Novel Quorum-Sensing Peptides Mediating Interspecies Bacterial Cell Death

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ABSTRACT *Escherichia coli* mazEF is a toxin-antitoxin stress-induced module mediating cell death. It requires the quorum-sensing signal (QS) “extracellular death factor” (EDF), the penta-peptide NNWNN (EcEDF), enhancing the endoribonucleolytic activity of *E. coli* toxin MazF. Here we discovered that *E. coli* mazEF-mediated cell death could be triggered by QS peptides from the supernatants (SN) of the Gram-positive bacterium *Bacillus subtilis* and the Gram-negative bacterium *Pseudomonas aeruginosa*. In the SN of *B. subtilis*, we found one EDF, the hexapeptide RGQNE, called BsEDF. In the SN of *P. aeruginosa*, we found three EDFs: the nonapeptide INEQTVVTK, called BsEDF-2, and APKLSDGAAAGYVTKA, called BsEDF-3. When added to a diluted *E. coli* cultures, each of these peptides acted as an interspecies EDF that triggered mazEF-mediated death. Furthermore, though their sequences are very different, each of these EDFs amplified the endoribonucleolytic activity of *E. coli* MazF, probably by interacting with different sites on *E. coli* MazF. Finally, we suggest that EDFs may become the basis for a new class of antibiotics that trigger death from outside the bacterial cells.

IMPORTANT Bacteria communicate with one another via quorum-sensing signal (QS) molecules. QS provides a mechanism for bacteria to monitor each other’s presence and to modulate gene expression in response to population density. Previously, we added *E. coli* EDF (EcEDF), the peptide NNWNN, to this list of QS molecules. Here we extended the group of QS peptides to several additional different peptides. The new EDFs are produced by two other bacteria, *Bacillus subtilis* and *Pseudomonas aeruginosa*. Thus, in this study we established a “new family of EDFs.” This family provides the first example of quorum-sensing molecules participating in interspecies bacterial cell death. Furthermore, each of these peptides provides the basis of a new class of antibiotics triggering death by acting from outside the cell.

Toxin-antitoxin modules are found on the chromosomes of most bacteria, including pathogens (1–7). Each of these modules consists of a pair of genes, of which the downstream gene generally codes for a stable toxin and the upstream gene codes for a labile antitoxin. In *Escherichia coli*, seven well-established toxin-antitoxin systems have been described (8–14). Among these, the most studied is mazEF, which was the first to be described as capable of regulation and responsible for bacterial programmed cell death (1, 8). *E. coli* mazF specifies for the stable toxin MazF, and mazE specifies for the labile antitoxin MazE. *In vivo*, MazE is degraded by the ATP-dependent ClpAP serine protease (8). MazF is a sequence-specific endoribonuclease that preferentially cleaves single-stranded mRNAs at ACA sequences (15, 16). MazE counteracts the action of MazF. The most intimate interaction between MazE and MazF is between tryptophan 73 of the C-terminal portion of MazE and a hydrophobic pocket located near the root of the S1-S2 loop on the MazF surface (17). 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 leads to the reduction of MazE in the cell, permitting toxin MazF to act freely. Such conditions include inhibition of transcription and/or translation by application of antibiotics for a short period (18), severe amino acid starvation and thus overproduction of ppGpp (8), and DNA damage (18, 19). Such stressful conditions have been found to act in *E. coli* through the mazEF module (18, 20, 21). We have recently shown that MazF cleaves at ACA sites at or closely upstream of the AUG start codon of some specific mRNAs and thereby generates leaderless mRNAs. Moreover, MazF also targets 16S rRNA within 30S ribosomal subunits at the decoding center, thereby removing the anti-Shine-Dalgarno (aSD) sequence that is required for translation initiation on canonical mRNAs. Thus, under stressful conditions, when MazF is triggered, alternative translation machinery is generated. The machinery consists of a subpopulation of ribosomes that selectively translate leaderless mRNAs (22). This stress-induced translation machinery is responsible for the selective synthesis of specific proteins due to MazF induction (23). 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. We have recently reported that *E. coli* mазEF-mediated cell death is a population phenomenon in which bacterial cells communicate with each other through the *E. coli* quorum-sensing (QS) factor EDF (extracellular death factor) (24, 25). Here we designate *E. coli* EDF EcEDF. Structural analysis revealed that EcEDF is the linear pentapeptide NNWNN required for triggering mазEF-mediated cell death (25). In addition, we have recently shown that the *E. coli* EcEDF enhances the endoribonuclease activity of *E. coli* MazF in vitro (26).

Here we report on the identification and characterization of EDFs from two different species of bacteria, *Bacillus subtilis* and *Pseudomonas aeruginosa*. *B. subtilis* produced one EDF, the hexapeptide (six-amino-acid) RGQQNE, which we designated BsEDF. *P. aeruginosa* produced three EDFs: one nonapeptide (nine amino acids), INEQTVVTK, and two hexadecapeptides (16 amino acids), VEVSDDGSGGNTSLSQ and APKLSDGAAGYVTKA, designated PaEDF-1, PaEDF-2, and PaEDF-3 (PaEDF-1/2/3), respectively. Under stressful conditions, each of these peptides acted as an interspecies extracellular death factor (EDF). When added to a diluted culture of *E. coli* or *B. subtilis*, they triggered mазEF- or ydcDE-mediated death, respectively. Here we introduce these various EDFs as belonging to a family of quorum-sensing peptides which permits one bacterial species to kill another when the first is at high population density. In addition, though their sequences are very different, each of these EDFs enhanced the endoribonuclease activity of *E. coli* MazF in vitro, probably by interacting with different sites of *E. coli* MazF.

**RESULTS**

The supernatant of a dense culture of *B. subtilis* or *P. aeruginosa* restores mазEF-mediated cell death when added to a diluted culture of *E. coli*. Previously, we reported that mазEF-mediated cell death takes place in dense cultures of *E. coli* (25). Moreover, the supernatant (SN) of a dense culture of *E. coli* restores mазEF-mediated cell death when added to a diluted culture of *E. coli* (25). Here we asked whether, when added to a diluted *E. coli* culture (2.5 × 10^6 cells/ml), the SN of a dense culture (about 2.5 × 10^8 cells/ml) of *B. subtilis* or of *P. aeruginosa* would lead to mазEF-mediated cell death in *E. coli*. We prepared SNs from dense cultures of *B. subtilis* or of *P. aeruginosa* and added them separately to diluted cultures of *E. coli* (see Materials and Methods). We added rifampin to induce mазEF-mediated cell death. We observed no cell death in diluted cultures to which we added no SNs. Adding either *B. subtilis* SN (Fig. 1A) or *P. aeruginosa* SN (Fig. 1B) led to mазEF-mediated death for these *E. coli* cells. Note that we observed no cell death when we added each of these SNs to diluted cultures of a ΔmазEF *E. coli* derivative. These results suggested to us that each of the SNs of *B. subtilis* and of *P. aeruginosa* contained an “extracellular death factor” (EDF) that caused the mазEF-mediated death of *E. coli* cells.

