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The Extracellular Death Factor: Physiological and Genetic Factors Influencing Its Production and Response in *Escherichia coli*▽

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Gene pairs specific for a toxin and its antitoxin are called toxin-antitoxin modules and are found on the chromosomes of many bacteria. The most studied of these modules is *Escherichia coli mazEF*, in which *mazF* encodes a stable toxin, MazF, and *mazE* encodes a labile antitoxin, MazE, which prevents the lethal effect of MazF. In a previous report from this laboratory, it was shown that *mazEF*-mediated cell death is a population phenomenon requiring a quorum-sensing peptide called the *e*xtracellular *d*eath *f*actor (EDF). EDF is the linear pentapeptide NNWNN (32). Here, we further confirm that EDF is a signal molecule in a mixed population. In addition, we characterize some physiological conditions and genes required for EDF production and response. Furthermore, stress response and the gene specifying MazEF, the Zwf (glucose-6-phosphate dehydrogenase) gene, and the protease ClpXP are critical in EDF production. Significant strain differences in EDF production and response explain variations in the induction of *mazEF*-mediated cell death.

Over the last few years, a great deal of attention has been focused on toxin-antitoxin modules that are found on the *Escherichia coli* chromosome and on the chromosomes of many other bacteria, including pathogens (12, 13, 23, 38, 41). Each of these modules consists of a pair of genes, of which generally the downstream gene encodes a stable toxin and the upstream gene encodes a labile antitoxin. In *E. coli*, seven toxin-antitoxin systems have been described (11, 14, 15, 20, 46). Among these, one of the most studied is the chromosomal toxin-antitoxin system *mazEF*, which was the first to be described as regulatable and responsible for bacterial programmed cell death (3, 15). *E. coli mazF* is specific for the stable toxin MazF, and *mazE* is specific for the labile antitoxin MazE. In vivo, MazE is degraded by the ATP-dependent ClpAP serine protease (but not by the proteases ClpXP or Lon) (3). MazF is a sequence-specific endoribonuclease that preferentially cleaves single-stranded mRNAs at ACA sequences (54, 55). MazE counteracts the action of MazF. Since MazE is a labile protein, preventing MazF-mediated action requires the continuous production of MazE. Thus, any stressful condition that prevents the expression of the chromosomally borne *mazEF* module will lead to the reduction of MazE in the cell, permitting toxin MazF to act freely. Such conditions include (i) short-term inhibition of transcription and/or translation by antibiotics such as rifampin, chloramphenicol, and spectinomycin (44); (ii) the overproduction of ppGpp, which inhibits *mazEF* transcription (3, 10); and (iii) DNA damage caused by thymine starvation (45) as well as by DNA-damaging agents, such as mitomycin C or nalidixic acid (15, 25, 32). These antibiotics and stressful conditions that are well known to cause bacterial cell death (1, 9) have been found to act through the *mazEF* module (25, 32, 44, 45). Clearly, a system that causes any given cell to die is not advantageous to that particular cell. On the other hand, the death of an individual cell may be advantageous for the bacterial population as a whole. It was therefore suggested that *mazEF*-mediated cell death is a population phenomenon in which bacteria communicate with each other (11, 15).

Bacteria communicate with one another via quorum-sensing signal molecules that are also called "autoinducers" (19; reviewed in references 4, 5, 6, 17, 27, 30, and 47). Quorum sensing provides a mechanism for bacteria to monitor one another's presence and to modulate gene expression in response to population density. In the simplest scenario, the accumulation of a threshold autoinducer concentration, which is correlated with increasing population density, initiates a signal transduction cascade that culminates in a population-wide alteration in gene expression. The most studied have been four main kinds of quorum-sensing signal molecules (autoinducers) that are specific for various processes. (i) Acylated homoserine lactones (AHLs) are typically synthesized by a LuxI-type enzyme (4, 27, 47) in gram-negative bacteria. When the AHL concentration reaches the threshold level, AHLs are bound by LuxR-type protein molecules. By binding to appropriate promoters, these LuxR-AHL complexes affect the transcription of quorum-sensing regulated target genes. One of the best studied AHL systems is the *lux* phenotype of *Vibrio* spp. (reviewed in references 17, 18, and 19). (ii) In addition to a typical AHL, designated AI-1, a second autoinducer, designated AI-2, which is a furanosyl borate diester, has more recently been discovered (8) to be involved in the bioluminescent quorum sensing of the marine bacterium *Vibrio harveyi*; it is involved in interspecies communication (4, 16, 47, 53). (iii) 2-Heptyl-3-hydroxy-4-quinolone is produced by the opportunistic pathogen *Pseudomonas aeruginosa* (33, 42). (iv) Finally, short modified peptides processed from precursors are the autoinducers in gram-positive bacteria. They are involved in many systems, including the development of competence in *Bacillus subtilis* (35, 48, 49, 50) and the virulence response in *Staphylococcus aureus* (28, 29, 34, 36, 39, 40). Signal transduc-

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tion occurs by a phosphorylation cascade that activates a DNA binding protein that controls the transcription of target genes. These autoinducers of gram-positive bacteria are highly specific because each oligopeptide sensor selects for a given peptide signal (reviewed in references 30, 34, and 52).

