RESEARCH ARTICLE

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 (*Ec*EDF), 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 RGQQNE, called *Bs*EDF. In the SN of *P. aeruginosa*, we found three EDFs: the nonapeptide INEQTVVTK, called *Pa*EDF-1, and two hexadecapeptides, VEVSDDGSGGNTSLSQ, called *Pa*EDF-2, and APKLSDGAAAGYVTKA, called *Pa*EDF-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.**

IMPORTANCE **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 (*Ec*EDF), 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.**

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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 mazEF*-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 *Ec*EDF. Structural analysis revealed that *Ec*EDF is the linear pentapeptide NNWNN required for triggering *mazEF*-mediated cell death (25). In addition, we have recently shown that the *E. coli Ec*EDF enhances the endoribonucleolytic 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 *Bs*EDF. *P. aeruginosa* produced three EDFs: one nonapeptide (nine amino acids), INEQTVVTK, and two hexadecapeptides (16 amino acids), VEVSDDGSGGNTSLSQ and APKLSDGAAAGYVTKA, designated *Pa*EDF-1, *Pa*EDF-2, and *Pa*EDF-3 (*Pa*EDF-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 *mazEF*- 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 endoribonucleolytic 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 *mazEF*-mediated cell death when added to a diluted culture of *E. coli*. Previously, we reported that *mazEF*-mediated cell death takes place in dense cultures of *E. coli* (25). Moreover, the supernatant (SN) of a dense culture of *E. coli* restores *mazEF*-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 (2.5×10^4 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 *mazEF*-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 *mazEF*-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 *mazEF*-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 Δ*mazEF 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 *mazEF*-mediated death of *E. coli* cells.

***Bs*EDF, the EDF of *B. subtilis*, is the hexapeptide RGQQNE.** To characterize the chemical nature of *Bs*EDF, we purified it from a large volume of a supernatant from a midlogarithmic 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 *B. subtilis* EDF activity in the fraction eluted at 27.8 min (Fig. 1E; see also Fig. S1A in the supplemental material and Materials and Methods). Edman degradation was carried out on the purified fraction (PF), and thereby the *B. subtilis* EDF, which we called *Bs*EDF, was identified as a hexapeptide (six amino acids) with the amino acid sequence RGQQNE.

To test if the RGQQNE hexapeptide was indeed the *Bs*EDF, we synthesized an identical peptide chemically and tested it for biological activity. When added to a diluted *E. coli* culture, this synthetic peptide enabled *mazEF*-mediated cell death induced by rifampin; this synthetic *Bs*EDF peptide acted just like the *Ec*EDF peptide (Fig. 2A). We observed no cell death when we induced the cultures of an *E. coli* Δ*mazEF* derivative strain by the use of rifampin (Fig. 2A). In addition, we asked if the EDF activity of *Bs*EDF required each of its six amino acids for its activity. We used synthetic *Bs*EDF mutants (m1 to m6) in which we replaced each of its amino acids with alanine (A) and studied the effects of these replacements on *mazEF*-mediated cell death in diluted cultures of *E. coli* (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 APKLSDGAAAGYVTKA.** 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 (*Pa*EDF-1), (ii) the hexadecapeptide (16-amino-acid) VEVSDDGSGGNTSLSQ (*Pa*EDF-2), and (iii) the hexadecapeptide APKLSDGAAAGYVTKA (*Pa*EDF-3). To test whether all of the three peptides, *Pa*EDF-1, *Pa*EDF-2, and *Pa*EDF-3, were indeed *Pa*EDFs, we synthesized identical peptides chemically and tested them for biological EDF activity. When added individually to diluted *E. coli* cultures, these synthetic peptides enabled *mazEF*-mediated cell death induced by rifampin; these synthetic *Pa*EDF peptides acted just like the synthetic *Ec*EDF peptide (Fig. 2B) and the synthetic *Bs*EDF peptide (Fig. 2B). Again, we observed no cell death when we induced the cultures of an *E. coli* Δ*mazEF* derivative strain by the use of rifampin (Fig. 2B).

***Bs*EDF, *Ec*EDF, and *Pa*EDFs trigger *ydcDE*-mediated cell death when added to a diluted culture of *B. subtilis*.** As we described above, *Bs*EDF, *Ec*EDF, and *Pa*EDFs all acted as extracellular death factors involved in *E. coli mazEF*-mediated cell death (Fig. 2A and B). In *B. subtilis*, *ydcDE* is an operon which encodes a TA module that belongs to the *mazEF* 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 can 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