BsEDF, the EDF of *B. subtilis*, is the hexapeptide RGQQNE. To characterize the chemical nature of BsEDF, we purified it from a large volume of a supernatant of a mid-logarithmic culture of *B. subtilis* grown in LB medium. After centrifugation, we collected the supernatant and separated fractions on a C-18 SepPak cartridge (Fig. 1C and Materials and Methods). We purified the active fractions by high-performance liquid chromatography (HPLC); we found *P. aeruginosa* EDF activity in the fraction eluted at 16.32 min (Fig. 1F; see also Fig. S1B in the supplemental material and Materials and Methods). We purified the active fractions by high-performance liquid chromatography (HPLC); we found *P. aeruginosa* EDF activity in the fraction eluted at 16.32 min (Fig. 1F; see also Fig. S1B in the supplemental material and Materials and Methods). Performing electrospray ionization-mass spectrometry (ESI-MS) on this fraction revealed three separate peptides (see Fig. S2 in the supplemental material): (i) the nonapeptide (9-amino-acid) INEQTVVTK (PaEDF-1), (ii) the hexadecapeptide (16-amino-acid) VEVSDDGSGGNTSLSQ (PaEDF-2), and (iii) the hexadecapeptide APKLSDGAAGYVTKA (PaEDF-3). To test whether all of the three peptides, PaEDF-1, PaEDF-2, and PaEDF-3, were indeed PaEDFs, we synthesized identical peptides chemically and tested them for biological EDF activity. When added individually to diluted *E. coli* cultures, these synthetic peptides enabled mазEF-mediated cell death induced by rifampin; these synthetic PaEDF peptides acted just like the EcEDF peptide (Fig. 2A). We found that, to cause death in an *E. coli* culture, there was an absolute requirement for the second (glycine) and the fifth (asparagine) amino acids. Replacing the third (glutamine), fourth (glutamine), and sixth (glutamic acid) amino acids resulted in partially reduced activity. Replacing the first amino acid (arginine) did not affect the EDF activity at all (Fig. 2A).

**P. aeruginosa** produces three different EDFs: INEQTVVTK, VEVSDDGSGGNTSLSQ, and APKLSDGAAGYVTKA. To characterize the chemical nature of *P. aeruginosa* EDF, we purified it from a large volume of a supernatant of a mid-logarithmic-phase culture of *P. aeruginosa* grown in M9 minimal medium. After centrifugation, we collected the supernatant and separated fractions on a C-18 SepPak cartridge (Fig. 1D and Materials and Methods). We purified the active fractions by high-performance liquid chromatography (HPLC); we found *P. aeruginosa* EDF activity in the fraction eluted at 16.32 min (Fig. 1F; see also Fig. S1B in the supplemental material and Materials and Methods). Performing electrospray ionization-mass spectrometry (ESI-MS) on this fraction revealed three separate peptides (see Fig. S2 in the supplemental material): (i) the nonapeptide (9-amino-acid) INEQTVVTK (PaEDF-1), (ii) the hexadecapeptide (16-amino-acid) VEVSDDGSGGNTSLSQ (PaEDF-2), and (iii) the hexadecapeptide APKLSDGAAGYVTKA (PaEDF-3). To test whether all of the three peptides, PaEDF-1, PaEDF-2, and PaEDF-3, were indeed PaEDFs, we synthesized identical peptides chemically and tested them for biological EDF activity. When added individually to diluted *E. coli* cultures, these synthetic peptides enabled mазEF-mediated cell death induced by rifampin; these synthetic PaEDF peptides acted just like the synthetic EcEDF peptide (Fig. 2B) and the synthetic BsEDF peptide (Fig. 2B). Again, we observed no cell death when we induced the cultures of an *E. coli* ΔmазEF derivative strain by the use of rifampin (Fig. 2B).

BsEDF, EcEDF, and PaEDFs trigger ydcDE-mediated cell death when added to a diluted culture of *B. subtilis*. As we described above, BsEDF, EcEDF, and PaEDFs all acted as extracellular death factors involved in *E. coli* mазEF-mediated cell death (Fig. 2A and B). In *B. subtilis*, ydcDE is an operon which encodes a TA module that belongs to the mазEF family (27). The toxin encoded by ydcE is an RNase called EndoA, which is an endoribonuclease whose cleavage specificity is different from that of *E. coli* MazF (27). As we asked in the case of *E. coli*, here also we asked whether these different EDFs could also lead to ydcDE-mediated cell death of *B. subtilis*. We have shown that *B. subtilis* cell death is density dependent; it occurred only in dense cultures and not in diluted cultures (Fig. 2C and D). Also, it is triggered by stressful...
conditions, as shown here by the addition of chloramphenicol (Fig. 2C and D). In addition, it is mediated by the ydcDE system, since death does not occur in a dense culture of a B. subtilis ΔydcDE derivative (Fig. 2C and D). Here we showed that adding each of the EDFs from E. coli (Fig. 2C) or B. subtilis (Fig. 2C) or P. aeruginosa (Fig. 2D) to diluted B. subtilis cultures resulted in ydcDE-mediated cell death. Thus, in the case of B. subtilis, cell death can be triggered not only by the BsEDF but also by the EDFs of the other two bacterial species, E. coli and P. aeruginosa. In addition, as we asked in the case of E. coli, we asked here if the activity of the BsEDF hexapeptide required each of its six amino acids. To cause cell death when added to cultures of either E. coli or B. subtilis, the first amino acid (arginine) of BsEDF was not required, while the second amino acid (glycine) was absolutely required (Fig. 2A and C). However, there the similarity ended (compare Fig. 2A and C). On the other hand, it seems that for the action

FIG 1 The effect of the supernatants from dense cultures of B. subtilis (A) or P. aeruginosa (B) on mazEF-mediated cell death in E. coli and the purification (C and D) and analysis of the activities (E and F) of the EDFs from those cultures. (A and B) Wild-type E. coli MC4100relA⁺ (WT) or E. coli MC4100relA⁺ΔmazEF (ΔmazEF) was grown as described in Materials and Methods. Cells were either not diluted (Dense) or diluted to a density of 3 × 10⁴ cells/ml in prewarmed M9 medium (M9) or in a prewarmed supernatant of a dense culture (SN) of B. subtilis PY79 (A) or of P. aeruginosa PA14 (B). The samples were incubated without shaking at 37°C for 10 min and for another 10 min with rifampin (10 µg/ml). Loss of viability was determined by CFUs. (C and D) Purification of BsEDF (C) or PaEDF (D): milliabsorbance at 220 nm was determined during elution from the HPLC column of purified supernatant. (E and F) BsEDF activity (E) or PaEDF activity (F) plotted as a function of the elution time from the HPLC column as marked by the arrows in panels C and D.
of BsEDF in enabling B. subtilis cell death to occur, there is a more absolute requirement of the wild-type (WT) BsEDF sequence than is the case for E. coli cell death. In BsEDF, in addition to the second (glycine) amino acid, the third (glutamine), fourth (glutamine), and sixth (glutamic acid) amino acids were required, and the fifth (asparagine) amino acid was partially required (Fig. 2C). As in the case of E. coli mazEF-mediated cell death, also, in cultures of B. subtilis, each of the three PaEDFs enabled ydcD-EDF-mediated cell death (compare Fig. 2B and D).

