Antibiotic Resistance Mechanisms in Benzoate-Evolved *Escherichia coli*

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Abstract

We evolved 24 populations of *Escherichia coli* K-12 in benzoate, a salicylate analog, for 2000 generations. We sequenced and analyzed 16 isolates using *breseq*, a computational pipeline for identifying mutations relative to a reference. Each isolate from this evolution experiment showed several mutations that implied benzoic acid stress reduced antibiotic resistance. Isolate G5-2 showed the most susceptibility to chloramphenicol and had a mutation in the *rob* regulon, a known antibiotic resistance mechanism. When the *rob* mutation was reverted to wild-type in the G5-2 strain, we saw no change in growth compared to G5-2, suggesting the existence of other antibiotic resistance mechanisms that are not yet characterized. We tested benzoate and chloramphenicol tolerance of single knockout strains of genes *hfq, add*, and *pqqL* that were constructed via P1 phage transduction. While Δ*hfq* and Δ*add* strains showed no increase in benzoate tolerance, they both demonstrated decreased chloramphenicol resistance and had growth curves similar to those of G5-2. Our results suggest that *hfq* and *add* do not play a role in benzoate survivability but may in antibiotic resistance. In future research, we will include phenotype strains with single and multiple knockouts to further search for these mechanisms, as well as test the growth of our evolved strains in other drugs and antibiotics.

Introduction

Benzoate Evolution

We evolved 24 populations of *E. coli* in benzoate for 2000 generations. Benzoate is a membrane-permeant acid which poses a challenge to cytoplasmic pH regulation, inflicting energy stress on the cell. Isolate G5-2 showed the most susceptibility to chloramphenicol, a bacteriostatic antibiotic that interferes with protein synthesis (1).

Antibiotic Resistance

Drug-resistant bacteria are a worldwide health concern as many human pathogens no longer respond to available treatments. Many systems are involved in antibiotic resistance, most of which are regulated by the *Mar* and *Rob* regulons (2). Because there was no phenotypic difference between growth of G5-2 and G5-2 with a transduced functional *rob* gene, we believe there are other, antibiotic resistance mechanisms regulated by aromatic acids that are not yet characterized.

Background on *hfq, add* and *pqqL*

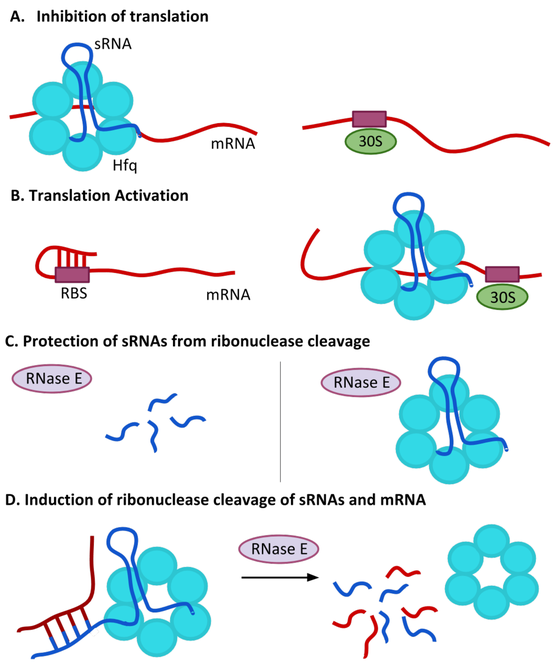


Figure 1. Accepted models of Hfq activity. Hfq is an RNA binding protein that facilitates pairing of sRNAs and mRNA. (A) Hfq can inhibit translation by blocking the ribosomal binding site (RBS). (B) Hfq can activate translation by by exposing the translational initiation region. Hfq can both protect from or induce ribonuclease cleavage of some sRNAs and their target mRNAs by ribonuclease E (C and D respectively) (3).

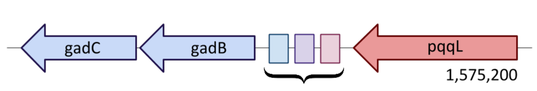


Figure 2. In the *E. coli* genome, *pqqL* is next to DNA binding regulators GadE, GadX, and GadW which can both activate and repress transcription, as well as the GadBC operon which confers resistance to extreme acid conditions (4).

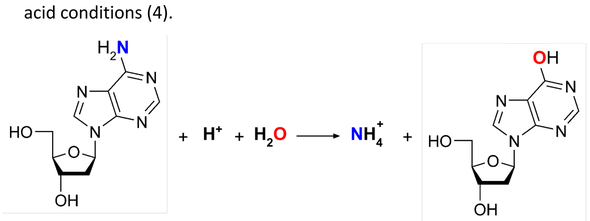
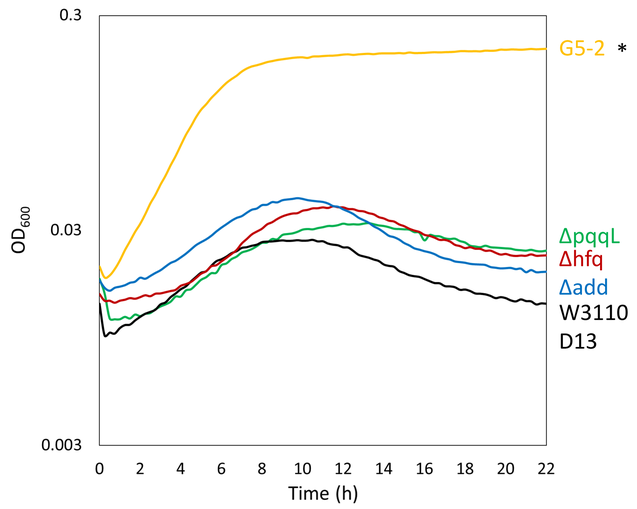


Figure 3. *add* encodes adenosine deaminase, an enzyme participating in the pathway that converts 2’-deoxyadenosine to 2’-deoxyinosine. The reaction is shown in the favored direction (5).