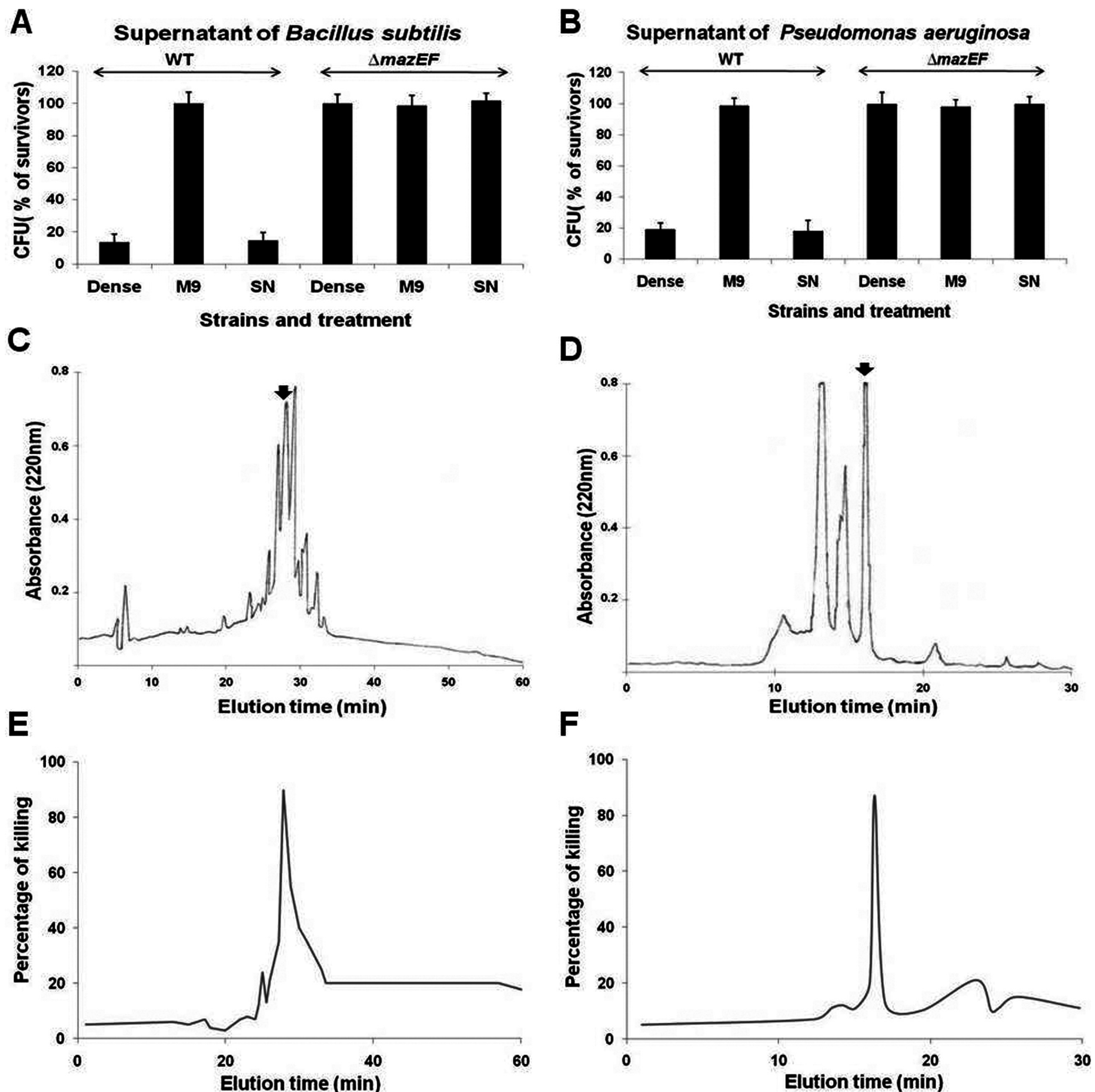


FIG 1 The effect of the supernatants from dense cultures of *B. subtilis* (A) or *P. aeruginosa* (B) on *mazEF*-mediated cell death in *E. coli* and the purification (C and D) and analysis of the activities (E and F) of the EDFs from those cultures. (A and B) Wild-type *E. coli* MC4100*relA*$^+$ (WT) or *E. coli* MC4100*relA*$^+$ Δ*mazEF* (Δ*mazEF*) was grown as described in Materials and Methods. Cells were either not diluted (Dense) or diluted to a density of 3×10^4 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 *Bs*EDF (C) or *Pa*EDF (D): milliabsorbance at 220 nm was determined during elution from the HPLC column of purified supernatant. (E and F) *Bs*EDF activity (E) or *Pa*EDF activity (F) plotted as a function of the elution time from the HPLC column as marked by the arrows in panels C and D.

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* Δ*ydcDE* 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 *Bs*EDF 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 *Bs*EDF 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 *Bs*EDF 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

