Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6059–6063, June 1996
Genetics

An *Escherichia coli* chromosomal "addiction module" regulated by 3′,5′-bispyrophosphate: A model for programmed bacterial cell death

(stringent response/protein degradation/nutritional stress/programmed cell death)

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Communicated by Carol A. Gross, University of California, San Francisco, CA, February 9, 1996 (received for review July 13, 1995)

ABSTRACT **"Addiction modules" consist of two genes. In most of them the product of one is long lived and toxic while the product of the second is short lived and antagonizes the toxic effect; so far, they have been described mainly in a number of prokaryotic extrachromosomal elements responsible for the postsegregational killing effect. Here we show that the chromosomal genes *mazE* and *mazF*, located in the *Escherichia coli rel* operon, have all of the properties required for an addiction module. Furthermore, the expression of *mazEF* is regulated by the cellular level of 3′,5′-bispyrophosphate, the product of the RelA protein under amino acid starvation. These properties suggest that the *mazEF* system may be responsible for programmed cell death in *E. coli* and thus may have a role in the physiology of starvation.**

The synthesis of 3′,5′-bispyrophosphate (ppGpp) in *Escherichia coli* is governed by at least two pathways. The first is by the activation of the synthesis of ppGpp during the stringent response to amino acid deprivation. The enzyme responsible for this pathway is encoded by the *relA* gene; it is activated by uncharged tRNA and thereby by limitation of amino acid availability or by inhibition of amino acylation (1). The second pathway for ppGpp synthesis is activated by carbon source limitation. This pathway is active also in a *relA* null mutant (1) and therefore it is independent on the *relA* gene. Cloning of the *relA* open reading frame (ORF) or part of it under the inducible P_{tac} promoter revealed a nutritional independent correlation between changes in the cellular levels of ppGpp and the pattern of gene expression (2). The latter includes inhibition of rRNA accumulation, slowing of growth rate, inhibition of DNA replication, and changes in the pattern of gene expression as observed by two-dimensional electrophoresis of cellular proteins (2, 3).

It was shown that the *E. coli relA* gene is part of an operon in which a pair of genes called *mazE* and *mazF* are located downstream from the *relA* gene (4). Sequence analysis revealed that they are partly homologous to the protein products of the genes *pemI* and *pemK* "addiction module" carried on plasmid pR100 (5). The *rel* operon *mazEF* pair of genes was also called *chpA* (5). These genes encode for 9.4- and 12.1-kDa proteins, respectively (4, 5).

Until now, addiction modules have been described as functioning mainly in a number of *E. coli* extrachromosomal elements (6–16). Among these the most studied are the *phd-doc* of bacteriophage P1 (10), the *ccdA-ccdB* of factor F (13), and the *pemI-pemK* of plasmid R100 (15). In all of these cases, an addiction module consists of two genes: the product of one is long lived and toxic, while the product of the second is short lived and antagonizes the toxic effect. The cells are "addicted" to the short lived polypeptide, since the *de novo* synthesis of the short lived polypeptide is essential for cell survival. When the bacteria lose the extrachromosomal element, the degradation of the antitoxic protein accounts for the selective killing of the cured cells. A different group of "addiction modules" is represented by the *hok-sok* system. The products of the *sok* gene of plasmid R1 and of its homologs in other plasmids are labile antisense RNA, subject to rapid degradation by nucleases (16). Two sets of chromosomal homologs of the plasmid addiction modules *hok-sok* (17) and of *pemI-pemK* (5) have been described. The latter is the *rel* operon *mazEF* (4), also called *chpA* (5). In addition, another chromosomal homologue of the *pem* pair called *chpB* (located at 100 min) has been described (5).