**BsEDF and PaEDFs enhance the in vitro endoribonucleolytic activity of E. coli MazF.** Previously, we showed that EcEDF triggers mazEF-mediated cell death (25) and enhances the in vitro endoribonucleolytic activity of E. coli MazF (26). Having shown here that BsEDF and each of the PaEDFs triggered mazEF-mediated cell death in E. coli (Fig. 2A and B), we asked if they would also enhance the in vitro endoribonucleolytic activity of E. coli MazF. To examine the effect of BsEDF or each of the PaEDFs on E. coli MazF activity, we used a highly purified preparation of E. coli MazF containing no other protein. Measuring MazF activity using a continuous fluorometric assay (Fig. 3A) (25), as described in reference 28, enabled us to make a real-time quantification and kinetic analysis of the MazF endoribonucleolytic activity. As a substrate for MazF, we used chimeric oligonucleotides composed of a single RNA base (Fig. 3A) or five RNA bases (Fig. S3A) flanked by DNA nucleotides labeled with a fluorophore molecule (6-carboxyfluorescein [FAM]) at their 5′ ends and a quencher molecule (black hole quencher-1 [BHQ1]) at their 3′ ends. We found that, in vitro, adding BsEDF and each of three PaEDFs led to increased MazF activity in a concentration-dependent manner (Fig. 3B to E; see also Fig. S3B and C in the supplemental material, left panels). The addition of 1.5, 3.75, and 7.5 μM BsEDF led to enhancement of MazF activity by approximately 34%, 75%, and 100%, respectively. Adding 7.5 μM EcEDF or 7.5 μM BsEDF led to nearly equal levels of MazF enhancement (Fig. 3B, right panel). Similarly, each of the three EDFs from P. aeruginosa, PaEDF-1, PaEDF-2, and PaEDF-3, also enhanced the in vitro activity of MazF but did so less efficiently than did the BsEDF. Adding 1.5, 3.75, or 7.5 μM PaEDF-1, PaEDF-2, or PaEDF-3 increased in vitro MazF activity by only about 20%, 35%, or 47%, respectively (compare Fig. 3B with Fig. 3C to E). Thus, PaEDF-1, PaEDF-2, and PaEDF-3 each enhanced the in vitro MazF endoribonucleolytic activity but did so less efficiently than did the EcEDFs or BsEDF (Fig. 3C to E, right panel). We confirmed these results in two ways: by testing another chimeric oligonucleo-
otide composed of five RNA bases at and near the cleavage site (Fig. S3A) and by using an S30 cell-free extract in vitro protein synthesis system (Fig. S4A and C) which revealed similar results. Here we also asked if each amino acid in BsEDF was required for the in vitro enhancement of E. coli MazF activity. To examine this, we observed the activities of the synthetic BsEDF mutants in which we had replaced each of the original amino acids with an alanine (A) residue (see above). We found that replacing either the second (glycine) or the fifth (asparagine) amino acid completely prevented the ability of BsEDF to enhance the activity of E. coli MazF (Fig. 4B and E). On the other hand, replacing the fourth (glutamine) or the sixth (glutamic acid) amino acid only partially prevented the ability of BsEDF to enhance the activity of E. coli MazF (Fig. 4D and F). However, replacing the first (arginine) or the third (glutamine) amino acid had only a minimal effect (Fig. 4A and C). Thus, we found that for enhancing MazF activity, the second and the fifth amino acids of BsEDF are completely required, the fourth and six are only partially required, and the first and third are not required at all. More specifically, for both the in vivo effects of BsEDF on E. coli cell death (Fig. 2A) and the in vitro effects of BsEDF on E. coli MazF activity (Fig. 4; see also Fig. S3B in the supplemental material), the second and fifth amino acids are required absolutely, but the first amino acid is not required at all.

We also found that BsEDF bound directly to E. coli MazF. We confirmed this direct interaction using an affinity column to which we coupled either the synthetic WT BsEDF or the synthetic mutant in which we substituted A in position 2 (RAQQNE). Through this column, we passed either highly purified E. coli MazF or the antitoxin MazE; we analyzed the fractions by SDS-PAGE (Fig. 5A) and the Bradford assay (Fig. 5B). SDS-PAGE analysis revealed that MazF (but not MazE) had a high affinity for the

FIG 3 BsEDF and each of PaEDFs enhance the in vitro MazF endoribonucleolytic activity. (A) Illustration of the reaction used for studies on the endoribonucleolytic activity of MazF. Cleavage of the chimeric fluorescent oligonucleotides by MazF was expressed as an increase of fluorescence emission of the fluorophore FAM. “G” represents an RNA base. The cleavage site was at the 5′ side of ACA. (B) The effect of BsEDF (RGQQNE) on MazF activity. (Left panel) BsEDF at 1.5 μM or 3.75 μM or 7.5 μM was added to a reaction mixture containing 0.3 μM MazF, and the activity of MazF was determined. (Right panel) The relative (%) increase of MazF activity induced by BsEDF. MazF activity in the presence of 7.5 μM EcEDF (NNWNN) was defined as the positive control. MazF activity without the addition of BsEDF was assigned a value of 100%. (C) The effect of PaEDF-1 on MazF activity. Different concentrations of PaEDF-1 were added to the same reaction mixture as described for panel B. (D) The effect of PaEDF-2 on MazF activity. Various concentrations of PaEDF-2 were added to the same reaction mixture as described for panel B. (E) The effect of PaEDF-3 on MazF activity. Various concentrations of PaEDF-3 were added to the same reaction mixture as described for panel B. Left and right panels and experimental conditions are as described for panel B. Error bars indicate standard deviations. *, P < 0.05; **, P < 0.1; ***, P < 0.001.
BsEDF-coupled column (compare lanes 5 and 15 in Fig. 5A). In contrast, MazF had no affinity for the mutant BsEDF-coupled column (Fig. 5A, lane 10). We found similar results using the Bradford assay (Fig. 5B).