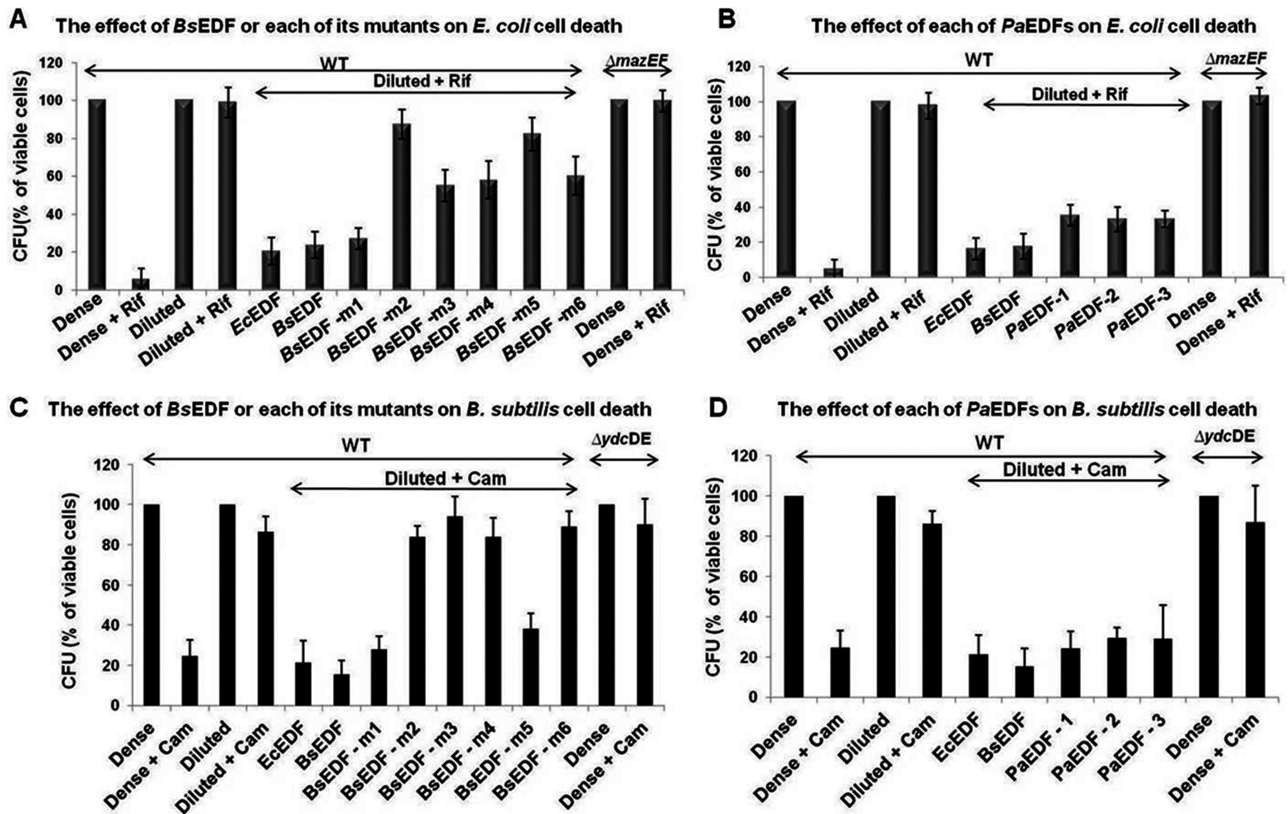


FIG 2 Application of chemically synthesized *Bs*EDF and each of *Pa*EDFs leads to *mazEF*-mediated cell death in *E. coli* (A and B) and *ydcDE*-mediated cell death in *B. subtilis* (C and D). (A and B) Wild-type *E. coli* MC4100*relA*$^+$ (WT) and *E. coli* MC4100*relA*$^+$ Δ*mazEF* (Δ*mazEF*) were grown and diluted (or not) as described in the legend to Fig. 1. To these diluted samples, we either applied or did not apply 500 ng/ml of (i) *Ec*EDF or (ii) *Bs*EDF or (iii) each of *Bs*EDF mutants (m1 to m6) (A) or (iv) each of *Pa*EDFs (B). Induction of cell death and loss of viability were determined as mentioned for Fig. 1. Rif, rifampin. (C and D) *B. subtilis* strain PY79 (WT) and *B. subtilis* PY79Δ*ydcDE* (Δ*ydcDE*) were grown with shaking in M9 glucose medium at 22°C for 20 h. Cells were diluted to an OD_{600} of 0.1 and grown aerobically to the mid-logarithmic phase ($OD_{600} = 0.5$) in M9-glycerol medium. Cultures were either not diluted (dense) or diluted to a density of 2×10^4 cells/ml in M9-glycerol medium. To the diluted samples, we either added or did not add 1 μg/ml of (i) *Ec*EDF or (ii) *Bs*EDF or (iii) each of the six *Bs*EDF mutants (m1 to m6) (C) or (iv) each of the *Pa*EDFs (D). These mixtures were incubated without shaking at 37°C for 10 min, and then chloramphenicol (Cam; 20 μg/ml) was added, and the mixture was incubated for a further 30 min. Loss of viability was determined by CFUs.

of *Bs*EDF in enabling *B. subtilis* cell death to occur, there is a more absolute requirement of the wild-type (WT) *Bs*EDF sequence than is the case for *E. coli* cell death. In *Bs*EDF, 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 *Pa*EDFs enabled *ydcDE*-mediated cell death (compare Fig. 2B and D).

***Bs*EDF and *Pa*EDFs enhance the *in vitro* endoribonucleolytic activity of *E. coli* MazF.** Previously, we showed that *Ec*EDF triggers *mazEF*-mediated cell death (25) and enhances the *in vitro* endoribonucleolytic activity of *E. coli* MazF (26). Having shown here that *Bs*EDF and each of the *Pa*EDFs 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 *Bs*EDF or each of the *Pa*EDFs 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 *Bs*EDF and each of three *Pa*EDFs 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 *Bs*EDF led to enhancement of MazF activity by approximately 34%, 75%, and 100%, respectively. Adding 7.5 μM *Ec*EDF or 7.5 μM *Bs*EDF led to nearly equal levels of MazF enhancement (Fig. 3B, right panel). Similarly, each of the three EDFs from *P. aeruginosa*, *Pa*EDF-1, *Pa*EDF-2, and *Pa*EDF-3, also enhanced the *in vitro* activity of MazF but did so less efficiently than did the *Bs*EDF. Adding 1.5, 3.75, or 7.5 μM *Pa*EDF-1, *Pa*EDF-2, or *Pa*EDF-3 increased *in vitro* MazF activity by only about 20%, 35%, or 47%, respectively (compare Fig. 3B with Fig. 3C to E). Thus, *Pa*EDF-1, *Pa*EDF-2, and *Pa*EDF-3 each enhanced the *in vitro* MazF endoribonucleolytic activity but did so less efficiently than did the *Ec*EDFs or *Bs*EDF (Fig. 3C to E, right panel). We confirmed these results in two ways: by testing another chimeric oligonucle-