In a previous report from this laboratory, it was shown that *mazEF*-mediated cell death is a population phenomenon requiring a quorum-sensing factor that we call the *e*xtracellular *d*eath *f*actor (EDF) (32). We also characterized the chemical nature of EDF to be the linear pentapeptide NNWNN. Each of the five amino acids in EDF is important for its *mazEF*-mediated killing activity, and the terminal asparagines are the most crucial (32). The quorum-sensing process involved in *mazEF*-mediated cell death and the quorum-sensing peptide EDF are particularly interesting not only because no other peptide has apparently been reported to be involved in quorum sensing in *E. coli* but also because EDF appears to be a distinct type of molecule related to the quorum-sensing peptides of gram-positive bacteria. Here, we further confirm that EDF is a signal molecule in a mixed population. Furthermore, our experiments reveal that *mazEF* is required for both EDF production and response. In addition, stress response and genes encoding Zwf (glucose-6-phosphate dehydrogenase) and the protease ClpXP are involved in EDF production.

MATERIALS AND METHODS

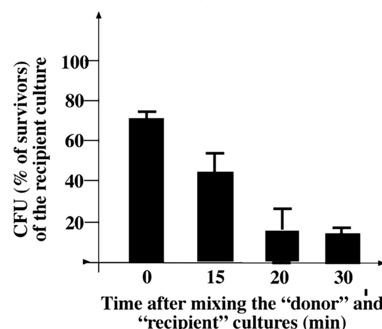
Bacterial strains and plasmids. We used the following sets of *E. coli* strains: (i) MC4100*relA1* (7) and MC4100*relA*$^+$ (10) and their Δ*mazEF*::*kan* derivatives (10) and *E. coli* strain K38 (43) and its Δ*mazEF* derivative (24); (ii) MC4100*relA1*Δ*clpP*, MC4100*relA1*Δ*clpA*, MC4100*relA1*Δ*clpX*, and MC4100*relA1*Δ*lon* (3); (iii) W3110 and MG1655 (22) and their Δ*mazEF*::*kan* derivatives, which we constructed by P1 transduction from strain MC4100*relA1*Δ*mazEF*::*kan*; and (iv) MC4100*relA*$^+$Δ*zwf*, MC4100 *relA*$^+$Δ*clpP*, and MC4100*relA*$^+$Δ*ygeO*, constructed by us using PCR deletion (32), pBAD33 carrying a chloramphenicol resistance gene (21), pQE30 (Qiagen) carrying an ampicillin resistance gene, pQE-*mazF* carrying *mazF* under the control of the *lac* operator and also *lacZ*, pKK223-*mazEF* carrying *mazEF* under the *tac* promoter (24), and pQE*zwf* and and pQE*ygeO*, which we constructed as follows. *zwf* and *ygeO* genes were PCR amplified from strain W3110 and cloned using EcoRI and HindIII sites into the plasmid pQE32*lacI*^{q} (kindly provided by the laboratory of Orna Amster-Choder) bearing an ampicillin resistance gene, downstream of the T5 promoter.

Materials and media. Bacterial cultures were grown in liquid M9 minimal medium with 1% glucose and a mixture of amino acids (10 μg/ml each) (37) and plated on rich LB agar plates as described previously (23). Isopropyl-β-D-thiogalactopyranoside (IPTG), nalidixic acid, mitomycin C, trimethoprim, rifampin, chloramphenicol, spectinomycin, serine hydroxamate, and Trizma base were obtained from Sigma (St. Louis, MO). Ampicillin was obtained from Biochemie GmbH (Kundl, Austria). Chemically synthesized EDF peptide (98% purity) was synthesized for us by GenScript Corporation (Piscataway, NJ).

Production of supernatants from dense cultures. A culture of an *E. coli* strain that served as an EDF donor was grown in M9 medium with shaking (160 rpm) at 37°C for 12 h. The cells were diluted 1:100 in M9 medium and grown with shaking (160 rpm) at 37°C to mid-logarithmic phase (optical density at 600 nm [OD_{600}] of 0.6; 2.5×10^8 cells/ml). Cells were then centrifuged at 14,000 rpm for 5 min. The supernatant was removed and filtered through a 0.22-μm filter; the filtrates were stored at 4°C.

Induction of *mazEF*-mediated cell death. *E. coli* cells were grown as described in the legend to each figure. When the cultures reached a density of 3×10^8 cells/ml, samples were treated and stressful conditions were induced as described in the figure legends. Samples were centrifuged at 14,000 rpm for 5 min and washed in preheated saline. The number of CFU was detected by plating the washed samples on prewarmed LB plates that were then incubated at 37°C overnight. The percentage of surviving CFU is represented by the ratio of "treated cells" to "untreated cells."