Like EcEDF, BsEDF, but not PaEDFs, overcomes the in vitro inhibitory effect of MazE on E. coli MazF endoribonucleolytic activity. We have previously reported that EcEDF can overcome the inhibitory effect of MazE on E. coli MazF (26). Here we asked whether BsEDF or the three PaEDFs could overcome the MazE inhibitory activity on E. coli MazF. To a reaction mixture containing highly purified MazF we added MazE with one of BsEDF, PaEDF-1, PaEDF-2, or PaEDF-3. We found that BsEDF could overcome the inhibitory effect of the antitoxin E. coli MazE on E. coli MazF (Fig. 6A; see also Fig. S5A in the supplemental material). Note that BsEDF overcame the inhibitory effect of MazE on MazF less efficiently than did EcEDF (NNWNN) (Fig. 6A). Moreover, similar amino acids of BsEDF were required for its ability to overcome the inhibitory effect on MazE (Fig. 6A; see also Fig. S5A in the supplemental material) and for enhancing the E. coli MazF activity in vitro (Fig. 4). In both cases, the second and fifth amino acids of BsEDF were absolutely required, the fourth and sixth were only partially required, and the first and third were not required at all.

EcEDF, BsEDF, and each of the PaEDFs could enhance the endoribonucleolytic activity of E. coli MazF, probably by interacting at different sites on MazF. We previously showed that EcEDF could enhance the in vitro endoribonucleolytic activity of E. coli MazF (26), and here we have shown that BsEDF and each of PaEDFs can do so as well (Fig. 3B to E). This was a little surprising, since the amino acid compositions of these EDFs are not at all
similar. Here we asked if they interacted at the same site or at different sites on E. coli MazF. Our first attempt to answer this question was based on our experiment showing that a mutant of EcEDF (NNGNN) interfered with the ability of WT EcEDF to enhance the activity of E. coli MazF (Fig. 7A). Therefore, we called it EciEDF. Similarly, the mutant of BsEDF RAQQNE, which we called BsiEDF, interfered with the ability of WT BsEDF to enhance the activity of E. coli MazF (Fig. 7B). However, we found that iEDFs from each species prevented the activity of the EDF of its own species but not that of the EDFs of other species. EciEDF did not affect the EDF activity of BsEDF (Fig. 7A) or any of the three PaEDFs (see Fig. S6 in the supplemental material). Similarly, BsiEDF did not affect the EDF activity of EcEDF (Fig. 7B) or any of the PaEDFs (Fig. S6). In all these cases, the concentrations of the EDF mutants were up to 14 times higher than the concentration of the wild-type EDFs. These results suggest that the EDFs of E. coli, B. subtilis, and P. aeruginosa acted on different sites in E. coli MazF (see the Discussion).

**DISCUSSION**

EDFs as a family of quorum-sensing peptides mediating cell death in bacteria. Bacteria communicate with one another via quorum-sensing signal (QS) molecules (29–36). QS provides a mechanism for bacteria to monitor each other’s presence and to modulate gene expression in response to population density. Four kinds of QS molecules have been identified. (i) In Gram-negative bacteria, the most commonly found QS signals are acylated homoserine lactones (AHLs) (29, 31, 32, 35). (ii) The bioluminescent marine bacterium Vibrio harveyi produces two kinds of QS molecules: AI-1, a typical Gram-negative-like AHL, and AI-2, a furanosyl borate diester involved in interspecies communication (37). (iii) The pathogen P. aeruginosa produces 2-heptyl-3-hydroxy-4-quinolone (PQS) (38). (iv) In Gram-positive bacteria, quorum-sensing molecules are short, usually modified peptides processed from precursors and involved in many systems, including the development of competence in B. subtilis (29). These QS molecules of Gram-positive bacteria are highly specific because each sensor oligopeptide selects for a given peptide signal (33, 34).

Previously, we added E. coli EDF (EcEDF) to this list of QS peptides (24, 25). EcEDF (NNNWW) is particularly interesting not only because no other peptide has apparently been reported to be involved in quorum sensing in E. coli but also because EcEDF appears to be a type different from the known molecules of the quorum-sensing peptides of Gram-positive bacteria. Unlike the QS peptides of Gram-positive bacteria, E. coli EDF is not derived from a small open reading frame but is rather derived from the enzyme Zwf (24, 25). Finally, we have revealed that EcEDF is peculiar because of its involvement in bacterial PCD: it triggers E. coli mazEF-mediated cell death (see the introduction).

Here we extended the group of QS peptides that can trigger E. coli mazEF-mediated cell death. We discovered that E. coli mazEF-mediated cell death could be triggered by QS peptides from the SNs of two other bacterial species, the Gram-positive bacterium B. subtilis and the Gram-negative bacterium P. aeruginosa. We were intrigued to find that these novel peptides are unlike EcEDF. In the supernatant of B. subtilis, we detected BsEDF—RGQQNE (Fig. 1A, C, and E; see also Fig. S1A in the supplemental material); in the supernatant of P. aeruginosa, we detected PaEDF-1—INEQTVVTK, PaEDF-2—VEVSDGSGSGLTLQ, and PaEDF-3—APKLSDGGAAGYVTKA (Fig. 1B, D, and F; see also Fig. S1B and S2 in the supplemental material). Under stressful conditions, when added to a diluted culture of E. coli, each of these EDFs triggered mazEF-mediated cell death (Fig. 2A and B). Thus, in addition to EcEDF, we found that these novel QS peptides of B. subtilis and P. aeruginosa were also involved in E. coli mazEF-mediated cell death.

As in E. coli, we have also found in B. subtilis a density-dependent stress-induced cell death mechanism in which BsEDF (Fig. 2C) or EcEDF (Fig. 2C) or any of the PaEDFs (Fig. 2D) can serve as a factor for ykdDE-mediated cell death. This is the first report showing that ykdDE, belonging to the mazEF family (27), triggers cell death in B. subtilis. In addition, like E. coli mazEF-mediated cell death (25), B. subtilis ykdDE-mediated cell death is

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**FIG 5** MazF directly binds to BsEDF. MazF and MazE were passed through an affinity column to which either BsEDF (RGQQNE) or BsiEDF (RAQQNE) was coupled. Fractions were analyzed by SDS-PAGE (A) and Bradford assay (B). See also Materials and Methods and main text.
also a population phenomenon that occurs only in a dense and not in a diluted culture. Also, under stressful conditions, through BsEDF, ydcDE mediates B. subtilis’s own cell death. Thus, like EcEDF, BsEDF also is a QS factor involved in the ydcDE-mediated cell death of B. subtilis: it can be replaced by the EDF of E. coli (EcEDF) or any of those of P. aeruginosa (PaEDFs). Based on the fact that PaEDFs can induce density-dependent cell death in either E. coli or B. subtilis, we also identified PaEDF-1, PaEDF-2, and PaEDF-3 as QS factors, although they are not involved in the density-independent cell death of P. aeruginosa itself (see Fig. S7 in the supplemental material). Note that P. aeruginosa is one of the rare bacteria that do not carry the mazEF module on a chromosome (P. aeruginosa genomic database). On the other hand, we are intrigued to report that, in the chromosome of P. aeruginosa, we have detected gene fliD that specifies for the flagellum-capping protein, FlID, from which all three of PaEDF-1, PaEDF-2, and PaEDF-3 are derived by proteolysis (NCBI reference sequence: YP_792175.1). The idea that all three PaEDF peptides originate from the product of fliD is further supported by our experiments showing that neither the supernatant nor the purified fractions (PF) from the /H9004 fliD derivative strain of P. aeruginosa had EDF activity (Fig. S8). On the other hand, we were not able to detect the source of BsEDF, as shown by the following approaches. (i) B. subtilis chromosome does not carry a gene that would be able to