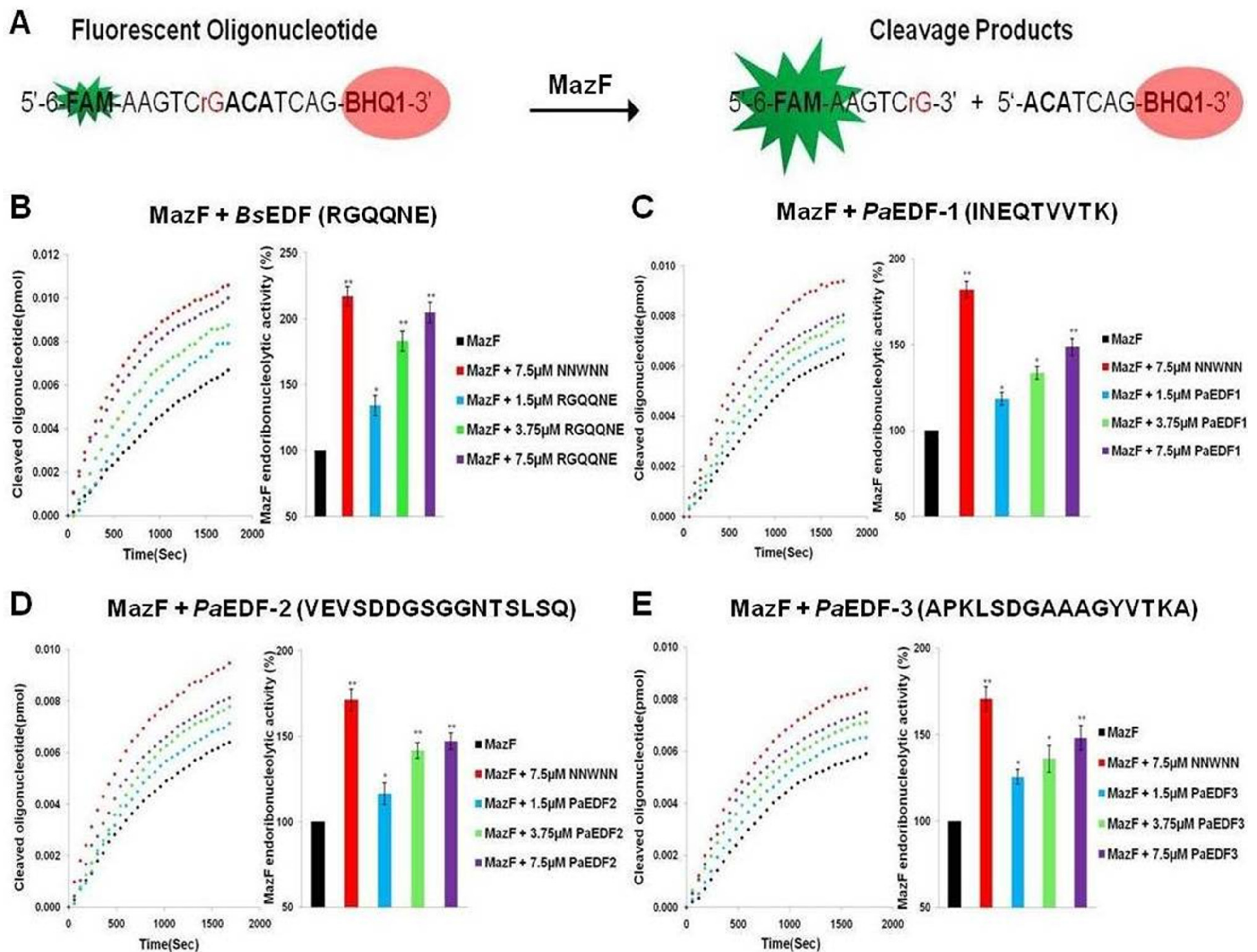


FIG 3 *Bs*EDF and each of *Pa*EDFs enhance the *in vitro* MazF endoribonucleolytic activity. (A) Illustration of the reaction used for studies on the endoribonucleolytic activity of MazF. Cleavage of the chimeric fluorescent oligonucleotides by MazF was expressed as an increase of fluorescence emission of the fluorophore FAM. "rG" represents an RNA base. The cleavage site was at the 5′ side of ACA. (B) The effect of *Bs*EDF (RGQQNE) on MazF activity. (Left panel) *Bs*EDF 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 *Bs*EDF. MazF activity in the presence of 7.5 μM *Ec*EDF (NNWNN) was defined as the positive control. MazF activity without the addition of *Bs*EDF was assigned a value of 100%. (C) The effect of *Pa*EDF-1 on MazF activity. Different concentrations of *Pa*EDF-1 were added to the same reaction mixture as described for panel B. (D) The effect of *Pa*EDF-2 on MazF activity. Various concentrations of *Pa*EDF-2 were added to the same reaction mixture as described for panel B. (E) The effect of *Pa*EDF-3 on MazF activity. Various concentrations of *Pa*EDF-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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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 *Bs*EDF was required for the *in vitro* enhancement of *E. coli* MazF activity. To examine this, we observed the activities of the synthetic *Bs*EDF 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 *Bs*EDF 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 *Bs*EDF 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 *Bs*EDF 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 *Bs*EDF on *E. coli* cell death (Fig. 2A) and the *in vitro* effects of *Bs*EDF 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 *Bs*EDF bound directly to *E. coli* MazF. We confirmed this direct interaction using an affinity column to which we coupled either the synthetic WT *Bs*EDF 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

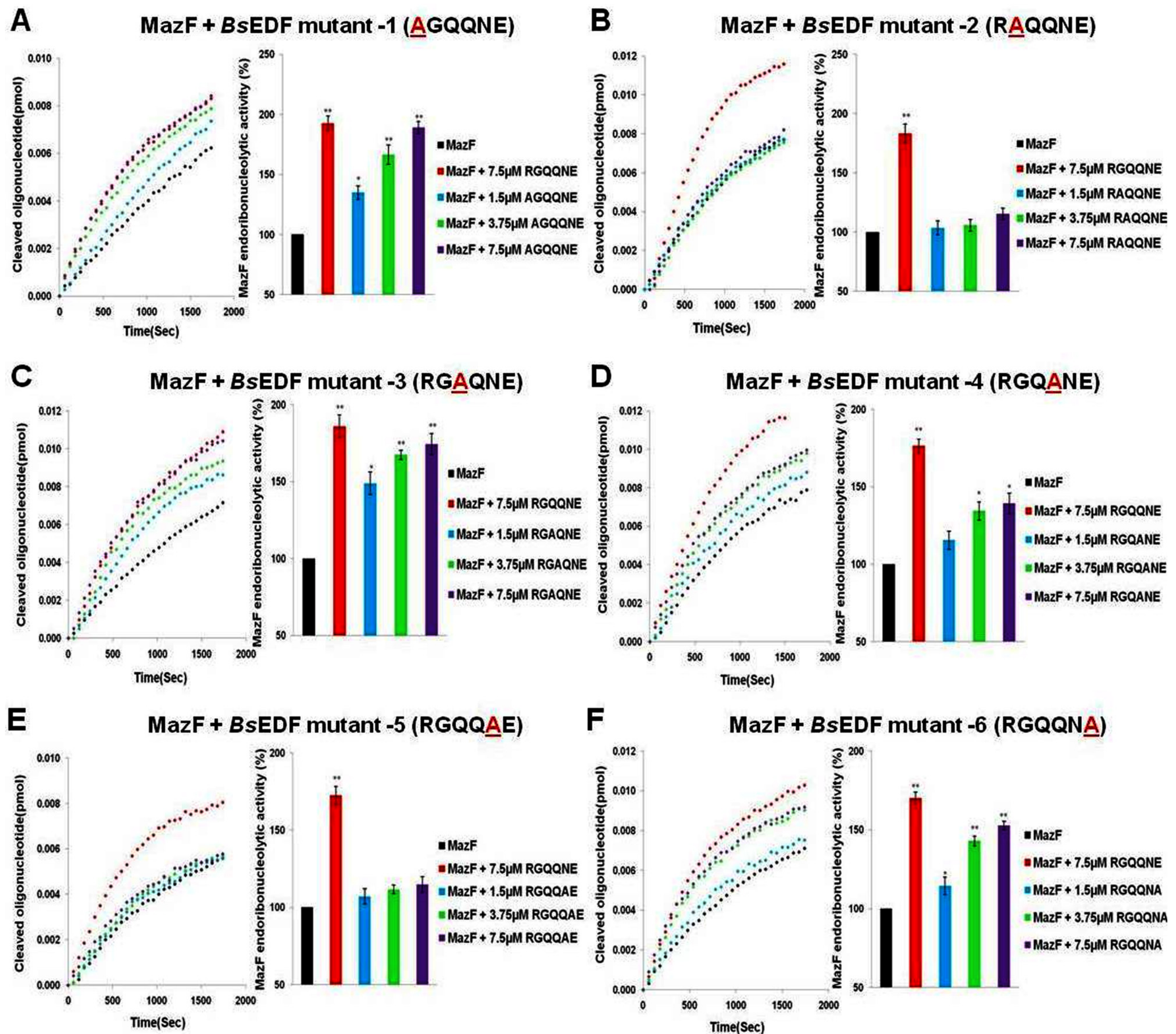
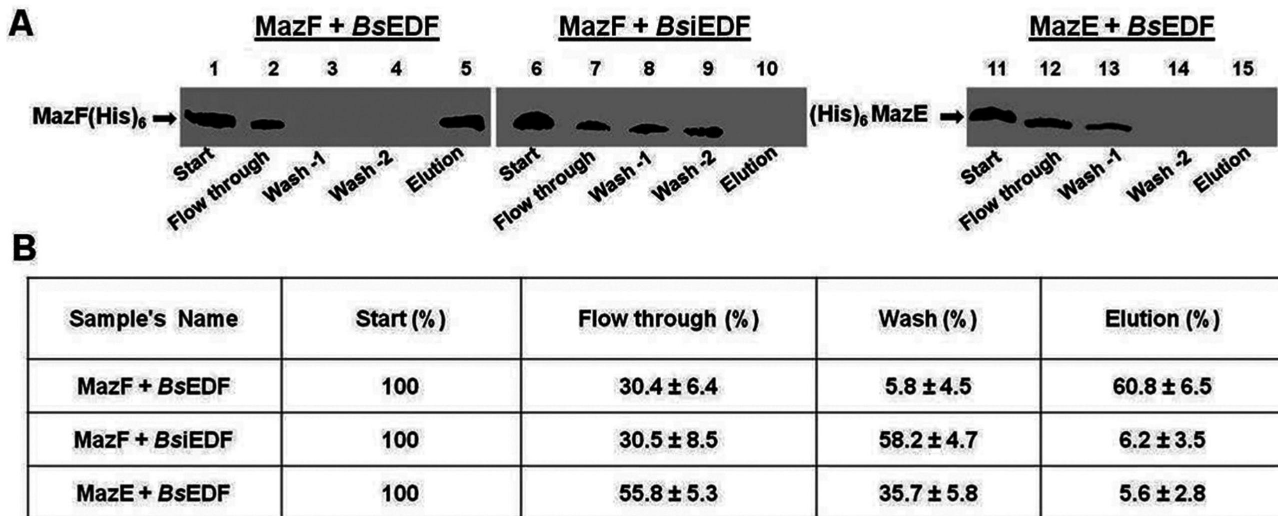