Quantification of EDF activity. The supernatant of MC4100*relA*$^+$ (dense culture of 2.5×10^8 cells/ml), serving as a donor, was titrated for EDF activity at different dilutions in Tris buffer (pH 7.0). A diluted culture (2.5×10^4 cells/ml) of MC4100*relA*$^+$ served as a recipient. A dilution factor of 25 (which is found in the linear range of the curve) permits 70% loss of viability. Therefore, 1 unit of EDF corresponds to a dilution factor of 25.



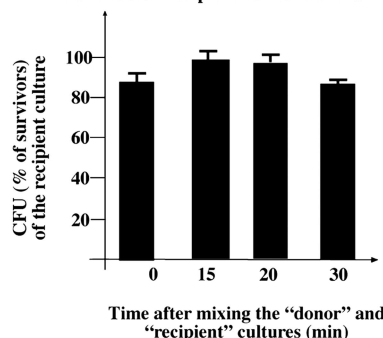


FIG. 1. EDF is a signal molecule that can trigger *mazEF*-mediated cell death. We used strains MC4100*relA*$^+$/pBAD (Cam^r), and MC4100*relA*$^+$/pQE30 (Cam^s). Each strain was grown separately in M9 minimal medium containing the relevant antibiotic. When the cultures reached mid-logarithmic phase (OD_{600} of 0.4), they were washed and resuspended in M9 minimal medium. (A) A mixture of two strains was prepared in M9 medium such that the final concentrations were 10^8 cells/ml of a "donor" Cam^r culture (carrying Cam^r/pBAD) and 10^4 cells/ml of "recipient" Cam^s Amp^r culture (WT or Δ*mazEF* carrying Amp^r pQE30). (B) Both "donor" Cam^r culture (carrying Cam^r/pBAD) and the "recipient" Cam^s Amp^r culture (carrying Amp^r pQE30) were diluted in M9 medium; a mixture of the two was prepared in which the final concentration of each strain was 10^4 cells/ml. At various times, samples were removed and preincubated without shaking at 37°C for 10 min, after which chloramphenicol (45 μg/ml) was added to induce cell death. A culture to which no chloramphenicol was added served as a control. The cultures were washed and resuspended in preheated (37°C) saline. CFU were determined by plating on LB medium plates with either chloramphenicol or ampicillin that were then incubated at 37°C for 12 h. Here, we present only the Amp^r Cam^s subculture survivors, which we determined by comparing the number of chloramphenicol-induced Amp^r colonies versus the number of uninduced control colonies on LB medium plates with ampicillin. In this figure and in all the following figures, the results are the averages from three independent experiments that were carried out in triplicate.

RESULTS

EDF is a signal molecule. We wished to examine whether EDF is a signal molecule in a population context. To this aim, we constructed a new experimental system based on the observation that a brief induction with chloramphenicol causes *mazEF*-mediated cell death (31). In the new system, we mixed two cultures of *E. coli* strain MC4100*relA*$^+$: a dense culture of MC4100*relA*$^+$Cam^r, to serve as the EDF "donor," and a di-

