mazEF: a chromosomal toxin-antitoxin module that triggers programmed cell death in bacteria

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Summary

***mazEF* is a toxin-antitoxin module located on the *Escherichia coli* chromosome and that of some other bacteria, including pathogens. *mazF* specifies for a stable toxin, MazF, and *mazE* specifies for a labile antitoxin, MazE, that antagonizes MazF. MazF is a sequence-specific mRNA endoribonuclease that initiates a programmed cell death pathway in response to various stresses. The *mazEF*-mediated death pathway can act as a defense mechanism that prevents the spread of bacterial phage infection, allowing bacterial populations to behave like multicellular organisms.**

Key words: Programmed cell death, Bacterial toxin-antitoxins, Stressful conditions

Introduction

Programmed cell death (PCD) is as an active process that results in cell suicide and is an essential mechanism in multicellular organisms. Generally, PCD is required for the elimination of superfluous or potentially harmful cells (reviewed by Jacobson et al., 1997; Nagata, 1997). In eukaryotes, the classical form of PCD is apoptosis (Kerr et al., 1972), a term that originally defined the morphological changes that characterize this form of cell death. Today, the phrase PCD is used to refer to any form of cell death mediated by an intracellular program, no matter what triggers it and whether or not it displays all of the characteristics of apoptosis (reviewed by Jacobson et al., 1997; Raff, 1998; Hengartner, 2000).

In bacteria, the best-studied PCD systems use unique genetic modules. These modules, called addiction modules or toxin-antitoxin systems, consist of a pair of genes that encode two components: a stable toxin and an unstable antitoxin that interferes with the lethal action of the toxin. Initially, such genetic systems for bacterial PCD were found mainly in *E. coli* on low-copy-number plasmids, where they are responsible for what is called the post-segregational killing effect. When a bacterium loses such a plasmid (or other extrachromosomal element), the cell dies because the unstable antitoxin is degraded faster than the more-stable toxin (reviewed by Jensen and Gerdes, 1995; Yarmolinsky, 1995; Couturier et al., 1998; Engelberg-Kulka and Glaser, 1999; Hayes, 2003; Gerdes et al., 2005). The cells can be thought of as 'addicted' to the short-lived product, since its de novo synthesis is essential for their survival. Thus, addiction modules maintain the stability in the host of the extrachromosomal elements on which they are borne.

Toxin-antitoxin systems, some of which are homologous to extrachromosomal addiction modules, have been found on the chromosomes of many bacteria (Mittenhuber, 1999; Engelberg-Kulka et al., 2004; Pandey and Gerdes, 2005). In *E. coli*, there are several toxin-antitoxin systems, including *mazEF* (Metzger et al., 1988; Masuda et al., 1993; Aizenman et al., 1996; Engelberg-Kulka et al., 2004), *chpBIK* (Masuda et al., 1993; Masuda and Ohtsubo, 1994), *relBE* (Bech et al., 1985; Gotfredsen and Gerdes, 1998; Gerdes et al., 2005), *yefM-yoeB* (Grady and Hayes, 2003; Cherney and Gazit, 2004; Christensen et al., 2004) and *dinJ-yafQ* (Hayes, 2003). The most studied among these is *mazEF*, which was the first to be discovered and described as regulatable and responsible for bacterial PCD (Metzger et al., 1988; Masuda et al., 1993; Aizenman et al., 1996). Among chromosomal toxin-antitoxin modules other than *mazEF*, only *relBE* has been studied extensively. It is not homologous to *mazEF*, and the structure and mode of action of the *relBE* products are unlike those of *mazEF*. Here, we focus on the *mazEF* system and on its relationship to PCD. In addition, we compare the modes of action and the structures of *mazEF* and *relBE* products.

