF* addiction module can also be triggered by several antibiotics that are general inhibitors of transcription and/or translation. These antibiotics inhibit the continuous expression of the labile antitoxin MazE, and as a result, the stable toxin MazF causes cell death. This finding, together with the observation that the toxic protein Doc of the P1 *phd-doc* system inhibits protein synthesis (7; M. B. Yarmolinsky, personal communication), prompted us to ask whether the P1 *phd-doc* system exerts its postsegregational killing effect through the chromosomal *E. coli mazEF* system.

Here we show that the postsegregational killing effect mediated by the P1 *phd-doc* addiction module does depend on the presence of the *E. coli* chromosomal addiction module *mazEF*. In addition, we found that under conditions of P1 *phd-doc* postsegregational killing, protein synthesis of *E. coli* is inhibited. Therefore we suggest that the toxic protein Doc triggers cell death through the *mazEF* system by the inhibition of *E. coli* protein synthesis.

MATERIALS AND METHODS

***E. coli* strains and plasmids.** The *E. coli* strains used in this study included MC4100 (genotype, *araD139* Δ(*argF-lac*)*205 flb-5301 pstF25 rpsL150 deoC1 relA1*) (2) and its derivatives, MC4100 *mazEF::kan* (Δ*mazEF*) (1). The plasmid pGB2ts::*phd-doc* (9) is a pGB2 derivative, thermosensitive for replication, carrying *phd-doc* genes and spectinomycin resistance genes (4). Plasmid pKK223*mazEF* was constructed by inserting the open reading frame of *mazEF* into the *Bam*HI and *Hin*dIII sites of the overexpression plasmid pKK233-3 (Amersham Pharmacia Biotech).

* Corresponding author. Mailing address: Department of Molecular Biology, The Hebrew University-Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel. Phone: 972-2-675-8250. Fax: 972-2-678-4010. E-mail: hanita@cc.huji.ac.il.

Materials and media. [^{35}S]methionine (>800 Ci/mmol [1 Ci = 37 GBq]) was obtained from Amersham (Little Chalfont, England). Bacteria were grown in M9 medium (14) with a mixture of amino acids (20 μg/ml each) or in Luria-Bertani medium (LB) (14).

Conditions for the loss of plasmids pGB2ts and pGB2ts::*phd-doc* from the cells. We used plasmid pGB2ts or pGB2ts::*phd-doc* to transform *E. coli* strain MC4100 and its Δ*mazEF* derivative. Transformants were selected on LB agar plates supplemented with spectinomycin (100 μg/ml) at 30°C. The postsegregational killing effect of the *phd-doc* addiction module was studied by growing the bacteria on LB agar plates, at 30 or 42°C or in liquid media. In liquid media, the cells were grown in LB medium or M9 medium at 30°C overnight. Plasmid loss was achieved by growing the cultures for at least eight generations at 42°C. For this purpose we grew the cultures from an optical density at 600 nm (OD_{600}) of 0.07 to 0.7 at 42°C and diluted them 1:10; this procedure was repeated at least twice. Plasmid loss was confirmed by plating the cultures on spectinomycin plates.

Assay for protein synthesis under conditions of the loss of plasmids pGB2ts and pGB2ts::*phd-doc*. We measured the incorporation of [^{35}S]methionine into a trichloroacetic acid (TCA)-insoluble fraction. In these experiments we used *E. coli* strain MC4100 and its Δ*mazEF* derivative carrying plasmid pGB2ts::*phd-doc* or pGB2t as a control. We grew the tested *E. coli* cultures in LB liquid medium under conditions of plasmid loss as described in the previous section. The cells were washed, resuspended in M9 medium without methionine, and grown at 42°C for 1 h. The culture was labeled with 0.2 μCi/ml in [^{35}S]methionine in a final concentration of 2 μg of unlabeled methionine/ml. At various time intervals, the reactions were stopped by the addition of TCA to a final concentration of 10%, after which the reaction tubes were put in ice. The samples were centrifuged at 14,000 rpm for 5 min in Eppendorf centrifuge 5417C. The pellets were washed twice with 5% TCA and then twice with acetone. The TCA-insoluble counts were determined by using a scintillation counter (BETAmatic I/II; KONTRON).

