Escherichia coli Mutant Strains Mediate Cellular Response to Benzoic Acid

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Abstract

The intestinal bacterium *Escherichia coli* is able to survive and protect its cytoplasmic contents from a wide range of acidic and basic conditions from low pH in the stomach to higher pH levels in the small intestine. While many studies have investigated the response and recovery of *E. coli* to external pH stress, there has been little research of *E. coli* response to permeant acid stress and the subsequent identification of specific genes that may be candidates for direct response to cytoplasmic stress by benzoic acid. We tested the importance of eight genes that were previously seen to be up-regulated during rapid permeant acid stress (*fimB*, *ygaC*, *yhcN*, *yhjX*, *ymgA*, *ariR*, *ymgC*, and *zinT)* for survival after extreme acid challenge and growth in acidic, basic, and permeant acid conditions. We found that *ymgA* showed decreased survival in extreme acid challenge and low growth rate in low pH conditions with and without potassium and sodium benzoate. Finally, we initiated an adaptive laboratory evolution procedure that incorporates a long-term challenge of *E. coli* grown and diluted daily in increasing concentrations of potassium benzoate. The extended adaptive laboratory evolution protocol will allow us to compare continuously grown *E. coli* in moderately acidic conditions, a previously ongoing project, with those grown in potassium benzoate with the intention of eventually identifying spontaneous mutations important for acid and permeant acid resistance by genetic sequencing.

Introduction

***E. coli* survives acidic environments, challenged by membrane-permeant weak acids like benzoic acid**

- *E. coli* travel through the gastrointestinal tract which ranges from acidic conditions in the stomach to alkaline conditions in the small intestine (1, 2)
- Specific genes in the *E. coli* genome express products that regulate the internal cytoplasmic pH to an approximate pH of 7.6 (4)
- Permeant acids, byproducts of digestion and fermentation, are another challenge which acidifies the cytoplasm of *E. coli*; Figure 1. (3)

Membrane-permeant acids are used prevalently in food industry

- Weak acids like sodium and potassium benzoate are used as preservatives in a wide variety of foods (pickling, juices, etc.) to prevent growth of contaminating organisms (3)

Eight candidate genes showed direct response to rapid cytoplasmic acidification

- Permeant acid stress on *E. coli* has not been extensively studied and mechanisms of cytoplasmic pH regulation with response to benzoic acid challenge are unknown
- *fimB, ygaC, yhcN, yhjX, ymgA, ariR, ymgC,* and *zinT* genes up-regulated after rapid cytoplasmic acidification, could be responsible for *E. coli* cytoplasmic acid stress responses (2)

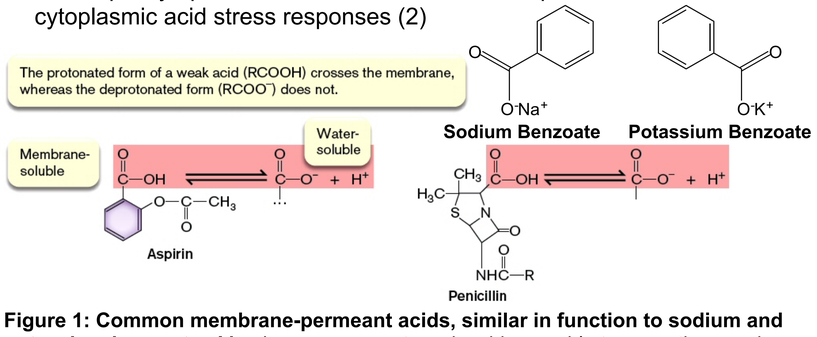

Figure 1: Common membrane-permeant acids, similar in function to sodium and potassium benzoate. Membrane-permeant weak acids are able to cross the membrane in their uncharged form and disassociate once inside the cell, thus acidifying the cytoplasm. Slonczewski, J.L. and Foster, J.W. 2013. *Microbiology: An Evolving Science.* Third Ed. W. W. Norton & Company. pg.88

