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GABA Production Through Acid Resistance Mechanisms in Escherichia coli

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Abstract

Escherichia coli, one of the most common gut microbes, must survive through the highly acidic environment of the stomach at a mean of pH 2 before reaching the intestines. One method by which *E. coli* survive the acidity is by expelling protons from the cell, raising the intracellular pH to a more viable level, most commonly with the decarboxylation of glutamate into γ-aminobutyric acid (GABA) via glutamate decarboxylase. The GABA expelled from the cell contains more protons than the glutamate or glutamine pumped in which, in turn, raises intracellular pH. Because GABA functions as a neurotransmitter in humans, excess or decreased expression of glutamate decarboxylase may yield behavioral effects. We aimed to investigate GABA production in different strains of laboratory-evolved *E. coli* after two hours of acid stress at pH 2 in media with varying concentrations of either glutamine or glutamate to further understand what happens to GABA production in different conditions. Statistical analysis reveals the addition of glutamine to the media yields a similar concentration of extracellular GABA as the addition of glutamate does (p=0.6527). Interestingly, none of the laboratory-evolved *E. coli* strains produced more GABA than the ancestral strain. Four strains evolved in pH 4.6 media lost all GABA production, as did two strains evolved in pH 6.5 media with benzoate. Three other benzoate-evolved strains showed reduced production of extracellular GABA when compared to the ancestral strain (p<0.001). These results suggest variations in *E. coli* evolution under stress conditions may yield reduced production of GABA.

Introduction

- Microbes entering the human gut must resist pH levels around pH 2 in the stomach, making acid resistance mechanisms essential for survival. (1)
- Glutaminase functions as an acid resistance mechanism by deaminating glutamine into glutamate. The released ammonia is protonated into ammonium and expelled from the cell, lowering intracellular pH. (2)
- Glutamate decarboxylase also functions as an acid resistance mechanism by replacing glutamate’s α-carboxyl group with a proton, forming GABA, which is then expelled from the cell, raising the intracellular pH. (1,3)
- Glutamate decarboxylase is most active when the cell is stressed in an environment at pH 2. (1)
- GABA acts as a neurotransmitter in humans and may be absorbed from the intestines into the vagus nerve, making GABA expelled from gut flora possibly influential on behavior. (4)
- Strains adapted to growth in media at pH 4.6 seem to have higher relative fitness than the ancestral strain when grown in pH 4.6 media. (5)
- We aim to see if strains adapted to growth in more acidic environments produce more extracellular GABA than the ancestral strain and if this is also the case in strains adapted to benzoate media buffered to pH 6.5.

Methods

Cultures. Seventeen different strains of *E. coli* were used, all of which were K-12 derivatives. These strains included eight strains evolved to media buffered to pH 4.6, eight evolved to media with benzoate at pH 6.5, and one wild type (WT) control strain, W3110-D13.

Culture Growth. All strains were grown overnight in Luria Broth medium with NaCl, 10 mM glutamine, and buffered with 100 mM MES to pH 5.5. The cultures were grown in screw cap test tubes filled to the lip with media for somewhat anaerobic conditions for 18 hours at 37 °C before being removed from incubation. The pH of the media was stressed to pH 2 using HCl and the samples were then placed back in the incubator for two hours.

Separation of Cells from Media. After two hours of acid stress, 1 mL of each sample was centrifuged for 5 minutes at 13,000 RPM. The supernatant was then collected via filtration using 25mm syringe filters. Amino acid concentrations were measured via gas chromatography/mass spectrometry (GC/MS) after derivitization. This was performed with the EZ:faast: Free Amino Acid Analysis kit (Phenomenex, Torrance, CA). With each set of *E. coli* cultures grown, a negative control solution with no *E. coli* was exposed to the same conditions and analyzed via the same derivitization and GC/MS protocol.

Data Analysis

A standard solution was prepared and analyzed using the same GC/MS protocol each time a set of cultures and negative control was ran. The standard consisted of glutamine, glutamic acid, GABA, and tryptophan in water all concentrated at 200 nmol/mL. Using the relative peak integrations of each amino acid in the gas chromatogram of the standard solution, a response factor was calculated for these amino acids to convert the relative peak integration into concentration (equations shown below). Variations in calculated GABA concentrations were analyzed using either a student’s t-test or an ANOVA with Tukey’s Honest Significant Difference test.

