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ORIGINAL PAPER

Bacillus globigii cell size is influenced by variants of the quorum sensing peptide extracellular death factor

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Abstract Toxin-antitoxin modules are necessary for the mode of action of several antibiotics. One of the most studied toxin-antitoxin modules is the quorum sensing—dependent MazEF system in *Escherichia coli*. The quorum sensing factor in this system is called the extracellular death factor (EDF), a linear pentapeptide with the sequence NNWNN. In spite of the extensive research on the mazEF system and the involvement of the quorum sensing factor EDF, the effect of EDF itself on bacteria has not yet been studied. In this research, we determined the effect of EDF and variants on cell growth in the Gram-negative bacterium *E. coli* and the Gram-positive *Bacillus globigii*. By aligning the *zwf* gene (from where EDF originates) of different bacterial species, we found 27 new theoretical variants of the peptide. By evaluating growth curves and light microscopy we found that three EDF variants reduced bacterial cell size in *B. globigii*, but not in *E. coli*. The D-peptides did not affect cell size, indicating that the effect is stereospecific. Peptides wherein tryptophan was substituted by alanine also did not affect cell size, which indicates that the effect seen is mediated by an intracellular target.

Keywords Quorum sensing · EDF · *Bacillus* · Cell size

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Introduction

Toxin-antitoxin (TA) systems have been found in a wide variety of bacteria such as *Escherichia coli, Bacillus subtilis*, and *Bacillus thuringiensis,* and are also found on plasmids in enteropathogenic *Yersinia enterocolitica* biotype 1a strains (Liu et al. 2008; Lepka et al. 2009; Florek et al. 2011; Fico and Mahillon 2006; Hayes 2003). TA systems consist of a stable toxin and a less stable antitoxin, which are usually expressed in one operon. The stable toxin is neutralized by the antitoxin. When expression is stopped the antitoxin is degraded and the toxin may kill the bacteria. TA systems are therefore considered as a bacterial system for apoptosis.

One of the most studied chromosomal TA systems is the quorum-sensing dependent MazEF system in

E. coli. In the MazEF system, the MazF toxin is a sequence-specific endoribonuclease (Zhang et al. 2003) and the MazE antitoxin an unstable dimeric protein which is sensitive to degradation by the ClpAP serine protease system (Aizenman et al. 1996). Both MazF and MazE form dimers, whereupon one MazE dimer binds two MazF dimers (Zhang et al. 2003; Kamada et al. 2003). MazF activity can be induced by several antibiotics such as rifampicin and chloramphenicol (Sat et al. 2001) as these antibiotics inhibit transcriptions of the *mazE* antitoxin. Lethality is dependent on the cell density of the bacteria (Amitai et al. 2004; Pedersen et al. 2002).

Previously a novel quorum sensing peptide was discovered, which plays a crucial role in the MazEF cascade (Kolodkin-Gal and Engelberg-Kulka 2008). This peptide, the extracellular death factor (EDF), is a pentapeptide with the amino acid sequence NNWNN. It was found that EDF originates from the *zwf* gene, which codes for glucose-6-phosphate dehydrogenase, and is involved in the pentose phosphate pathway. The pro-peptide NNWDN is released after cleavage by the ClpXP protease system and amidated to NNWNN (Kolodkin-Gal and Engelberg-Kulka 2008; Kolodkin-Gal et al. 2007). EDF modifies MazF activity by directly binding to MazF and thus preventing MazE to bind and silence MazF activity (Belitsky et al. 2011).

In spite of extensive research in the MazEF system and the role of the quorum sensing factor EDF, the effect of EDF itself on bacteria has not yet been studied. Considering that EDF originates from the highly conserved *zwf* gene, this factor might be present in a wide variety of bacterial species. In this study, we determined the effect of EDF on cell growth in *E. coli* and *Bacillus globigii*, which is closely related to *B. subtilis*. When *B. globigii* was cultured in the presence of EDF for 16 h, cell size was significantly smaller. An alanine substitution scan revealed that the highly conserved tryptophan residue was crucial for this effect. The all-D variant of EDF had no effect, suggesting that stereospecific interactions underlie the effect of EDF on cell-size.