Here we show that the chromosomal genes *mazE* and *mazF*, located in the *E. coli rel* operon, have all of the properties required for an addiction module. MazE is a labile protein degraded by the *clpPA* serine protease, it protects the bacterial cells from the toxic effect of the stable MazF protein. In addition, the expression of *mazEF* is regulated by the cellular levels of ppGpp. Based on these properties of the *mazEF* system, we suggest a model for programmed cell death in *E. coli*. Furthermore, ppGpp exerts toxicity on cell survival, which is partially prevented by the deletion of the chromosomal *mazEF* genes or by a mutation in *clpP*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strains were MC4100 (18) or its derivative MC4100(λΔ*Bam*Δ*H1*) (19) carrying the temperature-sensitive repressor λ*c*I857. MC4100(λΔ*Bam*Δ*H1*) was constructed by P1 transduction from strain N99(λΔ*Bam*Δ*H1*) (19). We used these lysogens as hosts for the λP_L-containing pKC30 plasmid derivatives.The *mazEF* null alleles were constructed in a plasmid by deleting the structural genes and inserting a kanamycin resistance gene. Insertion of this into the chromosome was performed as described previously (20). The mutations in the genes coding for the various proteases were kindly provided by Dr. S. Gottesman (National Institutes of Health) from her strain collection. MC4100(λΔBamΔ*H1*) *clpP*::*cat* and MC4100(λΔ*Bam*Δ*H1*) *clpX*::*kan* are strains SG22098 and SG22101, respectively (21, 22), which were lysogenized with (λΔ*Bam*Δ*H1*). MC4100(λΔ*Bam*Δ*H1*) *clpA319*::*kan* and MC4100(λΔ*Bam*Δ*H1*) *lon*$^-$ were generated by P1 transduction from strains SG12044 and SG1117 (22), respectively. Cells were either grown in Luria–Bertani medium (LB) or in M9 minimal medium (23) supplemented with 0.2% glucose and a mixture of all amino acids except methionine and cysteine (23). The following antibiotics were added as appropriate: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 30 μg/ml.

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Abbreviations: ppGpp, 3′,5′-bispyrophosphate; ORF, open reading frame; IPTG, isopropyl β-D-thiogalactoside; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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Plasmid pGP1–2, carries the T7 RNA polymerase gene under the λP_L promoter and λcI857 gene (24), and pSP72 carries the T7 promoter (24). Using the appropriate DNA primers, PCR fragments that carry the ORF of the genes *mazE*, *mazF*, or *mazEF* were synthesized. These PCR products were used for cloning *mazE*, *mazF*, or *mazEF* under the λP_L promoter present on a pBR322 derivative (25). The resulting plasmids were called pRSE, pRSF, and pRSEF, respectively. All three plasmids carry *colE1* replicon, and *mazE* and *mazF* carry their own ribosomal binding sites.The same PCR fragments were also cloned into the *Bam*HI–*Hin*dIII sites of pSP72 (24) downstream of the T7 promoter. pALS13 that carries the truncated *relA* gene under the inducible *tac* promoter was described previously (2, 26).

Cloning Procedures and DNA Sequencing. Commercially prepared restriction endonucleases were used as specified by the suppliers. Plasmid DNA was prepared by a rapid alkaline standard methods (27). Sequenase (United Research Biochemical) and standard primers (Bethesda Research Laboratories) or appropriate primers synthesized on MilliGen model 8750 oligonucleotide synthesizer were used for DNA sequencing on double-stranded template DNA (27).

Lifetime of MazE and MazF. *E. coli* MC4100($\lambda\Delta Bam\Delta HI$) and its derivatives carrying mutations in energy-dependent proteolytic pathways, transformed with pRSE or pRSF, were grown in M9 medium at 30°C to mid log phase. The culture was shifted to 42°C until the end of the experiment. 15 min after the temperature shift, the cells were labeled for 5 min with [^{35}S]methionine at a concentration of 22.5 μCi/ml. Unlabeled methionine was then added to a final concentration of 2.5 mg/ml. At various times after the addition of unlabeled methionine, 200-μl samples were withdrawn and immediately frozen in liquid nitrogen. A 30-kDa protein was expressed from T7 promoter in the presence of rifampicin. Cells containing this labeled protein were added as an internal standard. Samples were washed with 10 mM Tris·HCl (pH 8.0), lysed, and applied to 16% Tricine-SDS polyacrylamide gels for electrophoresis as described by us previously (28). Labeled proteins on dried gels were detected by phosphorimaging. The positions of MazE and MazF in the gels were determined according to their known molecular weights and with respect to the dominant proteins observed when these proteins were overproduced from their respective plasmids.