![Graph A](image1)

**FIG 6** BsEDF and its mutants at positions 1, 3, 4, and 6 (A) but not the PaEDFs (B) overcome the inhibitory effect of MazE on the *in vitro* endoribonucleolytic activity of MazF. (A) Left panel: 0.03 μM MazE with 0.0 μM or 15 μM BsEDF (RGQQNE) or each of its six mutants (the mutated amino acid is underlined) was individually added to preparations of MazF (0.3 μM). MazF activity in the presence of 0.03 μM MazE with 15 μM EcEDF (NNWNN) was defined as a positive control. Activity overcoming the effect of MazF was determined by the use of chimeric fluorescent oligonucleotide. Right panel: the relative (%) levels of activity by BsEDF and its mutants overcoming the effect of MazF. MazF activity without the addition of MazE or BsEDF or its mutants was assigned a value of 100%. (B) Left panel: 0.03 μM MazE with 0.0 μM or 15 μM PaEDF-1 or PaEDF-2 or PaEDF-3 was individually added to preparations of MazF (0.3 μM). MazF activity in the presence of 0.03 μM MazE with 15 μM EcEDF (NNWNN) was defined as a positive control. Activity overcoming the effect of MazF was determined by the use of chimeric fluorescent oligonucleotide. Right panel: the relative (%) levels of activity by PaEDF-1, PaEDF-2, and PaEDF-3 overcoming the effect of MazF. MazF activity without the addition of MazE or PaEDF-1 or PaEDF-2 or PaEDF-3 was assigned a value of 100%. Error bars indicate standard deviations, *, P < 0.05; **, P < 0.01; ***, P < 0.001.
specify for its EDF (BsEDF). (ii) The unique BsEDF peak at 27.8 min (Fig. 1C) did not appear when LB medium was purified by the procedure used for BsEDF (data not shown). (iii) BsEDF was not derived from the proteins tryptone and those in yeast extracts; we did not find a BsEDF sequence either in tryptone or in the yeast chromosome, which specifies the proteins in yeast extract. (iv) LB medium digested with any one of the proteolytic enzymes trypsin and chymotrypsin and proteinase K did not produce BsEDF activity (Fig. S9). Therefore, we assume that BsEDF is derived by a nonribosomal mechanism as was previously reported to take place in the synthesis of some B. subtilis proteins and peptides (39, 40).

EDFs as a family of quorum-sensing peptides that enhance the in vitro endoribonucleolytic activity of E. coli MazF. Previously, we reported that, in vitro, EEDF interacts directly with E. coli MazF, enhancing its endoribonucleolytic activity (26). Here, we were surprised to find that, in spite of the sequence differences among EEDF, BsEDF, and PatEDF-1/2/3, each of these peptides enhanced the in vitro endoribonucleolytic activity of E. coli MazF (Fig. 3B to E; see also Fig. S3B and C in the suppl-

**FIG 7** The iEDF (NNGNN) of EcEDF (NNWNN) and the iEDF (RAQQNE) of BsEDF (RGQQNE) each inhibit the in vitro E. coli MazF enhancement activity of its respective EDF only. In the labels at that top of the panels, the green color represents the EDF and the iEDF of E. coli and the red color represents those of B. subtilis. (A) The effects of E. coli iEDF on the enhancement of E. coli MazF activity by EcEDF or BsEDF. (Left panel) EcEDF (15 μM or 30 μM or 50 μM) was added to a reaction mixture containing MazF (0.3 μM) with EcEDF (3.75 μM) or with BsEDF (3.75 μM). E. coli MazF activity in the presence of 3.75 μM EcEDF or 3.75 μM BsEDF was defined as the positive control for its respective EDF activity with an EcEDF with respect to MazF. MazF activity was determined by using a chimeric fluorescent oligonucleotide. (Right panel) The relative (%) increase of MazF activity caused by the addition of EcEDF or BsEDF in the presence of EcEDF MazF activity without the addition of EcEDF or BsEDF was assigned a value of 100%. (B) The effect of BsEDF on the enhancement of E. coli MazF activity by BsEDF or EcEDF. Various concentrations of BsEDF were added to the same reaction mixture as described for panel A. The left and right panels and experimental conditions were as described for panel A. Error bars indicate standard deviations. *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001.
mental material). In the case of BsEDF, we found that this effect was also sequence specific: the second (glycine) and fifth (asparagine) amino acids were definitely required (Fig. 4B and E; see also Fig. S3B in the supplemental material), closely matching those required for the in vivo effect of BsEDF on E. coli cell death (Fig. 2A). Furthermore, as we have described for EcEDF—previously called EDF (26)—here, BsEDF also overcame the inhibitory effect of E. coli MazE on E. coli MazF. This inhibitory effect was dependent on the same EDF amino acids that were required for its ability to enhance MazF activity in vitro (Fig. 6A; see also Fig. S5A in the supplemental material) and for its effect on E. coli mazEF-mediated cell death (Fig. 2A). As we previously found with respect to EcEDF (26), BsEDF bound directly to E. coli MazF in a sequence-specific manner (Fig. 5). However, as we found here, EcEDF and BsEDF affected different sites on E. coli MazF (Fig. 7; see also Fig. S6 in the supplemental material). An excess of the inhibitor of EcEDF (EcEDF NNGNN) interfered with the ability of EcEDF to enhance the action of MazF; EcEDF did not inhibit such enhancement by BsEDF (Fig. 7A). In parallel, an excess of the inhibitor of BsEDF (BsEDF RAQQQNE) inhibited the enhancement of MazF activity only by BsEDF and not of that by EcEDF (Fig. 7B). Similar competition experiments that we carried out for each of PaEDF-1/2/3 (Fig. S6) also indicated that each of these EDFs affected a particular site(s) on E. coli MazF and that these sites were different from those affected by either EcEDF or BsEDF (Fig. 7A and B; see also Fig. S6 in the supplemental material). In the case of PaEDF-1/2/3, our conclusion was further supported by our results showing that, in contrast to EcEDF and BsEDF, PaEDF-1/2/3 did not overcome the inhibitory effect of MazE on MazF (Fig. 6B; see also Fig. S5B in the supplemental material).