FIG 4 The importance of each amino acid in *Bs*EDF for increasing the *in vitro* endoribonucleolytic activity of *E. coli* MazF. Each of the six amino acids in the *Bs*EDF sequence was individually replaced by alanine as follows: AGQQNE (A); RAQQNE (B); RGAQNE (C); RGQANE (D); RGQQAE (E); RGQQNA (F). Left and right panels and experimental conditions are as described for Fig. 3B. Error bars indicate standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

*Bs*EDF-coupled column (compare lanes 5 and 15 in Fig. 5A). In contrast, MazF had no affinity for the mutant *Bs*EDF-coupled column (Fig. 5A, lane 10). We found similar results using the Bradford assay (Fig. 5B).

Like *Ec*EDF, *Bs*EDF, but not *Pa*EDFs, overcomes the *in vitro* inhibitory effect of MazE on *E. coli* MazF endoribonucleolytic activity. We have previously reported that *Ec*EDF can overcome the inhibitory effect of MazE on *E. coli* MazF (26). Here we asked whether *Bs*EDF or the three *Pa*EDFs could overcome the MazE inhibitory activity on *E. coli* MazF. To a reaction mixture containing highly purified MazF we added MazE with one of *Bs*EDF, *Pa*EDF-1, *Pa*EDF-2, or *Pa*EDF-3. We found that *Bs*EDF 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). In contrast, none of the *Pa*EDFs overcame the inhibitory effect of MazE on *E. coli* MazF (Fig. 6B; see also Fig. S5B in the supplemental material). Note that *Bs*EDF overcame the inhibitory effect of MazE on MazF less efficiently than did *Ec*EDF (NNWNN) (Fig. 6A). Moreover, similar amino acids of *Bs*EDF 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 *Bs*EDF were absolutely required, the fourth and six were only partially required, and the first and third were not required at all.

***Ec*EDF, *Bs*EDF, and each of the *Pa*EDFs could enhance the endoribonucleolytic activity of *E. coli* MazF, probably by interacting at different sites on MazF.** We previously showed that *Ec*EDF could enhance the *in vitro* endoribonucleolytic activity of *E. coli* MazF (26), and here we have shown that *Bs*EDF and each of *Pa*EDFs 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



Sample's Name	Start (%)	Flow through (%)	Wash (%)	Elution (%)
MazF + *Bs*EDF	100	30.4 ± 6.4	5.8 ± 4.5	60.8 ± 6.5
MazF + *Bsi*EDF	100	30.5 ± 8.5	58.2 ± 4.7	6.2 ± 3.5
MazE + *Bs*EDF	100	55.8 ± 5.3	35.7 ± 5.8	5.6 ± 2.8

FIG 5 MazF directly binds to *Bs*EDF. MazF and MazE were passed through an affinity column to which either *Bs*EDF (RGQQNE) or *Bsi*EDF (RAQQNE) was coupled. Fractions were analyzed by SDS-PAGE (A) and Bradford assay (B). See also Materials and Methodss and main text.