Materials and methods

Bacterial strains

Three laboratory strains of *E. coli* (HB101, DHSα, and BL21) and two clinical isolates (o585 and o690) and *B. globigii* BM013 (TNO library) were used in our studies. Both bacteria were cultured under aerobic conditions in Tryptic Soy Broth (TSB, Fluka) or on Tryptic Soy Agar (TSA, Fluka) plates at 37 °C. Specific culture conditions are described in the experiments.

zwf Alignment

Based on sequence homology the *zwf* gene products of different bacterial species were aligned using CLC Main workbench (www.clcbio.com). The *E. coli* NNWDN sequence was used as template sequence for other potential EDFs (Table 1).

Peptide synthesis and purification

All peptides (Table 2, including all EDFs, D-EDFs and alanine substituted variants) were synthesized by solid phase peptide synthesis using Fmoc chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Biosearch, Bedford, MA, USA) and purification by reverse phase-HPLC was conducted as described previously (Veerman et al. 2007). The purity of all peptides was >90 %. Identity of the peptides was confirmed using mass spectrometry (MALDI-TOF MS, Microflex, Bruker Daltonics, Bremen, Germany).

Evaluation of growth and viability

E. coli and *B. globigii* were diluted 1:1,000 in fresh TSB and incubated in the presence of the peptides which were dissolved in TSB and serially diluted in a 96 well plate (Greiner, 96-wells, sterile). Plates were sealed using self-adhesive sterile seals (732–2,610, NUNC). The absorbance at 600 nm was measured every 10 min for 14 h at 37 °C with shaking before each measurement using a spectrophotometer (Spectramax M2, Molecular Devices).

After 16 h cultures which were treated with 62.5 μM peptide were tenfold diluted and plated on Tryptic Soy Agar (TSA, Fluka) plates. After 24 h growth under aerobic conditions at 37 °C the number of colony forming units (CFUs) was determined.

Microscopic analysis

Escherichia coli and *B. globigii* were cultured overnight with 62.5 μM peptide in TSB under aerobic

Table 1 Alignment of the *zwf* gene

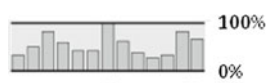
Strain	Sequence	Place in ZWF
Escherichia coli str. K-12 substr. MG1655	SLFV**NNWDN**RTID	207
Shigella dysenteriae Sd197	SLFV**NNWDN**RTID	207
Salmonella enterica subsp. Enterica serovar Newport str. SL254	SLFV**NNWDN**RTID	207
Klebsiella pneumoniae 342	SLFV**NNWDC**RTID	207
Proteus mirabilis	SLFV**NNWDN**KTID	210
Yersinia pestis Angola	SLFA**SNWDN**RTID	210
Yersenia enterocolitica subsp. Enterocolitica 8081	SLFA**SNWDN**RTID	207
Legionella pneumophila str-1. corby	SIFS**TNWDN**RVID	203
Pseudomonas aeruginosa PAO1	SLFE**TQWNQ**NHIS	203
Lactobacillus gasseri JV-V03	PIVN**NIWCK**KYIA	210
Borrelia burgdorferi 64b	SIFE**NIWNN**RYVD	210
Streptococcus gordonii str. Challis substr. CH1	LLFE**NIWNR**EYID	207
Streptococcus pneumoniae D39	LIFE**NVWNK**DFID	207
Burkholderia multivorans ATCC 17616	PIFG**PLWQA**PSIR	205
Eikenella corrodens ATCC 23834 (hypothetical protein)	RLFE**PLWCA**AQIR	206
Burkholderia mallei ATCC 23344	ALFE**PLWRR**EWVE	210
Brucella abortus bv. 1 str. 9-941	ALYE**PLWNS**AHID	210
Agrobacterium tumefaciens str. C58	ALYE**PLWNS**AHID	213
Bartonella henselae str. Houston-1	TLYE**PLWNS**NYID	207
Vibrio cholerae V51	GMFE**PLWNR**NFID	247
Pasteurella multocida subsp. Multocida str. Pm70	GWFE**PLWNR**NFID	202
Bacteroides fragilis NCTC 9343	GIFE**PLWNR**NYID	202
Prochlorococcus marinus str. MIT 9515	TIFE**PIWNR**NYIS	207
Propionibacterium acnes SK187	QIFE**PIWNN**HYVS	207
Neisseria meningitidis Z2491	VMFE**PLWNN**KYIE	207
Staphylococcus aureus subsp. aureus MRSA252	AMFE**PLWNN**KYIS	209
Mycobacterium avium subsp-1. paratuberculosis K-10	NALA**KLWDR**DSIS	212
Lactobacillus salivarius UCC118	NIFR**ALWNN**RYID	212
Lactobacillus casei BL23	AIFE**GVWNK**DYID	210
Bacillus anthracis str. Ames	PVLQ**SIWNK**EHIA	202
Consensus	SLFE**PLWNN**RYID	
Conservation		
Sequence logo		