Results

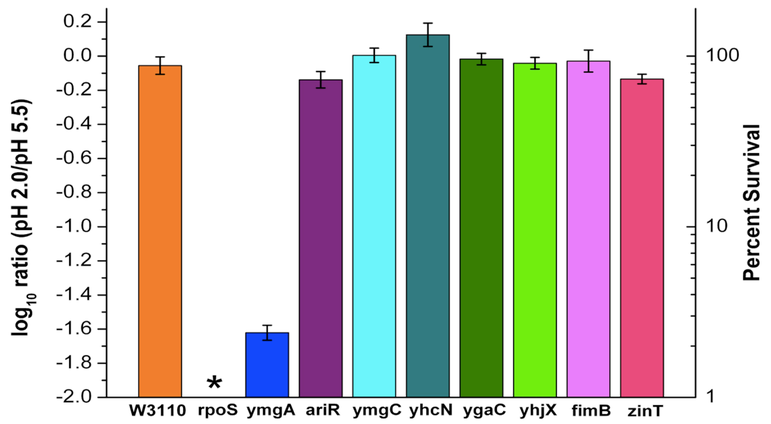

Figure 2: Acid survival of wild type, *rpoS, ymgA, ariR, ymgC, yhcN, ygaC, yhjX, fimB,* and *zinT* strains under aerobic conditions. Strains were cultured overnight and exposed to pH 2 for two hours with aeration. Dilutions were plated and colony counts were log transformed to calculate the ratio of acid-exposed to non-exposed strains. Error bars=SEM; * indicates zero percent survival.

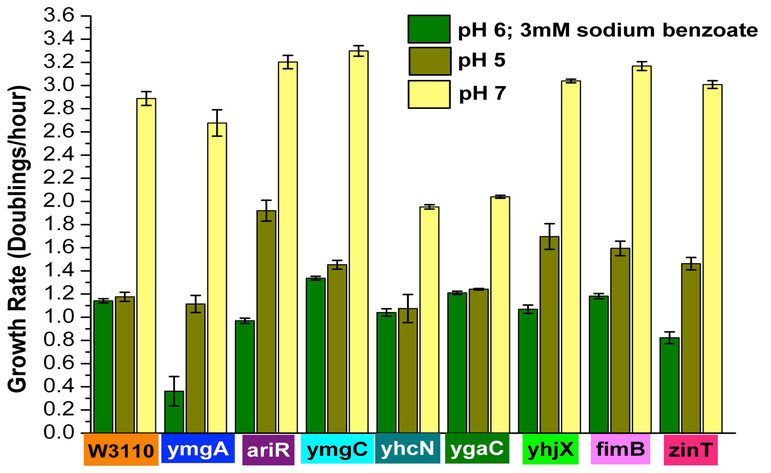

Figure 3: Growth rate of wild type, *ymgA, ariR, ymgC, yhcN, ygaC, yhjX, fimB,* and *zinT* strains in sodium benzoate/pH 6, pH 5, and pH 7 under aerobic conditions. Strains were cultured overnight and diluted 1:400 in flasks rotated at 37°C for two to three hours with OD_{600} measurements taken every 20 minutes with a spectrophotometer. Growth rate was calculated from the logarithmic linear growth of the cultures. Error bars=SEM.

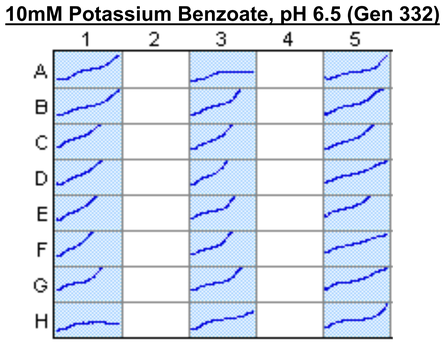

Figure 4: Plate geometry for daily potassium benzoate culture dilutions 1:100; Generation 332. Image represents half a layout of a 96-well plate. Growth curves represent daily cultures growing in LBK, 100mM PIPES buffered at pH 6.5, with 10mM potassium benzoate over 22 hour with readings every 15 minutes.