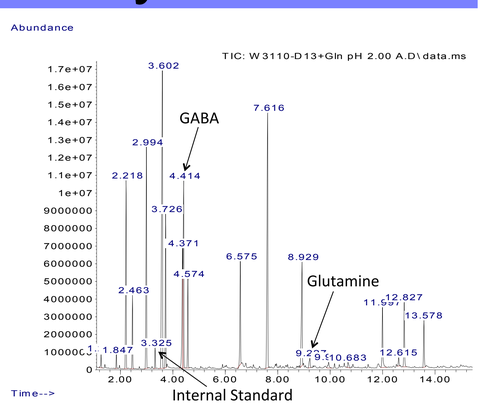


Figure 1. Gas chromatograph for WT *E. coli* after acid stress at pH 2 in 10 mM glutamine LBNaCl media.

$$\frac{\frac{concentration\ of\ specified\ amino\ acid}{total\ volume\ of\ sample} * 100}{\frac{area\ of\ internal\ standard\ in\ the\ standard}{area\ of\ specified\ amino\ acid\ peak\ in\ the\ standard} * 100} = response\ factor$$

$$concentration\ of\ specified\ amino\ acid = response\ factor * total\ volume\ of\ sample * \frac{area\ of\ specified\ amino\ acid\ peak}{area\ of\ internal\ standard\ peak}$$

GABA Production

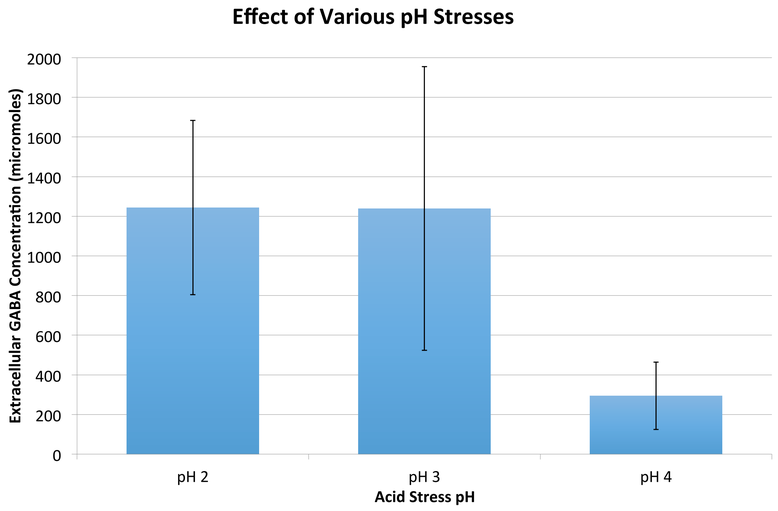


Figure 2. Mean amount of extracellular GABA produced by WT *E. coli*. Cultures were grown overnight in 10 mM glutamine LBNaCl media and then stressed for two hours at either pH 2, 3, or 4. Stressing *E. coli* to pH 2 and pH 3 caused an increase in extracellular GABA concentrations compared to stressing *E. coli* to pH 4 (student’s t-tests, p<0.05). Error Bars=SEM, n=8,3,3

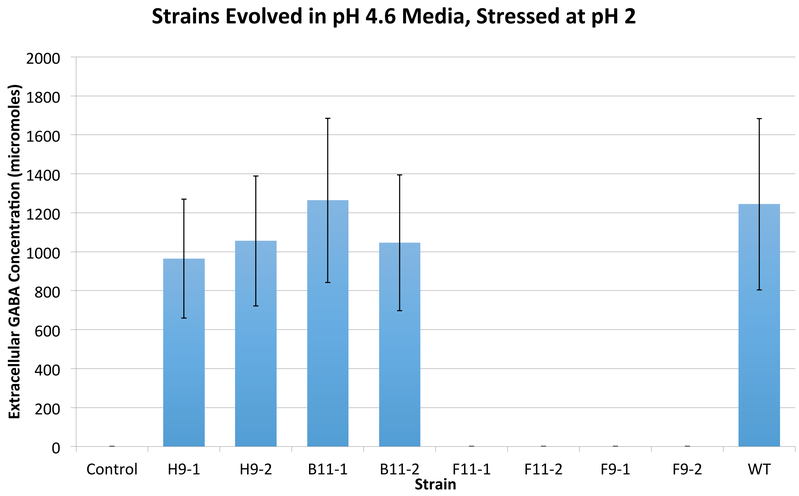


Figure 3. Mean amount of extracellular GABA produced by *E. coli* strains evolved to survival at pH 4.6. Cultures were grown overnight in 10 mM glutamine LBNaCl media and then stressed for two hours at pH 2. Four of the strains yielded no extracellular GABA, while the other four have concentrations of extracellular GABA similar to the WT strain (ANOVA, F=70.789, Df=9, p<0.001; Tukey HSD, 95% CI). Error Bars=SEM, n=38,10,10,9,9,10,10,10,10,8.