Table 2 Synthesized peptides and modifications

Sequence found in the *zwf*-alignment	Amidated	W → A	D-peptides
NNWDN	NNWNN		
NNWDC	NNWNC		
SNWDN	SNWNN		
TNWDN	TNWNN		
NVWNK		NVANK	D-NVWNK
NIWNR		NIANR	D-NIWNR
PLWCA		PLACA	D-PLWCA
SCWNS			
SIWNK			
NIWNN			
KLWDR			
KLWNR			
NVWNR			
NIWCK			
GVWNK			
ALWNN			
PIWNN			
GVWNN			
PLWQA			
TQWNQ			
PLWRR			
PLWNN			
PLWNS			
PLWNR			
PIWNR			

conditions at 37 °C until late stationary phase. Subsequently, of each sample, three different pictures were taken using a microscope (Zeiss Axioskop and Axiocam MRc), at a 1,000× magnification. The dimensions of 180 bacteria of each sample were determined using Adobe Photoshop CS4 measurement tool.

Statistical analysis

Statistical analysis was performed using one-way ANOVA and Bonferroni's multiple comparisons test. All samples (CFU counts and cell size) were analyzed at least in duplicate and each test was repeated three times. A p value of <0.05 % was considered significant. The level of significance is mentioned in the figure legends.

Results

The EDF sequence in the *zwf* gene product is highly conserved among bacteria

To generate a database of EDF peptide variants, we compared the *zwf* gene product of 30 microorganisms using CLC Main Workbench. The *zwf* alignment, as shown in Table 1, shows that the EDF sequence, and especially the central tryptophan, was highly conserved. The consensus sequence resulting from this comparison is PLWNN.

We deduced 23 new sequences from the *zwf* alignment of EDF. In addition to these sequences, we synthesized three amidated forms that might be present in vivo as was described for the original EDF (Kolodkin-Gal et al. 2007). Together with the known EDF peptide from *E. coli* NNWDN and its in vivo amidated form NNWNN, this resulted in a library of in total 29 EDF peptide variants (Table 2).

EDF variants lower the optical density of bacteria in stationary phase

We evaluated the growth of *E. coli* and *B. globigii* in the presence of EDF peptides. All peptides were tested on all bacterial strains. During 14 h the absorbance at 600 nm was determined with 10 min intervals. Culturing *B. globigii* in the presence of three of the EDF variants resulted in a lower absorbance in the stationary phase than the control (Fig. 1a–c). This effect was more pronounced at higher concentrations of the EDF variants. The EDF variant with the original sequence NNWNN showed no effect on the absorbance at 600 nm (data not shown). No effect on the growth curve of *E. coli* was observed.

To verify if the observed decrease in absorbance was due to growth inhibition, the number of CFU/ml was determined. This revealed that treatment with the peptide did not lead to a clear decrease in CFU/ml, even though the absorption at 600 nm was lower (Fig. 1D). This result leads to the hypothesis that culturing in the presence of EDF, might affect the dimensions, rather than the number, of the bacteria.

Bacteria grown in presence of EDF variants are smaller

To examine the cell size of treated bacteria, cultures were examined live using a microscope. The effect on