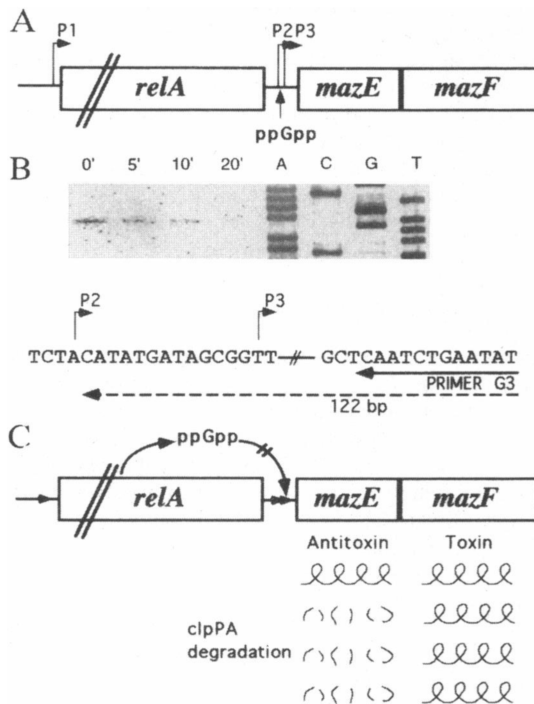

FIG. 1. The *rel* operon addiction module: structure and regulation. (*A*) The *rel* operon; (*B*) regulation of the P_2 promoter. *E. coli* MC4100 (18) with plasmid pALS13 (26) carrying a truncated *relA* gene under an inducible *tac* promoter (2) were grown in LB medium at 37°C to mid log phase. Samples for RNA purification were taken just prior, 5, 10, and 20 min after the addition of 1 mM IPTG to the cell culture. RNA purification was done using Qiagen kit RNeasy. RNA analysis was performed by primer extension as described previously (29) using the primer shown in the figure, which is located 122 bp downstream to the P_2 promoter. The left side of the figure shows the position in the gel of the 122-nt product, and the right side shows the DNA sequence obtained using the same primer (G3). This sequence TGTATACT is complementary to ACATATGA of the P_2 starting region. (*C*) A model for programmed cell death by the *mazEF* addiction module.

RESULTS

***mazE* and *mazF* Are Driven by P_2 Promoter, Which Is Regulated by ppGpp.** In the *rel* operon, downstream from the *relA* gene, are the two ORFs, *mazE* and *mazF* (Fig. 1*A*). Upstream from the *mazEF* genes, we found two promoters 13 nucleotides apart. These promoters, which we call P_2 and P_3, were active *in vitro* (data not shown). However, only P_2, the upstream promoter, was active in exponentially growing cells.