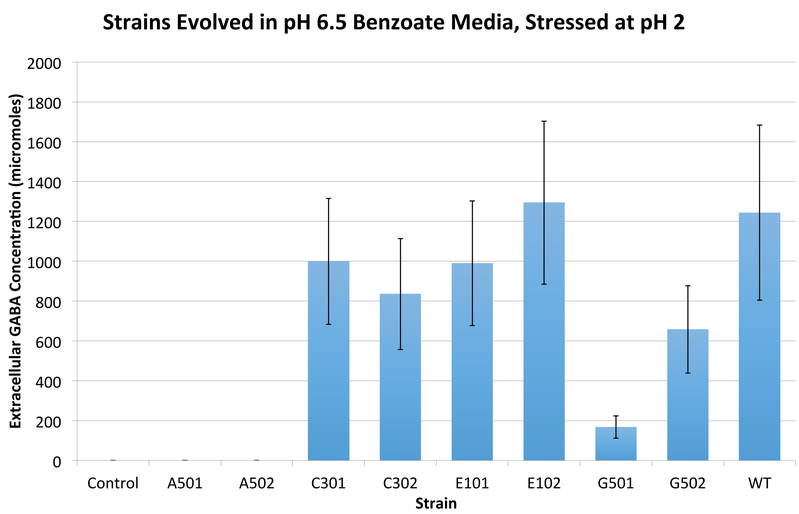
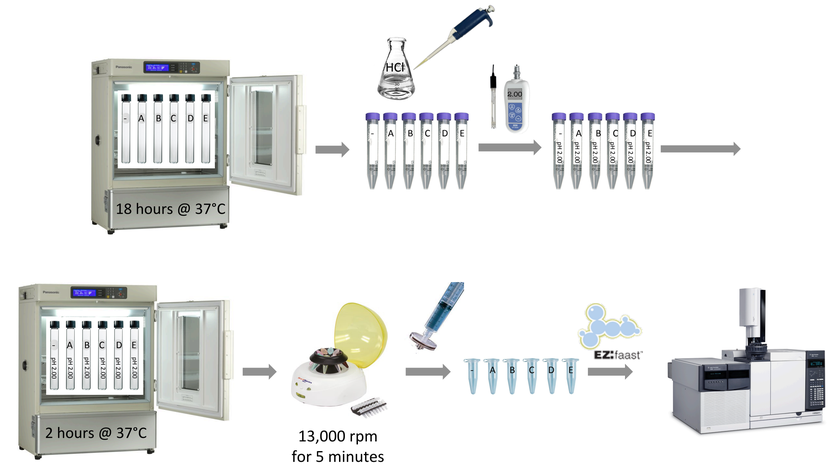


Figure 4. Mean amount of extracellular GABA produced by *E. coli* strains evolved to survival at pH 6.5 with benzoate. Cultures were grown overnight in 10 mM glutamine LBNaCl media and then stressed for two hours at pH 2. Two strains yielded no extracellular GABA while three strains had significant decreases in extracellular GABA concentrations compared to the WT strain (ANOVA, F=45.744, Df=9, p<0.001; Tukey HSD, 95% CI). Error Bars=SEM, n=38,10,10,10,9,10,10,10,9,8.

Experimental Design



Conclusions

GABA production does not vary between glutamine and glutamate supplemented media

- WT *E. coli* produced just as much GABA in 10 mM glutamine LBNaCl media stressed to pH 2 as in 10 mM glutamate LBNaCl media (results not shown).

GABA production increases with acidity

- Specifically, there is a drastic change in production between pH 4 and pH 3. This aligns with the findings of Foster and Richard (2004).

GABA production does not exceed WT production in any strain

- It is not beneficial to have glutaminase or glutamate decarboxylase hyperactivity.

Multiple strains completely lost GABA production

- This could be a result of loss of a necessary gene or extremely reduced expression of a gene.
- Similar acid resistance mechanisms, such as lysine decarboxylase and arginine decarboxylase, also appear to have lost functionality (Slonczewski et al., unpublished).
- This mechanism may require a large energy input that is inefficient for strains evolved in constant stress.

Mutations in GadX (in G501 and G502) lead to reduced extracellular GABA concentrations

- GadX regulates expression of GadA, GadB, and GadC.

rpoC mutation (F9-1, F9-2) is not responsible for lack of GABA production (results not shown)

- Codes for RNA polymerase ß subunit
- Transduction into a WT background did not change GABA production

Future Directions

- Analyze how the concentration of glutamine and glutamate in the media effect extracellular GABA concentration
- Compare final pH values after 2 hours of acid stress to investigate if added glutamine in the media allows *E. coli* to reach a higher intracellular pH
- Perform survival assays after acid stress to see if variations in GABA levels are a result of reduced gene expression or a higher cell mortality rate
- Investigate potential mutations responsible for absence of extracellular GABA, such as rpoD (F11-1, F11-2)

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