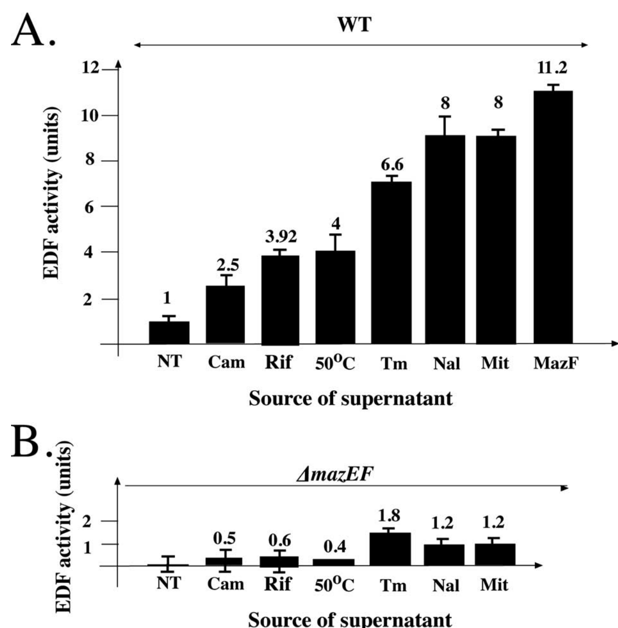


FIG. 2. Effect of various stressful conditions on EDF production. (A) *E. coli* MC4100*relA*$^+$ WT and (B) MC4100*relA*$^+$Δ*mazEF* were grown in M9 medium (containing 0.5% glucose) with shaking (160 rpm) at 37°C for 12 h. Cells were then diluted 1:100 in M9 medium and were grown with shaking (160 rpm) at 37°C to mid-logarithmic phase (OD_{600} of 0.6). The cells were incubated without shaking for 10 min and then one of the following stressful conditions was applied: (i) incubation at 37°C with chloramphenicol (45 μg/ml) for 20 min (Cam); rifampin (20 μg/ml) (Rif), nalidixic acid (1,000 μg/ml) (Nal), or mitomycin C (0.25 μg/ml) (Mit) for 10 min; or trimethoprim (2 μg/ml) (Tm) for 1 h; (ii) incubation for 10 min at 50°C; or (iii) overexpression of MazF. MC4100*relA*/pQE*mazF* was induced with IPTG (1 mM) at 37°C for 30 min. Supernatants were obtained as described in Materials and Methods. The supernatants from cultures that were induced by antibiotics were dialyzed in Tris buffer (1 mM) at 24°C for 8 h, followed by incubation at 4°C for 12 h. The EDF activities of the supernatants were quantified as described in Materials and Methods. The supernatants of an untreated culture (NT) served as a control.

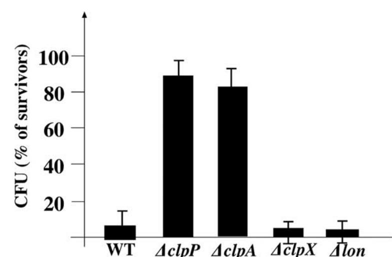


FIG. 3. Effect of various *E. coli* proteases on the EDF response. *E. coli* strains MC4100*relA1* (WT), MC4100*relA1*Δ*clpP* (Δ*clpP*), MC4100*relA1*Δ*clpA* (Δ*clpA*), MC4100*relA1*Δ*clpX* (Δ*clpX*), and MC4100*relA1*Δ*lon* (Δ*lon*) were grown as described in Materials and Methods. When the cultures reached a density of 2.5×10^8 cells/ml, duplicate samples were removed and diluted to 3×10^4 cells/ml in prewarmed (37°C) M9 medium containing chemically synthesized EDF (0.05 μg/ml). The samples were incubated without shaking at 37°C for 10 min. Samples were further incubated without shaking at 37°C with rifampin (25 μg/ml). For the rest of the experiment, see Materials and Methods.

luted culture of MC4100*relA*$^+$Cam^s, to serve as the EDF "recipient." As shown in Fig. 1A, in the presence of EDF produced by the donor subpopulation, chloramphenicol triggered *mazEF*-mediated cell death in the Cam^s recipient subpopulation. On the other hand, *mazEF*-mediated cell death did not occur when the donor subpopulation was diluted (Fig. 1B). Our results suggest that the EDF produced by the dense, Cam^r subpopulation could act as a signal molecule for the *mazEF*-mediated cell death of cells in the diluted, Cam^s subpopulation.

***mazEF* affects both EDF production and response.** Stressful conditions have been previously shown to prevent the expression of chromosomally borne *mazE*, leading to the reduction in MazE and thereby permitting MazF to exert its toxic effect (24, 44, 45). We call the effect of such stressful conditions "activation of *mazEF*." Here, we wished to compare the EDF activities of the supernatants of dense cultures that had been subjected to various stressful conditions. To this end, we developed an assay to quantify the EDF activity of the supernatant. This assay compares the dilution factor of the supernatant that permits 70% loss of viability of MC4100*relA*$^+$ (wild type [WT]) that serves as a recipient (diluted culture). We triggered the *mazEF* module by subjecting bacterial cultures to various specific stressful conditions by subjecting the donor strain MC4100*relA*$^+$ (dense culture) to them and then determined the resulting levels of EDF production by measuring the EDF activity of its supernatant. As shown, the level of EDF is significantly increased by all applied stressful conditions, although not always at the same level (Fig. 2A). Inducing *mazEF* by subjecting the cultures to a high temperature or to the chemical inhibitors of transcription and/or translation by rifampin or chloramphenicol led to an intermediate level (2.5 to 4.0 units/ml) of EDF production. Inducing *mazEF* by subjecting the cultures to agents damaging DNA either directly or indirectly, such as trimethoprim, nalidixic acid, or mitomycin C, led to a high level (6.6 to 8.0 units/ml) of EDF production (Fig. 2A). Overproducing MazF from a plasmid led to the highest level (11.0 units/ml) of EDF production (Fig. 2A). Thus, the activation of *mazEF* causes a significant increase in EDF production. Additional evidence for the role of *mazEF* in EDF production is our finding that the supernatant of logarithmic Δ*mazEF* cells lacked EDF activity (Fig. 2B). However, under various stressful conditions, about 10% of EDF activity was still detected in the supernatant of the Δ*mazEF* strain, compared to that of the WT (Fig. 2B). These results imply that EDF production is affected by the *mazEF* module but is not completely dependent on it.

Concerning the response to EDF, it was previously shown that it is completely dependent on *mazEF* at EDF concentrations of 1 to 200 μg/ml (32).

