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Gramicidin D enhances the antibacterial activity of fluoride

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Abstract

Fluoride is a toxic anion found in many natural environments. One of the major bacterial defenses against fluoride is the cell envelope, which limits passage of the membrane-impermeant fluoride anion. Accordingly, compounds that enhance the permeability of bacterial membranes to fluoride should also enhance fluoride toxicity. In this study, we demonstrate that the pore-forming antibiotic gramicidin D increases fluoride uptake in *B. subtilis* and that the antibacterial activity of this compound is potentiated by fluoride. Polymyxin B, another membrane-targeting antibiotic with a different mechanism of action, shows no such improvement. These results, along with previous findings, indicate that certain compounds that destabilize bacterial cell envelopes can enhance the toxicity of fluoride.

Keywords

antibiotic; Bacillus subtilis; fluoride reporter; riboswitch; polymyxin B

The development of novel treatments for multidrug-resistant bacterial infections is of critical importance given the limited pipeline of new antibiotic scaffolds currently in development.[1] Alternatively, it might be practical to use a combination therapy that includes a proven antibiotic and an additional toxic substance, such as fluoride. Fluoride derives its antibacterial function in part from its ability to mimic the γ-phosphate of ATP or GTP in conjunction with a divalent metal ion and ADP or GDP.[2,3] As such, fluoride can inhibit a

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wide range of metabolic enzymes,[4] including enolase,[5] and therefore is broadly toxic to organisms in all three domains of life.

If fluoride is to be more widely used as an antibacterial agent or as an agent to increase the potency of known antibiotics, separating its antibacterial activity from its toxicity to the host organism will be critical. This could potentially be done by using fluoride in conjunction with certain antibiotics that selectively target bacterial membranes. Such compounds would increase the membrane permeability of fluoride, which typically enters cells as the protonated HF. Selectively increasing the concentration of fluoride to bacterial cells would increase its antibacterial effects while presumably avoiding unwanted toxicity to host cells. Indeed, both the pore-forming antifungal compound amphotericin B and the antimicrobiral magainin peptides have been shown to enhance fluoride toxicity.[6, 7]

To further investigate this hypothesis, we examined the ability of fluoride to enhance the activity of a variety of antibiotics in Gram-positive and Gram-negative bacteria. While we reasoned that membrane-targeting antibiotics and fluoride would prove to be synergistic, we wanted to conclusively determine if these compounds increase the levels of fluoride in bacteria. To detect intracellular fluoride, a representative of a recently identified fluoride-responsive riboswitch class found in many species of bacteria and archaea[8] was exploited.

Riboswitches are small, structured RNA motifs found within messenger RNAs. Each riboswitch recognizes a small molecule or ion and regulates the expression of downstream genes.[9] Following transformation of *Bacillus subtilis* and *Escherichia coli* bacteria with a plasmid containing a *lacZ* reporter gene under the control of a fluoride riboswitch (**Fig. S1**) and subsequent optimization of experimental conditions, the cells report changes in fluoride levels as changes in β-galactosidase gene expression upon the addition of 4-methyl-umbelliferyl-β-D-galactopyranoside.[10, 11]

The first antibiotic we chose to examine was gramicidin D. Gramicidin D is actually a mixture of gramicidins A, B, and C. These compounds are members of a small peptide family that form β-helices in hydrophobic environments.[12, 13] Once formed, these helices assemble into a supramolecular structure within the phospholipid membrane of certain Gram-positive bacteria, resulting in the formation of a pore roughly 5 Å in diameter.[13-17] Gramicidins are known to facilitate the transport of a variety of monocationic ions, including Na^+, K^+, and Cs^+.[18] We hypothesized that fluoride should be able to pass through this pore, unless the gramicidin complex is able to discriminate based on charge, because F^- and K^+ are of roughly equivalent size. Indeed, upon addition of 0.95 μg mL^{-1} gramicidin D, a roughly five-fold increase in fluoride riboswitch-mediated gene expression is observed in *B. subtilis* (**Fig. 1A**). Although gramicidin D and fluoride independently exhibit antibacterial activity, gramicidin D enhances the activity of fluoride (**Fig. 1B**) and likewise fluoride enhances the activity of gramicidin D (**Fig. 1C**). Thus, the two antibacterial agents function synergistically, as expected if gramicidin D facilitates fluoride uptake by cells.

Similarly, polymyxin B,[19] another membrane-targeting antibiotic that is selective for Gram-negative bacteria,[20] was also examined for evidence of synergy with fluoride. Polymyxin B consists of a circular peptide that targets lipopolysaccharide (LPS, a major component of the

gram-negative membrane envelope) and a hydrophobic tail that inserts into the membrane.[20] The exact mechanism of action of polymyxin B is still uncertain, but it appears to cause disruption and mixing of the outer and inner membranes.[21] Addition of this antibiotic to *E. coli*, however, did not result in enhanced fluoride uptake or retention (**Fig. 2**) and, not surprisingly, there was no concomitant increase in fluoride-dependent cytotoxicity (data not shown).