RESULTS

P1 *phd-doc* postsegregational killing is triggered by the loss of pGB2ts::*phd-doc*. We used plasmid pGB2ts::*phd-doc* bearing the P1 *phd-doc* addiction module and pGB2ts as a control. Both plasmids are temperature sensitive for replication and carry a gene for spectinomycin resistance (9). As previously reported for *E. coli* MC4100 cells (10, 6) and confirmed here also for the Δ*mazEF* derivative, pGB2ts and pGB2ts::*phd-doc* are retained at a low temperature (30°C) but are lost when the cells are grown at a high temperature (42°C) (Fig. 1A). The presence of either plasmid in the cells was tested by plating the bacteria on LB plates supplemented with spectinomycin (Fig. 1A). Without spectinomycin the pattern of bacterial growth in LB liquid medium at 42°C (conditions of plasmid loss) depends upon the presence of *phd-doc* on the plasmid. This is shown in Fig. 1B, where the growth of *E. coli* MC4100 and its Δ*mazEF* derivative harboring the plasmid pGB2ts::*phd-doc* is inhibited relative to that of cells harboring pGB2ts. The growth of the latter at 42°C is similar in MC4100 and its Δ*mazEF* derivative (data not shown). It should be emphasized that according to our experiments, in order to get conditions for plasmid loss the cells should grow at 42°C for at least eight generations. This was achieved by growing the cultures from an OD_{600} of 0.07 to 0.7 followed by a 1:10 dilution; this procedure was repeated twice. Figure 1B shows the growth curves obtained after the second dilution.

P1 *phd-doc* postsegregational killing is dependent on *E. coli mazEF*. We determined quantitatively the postsegregational killing mediated by the loss of pGB2ts::*phd-doc* from cells. This was done by the comparison of the number of the surviving cells grown at 42°C to those grown at 30°C in LB medium without spectinomycin. In the case of the wild-type strain *E. coli* MC4100, only 5% of the population escaped the post-

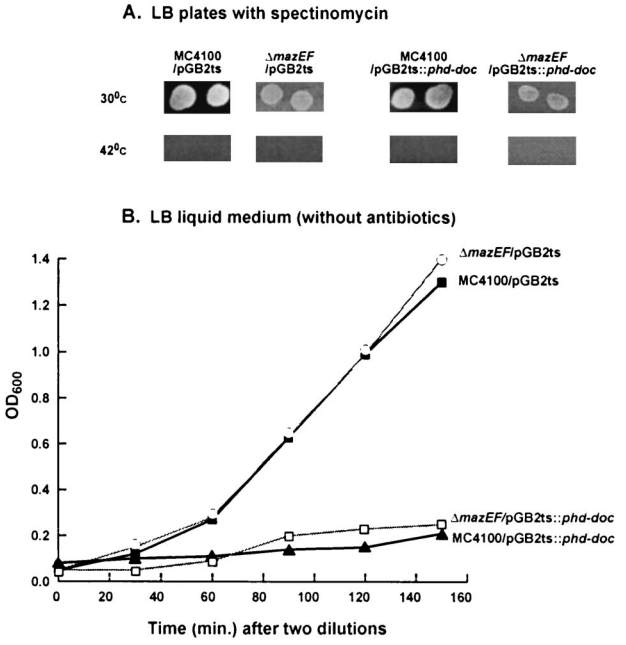

FIG. 1. The effect of the loss of the plasmids pGB2ts and pGB2ts::*phd-doc* on the growth of MC4100 and its Δ*mazEF* derivative. (A) Growth on LB plates with spectinomycin. *E. coli* MC4100 (wild type) and its Δ*mazE* derivative, both harboring either plasmid pGB2ts or pGB2ts::*phd-doc*, were plated on LB plates with 100 μg of spectinomycin/ml. The plates were incubated at 30 or at 42°C overnight. (B) Bacterial growth curves at 42°C in liquid LB medium without any added antibiotic. The bacteria were grown to an OD_{600} of 0.7 and then diluted to an OD_{600} of 0.07. This step was repeated twice in order to cure the cells from the plasmids. The curves represent the values after the second dilution. Results for MC4100 are illustrated by black lines, and those for its Δ*mazEF* derivative are illustrated by gray lines. Results with MC4100/pGB2ts (■), MC4100/pGB2ts::*phd-doc* (▲), Δ*mazEF*/pGB2ts (○), and Δ*mazEF*/pGB2ts::*phd-doc* (□) are shown.