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 *Ec*EDF (NNGNN) interfered with the ability of WT *Ec*EDF to enhance the activity of *E. coli* MazF (Fig. 7A). Therefore, we called it *Eci*EDF. Similarly, the mutant of *Bs*EDF RAQQNE, which we called *Bsi*EDF, interfered with the ability of WT *Bs*EDF 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. *Eci*EDF did not affect the EDF activity of *Bs*EDF (Fig. 7A) or of any of the three *Pa*EDFs (see Fig. S6 in the supplemental material). Similarly, *Bsi*EDF did not affect the EDF activity of *Ec*EDF (Fig. 7B) or of any of the *Pa*EDFs (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 (*Ec*EDF) to this list of QS peptides (24, 25). *Ec*EDF (NNWNN) 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 *Ec*EDF 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 *Ec*EDF 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 *Ec*EDF. In the supernatant of *B. subtilis*, we detected *Bs*EDF—RGQQNE (Fig. 1A, C, and E; see also Fig. S1A in the supplemental material); in the supernatant of *P. aeruginosa*, we detected *Pa*EDF-1—INEQTVVTK, *Pa*EDF-2—VEVSDDGSGGNTSLSQ, and *Pa*EDF-3—APKLSDGAAAGYVTKA (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 *Ec*EDF, 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 *Bs*EDF (Fig. 2C) or *Ec*EDF (Fig. 2C) or any of the *Pa*EDFs (Fig. 2D) can serve as a factor for *ydcDE*-mediated cell death. This is the first report showing that *ydcDE*, belonging to the *mazEF* family (27), triggers cell death in *B. subtilis*. In addition, like *E. coli mazEF*-mediated cell death (25), *B. subtilis ydcDE*-mediated cell death is

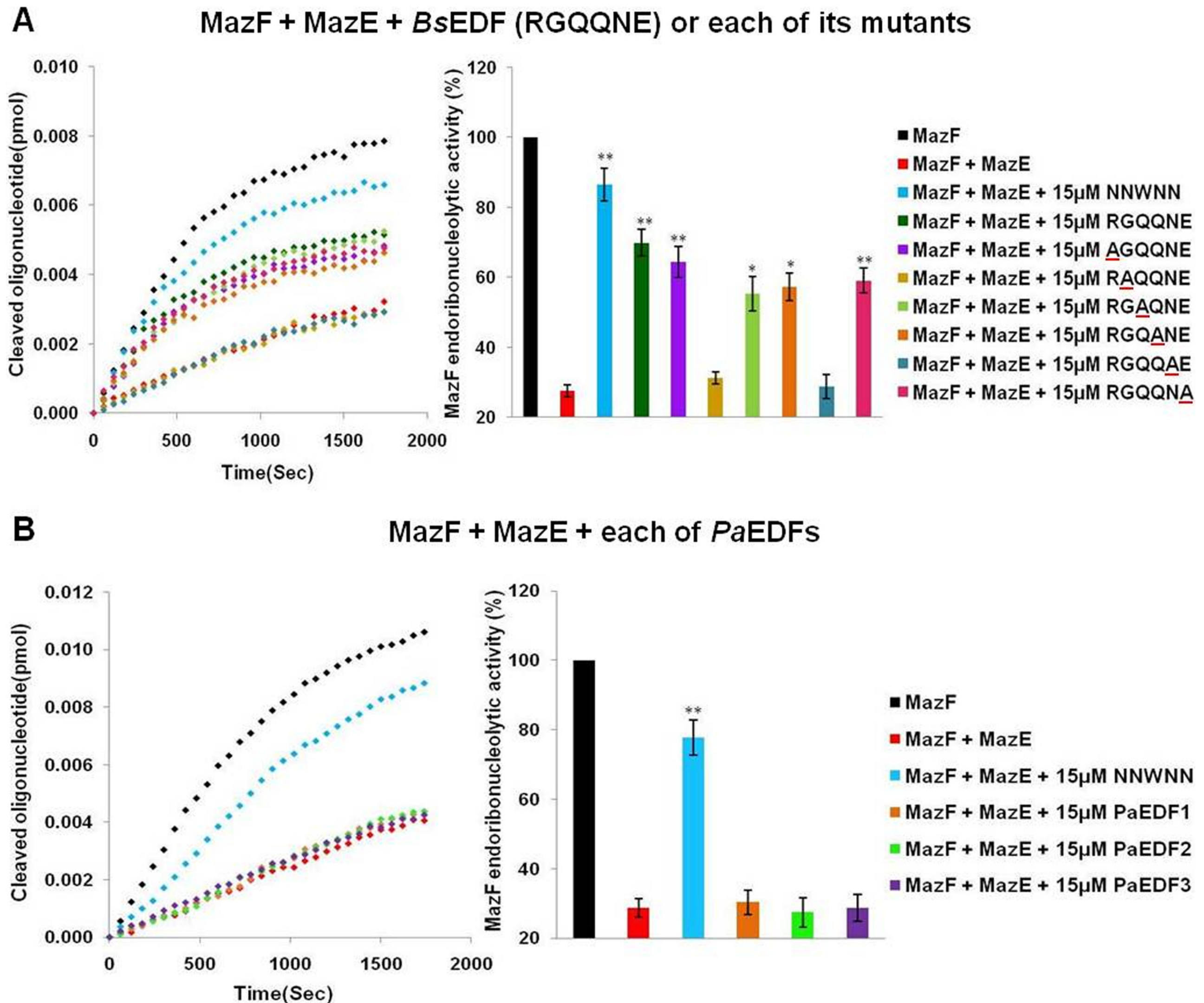


FIG 6 *Bs*EDF and its mutants at positions 1, 3, 4, and 6 (A) but not the *Pa*EDFs (B) overcome the inhibitory effect of MazE on the *in vitro* endoribonucleolytic activity of MazF. (A) Left panel: 0.03 μM MazE with 0.0 μM or 15 μM *Bs*EDF (RGQQNE) or each of its six mutants (the mutated amino acid is underlined) was individually added to preparations of MazF (0.3 μM). MazF activity in the presence of 0.03 μM MazE with 15 μM *Ec*EDF (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 *Bs*EDF and its mutants overcoming the effect of MazF. MazF activity without the addition of MazE or *Bs*EDF or its mutants was assigned a value of 100%. (B) Left panel: 0.03 μM MazE with 0.0 μM or 15 μM *Pa*EDF-1 or *Pa*EDF-2 or *Pa*EDF-3 was individually added to preparations of MazF (0.3 μM). MazF activity in the presence of 0.03 μM MazE with 15 μM *Ec*EDF (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 *Pa*EDF-1, *Pa*EDF-2, and *Pa*EDF-3 overcoming the effect of MazF. MazF activity without the addition of MazE or *Pa*EDF-1 or *Pa*EDF-2 or *Pa*EDF-3 was assigned a value of 100%. Error bars indicate standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