The results of our previous experiments examining the effect of EcEDF on E. coli MazF suggested that EcEDF competes with MazF for the binding site of the antitoxin MazE (26). Indeed, our E. coli peptide-protein interaction model suggests the presence of parallel contacts between EcEDF-MazF (26) and the MazE-MazF complex (17). In particular, our model suggests that EcEDF (NNWNN) and the binding site of MazE (locations 71 to 75; ID-WGE) compete directly for MazF. We conjecture that the critical tryptophan-3 residue in EcEDF maintains numerous hydrogen bond and electrostatic interactions that also would tether the important tryptophan-73 residue of MazE to the hydrophobic pocket of the toxin (26). Thus, it seems that the EcEDF sequence may mimic the native E. coli MazE antitoxin sequence remarkably well. Note also that the interaction of the C terminus of MazE with MazF inhibits MazF endoribonuclease activity, while the parallel interaction of EcEDF with MazF stimulates its action (26, 41, 42). These apparently opposing observations can be reconciled by the finding that MazF harbors two allosteric mRNA binding sites: when the MazE antitoxin occludes one of these positions, mRNA binding at the second site is simultaneously perturbed (42). Thus, by competitive inhibition of the MazE-MazF interaction at one site, EcEDF can promote mRNA cleavage by MazF at the second position. As the affinity of MazF for mRNA is decreased by EDF (26), this enhancement probably arises by permitting unfettered mRNA access to the second catalytic pocket.

We conjecture that E. coli MazF is an enzyme of a unique structural form that enables it to be a hub for various quorum-sensing peptides produced by various different bacteria. However, since, based on our results here, it seems likely that BsEDF and PaEDF-1/2/3 affected different sites of E. coli MazF than did EcEDF, it seems that the model that we have described above for the EcEDF-MazF interaction is not appropriate for these new EDF molecules from B. subtilis and P. aeruginosa that we have found here. We anticipate that crystallographic structural analysis would probably elucidate the precise interaction of each of the newly described EDFs with E. coli MazF.

The possible effect of EDFs on the survival of a specific bacterial species in a population of several bacterial species. In previous work, we described E. coli EDF-mazEF-mediated cell death as a population phenomenon enabling a mechanism for bacterial survival. EcEDF triggers a MazF-induced downstream pathway that leads to the death of most of the bacterial population and to the continued survival of a small subpopulation (23). That this phenomenon leads both to death and to survival offers an evolutionary rationale for mazEF-mediated cell death: under stressful conditions, most of the population undergoes programmed cell death, while a small part of the population remains to start a new population when the stressful conditions have subsided (23). Until now, our model was based on homogenous populations of a single bacterial species. Here, we have considered the possible interface between coexisting populations of two or more different bacterial species. We propose that the induction of the altruistic suicide mechanism by EDFs may be used in the “arms race” between different bacterial species in mixed populations. Here we discovered that each of the bacterial species studied, E. coli, B. subtilis, and P. aeruginosa, produced its own specific EDF(s) that we found to be involved in the induction of the mazEF of E. coli and ydcDE of B. subtilis. However, since cell death of P. aeruginosa is density independent (see Fig. S7 in the supplemental material), it seems that this highly aggressive bacterium uses its QS factors to kill other bacteria and not itself.

This pioneering research on the production and the role of the QS EDF peptides in interspecies bacterial cell death predicts the existence of an “EDF family” that would also be found in other bacterial species. We suggest that, as in E. coli (18), the synthesis of MazF- or MazE-like toxins is induced by stressful conditions and their endoribonucleolytic activities are enhanced by members of the EDF family. Thereby, under stress conditions, EDFs coordinate interspecies cell death of mixed bacterial populations. In addition, since bacterial resistance to known antibiotics has become a serious public health problem, our results here encourage us to predict that EDFs might help to solve this problem, as the basis for a new class of antibiotics that trigger PCD from outside the bacterial cells.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** We used the following sets of E. coli strains: (i) MC4100pA::mazEF-ydcDE (25) and its ∆mazEF::kan derivatives (25) and (iii) BL21(DE3) (Invitrogen, Carlsbad, CA) and TG1 (our strain collection). We used B. subtilis strain PT79 (from the collection of Sigal Ben-Yehuda) and P. aeruginosa strain PA14 and the P. aeruginosa ΔfilD transposon mutant (from the collection of Frederick M. Ausubel). B. subtilis PT79ΔydcDE was constructed by using standard methods (43). Long-flanking PCR mutagenesis was used to create a B. subtilis ΔydcDE::mazEF mutant. And DNA was introduced into laboratory strains by DNA-mediated transformation of competent cells (44). We constructed plasmid pET28a-mazEF(His)6 from pET28a (Novagen, San Diego, CA) to express MazE and MazF(His)6 in bicistronic expression under the control of the T7 promoter, using the Shine-Dalgarno (SD) sequence from the mazEF operon. Plasmid pET28a-(His)6 mazE was kindly provided by M. Inouye (Piscataway, NJ).
Media and materials. Cultures of E. coli, B. subtilis, or P. aeruginosa were grown in liquid M9 minimal medium with 1% glucose and a mixture of amino acids (10 μg/ml each) (25) and plated on rich LB agar plates as we have described previously (25). For some experiments, we grew E. coli and B. subtilis in LB medium. Rifampin, trypsin, chymotrypsin, and proteinase K were obtained from Sigma (St. Louis, MO). Ampicillin was obtained from Biochemie GmbH (Kundl, Austria). Primers were purchased from Hy-labs (Rehovot, Israel) and from Integrated DNA Technologies (IDT; Hudson, NH). To study E. coli MazF cleavage, we used an oligonucleotide with the sequence 5'-AAGTCrGACATCAG-3' and 5'-AAGTCrGArGrArUCAG-3' (lowercase letters indicate the use of ribonucleotides instead of deoxynucleotides) labeled with 6-carboxyfluorescein (FAM) on its 5' end and with black hole quencher-1 (BHQ1) on its 3' end and its corresponding oligonucleotide cleavage fragments (5'-FAM-AAGTCrG plus ACATCAG-BHQ1-3' and 5'-FAM-AAGTCrG plus ArCrArUUCAG-BHQ1-3'), respectively (Fig. 3A; see also Fig. S3A in the supplemental material). These oligonucleotides were purchased from Nunc (Thermo Fisher Scientific, Denmark). We purchased the synthetic B. subtilis and P. aeruginosa EDFs and their “mutant” derivative peptides from GenScript Corp. (Piscataway, NJ). Affinity columns (HiTrap N-hydroxyquinimide [NHS]-activated HP) were purchased from GE Healthcare (United Kingdom). Sephadex G-25 columns and phenyl Sepharose beads were purchased from GE Healthcare (United Kingdom). The E. coli S30 extract system for circular DNA was purchased from Promega (Madison, WI). Amicon ultrafilters were purchased from Millipore (Carrrigtowh, Ireland).

