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Effects of fluoride on bacterial growth and its gene/protein expression

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HIGHLIGHTS

- Bacterial growth and its gene/protein expression were detected at various concentrations of NaF.
- Fluoride has a classic rise/fall response of inducing *E. coli*-GFPuv growth and gene and protein expression of GFPuv at 1 mM.
- The effect of fluoride on bacterial growth may be from regulation of mRNA expression.
- Excessive fluoride may inhibit bacterial growth and/or disrupt its microbial balance in GI tract.

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ABSTRACT

To determine the effects of fluoride on bacterial growth, as well as upon its gene/protein expression, we grew *Escherichia coli* expressing GFPuv (*E. coli*-GFPuv) in Luria Bertani medium at different concentrations of NaF, 0, 0.1 mM, 1 mM, 10 mM and 100 mM. Results showed that *E. coli*-pGFPuv growth and expression of mRNA and protein of GFPuv were increased at 0.1 and 1 mM, but were inhibited at 10 and 100 mM, which demonstrated that fluoride has a classic rise/fall response of inducing *E. coli*-GFPuv growth and gene and protein expression of GFPuv at 1 mM. Our observation suggests that the effect of fluoride on bacterial growth may be from regulation of mRNA expression.

1. Introduction

Fluoride is a common chemical element that naturally exists in food and water. However, excessive absorption of fluoride is harmful and the most commonly affected organ is gastrointestinal (GI) tract. The primary symptoms of fluoride toxicity on the GI tract include nausea, vomiting, and abdominal pain (Susheela et al., 1992; Dasarathy et al., 1996; Sidhu and Kimmer, 2002). In the GI tract, trillions of microorganisms, gut flora, form a natural ecosystem (Guarner and Kimmer et al., 2003) which plays a very important role in digesting, stimulating cell growth, suppressing growth of harmful microorganisms, training immune system to respond only to pathogens, and preventing diseases (Sears, 2005; O'Hara and Shanahan, 2006). However, how fluoride affects microflora in the GI tract remain unclear. We hypothesized that excessive fluoride can damage GI tract by inhibiting its progenic bacterial growth and disrupting its microbial balance.

Green Fluorescent Protein (GFP) consists of 238 amino acids and has been widely used as a reporter in research. GFP can be quantified both *in vivo* and *in vitro* by different techniques, e.g., fluorescence microscopy, flow cytometry, and spectrofluorometry (Chalfie et al., 1994). GFPuv is a GFP variant with enhanced fluorescent intensity (Crameri et al., 1996). *Escherichia coli* is one of major bacteria presented in the GI tract (Guarner and Malagelada et al., 2003). In this study, we investigated the effect of fluoride on bacterial growth and the expression of GFPuv in *E. coli* at various concentrations of NaF *in vitro*.

2. Materials and methods

2.1. Materials and chemicals

E. coli BL21 (DE3) and *E. coli* BL21 carrying pGFPuv (*E. coli*-pGFPuv) were kindly provided by Dr. Hongquan Li at Shanxi Agriculture University. Trizol reagent and two-Step SYBR® QRT-PCR kit (Takara Biotechnology, China); RIPA lysis buffer and the BCA Protein Assay kit (Beyotime, China); and NaF (Sigma, USA) were purchased. 970 CRT spectrofluorometer (INESA, China), BX51TF Microscope (Olympus, Japan) and Mx3000P™ QRT-PCR system (Stratagene, USA) were used for analyzing the results.

2.2. Bacterial growth

The original stocks were streaked out on Luria Bertani (LB) agar plate with a supplement of 100 μg mL^{-1} ampicillin and grown

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overnight at 37 °C. A single colony was transferred into LB broth with 100 μg mL^{-1} ampicillin and incubated overnight at 37 °C. Bacteria were then diluted 10-fold into fresh LB broth with 100 μg mL^{-1} ampicillin and grown at 37 °C for 3 h in a shaking incubator until the OD_{600nm} of the mixture reached 0.6. The mixture was divided into five aliquots with supplementation of 1 mM isopropyl-β-D-thiogalactoside (IPTG) and various concentrations of NaF (0, 0.1 mM, 1 mM, 10 mM, and 100 mM) for an additional 6 h in culture. Finally, bacteria were collected by centrifugation (5000*g* for 15 min) and washed 3 times in PBS before performing further analyses.