mazEF is a stress-induced suicide module

The *mazEF* module consists of two adjacent genes, *mazE* and *mazF*, downstream of the *relA* gene (Metzger et al., 1988). *mazEF* has all the basic properties of an addiction module (Aizenman et al., 1996; Marianovsky et al., 2001): (1) MazF is a toxin and MazE is an antitoxin that antagonizes MazF; (2) MazF is long-lived, whereas MazE is a labile protein degraded in vivo by the ATP-dependent ClpPA serine protease; (3) MazE and MazF interact; (4) MazE and MazF are co-expressed; and (5) *mazEF* is negatively autoregulated at the transcriptional level by the combined action of both MazE and MazF proteins on the *mazEF* promoter P_2. Given these properties of *mazEF*, and the requirement for the continuous expression of MazE to prevent cell death, a model for *mazEF*-mediated PCD in response to severe nutrient starvation was proposed (Aizenman et al., 1996). Subsequently, this model was broadened to include various other stressful conditions (Sat et al., 2001;

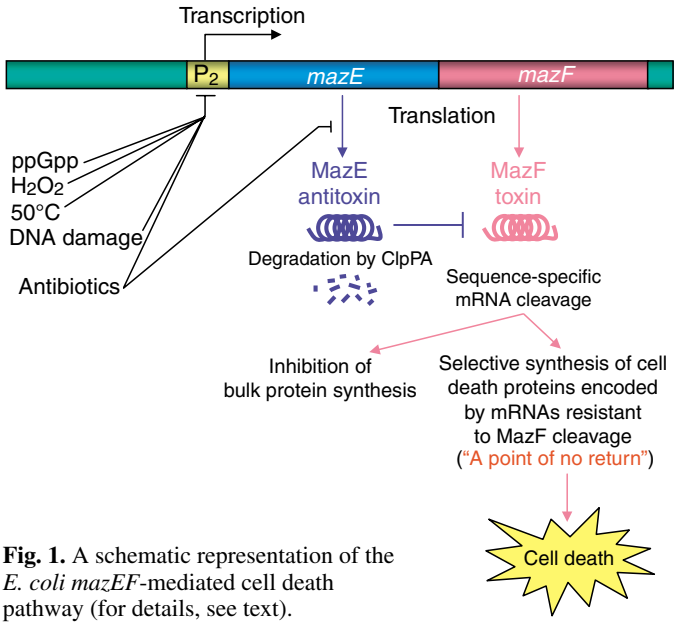

Fig. 1. A schematic representation of the *E. coli mazEF*-mediated cell death pathway (for details, see text).

Hazan et al., 2001; Sat et al., 2003; Hazan et al., 2004). Under such conditions, *mazEF* coexpression is inhibited (see below). Because MazE is a labile protein, its cellular concentration drops more rapidly than that of MazF, leaving MazF to exert its toxic effect, leading to cell death (Fig. 1).

Several experiments support this model by demonstrating that various agents that cause stressful conditions trigger the *mazEF* PCD system. (1) The artificial overproduction of guanosine 3′,5′-bispyrophosphate (ppGpp) (Aizenman et al., 1996; Engelberg-Kulka et al., 1998), the amino acid starvation signal molecule produced by the RelA protein (Cashel et al., 1996), triggers death; (2) several antibiotics (rifampicin, chloramphenicol and spectinomycin) that are general inhibitors of transcription and/or translation trigger *mazEF*-mediated death (Sat et al., 2001; Engelberg-Kulka et al., 2002); (3) Doc protein, which is the toxic product of the addiction module *phd-doc* of plasmid prophage P1 and is a general inhibitor of translation, drives post-segregational killing that requires the *E. coli mazEF* system (Hazan et al., 2001); (4) *mazEF*-mediated cell death is triggered by DNA damage caused by thymine starvation (Sat et al., 2003), mitomycin C, nalidixic acid and UV irradiation (Hazan et al., 2004); (5) oxidative stress (H_2O_2) and high temperature (50°C) also trigger *mazEF*-mediated death.

Note that most of the antibiotics and stresses considered in these studies have previously been shown to induce bacterial cell death (Rouviere et al., 1995; Ahmad et al., 1998; Davies and Webb, 1998; Storz and Zheng, 2000). The studies described above clearly show that cell death induced by these conditions is dependent on *mazEF*. In particular, the involvement of *mazEF* in cell death induced by thymine starvation deserves special attention: it provides a new insight to an old enigma concerning the mechanism underlying the well-known phenomenon thymine-less death (TLD) (reviewed by Engelberg-Kulka et al., 2004).