Effects of various *E. coli* proteases on EDF response and production. It was previously demonstrated that (i) activation of *mazEF* is required for the EDF response (32) and that (ii) ClpAP is responsible for MazE degradation (3); therefore, this protease permits MazF to act freely. So we were not surprised to find that neither Δ*clpP* nor Δ*clpA* recipient cultures responded to the chemically synthesized EDF (Fig. 3). On the other hand, the response to the chemically synthesized EDF (Fig. 3) was not affected in strains from which we deleted *clpX* or *lon*. These results further support the previous finding that neither ClpX nor Lon is responsible for MazE degradation in vivo (3).

To study the involvement of several *E. coli* proteases in the

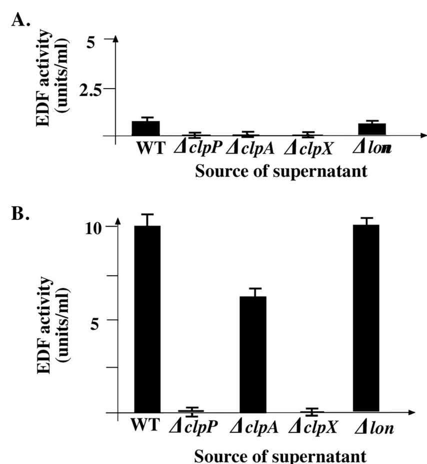


FIG. 4. Effect of various *E. coli* proteases on EDF production. (A) Supernatants of dense cultures of *E. coli* MC4100*relA1* (WT), MC4100*relA1* Δ*clpP* (Δ*clpP*), MC4100*relA1*Δ*clpA* (Δ*clpA*), MC4100*relA1*Δ*clpX* (Δ*clpX*), and MC4100*relA1*Δ*lon* (Δ*lon*) grown to mid-logarithmic phase (OD_{600} of 0.6) were prepared (see legend to Fig. 2), and EDF activities were quantified (see Materials and Methods). (B) Strains were as in panel A, but each of them harbored pQE-*mazF* to allow the overproduction of MazF (+MazF). These strains were grown in M9 with ampicillin to mid-logarithmic phase (as described for panel A). Cells were washed with and resuspended in prewarmed M9 and incubated without shaking at 37°C for 10 min. To induce *mazF*, IPTG (1 mM) was added, and the cells were incubated without shaking at 37°C for an additional 30 min. Supernatants were obtained from these cells as described for panel A.

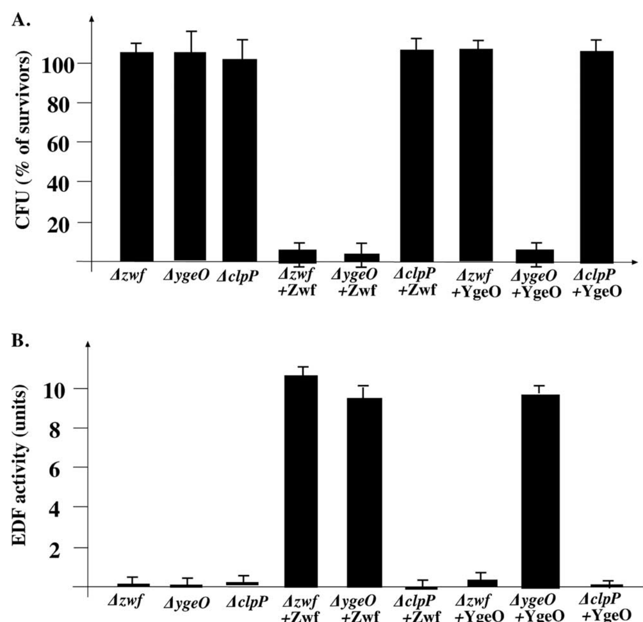


FIG. 5. *zwf* and *clpP* but not *ygeO* are required for cell death (A) and EDF production (B). (A) *E. coli* strains MC4100*relA*$^+$Δ*zwf* (Δ*zwf*), MC4100*relA*$^+$Δ*ygeO* (Δ*ygeO*), and MC4100*relA*$^+$Δ*clpP* (Δ*clpP*) with or without plasmids pQE*zwf* (+Zwf) or pQE*ygeO* (+YgeO) were grown to mid-logarithmic phase (OD_{600} of 0.6) as described for Fig. 4. When the cultures reached a density of 2.5×10^8 cells/ml, duplicate samples were removed, and cells containing the plasmids were induced using 1 mM of IPTG and incubated without shaking at 37°C for 10 min. Samples were further incubated without shaking at 37°C with rifampin (10 μg/ml). For the rest of the experiment, see Materials and Methods. (B) Cultures of the *E. coli* strains described for panel A were grown in M9 with or without ampicillin to mid-logarithmic phase. In order to examine the effect of YgeO or Zwf, these proteins were induced in a manner similar to that of MazF (as described for Fig. 4B). The EDF activity of the collected supernatants was examined as described for Fig. 4B.