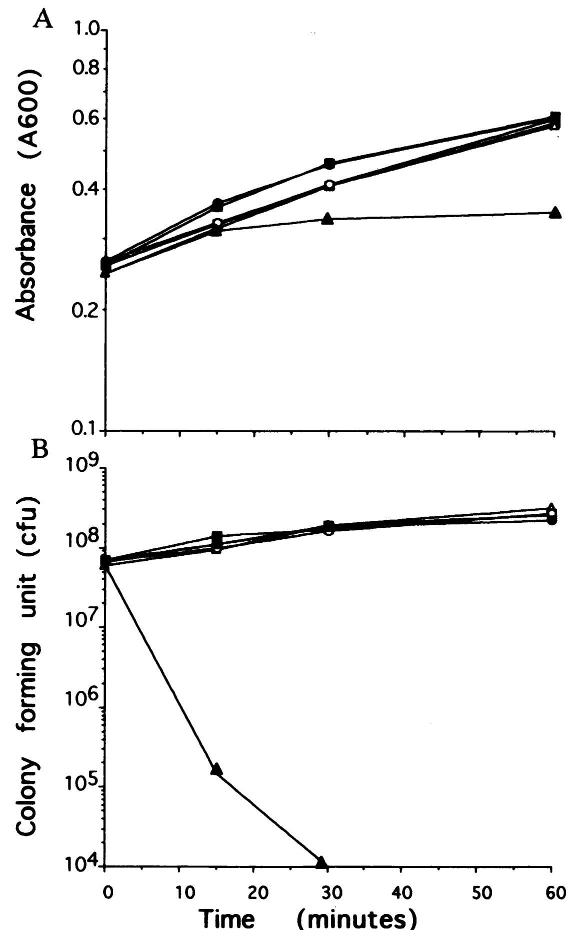

FIG. 2. The effect of MazE and MazF on the growth and survival of *E. coli* cells. *E. coli* strain MC4100($\lambda\Delta Bam\Delta HI$) (19) carrying the temperature-sensitive repressor λcI857, was transformed with plasmids pRSE, pRSF or pRSEF, respectively, carrying *mazE*, *mazF*, or *mazEF*, each under the control of the λP_L promoter. Cells were grown in LB medium at 30°C. At mid log phase, half of the culture was left at 30°C, while the rest was shifted to 42°C. Growth of the culture was followed by absorbency measurements at A_{600} (*A*) and by colony forming ability (*B*) measured in colony-forming units. Plating was carried out at 30°C.

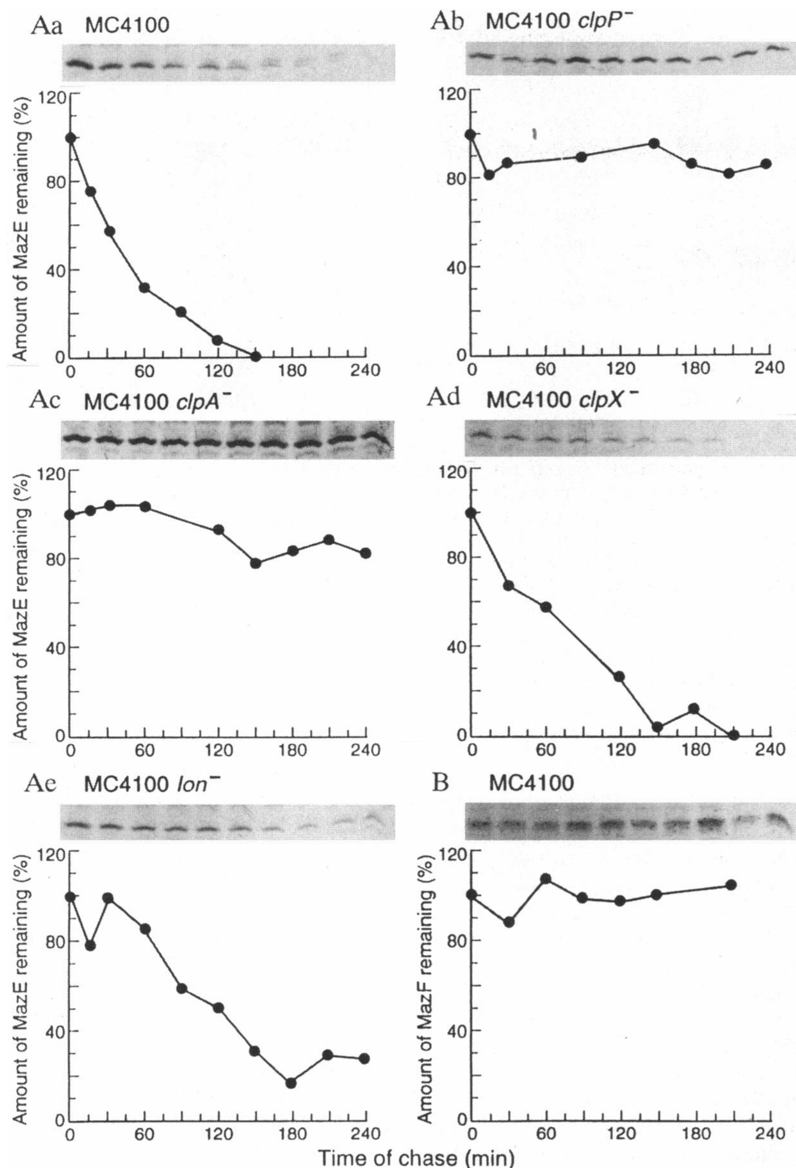

FIG. 3. Metabolic stability of proteins MazE (*A*) and MazF (*B*) in *E. coli*. MC4100(λΔ*Bam*Δ*HI*) (wt) and its derivatives carrying mutations in energy dependent proteolytic pathways. (*a*) wt, (*b*) *clpP*⁻, (*c*) *clpA*⁻, (*d*) *clpX*⁻, and (*e*) lon⁻. The strains were transformed with pRSE (*A*) carrying *mazE* or pRSF (*B*) carrying *mazF*. The cells were grown, labeled, and chased, and the samples were subjected to gel electrophoresis as described in *Materials and Methods*. The upper part of each panel represents the bands in the autoradiograph of MazE (*A*) or MazF (*B*), and the lower part represents the result of calculation of the amount of labeled protein based on phosphorimaging analysis of the specific band.