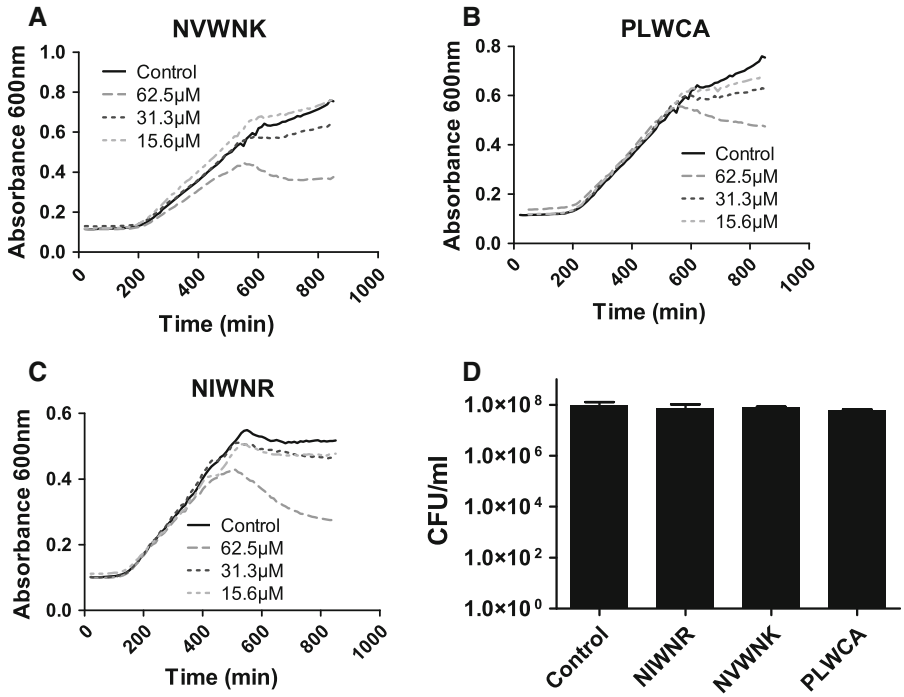


Fig. 1 The effect of NVWNK, NIWNR, and PLWCA on *B. globigii* growth. **a**–**c** Growth *curves* of the three active peptides NVWNK **a**, PLWCA **b**, and NIWNR **c**. The *solid lines* represent control values; the *dashed lines* represent three different concentrations of the peptides. Representative results are shown. **d** CFU counts after incubation with the peptides. No statistically significant differences were found

the absorbance was seen in mid-log phase and continued in the stationary phase. For optimal effects, further experiments were continued at the late-log phase.

After 16 h growth in presence or absence of 62.5 μM peptide, the length of the bacteria was determined (Fig. 2, black bars). The length of the bacteria treated with the peptides was approximately 15 % smaller than the untreated cells and cells treated with the original EDF with the sequence NNWNN. The diameter of both treated and untreated cells remained comparable. Typical examples of control cells and treated cells are shown in Fig. 2b.

Next we assessed whether this effect was irreversible or only during the presence of the peptides. For this, treated cells were 100× diluted in fresh medium without peptides and grown for another 6 h. Then, the cell size was determined again. The white bars in Fig. 2 represent this recovery and it can be seen that after 6 h of growth, the cell size of the bacteria was comparable to that of the untreated cells.

Tryptophan and L-stereoisomer of EDF is necessary for their effect

The functional amino acids within a peptide can be determined by using an alanine substitution of single amino acids. As the tryptophan is a highly conserved amino acid in all EDF sequences coded by *zwf* gene (Fig. 1), it is tempting to suggest that this residue might play a crucial role in its activity. To verify this we generated the active peptides with a tryptophan-to-alanine substitution that resulted in the peptides NV**A**NR, SI**A**NK and PL**A**CA. In addition, the full D-enantiomeric variants of the active peptides were prepared, to examine the stereospecificity of the effect.

Figure 3 shows that a substitution of the tryptophan by alanine results in loss of effect of the peptide on the absorbance at 600 nm. The D-enantiomers were also completely inactive, suggesting the involvement of a stereospecific process.