segregational killing effect (Fig. 2). As a control we used plasmid pGB2ts, which was also lost at 42°C (Fig. 1A), but cell growth was not inhibited (Fig. 1B) and the cells did not die (Fig. 2). We also studied the postsegregational killing effect mediated by the loss of plasmid pGB2ts::*phd-doc* on the Δ*mazEF* strain. As in the case of the wild-type strain, incubation at 42°C caused the loss of plasmids pGB2ts and pGB2ts::*phd-doc* from Δ*mazEF* cells (Fig. 1A), and the growth of Δ*mazEF*/pGB2ts::*phd-doc* cells was inhibited (Fig. 1B). However, in contrast to results with the wild-type strain MC4100, most of the Δ*mazEF* derivative cells (90%) survived the loss of the plasmid (Fig. 2). We also tested pGB2ts::*phd-doc* postsegregational killing in Δ*mazEF* cells in which the *mazEF* gene module was not present on the chromosome but was borne on the plasmid pKK223*mazEF*. In this case, we observed that postsegregational killing did take place and only 8% of the cells survived plasmid loss (Fig. 2). Thus, we found that the postsegregational killing effect of the *phd-doc* addiction module required the presence of the *mazEF* system.

***E. coli* protein synthesis is inhibited under conditions of pGB2ts::*phd-doc* plasmid loss.** Note that although the Δ*mazEF* derivative cells survive the loss of plasmid pGB2ts::*phd-doc* (Fig. 2), their growth is inhibited in liquid LB medium as is that of the wild-type cells (Fig. 1B). Thus, the loss of plasmid

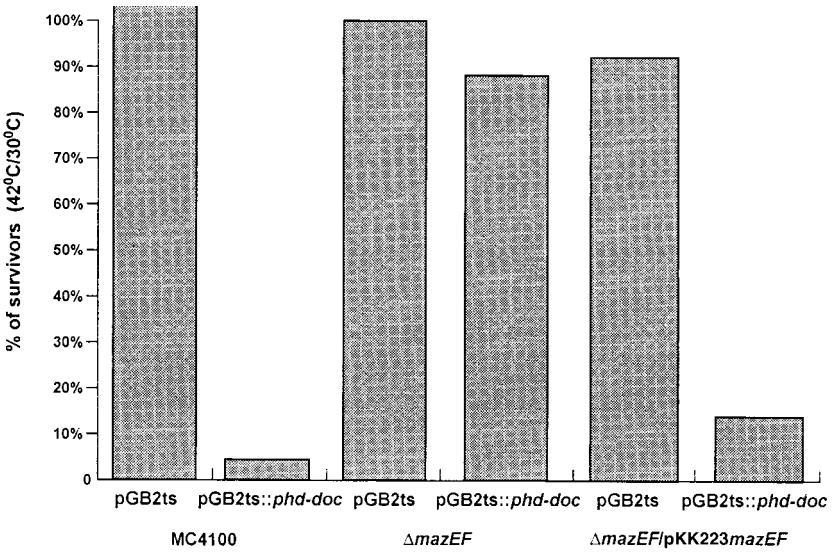

FIG. 2. The effect of the *E. coli mazEF* system on the postsegregational killing mediated by the P1 *phd-doc* addiction module. Plasmids pGB2ts and pGB2ts::*phd-doc* (Spt^r) were used to transform *E. coli* MC4100 (wild type) and its Δ*mazEF* derivative. The transformant MC4100Δ*mazEF*/pGB2ts::*phd-doc* was further transformed by plasmid pKK223*mazEF* (Amp^r). Transformants were grown in LB liquid medium at 30°C to mid-logarithmic phase and were plated on LB plates without antibiotics at 30 and 42°C. The percentage of cell survivors was calculated by comparing the numbers of CFU at 42°C versus 30°C.

pGB2ts::*phd-doc* is bactericidal for wild-type MC4100 cells but only bacteriostatic for the Δ*mazEF* derivative. This finding is similar to the effect of the translational inhibitor chloramphenicol which we show in the accompanying report (14a). There we found that in M9 minimal medium, chloramphenicol is bactericidal for the wild-type strain MC4100 but only bacteriostatic for the Δ*mazEF* derivative. Having considered our results described above, along with the observation that Doc inhibits protein synthesis (Yarmolinsky, personal communication) (reviewed in reference 7), we decided to study the effects of the loss of plasmid pGB2ts::*phd-doc* on the level of protein synthesis. We compared the level of protein synthesis in MC4100 and in its Δ*mazEF* derivative carrying pGB2ts::*phd-doc* or to the same cells carrying pGB2ts as a control. We determined the level of protein synthesis by measuring the incorporation of [^{35}S]methionine into a TCA-insoluble fraction. At 42°C, protein synthesis was drastically inhibited in both MC4100 and its Δ*mazEF* derivative carrying pGB2ts::*phd-doc* relative to that in cells carrying pGB2ts (Fig. 3). Thus, under conditions of plasmid loss (Fig. 1A), protein synthesis (Fig. 3) and bacterial growth (Fig. 1B) are inhibited.