2.3. Bacterial quantification

The numbers of bacteria in the culture medium was monitored by an plate count method with a slight modification (Jongenburger et al., 2010). Briefly, bacteria were diluted and spread on an agar medium plate to form individual colonies. Total numbers of colonies were counted to quantify bacteria (CFU) in the original media.

2.4. Fluorescence measurement

The bacteria were centrifuged (5000*g* for 20 min) and the pellets were washed twice in ice-cold PBS and then resuspended in ice-cold PBS at concentration of 1×10^8 CFU mL^{-1}. Fluorescence intensity was measured in a 970 CRT spectrofluorometer at an excitation wavelength of 395 nm and an emission at 509 nm.

2.5. GFPuv production in bacteria analyzed by SDS–PAGE

1 mL of bacteria at 2×10^8 CFU mL^{-1} were centrifuged and rinsed twice in PBS and the pellets were lysed in a certain volume of RIPA lysis buffer supplemented with 1 mM PMSF. The lysates were mixed with loading buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 5% SDS, 5% β-mercaptoethanol, and 0.25% bromophenol blue) to perform protein electrophoresis analyses using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 12%). Following the electrophoresis, the gel was then stained with Coomassie Brilliant Blue R-250 and washed in 5% (v/v) acetic acid. Using the AlphaView Image Analysis Software (Alpha Innotech, USA), the corresponding pixel intensity values of GFPuv protein was quantified.

2.6. Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR)

Total RNA was isolated from bacteria (1.0×10^8 CFU mL^{-1}) using Trizol reagent in accord with the manufacturer's instructions. Specific primers for GFPuv and 16S rRNA were designed using Primer 3.0 plus. The primer sequences and their corresponding PCR products, and gene accession numbers are listed in Table 1. QRT-PCR was conducted by using the two-Step SYBR® QRT-PCR kit and Mx3000P™ QRT-PCR system. Reverse transcription with random hexamer primers was performed at 37 °C for 15 min and at 85 °C for 5 s. Thermal cycling conditions were: 95 °C for 10 s, 40 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 6 s. The reaction was then subjected to a melting protocol from 55 °C to 95 °C with a 0.2 °C increment and 1 s holding at each increment to verify specificity of the amplified products. 16S rRNA was used as house keeping gene. Data was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

All data was analyzed by one-way ANOVA implemented using the GraphPad Prism 5 software. A p value less than 0.05 was considered to be significant.

3. Results

3.1. GFPuv expression in E. coli-pGFPuv

The GFPuv expression in *E. coli*-pGFPuv was observed under a fluorescence microscopy (Fig. 1), indicating that these bacteria are viable models for our studies.

3.2. Effect of NaF on growth of E. coli-pGFPuv

To study the effect of NaF on the growth of *E. coli*-pGFPuv, we investigated the dose response of NaF at 0.1, 1, 10, and 100 mM. Compared to the control, our data shows a classic biphasic growth response of NaF at 1 mM ($p < 0.01$) in *E. coli*-pGFPuv (Table 2).

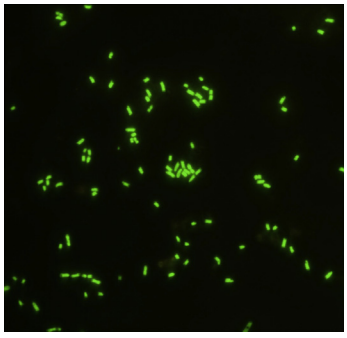

Fig. 1. GFPuv expression in *E. coli*-pGFPuv (×1000).

Table 2
Effect of fluoride on growth of *E. coli*-pGFPuv.

Concentrations of NaF (mM)	CFU mL^{-1} (×10^6)
0	221 ± 9.849
0.1	241 ± 4.359*
1	256.67 ± 7.024**
10	243.33 ± 5.508*
100	114.33 ± 6.506***

Note: Table 2 shows biphasic effects of fluoride on *E. coli*-pGFPuv growth at 1 mM. The lower concentrations of NaF increased CFU and higher concentration of NaF decreased CFU compared to the control group. Mean ± SD. $N = 3$.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 1
Primer sequences with corresponding PCR product size and position.

Gene	Primers (5′→3′)	Primer locations	Product (bp)	Genbank accession no.
GFPuv	GCGCAGATATCAGGAGGAAC AAGGAAGGAAACCCACACCT	88–176	89	NM_X83959
16S rRNA	AGTGGAGAGGGTGAAGGTGA GTTGGCCATGGAACAGGTAG	608–757	150	NM_JQ037847