also a population phenomenon that occurs only in a dense and not in a diluted culture. Also, under stressful conditions, through *Bs*EDF, *ydcDE* mediates *B. subtilis*'s own cell death. Thus, like *Ec*EDF, *Bs*EDF 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* (*Ec*EDF) or any of those of *P. aeruginosa* (*Pa*EDFs). Based on the fact that *Pa*EDFs can induce density-dependent cell death in either *E. coli* or *B. subtilis*, we also identified *Pa*EDF-1, *Pa*EDF-2, and *Pa*EDF-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 *Pa*EDF-1, *Pa*EDF-2, and *Pa*EDF-3 are derived by proteolysis (NCBI reference sequence: YP_792175.1). The idea that all three *Pa*EDF 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 Δ*fliD* derivative strain of *P. aeruginosa* had EDF activity (Fig. S8). On the other hand, we were not able to detect the source of *Bs*EDF, as shown by the following approaches. (i) *B. subtilis* chromosome does not carry a gene that would be able to

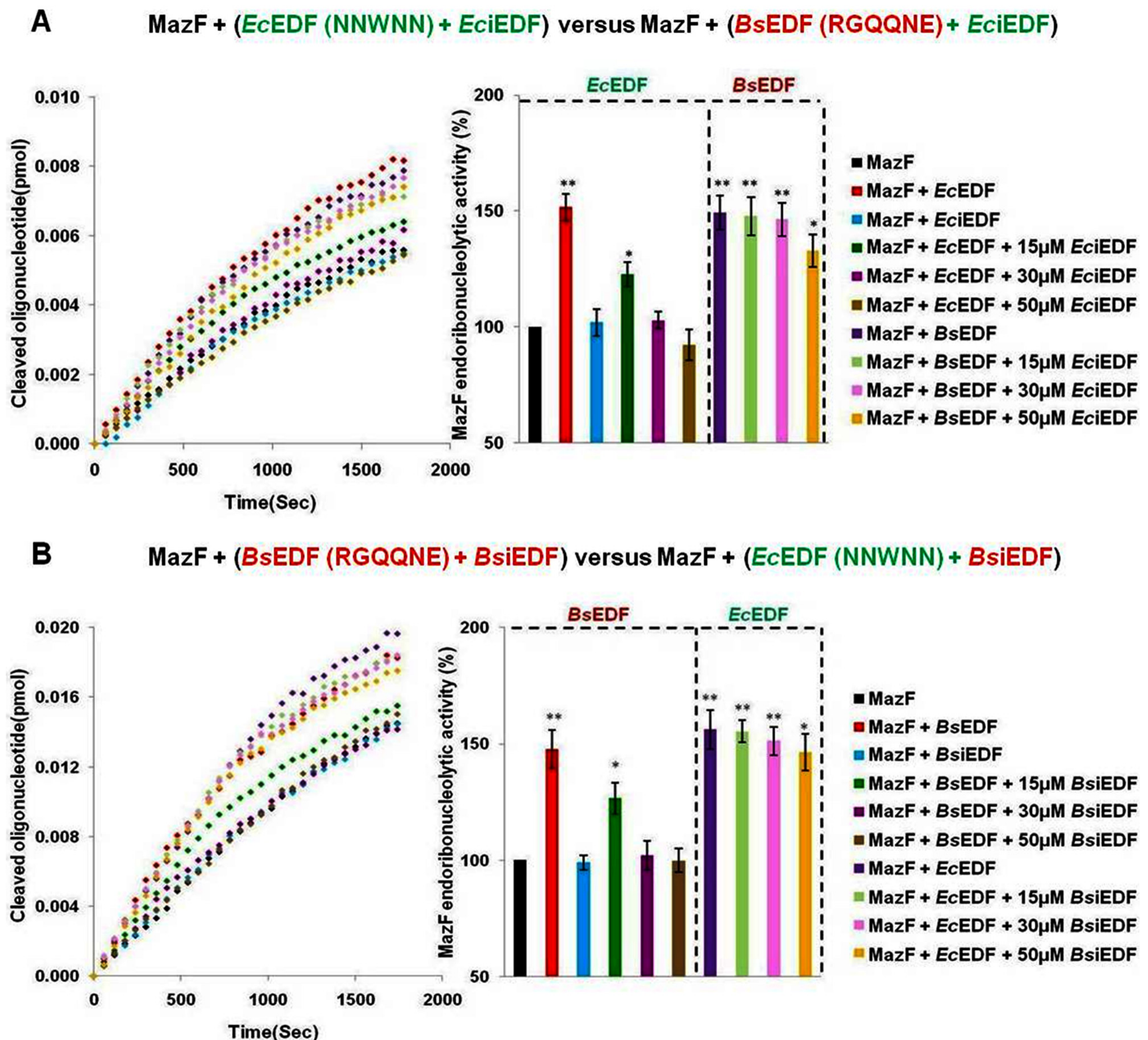


FIG 7 The iEDF (NNGNN) of *Ec*EDF (NNWNN) and the iEDF (RAQQNE) of *Bs*EDF (RGQQNE) each inhibit the *in vitro E. coli* MazF enhancement activity of its respective EDF only. In the labels at that top of the panels, the green color represents the EDF and the iEDF of *E. coli* and the red color represents those of *B. subtilis*. (A) The effects of *E. coli* iEDF on the enhancement of *E. coli* MazF activity by *Ec*EDF or *Bs*EDF. (Left panel) *Eci*EDF (15 μM or 30 μM or 50 μM) was added to a reaction mixture containing MazF (0.3 μM) with *Ec*EDF (3.75 μM) or with *Bs*EDF (3.75 μM). *E. coli* MazF activity in the presence of 3.75 μM *Ec*EDF or 3.75 μM *Bs*EDF was defined as the positive control for its respective EDF activity with an *Eci*EDF 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 *Ec*EDF or *Bs*EDF in the presence of *Eci*EDF. MazF activity without the addition of *Ec*EDF or *Bs*EDF was assigned a value of 100%. (B) The effect of *Bsi*EDF on the enhancement of *E. coli* MazF activity by *Bs*EDF or *Ec*EDF. Various concentrations of *Bsi*EDF 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