DISCUSSION

Addiction modules of extrachromosomal element (including P1 *phd-doc*) and their postsegregational killing effect have been thoroughly studied (reviewed in references 5, 7, 8, and 15). Here we found that P1 postsegregational killing is mediated by the *E. coli* chromosomal programmed cell death system *mazEF* (Fig. 2). Moreover, we show that while in *E. coli* MC4100 the loss of the pGB2ts::*phd-doc* plasmid is bactericidal, in its Δ*mazEF* derivative it is only bacteriostatic (Fig. 2). More specifically, under conditions of plasmid loss, protein synthesis (Fig. 3) and cell growth (Fig. 1B) were inhibited both in wild-type MC4100 cells and in Δ*mazEF* derivative cells; however, while the wild-type cells died, the Δ*mazEF* cells survived (Fig. 2). Furthermore, when we reintroduced the *mazEF* system on a plasmid to the Δ*mazEF* cells, the loss of the pGB2ts::*phd-doc* plasmid was again bactericidal (Fig. 2). In addition, we demonstrate that *E. coli* protein synthesis is inhibited under the conditions of postsegregational killing mediated by P1 *phd-doc* (Fig. 3).

Based on our results, we suggest a model in which *mazEF* is required for the postsegregational killing process mediated by P1 *phd-doc* (Fig. 4). The extrachromosomal module *phd-doc* and the chromosomal module *mazEF* function analogously: in both systems, the second gene specifies for a toxic stable pro-

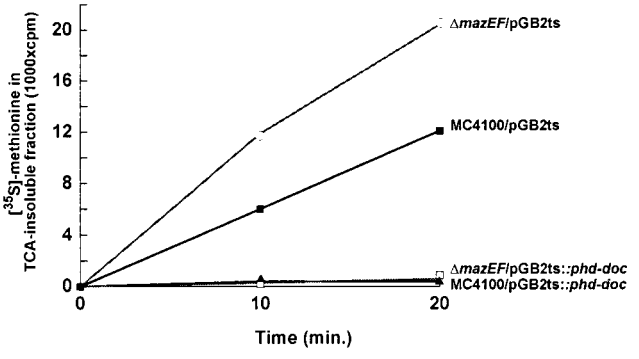

FIG. 3. The effect of the P1 *phd-doc* system on protein synthesis. Plasmid pGB2ts::*phd-doc* or pGB2ts (as a control) were used to transform *E. coli* MC4100 (wild type) and the MC4100 Δ*mazEF* derivative. Plasmid curing was carried out in LB liquid medium as described in the legend to Fig. 1. The rate of protein synthesis was determined as described in Materials and Methods. Results for MC4100 are illustrated by black lines, and those for its Δ*mazE* derivative are shown by gray lines. Results with MC4100/pGB2ts (■), MC4100/pGB2ts::*phd-doc* (▲), Δ*mazEF*/pGB2ts (❍), and Δ*mazEF*/pGB2ts::*phd-doc* (❑) are shown.