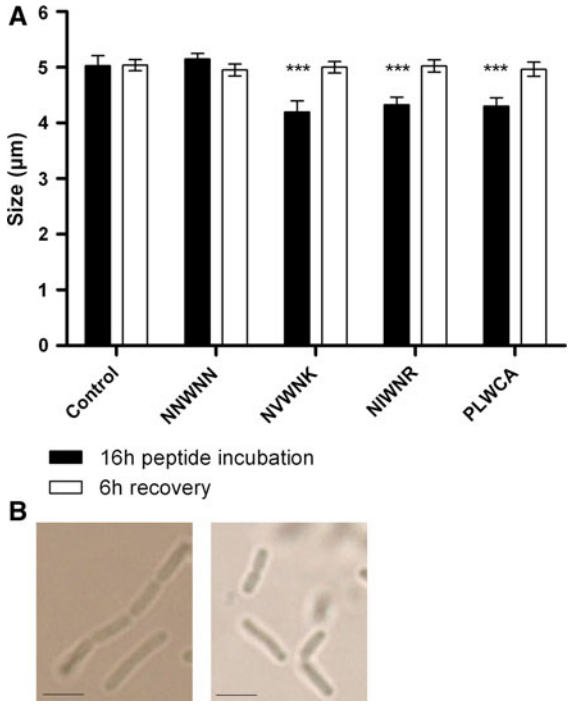


Fig. 2 **a** The effect of NNWNN, NVWNK, NIWNR and PLWCA on the size of *B. globigii* cells. *Filled columns* represent the results after 16 h incubation of *B. globigii* with the peptides; *open columns* represent the 6 h growth after the 16 h incubations were re-inoculated in fresh medium. *** $p < 0.001$. **b** Typical examples of control cells (*left*) and cells treated with an active peptide (*right*). The treated example was treated with NIWNR. *Scale bars* represent 5 µm

Discussion

In this study, we examined the effect of the EDF and theoretical variants of this peptide on the growth of *E. coli* and *B. globigii,* which is a member of the *B. subtilis* family.

We found that three variants of the EDF peptide i.e. NVWNK, NIWNR, and PLWCA, caused *B. globigii* cells to be shorter in the stationary phase. This effect on cell size appeared to be reversible. We also found that the effect of the peptides is stereospecific which may suggest that there is a specific target for these peptides. The highly conserved tryptophan residue is also important in this effect. By substituting the tryptophan by alanine the effect on the absorption was abolished. Tryptophan, which has a preference for the lipid-water interfases, contributes to the transmigration of peptides across membranes (Yau et al. 1998). Thus we speculate that substitution of tryptophan by alanine will hamper the entrance of EDF peptides into the cell, resulting in loss of activity. The effects that we have found with *B. globigii* are however in concentrations that are more than a thousand times higher than the effect found by Kolodkin-Gal et al. (2007). This indicates that the effects that we see are probably not related to MazF.

In contrast to *B. globigii*, none of the peptides, including the original EDF peptide, had an effect on the growth of one *E. coli* strain we analyzed. Since we used a different *E. coli* strain than the one used by Kolodkin-Gal and co-workers, we analyzed four additional *E. coli* strains to confirm our results. Again, no effect was found for any of the additional *E. coli* strains tested. To our best knowledge, there is nothing known about the MazEF system in the two clinical isolates and two of our lab strains: DH5α and HB101. A UniProt search revealed the BL21 has a complete MazF sequence (http://www.uniprot.org/uniprot/C6EJ90), but the status is unreviewed at the moment.

We had expected to find an effect, if any, with the original EDF peptide (NNWNN), as it was stated that this peptide functions as a quorum sensing peptide (Kolodkin-Gal et al. 2007). Even though this may seem contradictory, our experiments differ significantly from the two papers published on in vivo effects of EDF on *E. coli* (Kolodkin-Gal and Engelberg-Kulka 2008; Kolodkin-Gal et al. 2007). In the first publication on the discovery of EDF, it is found that MazEF mediated cell death by rifampicin is a population phenomenon. Later on it is stated that EDF is responsible for a successful MazEF mediated cell death by rifampicin. In their second publication on EDF and *E. coli* the researchers further investigate the role of EDF in mazEF mediated cell death by antibiotics.

The main difference between our research and the research published by Kolodkin-Gal et al. is that we do not use rifampicin or other antibiotics, but we focus mainly on the effect of EDF itself on the organism. It is therefore possible that the effect found in our research is not related to the *E. coli* MazEF TA system.

