

New Insight on the Response of Bacteria to Fluoride

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Fluoride has been used for decades to prevent caries and it is well established that this anion can inhibit the growth of bacteria. However, the precise effects that fluoride has on bacteria and the mechanisms that bacteria use to overcome fluoride toxicity have largely remained unexplored. Recently, my laboratory reported the discovery of biological systems that bacteria use to sense fluoride and reduce fluoride toxicity. These sensors and their associated genes are very widespread in biology, which has implications for a number of issues that are central to the use of fluoride for dental care. Below I provide a summary of our findings, comment on some of the key prospects for expanding our understanding of fluoride's effects on biology, and propose some future uses of this knowledge.

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action of fluoride in caries prevention is widely believed to be its ability to bond with enamel of teeth to form fluorhydroxyapatite [ten Cate, 1999]. However, fluoride is strongly antimicrobial, and this property has been proposed to contribute to the anticaries activity by reducing metabolism and growth of organisms such as *Streptococcus mutans* [e.g., Levine, 1976; Hamilton, 1990].

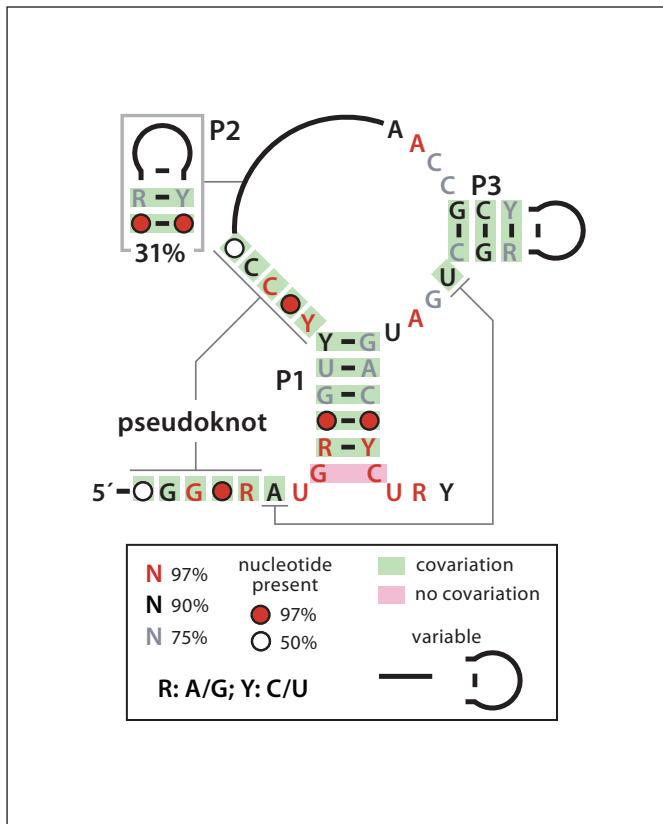
Although fluoride has been known for many decades to be antimicrobial [e.g., Leshner et al., 1977; Maltz and Emilson, 1982], there has been little understanding of its precise effects on cells or of the mechanisms that might be used by organisms to overcome this toxicity. Fluoride more efficiently enters bacterial cells at acidic pH values as HF and dissociates when exposed to the more neutral intracellular pH [Marquis et al., 2003]. Bacteria have evolved numerous strategies to alleviate the toxic effects of other ions, and yet analogous systems for fluoride toxicity mitigation were notably absent [Silver, 1996]. Our discovery of more than 2,000 examples of fluoride-sensing 'riboswitches' helps resolve this longstanding mystery and offers new opportunities for research and development.

Fluoride and Bacteria

Since the 1950s, fluoride has been widely used in oral hygiene products and has been added to municipal water supplies due to its strong anticaries function [Mullen, 2005; Slayton et al., 2006]. The primary mechanism of

RNA Molecules That Sense Fluoride

Riboswitches are structured parts of some bacterial messenger RNAs that bind metabolites or other ligands and regulate the expression of proteins encoded by the mRNA [Roth and Breaker, 2009]. Ligand binding chang-



es the folding of the riboswitch and these structural changes either activate or inhibit expression of the adjacent gene. In most instances, riboswitches turn off the expression of genes that make or transport the metabolite that is sensed by the RNA. When cells have sufficient metabolite, they save energy by sensing its abundance and deactivating expression of genes whose protein products would otherwise make or import more. However, there are some riboswitches that activate gene expression when they bind a ligand. For example, riboswitches that activate expression have been demonstrated to respond to glycine [Mandal et al., 2004], the bacterial second messenger c-di-GMP [Sudarsan et al., 2008; Lee et al., 2010], and adenine [Mandal and Breaker, 2004]. In the latter case, adenine binding occurs when this compound accumulated to toxic levels, which induces expression of a



Fig. 2. Control of β -galactosidase reporter gene expression in *E. coli* by a fluoride-responsive riboswitch from *Bacillus cereus*. Reporter construct (top) and Miller assay analysis (bottom) were as described previously [Baker et al., 2012].

transporter that exports adenine to reduce its cellular concentration.

While pursuing the discovery of new types of RNAs, we identified a novel riboswitch candidate called the 'crcB motif' RNA (fig. 1) [Weinberg et al., 2010]. Examples are present in many species of bacteria and archaea, and we found homologs of the riboswitch-associated bacterial and archaeal genes in fungi, plants, and other eukaryotes (but not in humans). These findings indicate that the ligand for this riboswitch class is important to many species from all three branches of the tree of life.