The *mazEF* system is thus a stress-induced suicide module. Stressful conditions can affect the continuous expression of MazE by preventing either its transcription and/or its translation. ppGpp inhibits transcription from the *mazEF* P_2 promoter (Aizenman et al., 1996). In addition, some antibiotics that are general inhibitors of transcription and/or translation can also affect MazE expression. For example, rifampicin inhibits the initiation of RNA synthesis through its interaction with the β-subunit of *E. coli* RNA polymerase (Wherli and Staehelin, 1971; Davies and Webb, 1998), and chloramphenicol and spectinomycin affect ribosomes and are, therefore, general inhibitors of translation (Spahn and Prescott, 1996; Davies and Webb, 1998). Thymine starvation, another stressful condition, provokes DNA damage that involves a unique breaking/twisting of the chromosome into a configuration that defies all the repair/protective mechanisms (Nakayama et al., 1994; Ahmad et al., 1998). Such serious damage to the DNA would be expected to reduce transcription from the *mazEF* promoter P_2 substantially. Indeed, experiments have shown that, under thymine starvation, the activity of the *mazEF* promoter P_2 is drastically reduced (Sat et al., 2003). *mazEF*-dependent death at high temperatures may also be induced by inhibition of *mazEF* expression. At such stressful temperatures, the normal transcription factor, σ^{70}, becomes inactivated; σ^{70} is replaced by periplasmic σ^{E} (Raina et al., 1995; Rouviere et al., 1995). Since the promoter recognition sites of σ^{E} (Yura et al., 2000) do not exist in *mazEF* promoter, σ^{E} should not initiate the transcription of the *mazEF* genes.

Modes of action and structures of MazF and RelE

The mechanisms of the actions of the toxins have recently received much attention in studies of *E. coli* chromosomal toxin-antitoxin systems. Ectopic over-expression of each toxin studied so far (MazF, ChpBK, RelE and YoeB) inhibits translation but not RNA or DNA synthesis (Christensen et al., 2001; Christensen et al., 2003; Christensen et al., 2004; Zhang et al., 2003). The mode of action of RelE was the first to be studied in vivo and in vitro (Pedersen et al., 2003). RelE causes cleavage of mRNA codons in the ribosomal A site. This is highly codon specific, and occurs between the second and third nucleotide (Pedersen et al., 2003) (Fig. 2B). Among stop codons, it exhibits a strong preference for UAG, intermediate for UAA and weak for UGA. Among the sense codons, its preference is for UCG and CAG**.** Cleavage of mRNA codons at the ribosomal A site now appears to be an intrinsic characteristic of the ribosome during its pausing. Rather than having ribonucleolytic activity, RelE might therefore simply modulate cleavage at the A site (Hayes and Sauer, 2003).

MazF also inhibits translation by cleaving mRNAs at specific sites (Christensen et al., 2003; Zhang et al., 2003; Münoz-Gomez et al., 2004). However, unlike RelE, MazF cleaves mRNAs in a ribosome-independent manner (Zhang et al., 2003). Thus, MazF appears to be a sequence-specific rather than codon-specific endoribonuclease that preferentially cleaves single-stranded mRNAs at ACA sequences**.** It does not degrade ACA-less mRNA (Suzuki et al., 2005). Zhang et al. (Zhang et al., 2004) have confirmed this mechanism of MazF action by following cleavage of DNA-RNA chimeric substrates containing XACA sequences. They showed that MazF cleaves 5′ or 3′ of the first A residue of the ACA sequence (Fig. 2A). It cleaves phosphodiester

bonds at the 5′ side, yielding a free 5′-OH group on the 3′-end cleavage product and a 2′,3′-cyclic phosphate on the 5′-end product. The 2′-OH group of the nucleotide preceding the ACA sequence is essential for MazF cleavage. Thus, though MazF and RNaseA have different sequence specificities, enzymatically they seem to function similarly.