specify for its EDF (*Bs*EDF). (ii) The unique *Bs*EDF peak at 27.8 min (Fig. 1C) did not appear when LB medium was purified by the procedure used for *Bs*EDF (data not shown). (iii) *Bs*EDF was not derived from the proteins tryptone and those in yeast extracts; we did not find a *Bs*EDF 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 *Bs*EDF activity (Fig. S9). Therefore, we assume that *Bs*EDF 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*, *Ec*EDF 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 *Ec*EDF, *Bs*EDF, and *Pa*EDF-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 supple-

mental material). In the case of *Bs*EDF, 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 *Bs*EDF on *E. coli* cell death (Fig. 2A). Furthermore, as we have described for *Ec*EDF—previously called EDF (26)—here, *Bs*EDF 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 *Ec*EDF (26), *Bs*EDF bound directly to *E. coli* MazF in a sequence-specific manner (Fig. 5). However, as we found here, *Ec*EDF and *Bs*EDF affected different sites on *E. coli* MazF (Fig. 7; see also Fig. S6 in the supplemental material). An excess of the inhibitor of *Ec*EDF (*Ec*iEDF NNGNN) interfered with the ability of *Ec*EDF to enhance the action of MazF; *Ec*iEDF did not inhibit such enhancement by *Bs*EDF (Fig. 7A). In parallel, an excess of the inhibitor of *Bs*EDF (*Bs*iEDF RAQQNE) inhibited the enhancement of MazF activity only by *Bs*EDF and not of that by *Ec*EDF (Fig. 7B). Similar competition experiments that we carried out for each of *Pa*EDF-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 *Ec*EDF or *Bs*EDF (Fig. 7A and B; see also Fig. S6 in the supplemental material). In the case of *Pa*EDF-1/2/3, our conclusion was further supported by our results showing that, in contrast to *Ec*EDF and *Bs*EDF, *Pa*EDF-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 *Ec*EDF on *E. coli* MazF suggested that *Ec*EDF 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 *Ec*EDF-MazF (26) and the MazE-MazF complex (17). In particular, our model suggests that *Ec*EDF (NNWNN) and the binding site of MazE (locations 71 to 75; IDWGE) compete directly for MazF. We conjecture that the critical tryptophan-3 residue in *Ec*EDF 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 *Ec*EDF 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 *Ec*EDF 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, *Ec*EDF 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 *Bs*EDF and *Pa*EDF-1/2/3 affected different sites of *E. coli* MazF than did *Ec*EDF, it seems that the model that we have described above for the *Ec*EDF-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: *Ec*EDF 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 MazF-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) MC4100*relA*$^+$ (25) and (ii) its Δ*mazEF*::*kan* derivatives (25) and (iii) BL21(DE3) (Invitrogen, Carlsbad, CA) and TG1 (our strain collection). We used *B. subtilis* strain PY79 (from the collection of Sigal Ben-Yehuda) and *P. aeruginosa* strain PA14 and the *P. aeruginosa* Δ*fliD* transposon mutant (from the collection of Frederick M. Ausubel). *B. subtilis* PY79Δ*ydcDE* was constructed by using standard methods (43). Long-flanking PCR mutagenesis was used to create a *B. subtilis* Δ*ydcDE*::mls 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 for cloning 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′-AAGTCrGrArCrArUCAG-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 rArCrArUCAG-BHQ1-3′), respectively (Fig. 3A; see also Fig. S3A in the supplemental material). These oligonucleotides were purchased from IDT (Hudson, NH). Nickel-nitrilotriacetate (Ni-NTA) resin was purchased from Qiagen (Hilden, Germany). Black 96-well plates 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*-hydroxysuccinimide [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 (Carrigtwohill, 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 PA14Δ*fliD* in M9 medium at 37°C to the mid-logarithmic phase (optical density at 600 nm [OD_{600}], 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 *Bs*EDF and *Pa*EDFs on *E. coli* cell death. To a diluted culture (2.5×10^4 cells/ml) of *E. coli* MC4100*relA*$^+$ we added the SN of *B. subtilis* or the SNs of *P. aeruginosa* (PA14 and PA14Δ*fliD*) 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 *Bs*EDF and *Pa*EDFs on *ydcDE*-mediated cell death in *B. subtilis*. *B. subtilis* strains PY79 and PY79Δ*ydcDE* were grown in M9 glucose medium at 22°C for 20 h. Subsequently, cells were diluted to $OD_{600} = 0.1$ and grown aerobically in M9-glycerol medium to the mid-logarithmic phase ($OD_{600} = 0.5$) with shaking at 180 rpm. Cultures were either diluted to a density of 2×10^4 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 *Ec*EDF or 1 μg/ml of chemically synthesized *Bs*EDF or 1 μg/ml of each of six chemically synthesized *Bs*EDF mutants (m1 to m6) or 1 μg/ml of each of the three *Pa*EDFs. 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 *Bs*EDF was eluted at the elution time of 27.8 min (Fig. 1C; see also Fig. S1A in the supplemental material). The *Bs*EDF sequence was determined by the Edmen degradation procedure using a Procise 492 protein sequencer (Applied Biosystems).

Identifying and purifying the *P. aeruginosa* EDFs. SNs from dense cultures of *P. aeruginosa* (PA14 and PA14Δ*fliD*) 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* PA14Δ*fliD*. 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. *Pa*EDF was eluted at elution time 16.32 min (Fig. 1D; see also Fig. S1B in the supplemental material). The *Pa*EDF 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 *Bs*EDF and *Pa*EDFs on *E. coli* MazF endoribonucleolytic activity *in vitro*. We determined *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 *Bs*EDF by using affinity chromatography. To measure the interaction between *Bs*EDF and *E. coli* MazF, we coupled WT *Bs*EDF (RGQQNE) 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 *Bsi*EDF (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.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 0.7 MB.
Figure S2, TIF file, 1 MB.
Figure S3, TIF file, 1 MB.
Figure S4, TIF file, 0.2 MB.
Figure S5, TIF file, 0.6 MB.
Figure S6, TIF file, 1.6 MB.
Figure S7, TIF file, 0.2 MB.
Figure S8, TIF file, 0.4 MB.
Figure S9, TIF file, 0.3 MB.

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