Preparing supernatants (SNs) from dense culture of B. subtilis and P. aeruginosa. We grew B. subtilis strain PY79 in LB medium at 37°C and P. aeruginosa strains PA14 and PA14ΔfliD in M9 medium at 37°C to the mid-logarithmic phase (optical density at 600 nm [OD600], 0.6) with shaking at 180 rpm. We prepared cell-free supernatants (SNs) by removing the cells from the growth medium by centrifugation at 14,000 rpm followed by successive filtrations through 0.2-μm-pore-size filters. The filtrates were stored at −20°C overnight.

Determining the effect of BsEDF and PaEDFs on E. coli cell death. To a diluted culture (2.5 × 10^7 cells/ml) of E. coli MC4100 strain we added the SN of B. subtilis or the SNs of P. aeruginosa (PA14 and PA14ΔfliD) or the HPLC-purified fractions from these SNs or the synthetic WT or mutant EDF peptides. We incubated the samples without shaking at 37°C for 10 min followed by another 10 min after adding a sublethal concentration of rifampin (10 μg/ml). When we added synthetic peptides, the initial incubation was 20 min. Loss of viability was determined by quantifying colony-forming units (CFUs).

Determining the effect of BsEDF and PaEDFs on ydcDE-mediated cell death in B. subtilis. B. subtilis strains PY79 and PY79ΔydcDE were grown in M9 glucose medium at 22°C for 20 h. Subsequently, cells were diluted to OD600 = 0.1 and grown aerobically in M9-glycerol medium to the mid-logarithmic phase (OD600 = 0.5) with shaking at 180 rpm. Cultures were either diluted to a density of 2 × 10^5 cells/ml (diluted) or not (dense). The dilution media were M9-glycerol medium (M9) without any additions or with an added 1 μg/ml of chemically synthesized EceEDF or 1 μg/ml of chemically synthesized BsEDF or 1 μg/ml of each of six chemically synthesized BsEDF mutants (m1 to m6) or 1 μg/ml of each of the three PaEDFs. In these media, cells were incubated without shaking at 37°C for 10 min followed by another 30 min after addition of a sublethal concentration of chloramphenicol (20 μg/ml). Loss of viability was determined by CFU.

Identifying and purifying the B. subtilis EDF. SNs from dense cultures of B. subtilis were prepared as described above. Fractions containing EDF activity were separated on a C-18 SepPak cartridge using 10 steps of a 10% to 80% methanol gradient. EDF activity was found in the fractions eluted with 40% methanol. The active fractions were concentrated by the use of a SpeedVac, and then the EDF active fraction was separated once again on a C-18 SepPak cartridge, this time using a stepwise gradient of 5% to 60% acetonitrile. The fractions with EDF activity were eluted with 40% acetonitrile; the active fraction was purified by HPLC using an acetonitrile gradient of 2% to 20% for 15 min, 20% to 30% for 15 min, and 30% to 98% at a rate of 2%/min. The BSEDF was eluted at the elution time of 27.8 min (Fig. 1C; see also Fig. S1A in the supplemental material). The BSEDF sequence was determined by the Edman degradation procedure using a Prociase 492 protein sequencer (Applied Biosystems).

Identifying and purifying the P. aeruginosa EDFs. SNs from dense cultures of P. aeruginosa (PA14 and PA14ΔfliD) were prepared as described above for cultures of B. subtilis. Fractions containing EDF activity were separated on a C-18 SepPak cartridge using 10 steps of a 10% to 80% methanol gradient. In this case, EDF activity was found in the fractions eluted with 30% methanol. No activity was found in any of the fractions of P. aeruginosa PA14ΔfliD. The active fractions were concentrated as described above for the EDF from B. subtilis. The fraction with EDF activity was eluted at 40% acetonitrile, and the active fraction was purified by HPLC, using an acetonitrile gradient of 2% to 98% at flow rate of 1.3%/min. PaEDF was eluted at elution time 16.32 min (Fig. 1D; see also Fig. S1B in the supplemental material). The PaEDF sequences were determined by using ESI-MS (the Q-ToF 2 Micromass instrument) as we have described previously (25) (also see Fig. S2 in the supplemental material).

Expression and purification of MazF and MazE. MazF and MazE were expressed and purified as we have described earlier (26).

Determining the effect of BsEDF and PaEDFs on E. coli MazF endoribonucleolytic activity in vitro. We determined the effect of E. coli MazF endoribonucleolytic activity quantitatively as we have described previously (26), as mentioned in the extended experimental procedure section in the supplemental material.

Determining the interactions between E. coli MazF and BsEDF by using affinity chromatography. To measure the interaction between BsEDF and E. coli MazF, we coupled WT BsEDF (RGOQNE) in an HiTrap NHS-activated HP affinity column and set the flow rate to 0.5 ml/min. As a control, we prepared an identical column using BsEDF (RAQQNE). The other experimental conditions were like those that we have described previously (26).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00314-13/-/DCSupplemental.

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REFERENCES
1. Engelberg-Kulka H, Amitai S, Kolodkin-Gal I, Hazan R. 2006. Bacterial programmed cell death and multicellular behavior in bacteria. PLoS Genet. 2:e135.
2. Engelberg-Kulka H, Glaser G. 1999. Addiction modules and programmed cell death and antideath in bacterial cultures. Annu. Rev. Microbiol. 53:43–70.
3. Fu Z, Donegan NP, Memmi G, Cheung AL. 2007. Characterization of MazF6a, an endoribonuclease from Staphylococcus aureus. J. Bacteriol. 189:8871–8879.

4. Hayes F. 2003. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 301:1496–1499.

5. Mittenhuber G. 1999. Occurrence of mazEF-like antitoxin/toxin systems in bacteria. J. Mol. Microbiol. Biotechnol. 1:295–302.

6. Pandey DP, Gerdes K. 2000. Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. Nucleic Acids Res. 33:996–976.

7. Ramage HR, Connolly LE, Cox JS. 2009. Comprehensive functional analysis of Mycobacterium tuberculosis toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. PLoS Genet. 5:e1000767.

8. Aizenman E,Engelberg-Kulka H, Glaser G. 1996. An Escherichia coli chromosomal “addiction module” regulated by guanosine [corrected] 3',5'-bispyrophosphate: a model for programmed bacterial cell death. Proc. Natl. Acad. Sci. U. S. A. 93:6059–6063.

9. Cheny I, Gazar E. 2004. The YeFm antitoxin defines a family of natively unfolded proteins: implications as a novel antibacterial target. J. Biol. Chem. 279:8252–8261.