The differences in the modes of action of MazF and RelE are probably a result of different structures. The structures of the *E.coli mazEF* products have now been solved. Loris and colleagues have determined the crystal structure of MazE (Loris et al., 2003; Lah et al., 2003), and Burley and colleagues have determined the crystal structure of the MazE-MazF antitoxin-toxin complex (Kamada et al., 2003). MazE, in its isolated state, consists of a structured N-terminal dimerization domain and an intrinsically unstructured C-terminal MazF-binding domain. Upon forming a complex with MazF, the C-terminal domain of MazE becomes ordered. It forms an extended structure that runs around the surface of MazF. In the crystal, MazE and MazF form a heterohexamer in which a MazE dimer is sandwiched between two MazF dimers (Fig. 2C). Each MazF homodimer has one binding site occupied with MazE; the other binding site remains free. Thus, in principle, it could form longer linear oligomers of alternating MazE and MazF homodimers. Lah and colleagues (Lah et al., 2005) have described a particular conformational adaptability of MazE, which seems to be essential for the specific recognition of MazF and DNA. The latter is a characteristic that is essential for autoregulation of *mazEF* at the transcriptional level (Marianovsky et al., 2001). Inouye and colleagues (Zhang et al., 2003) have hypothesized that the unstructured C-terminal region of MazE, which is highly negatively charged, might mimic the single-stranded RNA structure, allowing it to bind to a MazF dimer. This would disturb the MazF RNA-binding site and thus block its endoribonuclease activity.

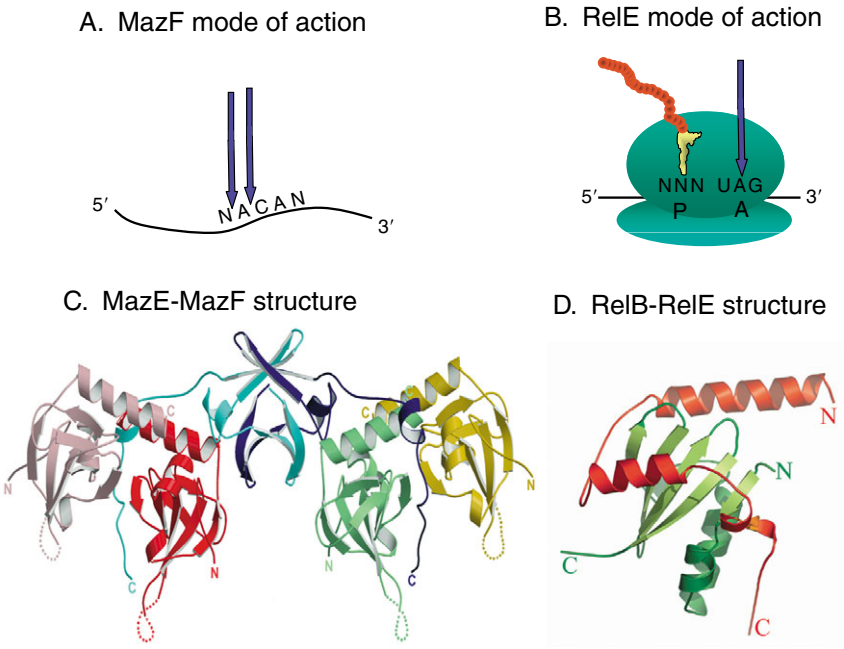

Fig. 2. MazF and RelE actions and structures. (A) *E. coli* MazF cleaves mRNAs in a sequence-dependent manner (see main text for details). (B) *E. coli* RelE affects mRNA cleavage, either directly or indirectly, in a ribosome- and codon-dependent manner (see main text for details). (C) The structure of the *E. coli* MazE-MazF complex: the two MazE molecules are shown in light and dark blue; the four MazF molecules are shown in yellow, green, pink and red. Reproduced with permission from Elsevier (Kamada et al., 2003). (D) The structure of the *P. horikoshii* RelB-RelE complex: RelE is shown in green and RelB is shown in red. Reproduced with permission from *Nat. Struct. Mol. Biol.* (Takagi et al., 2005).