Conclusions

- ***ymgA* may be important to external and internal acid resistance of *E.coli.*** *ymgA* strains showed decreased survival in extreme acid challenge and low growth rate in pH 5.0, pH 6.0 with 3mM sodium and potassium benzoate
- *ariR* and *zinT* strains showed decreased growth rate in sodium benzoate permeant acid conditions, but not in extreme external acid challenge and low pH conditions
- **Wild type *E.coli* was unable to survive after 120 minutes in moderate acid pH 4.0, 20mM sodium benzoate conditions**, with decreased percent survival after 90 minutes
- **An adaptive laboratory evolution procedure with potassium benzoate challenge of *E.coli* was established.** In the future, the benzoate challenged strains will be compared to ALE strains grown in moderate (pH 4.5, 4.6) external acid conditions for comparative spontaneous mutations for increased resistance to acidic conditions

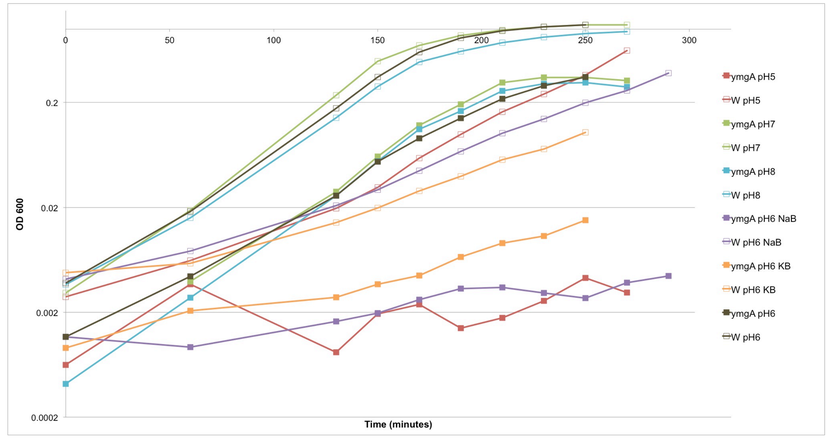

Figure 5: Growth curve of wild type and *ymgA* strains in pH 5, pH 7, 3mM sodium benzoate/pH 6, 3mM potassium benzoate/pH 6, and pH 6 under aerobic conditions. Strains were cultured overnight and diluted 1:400 in flasks rotated at 37°C for five hours with OD_{600} measurements taken every 20 minutes with a spectrophotometer.

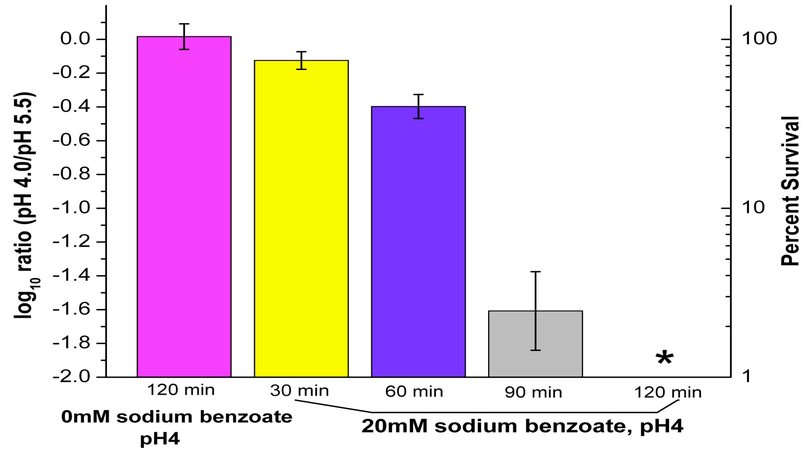

Figure 6: Permeant acid survival of wild type strain under aerobic conditions. Wild type strains were cultured overnight and exposed to 20mM sodium benzoate/pH 4 for 30, 60, 90, and 120 minutes with aeration. Dilutions were plated and colony counts were log transformed to calculate the ratio of permeant acid-exposed to non-exposed strains. Error bars=SEM; * indicates zero percent survival.

Methods

Preparation of the Strains *Escherichia coli* strains with the genes being studied in this experiment (*fimB, ygaC, yhcN, yhjX, ymgA, ariR, ymgC,* and *zinT*) were obtained from the Keio collection (5) with kanamycin resistance cassettes and transduced into the *E. coli* K-12 W3110 background strain via P1 phage lysate transduction.