production of EDF, we prepared supernatants of untreated dense cultures of *E. coli* strain MC4100*relA1* (WT) and of its derivatives with deletions of *clpP*, *clpA*, *clpX*, or *lon* (see Materials and Methods). We observed EDF production in the supernatants from WT and Δ*lon* donor cultures but not in the supernatants from Δ*clpP*, Δ*clpA*, or Δ*clpX* donor cultures (Fig. 4A), suggesting that Lon is not involved in EDF production. Since we found that the activation of *mazEF*, in which ClpAP is involved, is required for EDF production (Fig. 2), we were not surprised to see that deleting *clpP* or *clpA* from the dense donor cells resulted in supernatants with no EDF activity (Fig. 4A). However, since ClpX is not involved in *mazEF* activity (3), we were surprised to see that the supernatant from a Δ*clpX* donor also had no EDF activity (Fig. 4A). To test if ClpX might affect EDF production independently of the action of the *mazEF* module, we used donor strains that could overproduce MazF: the same *E. coli* strains described above, but this time harboring plasmid pQE-*mazF*, so that inducing them with IPTG resulted in an overproduction of MazF (see legend to Fig. 2). In the WT strain, overproducing MazF led to 10 times more EDF production than in an untreated culture (Fig. 2, compare NT with MazF). As we expected, we observed a similarly high level of EDF in the supernatant of the Δ*lon* culture (Fig. 4B) but, again as expected, we observed no EDF activity in the supernatant of the Δ*clpP* or Δ*clpX* donor cultures (Fig. 4B). These results support our hypothesis that ClpXP is required for EDF production but is independent of *mazEF* activation. In a Δ*clpA* culture that overproduces MazF, EDF production was reduced compared to that in the WT culture (Fig. 4B). However, the Δ*clpA* culture still produced significant amounts of EDF when MazF was overproduced (Fig. 4B). Therefore, it seems that ClpA does not directly participate in EDF production and that its effect is indirect.

Zwf, but not YgeO, is necessary for cell death and EDF production. The previously described data analysis and genetic experiments indicate that the genes *zwf* (encoding glucose-6-phosphate dehydrogenase) and *ygeO* (encoding an unknown protein) are involved in EDF production (32). It was suggested Zwf is the EDF precursor and YgeO is indirectly involved in EDF production (32). In order to support this idea, we cloned *zwf* and *ygeO* under the control of the *lac* operator and performed a series of complementation experiments using strains with deletions of either chromosomal *zwf* or *ygeO*. We studied loss of viability (Fig. 5A) and EDF production (Fig. 5B). As shown, overproduction of Zwf complemented these characteristics in both a Δ*zwf* strain and a Δ*ygeO* strain (Fig. 5). On the other hand, overproduction of YgeO which, as expected, complemented the chromosomal deletion of *ygeO*, did not complement a chromosomal deletion of *zwf* (Fig. 5). In addition, we have shown that the overproduction of neither Zwf nor YgeO was able to complement a chromosomal deletion of *clpP* (Fig. 5). These results indicate that Zwf and ClpP are involved primarily in EDF production, while YgeO has only a secondary role.

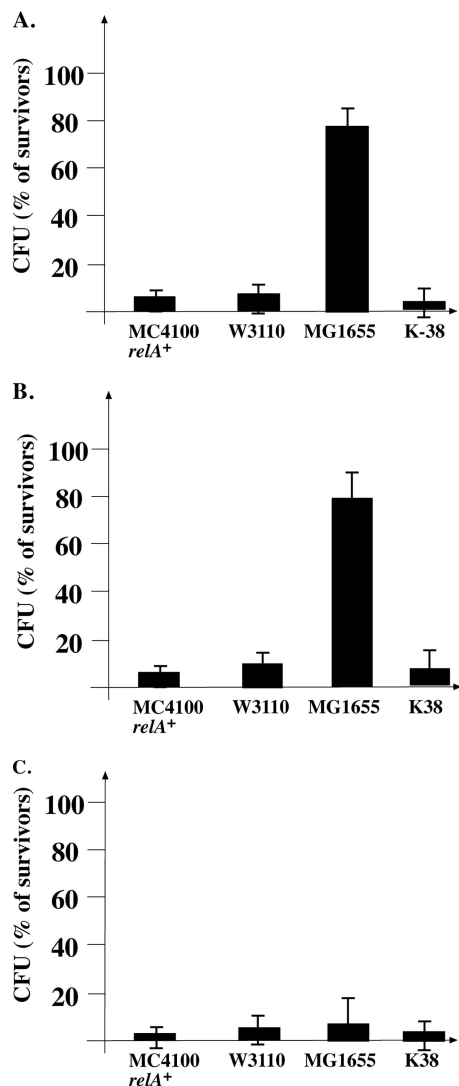


FIG. 6. Comparison of the EDF responses of various *E. coli* strains. *E. coli* strains MC4100*relA*$^+$, K-38, MG1655, and W3110 were grown to mid-logarithmic phase (OD_{600} of 0.6) (2.5×10^8 cells/ml). Duplicate samples were removed and diluted to 3×10^4 cells/ml in preheated supernatant of a dense culture of MC4100*relA*$^+$ (WT) (A), with chemically synthesized EDF in concentrations of 0.01 μg/ml (B) or 0.05 μg/ml (C). After incubation without shaking at 37°C for 10 min, rifampin (10 μg/ml) was added, and the culture was incubated without shaking at 37°C for an additional 10 min. For the rest of the experiment, see Materials and Methods.