The lack of synergy between polymyxin B and fluoride indicates that not all antibiotics that target membranes will enhance fluoride uptake by bacteria. It has been shown that amphotericin B, an antifungal agent, also acts in a synergistic fashion with fluoride,[7] and the antimicrobial magainin peptides have also been shown to have their effects potentiated by fluoride.[6] Interestingly, like gramicidin D, amphotericin B and the magainins are also pore-forming antibiotics. Perhaps other compounds that employ a pore-forming mechanism could be identified that increase fluoride uptake and toxicity.

Since fluoride cannot easily pass through the plasma membrane except as a neutral complex like HF, it is not surprising that bacteria that reside in alkaline environments are more resistant to the toxic effects of fluoride compared to mesophilic organisms. For example, we have observed that *Bacillus halodurans*, an extremophilic relative of *B. subtilis* that grows well at pH 10, shows no ill effects even as fluoride approaches its solubility limit at this pH (~1 M; data not shown). In contrast, *B. subtilis* has a minimal inhibitory concentration (MIC) for NaF of ~200 mM at neutral pH.[8] Upon treatment with both gramicidin D and fluoride, however, *B. halodurans* exhibits a six-fold reduction in growth (**Fig. 3**), which is a noticeably greater effect than that observed for gramicidin D combined with chloride.

As noted above, the exact mechanism by which gramicidin D enhances fluoride uptake is uncertain. While gramicidin D might directly facilitate the transport of fluoride into the cell, there are known cation binding sites within the gramicidin channel that could inhibit anion transport.[22] Although the gramicidins select against divalent cations in favor of monovalent ones, [18] gramicidin D has not been shown to discriminate against fluoride. In fact, some evidence of fluoride binding gramicidin A and affecting transport has been previously observed.[23] Thus, it is possible that these data could be the result of direct, gramicidin-facilitated transport of fluoride. However, other mechanisms for the observed increase in cellular fluoride uptake and/or retention are possible, such as disruption of membrane potential or interference with fluoride ion channel function. Regardless of the precise mechanism by which these existing compounds affect cellular fluoride concentrations, new compounds that affect fluoride toxicity resistance could conceivably be identified and serve as effective combination therapies with this toxic anion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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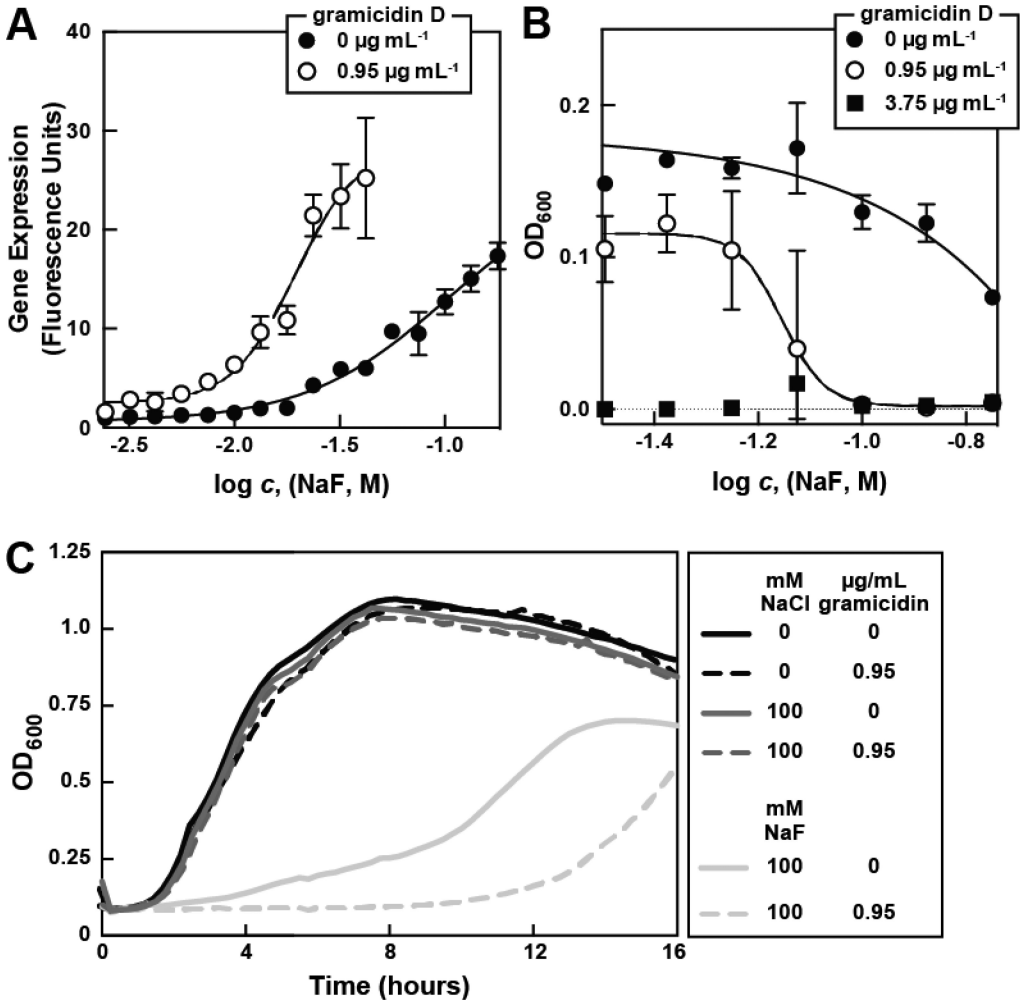

Figure 1.