More recently, Kimura and colleagues (Takagi et al., 2005) have determined the crystal structure of the RelB-RelE antitoxin-toxin complex of the hyperthermophilic archaeon *Pyrococcus horikoshii*. In contrast to the MazE-MazF complex, in which the ratio of the antitoxin-toxin molecules is 1:2, the ratio in the RelB-RelE complex is 1:1. Since two molecules of RelB-RelE complex are bound together in solution, a heterotetrameric structure has been suggested. In the complex, RelE has a simple ellipsoid architecture, composed of three α-helices and a five-stranded β-sheet. In the RelB-RelE complex, RelB is present as a polypeptide chain that lacks any distinct hydrophobic core and wraps around RelE. Like the C-terminal half of MazE, RelB is probably unstructured in isolation.

Most of the RelB chain makes contact with surface residues of RelE (Fig. 2D). However, site-directed mutagenesis indicates that the site predicted to be essential for RelE function does not interact directly with the antitoxin RelB. This is in contrast to nucleases such as barnase, colicin D (Buckle et al., 1994) and probably MazF (see above) that are blocked by their respective inhibitors through direct interactions with the active site of the enzyme. RelB therefore does not appear to prevent RelE from being active, but rather prevents RelE from entering the A site of the ribosome by increasing the size of the complex. It is interesting that the dimensions and shape of RelE are similar to those of domain IV of bacterial elongation factor G (EF-G), which recognizes the ribosomal A site by tRNA mimicry. This suggests that RelE enters the ribosome A site in a way similar to that in which the decoding domain of EF-G interacts with ribosomes (Takagi et al., 2005; Wilson and Nierhaus, 2005). Thus, the particular structure of RelE might contribute to its direct or indirect ribosome-dependent ribonucleolytic action, whereas MazF, which lacks such required properties, acts differently: it is a ribosome-independent ribonuclease.

A point of no return

On the basis of experiments on the *mazEF*, *chpBIK* and *relBE* modules, Pedersen et al. (Pedersen et al., 2002) have suggested that, rather than inducing PCD, chromosomal toxin-antitoxin systems induce a state of reversible bacteriostasis. They showed that ectopic over-expression of MazF or RelE inhibits translation and cell growth, which can resume if the cognate antitoxin is expressed at a later time (Pedersen et al., 2002). However, these experiments were carried out over a short time

window, within only five hours of MazF induction (Pedersen et al., 2002). More-recent studies using a similar ectopic over-expression system (Amitai et al., 2004) showed that MazE can indeed resuscitate *E. coli* cells within six hours of MazF overproduction. But, when this period is extended past six hours, the ability of MazE to resuscitate the cells drastically decreases. Moreover, the inability of MazE to reverse the bacteriocidic effect of MazF is even more dramatic when MazF is over-expressed in cells growing in liquid minimal medium, rather than in rich medium. There is thus a 'point of no return', which occurs sooner in minimal medium than in rich medium (Amitai et al., 2004). A point of no return is also reached after the induction of chromosomal *mazEF*-mediated cell death by various stressful conditions. Again, there is only a short time window during which the ectopic overexpression of MazE can reverse the lethal action of the chromosome-encoded MazF (I. Kolodkin and H.E.-K., unpublished).

As described above, MazF cleaves mRNAs (Christensen et al., 2003; Münoz-Gomez et al., 2004; Zhang et al., 2003; Zhang et al., 2004). In addition, MazF also cleaves tmRNA (Christensen et al., 2003), which is a tRNA-mRNA hybrid that can rescue ribosomes by binding to the A site of those containing a truncated mRNA. It then tags the corresponding nascent polypeptide chains with a protein degradation signal, while allowing translation to terminate normally (reviewed by Karzai et al., 2000). MazF-cleaved tmRNA probably cannot rescue ribosomes that are stalled by MazF-cleaved mRNAs; as a result, protein synthesis is inhibited.