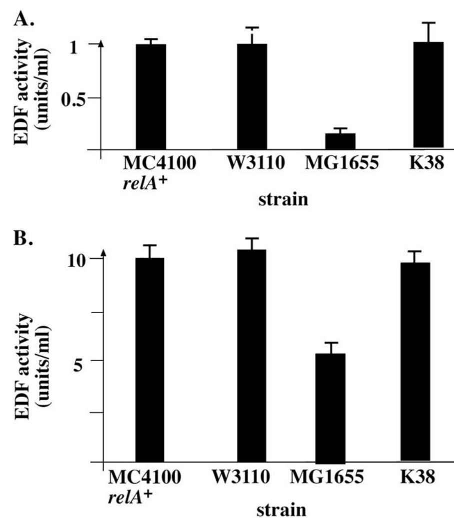


FIG. 7. Comparison of EDF production levels of various *E. coli* strains. *E. coli* strains MC4100*relA*$^+$, K-38, MG1655, and W3110 were grown to mid-logarithmic phase (OD_{600} of 0.6) as described in the legend to Fig. 2. Supernatants of these untreated dense cultures (NT) were obtained (see legend to Fig. 2), and their activities were quantified (see Materials and Methods) (A). Supernatants were also obtained from cultures of the parallel strains harboring pQE-*mazF* in which MazF was overproduced (+MazF), which were grown in M9 medium with added ampicillin to mid-logarithmic phase, and *mazF* was induced (see the legend to Fig. 4) (B). Finally, supernatants were obtained and EDF activities were quantified (see Materials and Methods). After incubation without shaking at 37°C for 10 min, rifampin (10 μg/ml) was added, and the culture was incubated without shaking at 37°C for an additional 10 min. For the rest of the experiment, see the legend to Fig. 2.

EDF production and response in various *E. coli* strains. Having done most of our work with *E. coli* strains developed from the parent strain MC4100, we also wished to compare EDF production and response levels in dense donor and dilute cultures of *E. coli* strains K38 (32), W3110, and MG1655 (Fig. 6 and 7). We found similar EDF responses and production levels for *E. coli* strains MC4100*relA*$^+$, K38, and W3110 (Fig. 6 and 7). However, *E. coli* strain MG1655 was found to be defective in both EDF response (Fig. 6) and production (Fig. 7). In this strain, *mazEF*-mediated cell death did not occur when we applied to its diluted culture a supernatant of a dense culture of MC4100*relA*$^+$ (Fig. 6A). EDF response was observed only with the addition of 0.05 μg/ml of chemically synthesized EDF (Fig. 6C), which is five times higher than the concentration required for an EDF response in other tested *E. coli* strains (Fig. 6B). In addition, in contrast to the other tested strains, EDF activity in the supernatant of untreated MG1655 culture was not observed (Fig. 7A). Only the overproduction of MazF, which induced very high levels of EDF activity in other strains, permitted the detection of EDF activity in the supernatant of *E. coli* strain M61655 (Fig. 7B). The defect of *E. coli* strain M61655 in both EDF response and production explains our failure (Fig. 8) and that of others (20, 51) to show *mazEF*-mediated cell death in MG1655. These results (Fig. 6 and 7) were obtained with MG1655 from our strain collection. Similar results for EDF production and response were obtained with MG1655 received from the laboratories of Marlene Belfort (Albany, NY) and Orna Amster-Choder (Jerusalem, Israel) (data not shown).

Though both MG1655 and W3110 are considered to be the strains most closely related to *E. coli* strain K-12 (22), we found that signaling by EDF required very high concentrations of EDF only in strain MG1655 but not in strain W3110. We are currently studying the nature of this difference between strains MG1655 and W3110.