This is demonstrated in Fig. 1*B*, where transcription from P_2 promoter was studied by (i) the synthesis of the primer extension product of 122 nt (sample 0′ at the left side of the figure) and (ii) by the complementary DNA sequence obtained using the same primer (at the right side of the figure). In addition, when cells were starved for amino acids, the expression of P_2 promoter was inhibited (not shown). Furthermore, Fig. 1*B* shows that transcription from P_2 is inhibited by high levels of cellular ppGpp, induced by the expression of a truncated *relA* ORF with isopropyl β-D-thiogalactoside (IPTG). The synthesis of the 122-nt extension product is drastically decreased already after 5 min of induction.

MazF Is Toxic in the *E. coli* Cells; MazE Is Antitoxic. The partial homology of MazE and MazF to the protein products of the *pem* locus of pR100 addiction module (5) and the concomitant control of the expression of these genes by nutritional supply suggested that these *E. coli* genes may act as a chromosomal addiction module. We tested the toxic effect of MazF by cloning *mazF* under the inducible λP_L promoter. Cloning this gene was possible only in cells containing both the chromosomal *mazEF* genes and the temperature-sensitive repressor λ*c*I857. When MC4100 cells containing plasmid pRSF, which carries *mazF* under λP_L control, were shifted from 30 to 42°C, there was a cessation of growth (Fig. 2*A*). Furthermore, induction of *mazF* caused a reduction in the cell count of about 3 orders of magnitude of the induced cells in about 20 min (Fig. 2*B*). In M9 medium, induction of *mazF* caused cell lysis (data not shown). We observed no effect on cell growth or viability when *mazF* and *mazE* were induced together or when *mazE* was induced alone (Fig. 2). Thus, in agreement with the results of Masuda *et al.* (5), we found that MazF affects cell growth; in our experiments MazF also affects viability.

MazE Is a Substrate for *clpPA* Degradation *in Vivo*. A few ATP-dependent proteases have been identified in *E. coli* (30). Lon is one well characterized ATP-dependent serine protease of *E. coli*, the other being the ClpP protease. The latter forms a family in which the proteolytic subunit, ClpP, can use at least two alternative protein components with ATPase activity: the

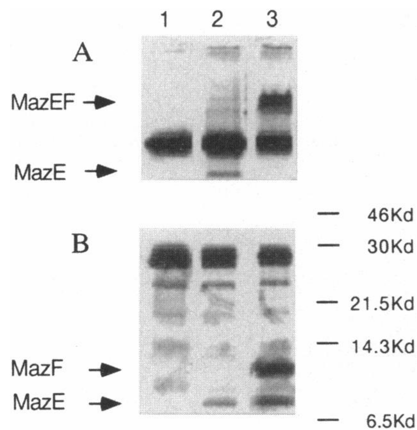

FIG. 4. Interaction between MazE and MazF. *E. coli* MC4100, carrying plasmid pGP1–2, which has T7 RNA polymerase under the inducible prophage λP_L promoter, and λcI857 gene, was transformed with pSP72 (24) (lane 1) or one of its derivatives carrying either *mazE* (lane 2), or *mazEF* (lane 3), under the control of the T7 promoter. Cells were grown at 30°C to mid log phase in M9 medium supplemented with glucose and amino acids as described in Fig. 3. The cultures were shifted to 42°C for 10 min, at which time rifampicin was added to a concentration of 250 μg/ml, and the cells were further incubated at 42°C for an additional 10 min. The cells were transferred to 30°C for 15 min and labeled with [^{35}S]methionine for 5 min. Samples were prepared for analysis on both 10% polyacrylamide native gels (*A*) and on 16% polyacrylamide denaturing gels (*B*).

product of either *clpA* or *clpX* (31–33). Fig. 3 shows the lifetimes of MazE and MazF, as they are affected by mutations in several *E. coli* genes that participate in various ATP-dependent degradation pathways. While MazF is stable over a period of 4 h (Fig. 3*B*), MazE is degraded with a halflife of only about 30 min (Fig. 3*A a*). MazE is not stabilized in a *lon*$^-$ mutant (Fig. 3*A e*), indicating that Lon is not responsible for MazE degradation. On the other hand, MazE is not degraded in *clpP*$^-$ and *clpA*$^-$ mutants (Figs. 3*A b* and *c*) but is degraded in a *clpX*$^-$ mutant (Fig. 3*A d*). Thus, our results indicate that MazE is degraded by the ClpPA protease.