10. Christensen SK, Mikkelsen M, Pedersen K, Gerdes K. 2001. REE, a global inhibitor of translation, is activated during nutritional stress. Proc. Natl. Acad. Sci. U. S. A. 98:14328–14333.

11. Grady R, Hayes F. 2005. Aze-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of Enterococcus faecium. Microbiol. 47:1419–1432.

12. Masuda Y, Miyakawa K, Nishimura Y, Ohtsubo E. 2003. chpA and chpB, Escherichia coli chromosomal homologs of the pem locus responsible for stable maintenance of plasmid R100. J. Bacteriol. 175:6850–6856.

13. Metzger S, Dror IB, Aizenman E, Schreiber G, Toome M, Friesen JD, Cashel M, Glaser G. 1988. The nucleotide sequence and characterization of the relA gene of Escherichia coli. J. Biol. Chem. 263:15699–15704.

14. Schmidt O, Schuennemann VJ, Hand NJ, Silhavy TJ, Martin J, Lupas AN, Djuranovic S. 2007. prfE and yhaV encode a new toxin-antitoxin system in Escherichia coli. J. Mol. Biol. 372:894–905.

15. Zhang Y, Zhang J, Hara H, Kato I, Inouye M. 2005. Insights into the mRNA cleavage mechanism by MazF, an mRNA ribonuclease. J. Biol. Chem. 280:3143–3150.

16. Zhang Y, Zhang J, Hoeflich PK, Ikura M, Q Ing G, Inouye M. 2003. MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in Escherichia coli. Mol. Cell 12:913–923.

17. Kamada K, Hanaoka F, Burley SK. 2003. Crystal structure of the MazE/MazF complex: molecular bases of antioxidant-toxin recognition. Mol. Cell 11:875–884.

18. Hazan R, Sat B, Engelberg-Kulka H. 2004. Escherichia coli mazEF-mediated cell death is triggered by various stressful conditions. J. Bacteriol. 186:3663–3669.

19. Godoy VG, Jarosz DF, Walker FL, Simmons LA, Walker GC. 2006. Y-Family DNA polymerases respond to DNA damage-independent inhibition of replication fork progression. EMBO J. 25:8868–8879.

20. Sat B, Hazan R, Fisher T, Kahaner H, Glaser G, Engelberg-Kulka H. 2001. Programmed cell death in Escherichia coli: some antibiotics can trigger mazEF lethality. J. Bacteriol. 183:2041–2045.

21. Sat B, Reches M, Engelberg-Kulka H. 2003. The Escherichia coli mazEF suicide module mediates thymineless death. J. Bacteriol. 185:1803–1807.

22. Vesper O, Amitai S, Belitsky M, Byrgazov K, Kamberdin AC, Engelberg-Kulka H, Moll I. 2011. Selective translation of leaderless mRNAs by specialized ribosomes generated by MazF in Escherichia coli. Cell 147:147–157.

23. Amitai S, Kolodkin-Gal I, Hananya-Meltbashi M, Sacher A, Engelberg-Kulka H. 2009. Escherichia coli MazF leads to the simultaneous selective synthesis of both “death proteins” and “survival proteins”. PLoS Genet. 5:e1000390.

24. Kolodkin-Gal I, Engelberg-Kulka H. 2008. The extracellular death factor: physiological and genetic factors influencing its production and response in Escherichia coli. J. Bacteriol. 190:3169–3175.

25. Kolodkin-Gal I, Hazan R, Gaathon A, Carmeli S, Engelberg-Kulka H. 2007. A linear pentapeptide is a quorum-sensing factor required for mazEF-mediated cell death in Escherichia coli. Science 318:652–655.

26. Belitsky M, Avshalom H, Erental A, Yelin I, Kumar S, London N, Sperber M, Schuler-Furman O, Engelberg-Kulka H. 2011. The Escherichia coli extracellular death factor EDF induces the endoribonucleolytic degradation of the toxin MazF. Cell 147:65–635.

27. Pellegrini O, Mathy N, Gogos A, Shapiro L, Condon C. 2005. The Bacillus subtilis ydkD operon encodes an endoribonuclease of the MazF/PemK family and its inhibitor. Mol. Microbiol. 56:1139–1148.

28. Wang NR, Hergenrother PJ. 2007. A continuous fluorometric assay for the assessment of MazF ribonuclease activity. Anal. Biochem. 371:173–183.

29. Bassler BL, Losick R. 2006. Bacterially speaking. Cell 125:237–246.

30. Camilli A, Bassler BL. 2006. Bacterial small-molecule signaling pathways. Science 311:1113–1116.

31. Fuqua C, Winans SC, Greenberg EP. 1996. Censs and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. 50:727–751.

32. Ng WL, Bassler BL. 2009. Bacterial quorum-sensing network architectures. Annu. Rev. Genet. 43:197–222.

33. Novick RP, Geisinger E. 2008. Quorum sensing in staphylococci. Annu. Rev. Genet. 42:541–564.

34. Okada M, Sato I, Cho SJ, Iwata H, Nishio T, Dubnau D, Sakagami Y. 2005. Structure of the Bacillus subtilis quorum-sensing peptide pheromone ComX. Nat. Chem. Biol. 1:23–24.

35. Parsek MR, Greenberg EP. 2005. Sociomicrobiology: the connections between quorum sensing and biofilms. Trends Microbiol. 13:27–33.

36. Waters CM, Bassler BL. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. 21:319–346.

37. Chen X, Schauder S, Potier N, Van Dorselaer A, Pelczer I, Bassler BL, Hughson FM. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545–549.

38. Lazdunski AM, Ventre I, Sturgis JN. 2004. Regulatory circuits and communication in gram-negative bacteria. Nat. Rev. Microbiol. 2:581–592.

39. Straight PD, Fischbach MA, Walsh CT, Rudner DZ, Kolter R. 2007. A singular enzymatic megacomplex from Bacillus subtilis. Proc. Natl. Acad. Sci. U. S. A. 104:305–310.

40. Zhang H, Wang Y, Pfeifer BA. 2008. Bacterial hosts for natural product production. Mol. Pharm. 5:212–225.

41. Agarwal S, Mishra NK, Bhatnagar S, Bhatnagar R. 2010. PemK toxin of Bacillus anthracis is a ribonuclease: an insight into its active site, structure, and function. J. Biol. Chem. 285:7254–7270.

42. Li GY, Zhang Y, Chan MC, Mal TK, Hoeflich KP, Inouye M, Ikura M. 2006. Characterization of dual substrate binding sites in the homodimeric structure of Escherichia coli mRNA interferase MazF. J. Mol. Biol. 357:139–150.

43. Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

44. Gryczan TJ, Contente S, Dubnau D. 1978. Characterization of Staphylococcus aureus plasmids introduced by transformation into Bacillus subtilis. J. Bacteriol. 134:318–329.