Given the point of no return in *mazEF*-mediated cell death (Amitai et al., 2004), one can envisage a model in which the endoribonucleolytic effect of MazF would be one of the initial steps in the PCD pathway. Such a step could still be reversed by the antagonistic effect of MazE on MazF (Fig. 1). Further cleavage of mRNAs and tmRNA by MazF would be prevented by MazE, and previously truncated mRNAs could be released from the ribosomes through the action of de novo synthesized, uncleaved tmRNA. However, we suggest that MazE cannot reverse downstream events already initiated by MazF. Thus, if the process is not stopped in time, cell death eventually becomes unavoidable. How might the inhibition of translation by MazF induce such a downstream cascade that leads to cell death? It might be because MazF cleaves mRNAs at specific sites (Zhang et al., 2003). The action of MazF could lead to selective synthesis of cell death proteins encoded by mRNAs resistant to cleavage by MazF (Fig. 1). These might not contain the ACA target site or be protected from MazF by some other mechanism. Indeed, *mazEF* could be part of a PCD network. In such a network, MazF would be a mediator rather than an executioner.

mazEF-mediated cell death as a defense mechanism that prevents the spread of phage infections

The presence of a PCD system on the bacterial chromosome raises an intriguing question: what is the role of such a system in unicellular organisms, as bacteria are traditionally considered to be? For an individual bacterium, PCD is clearly counterproductive, but it becomes effective when simultaneously operated by a group of cells (Engelberg-Kulka and Hazan, 2003). Supporting evidence for the view that bacterial PCD is beneficial for the whole culture rather than individual cells comes from experiments showing that *mazEF*-mediated death acts as a defense mechanism that prevents the spread of phage P1 (Hazan and Engelberg-Kulka, 2004). P1 phages exist in two forms: (1) virulent particles that develop in the host cells (*E. coli*) and are released by cell lysis; and (2) lysogenic prophages that replicate like plasmids because of their autonomous origin of replication. These phages encode a repressor that permits them to replicate in the host cells without entering into the lytic phase. If the repressor becomes inactivated, prophages enter into the lytic stage.

Studies of lysogenic *E. coli* cultures that harbor a heat-inducible dormant P1 phage have examined the effect of the *mazEF* system on phage growth (Hazan and Engelberg-Kulka, 2004). Upon heat-induction, most cells lacking the *mazEF* system (Δ*mazEF* cells) lyse, whereas only a small fraction of the wild-type (WT) cells lyse. The Δ*mazEF* cultures produce significantly more phages than do the WT cultures. Nevertheless, despite the differences in the levels of lysis and phage production, both *mazEF*$^{+}$ (WT) and Δ*mazEF* cells do not produce colonies upon phage induction. A virulent phage P1 gives similar results. A model involving two separate killing mechanisms has therefore been suggested. The Δ*mazEF* cells (and a small fraction of the cells of the parental strain) are killed by the release of mature progeny phage particles. However, since most of the WT cells do not lyse, they must be killed by a different mechanism: the *mazEF*-mediated PCD pathway (Fig. 3A).

To test this model, we have simulated invasion of a non-lysogenic culture by lysogenic cells by mixing lysogenic cultures of WT or Δ*mazEF* cells with the corresponding non-lysogenic cells. Whereas the Δ*mazEF* cells are susceptible to