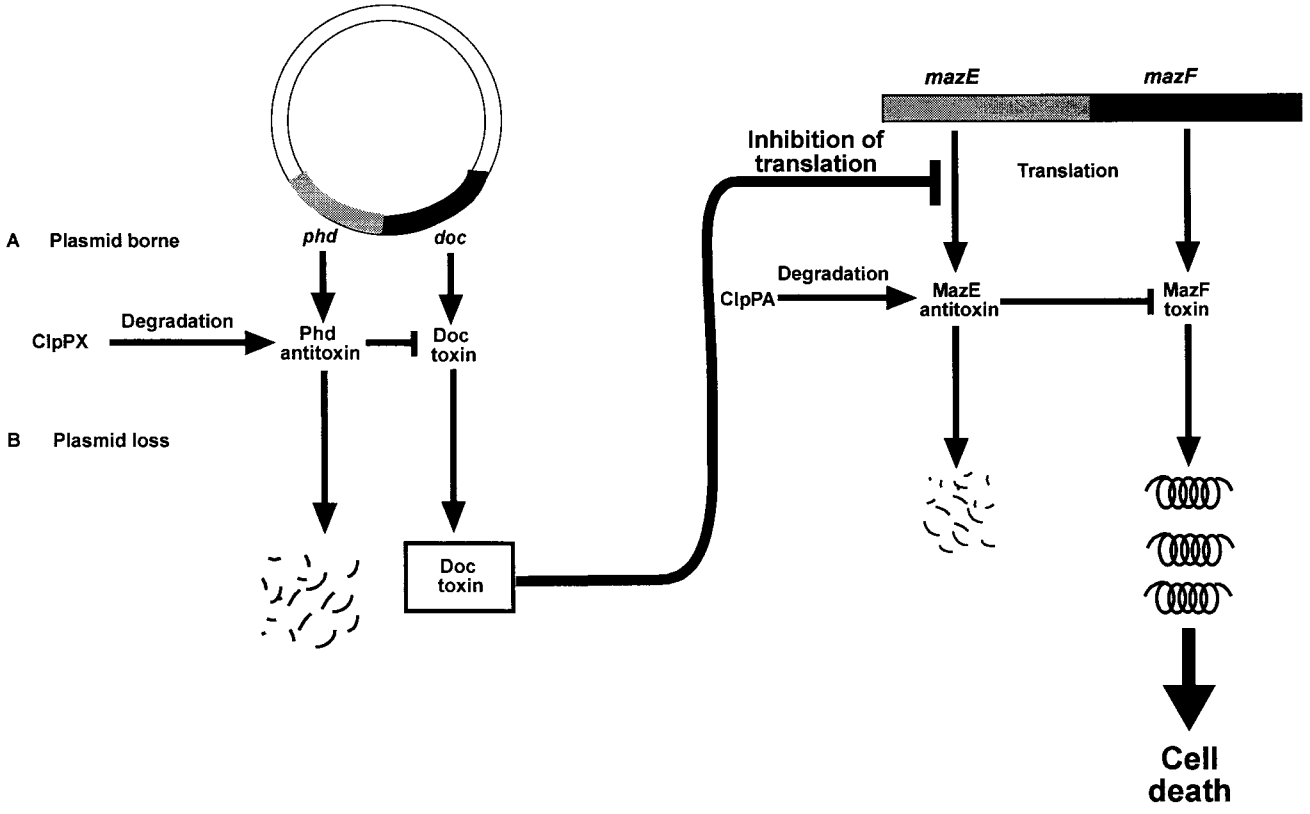

FIG 4. A schematic representation for the coupling of the extrachromosomal P1 *phd-doc* system and the chromosomal *mazEF* addiction module. (A) In the presence of plasmid-borne *phd-doc*. When the plasmid-borne *phd-doc* system is expressed, there is a balance between the expression of the antitoxic Phd and its degradation by ClpX. This balance permits the neutralization of the toxic protein Doc by Phd. (B) When the plasmid is lost. Under conditions of plasmid loss, the P1 *phd-doc* system mediates postsegregational killing. Under these conditions, the level of the labile antitoxin Phd decreases below the threshold required for neutralizing Doc. Doc inhibits the translational machinery and thereby triggers programmed cell death mediated by the *E. coli mazEF* system. When protein translation is inhibited, the continuous expression of the labile antitoxic MazE protein is prevented. As a result, the stable toxin MazF causes cell death (for further details, see the text).

tein and the first gene specifies for an antitoxic labile protein. Each of these systems is triggered when the continuous expression of its labile antitoxic component is inhibited. In the case of the extrachromosomal *phd-doc* system, such a triggering occurs by the loss of the extrachromosomal element (9, 10). On the other hand, in the case of the chromosomal *mazEF* system, we have previously shown that the inhibition of the antitoxin expression can be triggered at least in two ways: (i) inhibition of transcription by ppGpp (1, 6) or by rifampin (14a) and (ii) inhibition of translation by chloramphenicol or spectinomycin (14a). Here we suggest an additional possible trigger for *mazEF*-mediated programmed cell death: through its action as a translational inhibitor, the toxic protein Doc can trigger the *mazEF* system. In this respect, Doc can be considered an analogue to the antibiotics chlorampenicol and spectinomycin. Moreover, Doc seems to trigger the *mazEF* system even more efficiently than do the drugs chloramphenicol and spectinomycin. In an accompanying report (14a), we reported that these antibiotics trigger the *mazEF* system only in a minimal medium, and they were not able to do it in LB medium. In contrast, the bactericidal effect of the postsegregational killing mediated by Doc is manifested both in LB (10) (Fig. 2) and in M9 (data not shown), and in both cases it requires the *E. coli mazEF* system.

In summary, our results described here suggest that at least in the case of *phd-doc*, postsegregational killing is not a one-step process manifested by Doc. It seems that instead a "death cascade" is involved. Thus, our results suggest that by itself Doc is not a toxin; rather, it triggers the *E. coli mazEF* system in this cascade. The question of whether the toxic MazF is responsible by itself for the death or is also an intermediate in the death cascade is under investigation.

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