During our search for the natural ligand, we serendipitously determined that fluoride is selectively bound by the riboswitch and that this anion triggers riboswitch-mediated gene expression in cells (fig. 2). After numerous incredulous discussions and many validation experiments, we indeed confirmed that fluoride is the natural ligand for this riboswitch [Baker et al., 2012].

Proteins That Fight Fluoride Toxicity

The genes associated with fluoride riboswitches include enzymes such as enolase that have long been known to be inhibited by this anion [Hoorn et al., 1974].

Thus, fluoride buildup in bacterial cells is detected by a fluoride-responsive riboswitch, and this triggers expression of genes that produce more copies of the enzyme being inhibited. Even more intriguing, two of the most common genes (called *crcB* and *eriC^F*) associated with this riboswitch class appear to code for distinct fluoride transporters that we believe are exporting fluoride. Again, fluoride riboswitches activate expression of these new-found fluoride exporters that expel the anion from cells.

There are also many other types of genes associated with fluoride riboswitches. Some of these genes are annotated as stress response genes and DNA repair genes, while others encode for proteins of unknown functions. Not every bacterial species has the full spectrum of fluoride-induced genes, and therefore organisms appear to have developed multiple different strategies for overcoming the effects of fluoride. In general, riboswitch associations with various genes, whether the functions of their protein products are known or unknown, links them to fluoride resistance and thus reveals new paths to follow when investigating the effects of fluoride on cells.

The wide distribution of these riboswitches and associated genes reveals that fluoride toxicity is likely to be near universal and a very frequent occurrence, otherwise these systems would have been lost. Since fluoride is the 13th most common element in earth's crust, natural exposure of many organisms to fluoride is likely to be common. Curiously, humans and other metazoans appear to lack representatives of this riboswitch class and the other cellular components used so commonly by bacteria to overcome the adverse effects of fluoride. Perhaps humans have distinctive fluoride sensors and toxicity mitigation proteins that remain to be discovered. Regardless, it is clear that humans tolerate high levels of fluoride in the oral cavity, at least temporarily as delivered by fluoridated toothpastes and mouthwashes.

Prospects for Fluoride Riboswitch Research and Development

The various RNA and protein components used by cells to reduce fluoride toxicity conceivably could serve as targets for compounds that enhance fluoride uptake or retention by bacteria. The most common gene associated with fluoride riboswitches is *crcB*, and therefore compounds that block the putative fluoride exporter encoded by this gene might serve as broad-spectrum antimicro-

bial agents when combined with fluoride. Similarly, compounds that disrupt fluoride riboswitch function would prevent cells from activating the expression of fluoride toxicity mitigation genes. These compounds would sensitize bacteria to fluoride by causing its buildup in cells or otherwise by preventing cells from overcoming its inhibitory effects.

The second unrelated fluoride exporter protein is coded for by a mutant type of *eriC* (ClC) chloride channel gene we call *eriC^F*. Although *eriC^F* genes are far less common in bacteria, two homologs of this gene reside in tandem in the genome of *S. mutans*. Again, chemical agents that selectively block members of this fluoride ion exporter class should act as more targeted inhibitors of *S. mutans* growth when combined with fluoride. Moreover, *S. mutans* appears to lack a fluoride riboswitch, suggesting that another type of fluoride sensor system may be present in this organism and that this system also could be selectively targeted by compounds.

Those who are interested in manipulating cellular fluoride concentrations now can exploit components of the fluoride sensory and resistance systems. For example, fluoride-specific biochemical tools such as fluoride riboswitches can act as convenient in vivo sensors of fluoride concentration when fused to reporter genes (fig. 2). Likewise, model bacterial species cells can be genetically altered to be more sensitive or more resistant to fluoride by knocking out or overexpressing exporters or other toxicity mitigation proteins [Baker et al., 2012].

Among many other interesting questions to be addressed is whether *S. mutans* has always carried two copies of *eriC^F* genes, or whether this is a more recent adaptation made in response to the bursts of extremely high fluoride concentrations delivered with oral healthcare products. If *S. mutans* or other bacteria show evidence of recent adaptation, then there might indeed be an antimicrobial component to the therapeutic effects of fluoride on tooth decay. Moreover, these cells could have the evolutionary capacity to further improve their fluoride resistance [e.g., Brown et al., 1983; van Loveren et al., 1989], which might lessen the utility of fluoride over time, much like the emergence of resistance by bacteria to antimicrobial drugs.

Our data indicate that bacteria such as *Escherichia coli* [Baker et al., 2012] or fungi such as *Candida albicans* (unpublished data) cannot survive upon long exposure to fluoride approaching 250 mM, which is the concentration found in some prescription fluoride toothpastes. Perhaps there is a fluoride concentration beyond which no expression level of fluoride toxicity mitigation genes

such as enolase or ion channels can overcome. Regardless, it seems reasonable to expect that compounds that promote fluoride transport across bacterial membranes or that prevent the cells from detecting and ejecting cytoplasmic fluoride would selectively increase the antibacterial effects of this anion for the benefit of oral health.

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Disclosure Statement

The author is a cofounder of BioRelix, a biotechnology company that has licensed riboswitch intellectual property from Yale University.

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