MazE and MazF Interact Directly. Since MazE appears to protect the cell from the toxicity of MazF, we tested whether MazE and MazF interact directly. When extracts of cells expressing both MazE and MazF are run on native gels, a new, strong band is clearly observed (Fig. 4*A* lane 3). This band probably contains the MazE-MazF interaction product, since when it appeared, the band corresponding to MazE disappeared (Fig. 4*A* lane 3). Furthermore, when only *mazE* is overexpressed (Fig. 4*A* lane 2) the new band is absent.

The Chromosomal *mazEF* Addiction Module Is Necessary for Cell Death Induced by ppGpp. Finally we asked whether the chromosomal *mazEF* addiction module is related to cell death. The correlation between three parameters was tested: the effect of an abrupt increase in ppGpp, deletion of *mazEF* genes, and a mutation in *clpP*. Based on previous experiments (2), the intracellular level of ppGpp can be modulated by the induction of a truncated *relA* gene with IPTG. Here we used this system in order to study the effect of ppGpp on cell viability (Fig. 5). Under our experimental conditions, after an abrupt ppGpp induction only 15% of the cells survived. However, cell survival was significantly increased when the strain was either deleted for *mazEF* genes or mutated in *clpP*. In both cases, in spite of the induction of ppGpp, about 65% of colony-forming units were obtained. It should be noted that ppGpp toxicity is increased at 42°C as compared with 37°C (data not shown). Therefore the herein described ppGpp induction was carried out at 42°C (Fig. 5). This temperature dependent toxicity may be related to the heat shock property of *clpP* (34).

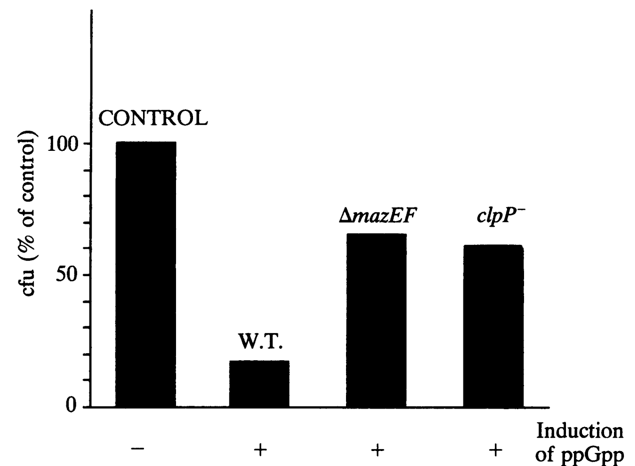

FIG. 5. The effect of *mazEF* and *clpP* on cell death induced by ppGpp. *E. coli* strains MC4100, MC4100Δ*mazEF* and MC4100 *clpP*::*cat* were transformed with plasmid pALS13 carrying a truncated *relA* gene under the inducible promoter P_{tac}. Cells were grown in LB medium at 30°C to mid log phase and shifted to 42°C for 10 min. IPTG was added to a concentration of 30 μM for 5 min, and cell survival was measured by colony forming ability.

DISCUSSION

Our results show that MazE and MazF have the properties required for products of an addiction module: (i) MazF is toxic (Fig. 2) and long lived (Fig. 3*B*); (ii) MazE is anti-toxic (Fig. 2) and short lived (Fig. 3*A a*); (iii) MazE and MazF interact (Fig. 4); (iv) they are co-expressed (Fig. 1*A*); and (v) *mazE*, the gene for the labile protein MazE, is located upstream from that of the stable protein MazF (Fig. 1*A*). Furthermore, the *mazEF* system has a unique property: its expression is inhibited by high concentrations of ppGpp (Fig. 1*B*). It seems, therefore, that the maintenance of an adequate level of MazE, which prevents the toxic effect of MazF, is possible only at low cellular levels of ppGpp.