Synergistic antibacterial function of fluoride and gramicidin D. (A) Gramicidin D increases intracellular fluoride levels in *B. subtilis*. Reporter gene expression values were normalized to account for culture growth (see Supplemental Material). Error bars are the standard deviation of three replicates. (B) Gramicidin D enhances the antibacterial activity of fluoride. Data points represent the growth of *B. subtilis* following 16 hours of incubation in the condition indicated for each experiment. Error bars represent the standard deviation of three measurements and the data is fit to a sigmoidal curve. (C) Fluoride enhances the antibacterial activity of gramicidin D. Culture growth is depicted as trend lines, which represent the average optical density of three replicates that were measured every fifteen minutes.

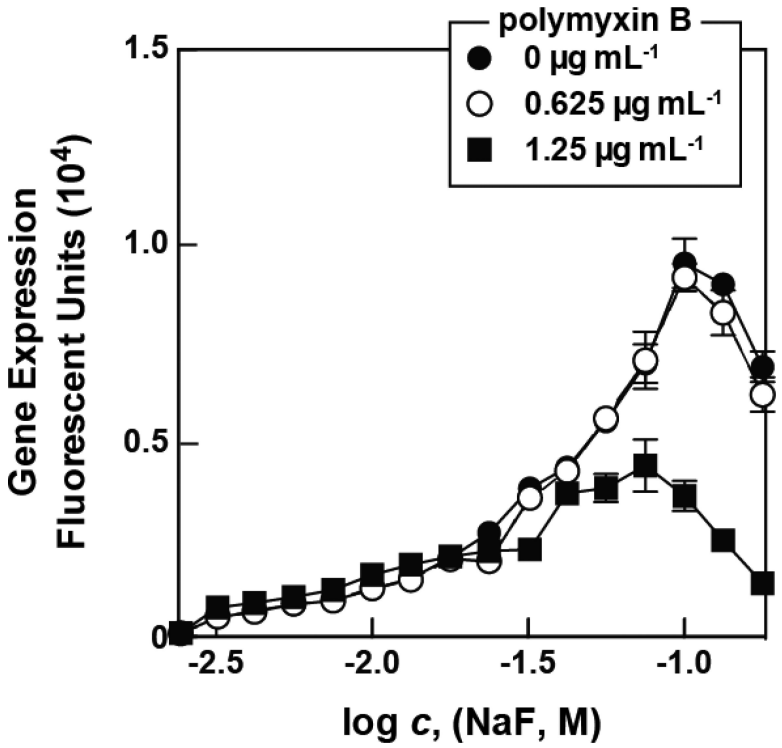

Figure 2.

No enhancement of fluoride uptake is observed upon addition of polymyxin B to *E. coli*. Data points are the average of three replicates and error bars represent the standard deviation, which is smaller than the data point symbols. No growth was observed under the experimental conditions with 2.5 μg mL^{-1} polymyxin B (data not shown).

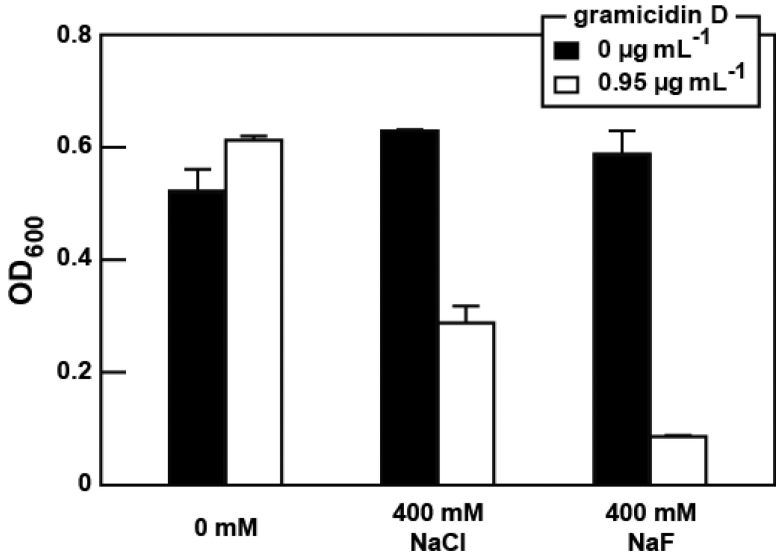

Figure 3.

Synergistic effect of fluoride and gramicidin D in *B. halodurans*. Data presented are the average of three replicates and error bars represent standard deviation.