DISCUSSION

In a previous report from this laboratory, it was shown (32) that *mazEF*-mediated cell death is a population phenomenon

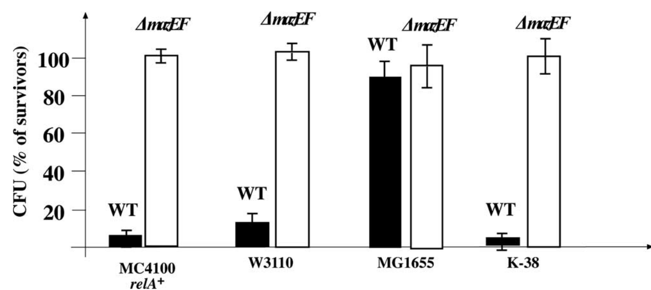


FIG. 8. Comparison of *mazEF*-mediated cell death in various *E. coli* strains. *E. coli* strains MC4100*relA*$^+$, K38, MG1655, and W3110 were grown to mid-logarithmic phase (OD_{600} of 0.6) (2.5×10^8 cells/ml) as described in the legend to Fig. 2. When the cultures reached this density, duplicate samples were removed; rifampin (10 μg/ml) was added, and the culture was incubated without shaking at 37°C for an additional 10 min. For the rest of the experiment, see Materials and Methods.

that depends on the density of the bacterial culture; death occurs in a dense culture, while not in a diluted one. In addition, *mazEF*-mediated cell death is dependent on a quorum-sensing factor that we named EDF. It was demonstrated that (i) EDF is required when the *mazEF*-mediated cell death is triggered by all studied stressful conditions, (ii) EDF is the linear pentapeptide NNWNN, and (iii) each of the five EDF amino acids is important for its *mazEF*-mediated killing activity (32). Here, we further showed that EDF is a signal molecule capable of conveying a signal from a dense subpopulation to a diluted subpopulation (Fig. 1).

Here, we were also interested to note that there was a positive feedback loop between the *mazEF* module and EDF signaling: EDF permitted the activation of MazF, and EDF action was dependent on the presence of the *mazEF* module in the recipient cells (32). We showed that MazF activation led to an increase in the production of EDF (Fig. 2), resulting in an increase in cell death. That EDF is an integral part of the *mazEF* system is supported by our results showing that both EDF response and EDF production were dependent on ClpAP (Fig. 3 and 4), the protease that is responsible for MazE degradation (3). Our finding of EDF response (reflected by *mazEF*-mediated cell death) as well as EDF production in a Δ*lon* strain (Fig. 3 and 4), further supports our previous results that Lon protease does not participate in MazE degradation (3).

Our results also suggest that ClpXP is involved in EDF production: in the supernatant of a Δ*clpX* strain, we found no EDF activity (Fig. 4), but we did observe an EDF response in a Δ*clpX* strain (Fig. 3A). Since ClpXP does not participate in MazF activation (3), this result is significant. Even when we used a Δ*clpX* donor culture that overproduced MazF, we observed no EDF activity (Fig. 4). Based on preliminary results (32), the *zwf* gene product (glucose-6-phosphate-dehydrogenase), carrying the amino acid sequence NNWDN may be the precursor of EDF. A subsequent amidation step may generate the full NNWNN sequence. Amidation may occur before or after cleavage of the precursor by one of the *E. coli* proteases (32). The herein-described results indicate that Zwf (Fig. 5) and ClpXP protease (Fig. 3 to 5) have a primary role in EDF production. We suggest that ClpXP is the Zwf-cleaving protease involved in the generation of EDF. The biochemical steps involved in the generation of EDF as well as the involvement of ClpXP are under current investigation in our laboratory.

Our previous results (32) and the results described herein clearly show that *mazEF*-mediated cell death is absolutely dependent on EDF. Therefore, we emphasize that studies on *E. coli mazEF*-mediated cell death (and maybe that of other bacteria as well) should use a protocol in which an active EDF is produced at the required concentrations. Therefore, the density of the bacterial culture (3×10^8 to 5×10^8 cells/ml) is crucial for the success of these experiments. This was one of our experimental conditions that was not followed by Tsilibaris and colleagues; therefore, they failed to show *E. coli mazEF*-mediated cell death (51). In addition, our experiments revealed that in contrast to *E. coli* strains MC4100, K38, and W3110, strain MG1655 does not produce EDF under normal growth conditions (Fig. 7A) and only weekly responds to EDF (Fig. 6). Therefore, *mazEF*-mediated cell death does not occur in this strain (Fig. 8). We found that in regard to EDF, MG1655 is defective. It may be that the laboratory strain MG1655 was derived by selection against genes promoting cell death.

So far, bioluminescence, virulence factor expression, biofilm formation, sporulation, mating, and competence for DNA uptake have all been described as being regulated by quorum sensing (see reviews mentioned above). The results of the work that we describe here add to this list another important biological phenomenon: bacterial programmed cell death. In previous work, we showed that *mazEF* prevents the spread of phage infection (26). Previously (32) and here, we show that *E. coli mazEF*-directed death is mediated by the communication factor EDF, which is the pentapeptide NNWNN. These findings firmly support our view that bacterial programmed cell death is a fundamental characteristic of the multicellular behavior of bacteria (15). Programmed cell death is unproductive when undertaken by an individual bacterium, but it might be beneficial as a group strategy in which a subpopulation of cells die and release nutrients (15) and/or signaling molecules (32). Extracellular signals such as EDF may be very helpful in coordinating or regulating such a group strategy.

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