Different proteolytic degradation pathways account for the ability of the antitoxic proteins of the addiction modules of different extrachromosomal elements. The protein product of *phd* of bacteriophage P1 is degraded by the ClpPX ATP-dependent serine protease (14), whereas, the protein products of *ccdA* of factor F (13) and *pemI* of plasmid R100 (15) are degraded by the Lon ATP-dependent serine protease. Our results show, that unlike the protein product of *pemI* of plasmid R100, which is partly homologous (34%) to *mazE* (5), MazE is not stabilized in a *lon*$^-$ mutant (Fig. 3*A e*), indicating that the Lon protease is not responsible for MazE degradation. Rather, our results indicate (Figs. 3*A b* and *c*) that the ATP-dependent serine protease ClpPA is responsible for the degradation of the short-lived component (MazE) of the cellular addiction module described here. This finding provides several new insights in relation to selective proteolysis in *E. coli*. (i) MazE, the substrate for ClpPA degradation, is a gene product encoded by the *E. coli* chromosome, whereas, most substrates for the various *E. coli* proteases identified so far, are encoded by bacteriophages, like λ, Mu, and P1 (31–33, 35, 36). (ii) MazE is the second cellular substrate for *clpPA*, which has so far been only described to be involved in the degradation of ClpA itself (21, 37). MazE being the smaller substrate of the two would be a promising substrate for studies of protease target specificity. (iii) The fact that PemI, which is partly homologous to MazE protein, is degraded *in vivo* by a different ATP-dependent protease, Lon, may be another clue in the understanding of protease specificity; and (iv) the degradation of MazE, the antitoxic protein product of the *rel* "addiction module" has obvious regulatory implications (see below).

The properties of the "*rel mazEF* module" described here suggest a model for programmed cell death in *E. coli* (Fig. 1*C*). Under conditions of nutritional starvation, the level of ppGpp increases. During amino acid starvation, this is achieved by the interaction of the product of *relA*, and during carbon limitation by an alternative pathway (1, 38). ppGpp inhibits the co-expression of *mazE* and *mazF* (Fig. 1). Because MazE is a labile protein (Fig. 3*A*), its cellular concentration is decreased more rapidly then that of MazF, and thereby MazF can exert its toxic effect and cause cell death. The results illustrated in Fig. 5 support the herein described model. (*i*) An abrupt increase in the level of ppGpp causes a significant decrease in cell viability. (*ii*) A deletion in chromosomal *mazEF* or a mutaion in *clpP* has a profound protective effect on ppGpp toxicity. It should be noted that the deletion of *mazEF* genes in the herein studied strain MC4100 does not affect growth in liquid or solid rich medium at 37°C. Characterization of the phenotype of the Δ*mazEF* strain at various growth conditions is under investigation.

As generally viewed for extrachromosomal elements, the addiction module renders the bacterial host addicted to the continued presence of the "dispensable" genetic element; its loss causes cell death (36). The new concept offered here for cellular genes is that the continued expression of the "addiction system" is required in order to prevent cell death. It is a matter of speculation whether a cellular gene has acquired the addiction module from an extrachromosomal element or the other way around. It is striking that the *mazE-mazF* module is located downstream to *relA* gene, which is responsible for the production of the signal molecule ppGpp that regulates this addiction module.

Here we have described a sophisticated addiction system that may have an important role in programming cell death in starving bacteria. We suggest that this novel addiction system serves as a mechanism for altruistic cell death: during extreme conditions of starvation part of the starved cells lyse, thereby enabling the survival of the rest of the cell population. The *rel mazEF* module is the first described chromosomal directed addiction system that is able to be regulated by a signal molecule. Other regulateable addiction systems with a similar suicidal effect may occur in *E. coli* and other organisms as well.

We thank Dr. S. Gottesman for kindly supplying us with bacterial strains used in this study. We also thank Dr. R. Schoulaker-Schwarz, Dr. H. Cedar, and F. R. Warshaw-Dadon, for critical readings of this manuscript. This study was supported by a grant of the United States-Israel Binational Science Foundation awarded to H.E.K. and a National Institutes of Health grant awarded to G.G.

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