The difference in observations for *B. globigii* and *E. coli* may lie in the fact that these bacteria regulate their cell size in a different way. The cell size of *B. subtilis* is strongly dependent on nutrient availability. When cultured in minimal medium, these bacteria are smaller than when they are grown under nutrient-rich conditions. A change in length of *B. subtilis* does not

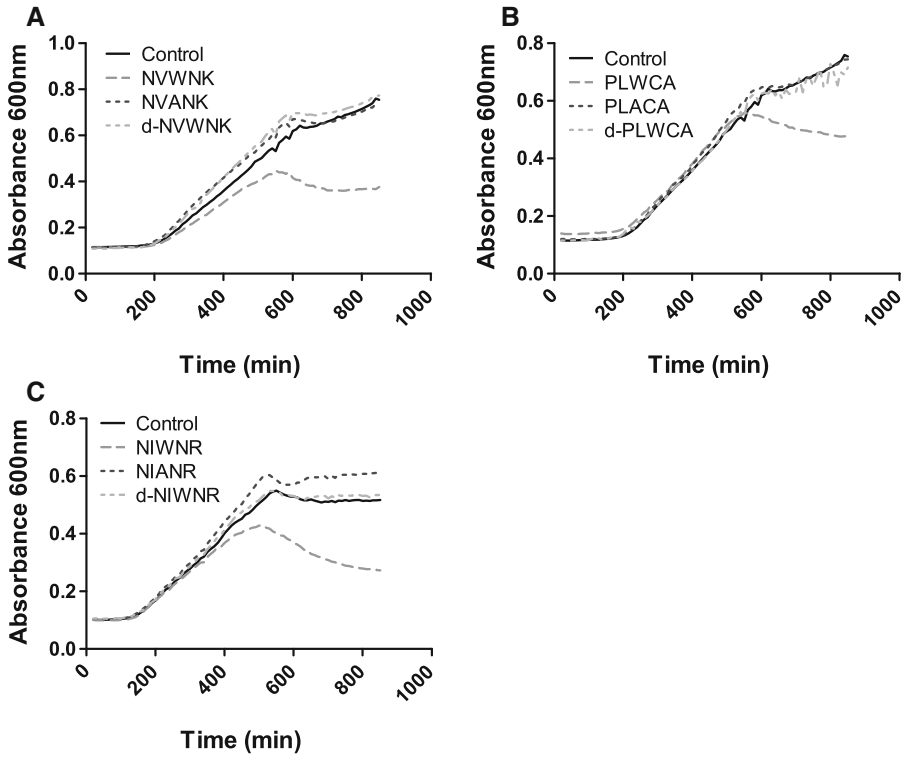


Fig. 3 The effect of D-peptides and W → A peptides of NVWNK **a**, PLWCA **b**, and NIWNR **c** on *B. globigii* growth. All peptides were tested in a concentration of 62.5 μM. The *solid lines* represent control values; the *dashed lines* represent three different concentrations of the peptides. Representative results are shown

influence its diameter, which was also seen in our study. (Trueba and Woldringh 1980).

The cell size of *B. subtilis* is linked to carbon source availability. The main determinant in this system is the nucleotide sugar UDP-glucose (UDP-glc), which may serve as a sensor for carbon excess, where carbon excess increases UDP-glc synthesis and cell size (Chien et al. 2012).

The effector of UDP-glc carbon sensing in *B. subtilis* is the glucosyltransferase UgtP. Disruption of this gene decreases cell size by 20 % when the bacteria are grown in rich medium. UgtP regulates cell size by interfering with the FtsZ ring formation, which controls cell division. In rich media, UgtP inhibits FtsZ ring formation, thus delaying division. In carbon-poor media, UgtP is randomly distributed in the cell and not localized at the FstZ ring site (Weart et al. 2007). As *B. globigii* is closely related to *B. subtilis* and is sometimes even considered as a subspecies of *B. subtilis* (*B. subtilis subsp niger*), we hypothesize that this system may be involved in cell-size control in *B. globigii*. A preliminary alignment of EDF peptides and the UgtP protein sequence of *B. subtilis* show a similarity between the peptides and part of the protein sequence (unpublished data). This might be an indication that the peptides are able to reduce the ability of UgtP to inhibit FtsZ ring formation by for instance blocking its access, leading to a decrease in cell size.

As there is no UgtP homologue in *E. coli*, an effect of the peptides on UgtP may explain why the peptides did not similarly affect the cell size of *E. coli*, even though *E. coli* cell size is influenced by UDP-glc signaling. To test this hypothesis, future experiments should evaluate the effect of the peptides on *ugtP* mutant *Bacillus* cells. This will give a straightforward answer whether this mechanism is influenced by the EDF peptides.

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