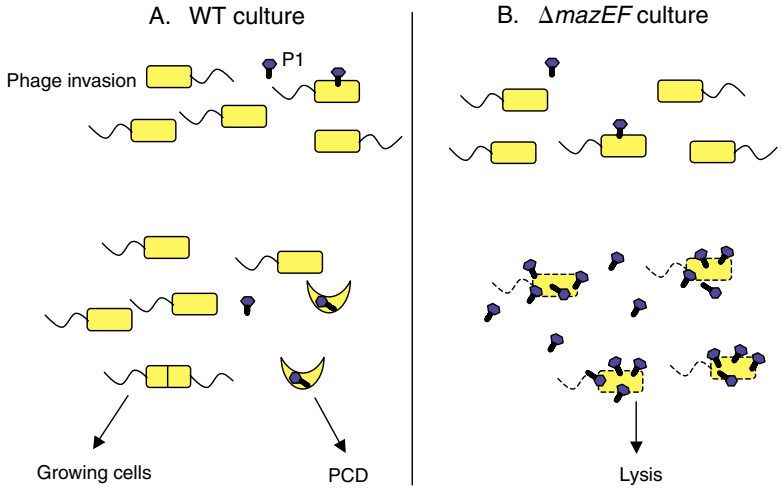

Fig. 3. A model for the behavior of WT and Δ*mazEF* cultures during phage P1 attack. (A) In WT cells, *mazEF* mediates the death of the infected cells. As a consequence, the development of the phage is restricted, the phage titer is low, and the culture survives. (B) In Δ*mazEF* cultures, nothing interferes with the phage infections: the infected cells lyse and spread infecting particles to the rest of the cells in the culture. Thus, the WT culture can survive phage infections whereas the Δ*mazEF* culture dies. PCD, programmed cell death.

infection and are totally lysed (Fig. 3B), the WT culture continues to grow and appears not to be infected by phages released from the induced lysogens (Fig. 3A). Since the phage cannot develop in cells that have already died because of the lethal action of the *mazEF* module, the infection cannot spread, and only the WT culture is protected from total collapse. Thus, although the *E. coli mazEF* module causes the death of individual cells, it might protect the culture as a whole by preventing the spread of infective phage (Hazan and Engelberg-Kulka, 2004).

Of course, there are probably additional roles for *mazEF*-mediated PCD. During the response to severe nutritional stress, the death of a subpopulation would provide food for the surviving cells (Aizenman et al., 1996). This occurs during sporulation of *Bacillus subtilis*, which has a novel PCD system that differs from *mazEF* and the other toxin-antitoxin systems (Gonzalez-Pastor et al., 2003; Engelberg-Kulka and Hazan, 2003). *mazEF* could also act as a guardian of the bacterial chromosome: when other systems fail, *mazEF*-mediated cell death might preserve genomic stability by causing the elimination of cells carrying genomic defects and mutations from the culture. The *mazEF* module therefore provides an evolutionary advantage for those bacteria that carry it.

Conclusions and perspectives

The *mazEF* system represents a form of toxin-antitoxin module that has adapted to respond to various stimuli and execute a cell death program in response to viral infection and several environmental stresses. The MazF endoribonuclease, which cleaves mRNAs at specific sequences, might in fact be a mediator rather than an executioner of PCD. It probably mediates cell death by inducing a pathway downstream of its primary endoribonucleolytic effect, but the details of this await elucidation.

So far, the *mazEF* toxin-antitoxin system has been studied only in *E. coli*. However, the system is not unique to *E. coli*, and systems similar to *mazEF* have been found on the chromosomes of many other bacteria (Mittenhuber, 1999; Engelberg-Kulka et al., 2002), including the pathogens *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Bacillus anthracis* (Engelberg-Kulka et al., 2004; Pandey and Gerdes, 2005). Future studies will reveal whether such *mazEF*-like modules mediate cell death in these pathogenic organisms. Of course, toxin-antitoxin systems unlike *mazEF*, both in *E. coli* and in other microorganisms (Pandey and Gerdes, 2005), might also play a role in cell death. Given the differences in the structures and modes of action of MazF and RelE, such systems could operate differently and have different roles in bacterial physiology. The products of *mazEF* (Engelberg-Kulka et al., 2004) and other bacterial toxin-antitoxin systems could be targets for the development of new antibiotics.

Clearly, a system that causes any given cell to commit suicide is not advantageous to that particular cell. However, death by suicide of an individual cell might be advantageous for the bacterial population as a whole. More and more experimental evidence supports the idea that bacterial cultures have many characteristics of multicellular organisms (Kaiser and Losick, 1993; Swift et al., 1996; Dworkin and Shapiro, 1997; Gray, 1997; Fuqua and Greenberg, 1998; Shapiro, 1998; Miller and Bassler, 2001; Henke and Bassler, 2004). Thus, we suggest that it is important to study the process of bacterial cell death mediated by toxin-antitoxin in relation to the multicellular characteristics of bacterial cultures.

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