Acid Survival Assays Strains were grown overnight in LBK buffered with 100mM MES at pH 5.5 to up-regulate acid response systems. Overnight cultures were diluted 1:200 in extreme acid pH 2.0 LBK for 2 hours at 37°C and then serially diluted in M63 minimal media pH 7.0 to a final dilution of 1:400,000. 50µL of the final dilutions were spread onto LBK agar plates. Overnight cultures were also serially diluted the same way as the acid-challenged cultures in M63 pH 7.0 and plated on LBK agar. The number of colonies on both the acid challenged and non-challenged culture plates were counted and compared to determine percent survival. A modified procedure using solely wild type W3110 *E.coli* exposed to pH 4.0, 20mM sodium benzoate LBK for 30-minute increments up to 120 minutes versus a control exposure of pH 4.0 LBK for 120 minutes was conducted to determine a baseline percentage survival for a modified extreme-permeant benzoic acid and moderate acid challenge for wild type *E.coli.*

Strain Growth Strains were grown overnight in LBK pH 5.5 buffered with 100mM MES. Overnight cultures were diluted into LBK pH 5.0 buffered with 100mM HOMOPIPES, LBK pH 7.0 buffered with 100mM MOPS, and LBK pH 6.0 3mM sodium benzoate buffered with 100mM MES for a final 1:400 dilution. Cultures were aerated at 37°C for two to five hours with OD_{600} measurements taken every 20 minutes with a spectrophotometer. Growth rate (doublings per hour) was determined from the linear growth of the cultures. A specialized strain growth procedure using buffered LBK pH 5.0, pH 7.0, pH 8 buffered with 100mM HOMOPIPES, LBK pH 6 both 3mM potassium benzoate and sodium benzoate buffered with MES was used to test *ymgA* growth in various conditions.

Adaptive Laboratory Evolution Procedure An overnight culture of W3110 was diluted 1:100 in LBK, pH 6.5 100mM PIPES, 5mM potassium benzoate. Growth was recorded over a twenty-two hour period with spectrophotometer OD_{450} readings every 15 minutes at 37°C. Daily 1:100 dilutions of the cultures into fresh potassium benzoate media occurred and eventually the concentration of potassium benzoate was raised to 6mM and then to 10mM (current potassium benzoate concentration).

Genes

zinT	JLS1302	Formerly *yodA*, renamed *zinT* for its zinc uptake regulating actions controlled by the Zur regulon. Codes for a binding site of zinc and cadmium; important role in the zinc homeostasis of *E.coli*, curli formation, and biofilm formation
ariR	JLS1311	Formerly *ymgB*; a regulator of acid resistance influenced by indole; contributes to acid resistance, decreased cell motility, and represses biofilm formation
yhjX	JLS1303	*oxlT* homolog in *E.coli*, an oxalate and formate antiporter in the major facilitator super family of membrane transporters, a possible acid response system including carbon dioxide removal to conserve protons, potential membrane exchange system for carboxylates
fimB	JLS1301	Independent regulatory protein in the control region of the phase variation of *fimA*, which codes for the expression of type 1 fimbrae in *E.coli*
ygaC	JLS1008	Regulated by the Fur regulon, repressed by iron
yhcN	JLS1007	Related to stress responses in *E.coli* to hydrogen peroxide, cadmium, and acid; related to biofilm formation
ymgA ymgC	JLS1300 JLS1008	Both regulated by the bluR repressor and rpoS; encode for biofilm development, stability, motility; increased expression observed in intermediate and developed biofilms

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References

1. Foster, J.W. 2004. Escherichia coli acid resistance: tales of an amateur acidophile. *Nat. Rev. Microbiol.*, **2**: p. 898-907.
2. Kannan G., Wilks J.C., Fitzgerald D.M., Jones B.D., BonDurant S.S., Slonczewski J.L. 2008. Rapid acid treatment of Escherichia coli: transcriptomic response and recovery. *BMC Microbiol.* 8:37.
3. Lin, J., Smith, M.P., Chapin, K.C., Baik, H.S., Bennett, G.N., Foster, J.W. 1996. Mechanisms of acid resistance in enterohemorrhagic Escherichia coli. *Appl Environ Micobiol* 62: 3094-3100.
4. Slonczewski J.L., Fujisawa M., Dopson M., Krulwich T.A. 2009. Cytoplasmic pH measurement and homeostasis in bacteria and archaea. *Adv. Microb. Physiol.* 55:1–79.
5. Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K.A. Datsenko, M. Tomita, B.L. Wanner, H. Mori 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2:1-11.