Growth of knockout strains in Benzoate and Benzoate with Chloramphenicol



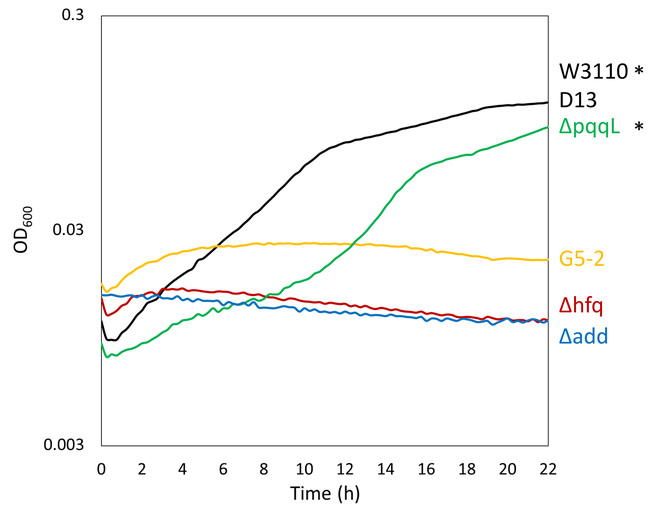


Figure 4. Growth of wild type (W3110D13), benzoate-evolved (G5-2) and knockout (Δadd, Δhfq, ΔpqqL) strains in 20 mM benzoate pH 6.5 (left) and 8 µg/mL chloramphenicol 5 mM benzoate pH 7.0 (right). Strains were grown overnight, diluted 1:200 in a 96-well plate, and placed in a Spectramax with reads taken every 15 minutes for 22 hours. Stars (*) show statistically significant differences in endpoints determined by one-way ANOVA and Tukey HSD tests (n=16).

Antibiotic Sensitivity in G5-2 (No Inducer)

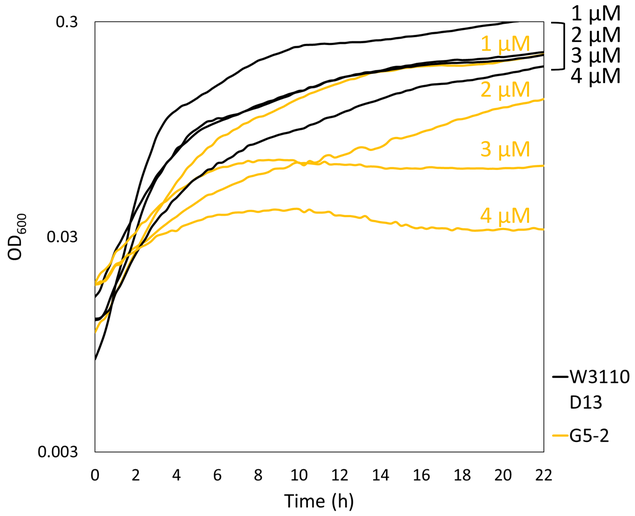


Figure 5. Growth of wild type W3110D13 (black) and benzoate-evolved strain G5-2 (orange) in 1, 2, 3, and 4 µg/mL chloramphenicol Strains were grown overnight, diluted 1:200 in a 96-well plate, and placed in a Spectramax with reads taken every 15 minutes for 22 hours. W3110D13 and G5-2 had significant differences in endpoints at each condition, determined by one-way ANOVA and Tukey HSD tests (n=8).

Mutations

Gene	Mutation	Description
[hscC]-glt	Δ5116 bp	membrane components of glutamate ABC transporter
ltaE←	G→A	L-allo-threonine aldolase, PLP-dependent
cysB→/→acnA	G→A	DNA-binding transcriptional dual regulator/aconitate hydratase 1
hrpA→	C→A	ATP-dependent helicase
pqqL←	insH-4 IS5 (+) +4 bp	zinc peptidase
yneL←/←hipA	C→A	predicted transcriptional regulator/regulator with hipB
add→	Δ1 bp	adenosine deaminase
yeaR←	insL-3 IS186/IS421 (+) +6 bp	hypothetical protein
mdtA→	C→A	multidrug efflux system, subunit A
fabB←/→trmC	C→T	beta-ketoacyl ACP synthase/5-methylaminomethyl-2-thiouridine
nupG→	G→A	Nucleoside transporter
rpoD→	A→C	RNA polymerase, sigma 70 (sigma D) factor
gadX→	Δ78bp	DNA-binding transcriptional dual regulator
yrfF←/→nudE	C→A	inner membrane protein/ADP-ribose diphosphatase
glyV→/→glyX	C→T	tRNA-Gly/tRNA-Gly
glyV→/→glyX	A→G	tRNA-Gly/tRNA-Gly
hfq→	G→A	HF-I, host factor for RNA phage Q beta replication
yjgN→	insH IS5 (-) +4 bp	inner membrane protein
rob←	A→G	DNA-binding transcriptional activator

Table 1. Mutations in the benzoate-evolved strain G5-2 were detected by aligning the genome sequence of G5-2 with the sequence of the ancestral strain, *E. coli* K-12 W3110 reference genome using the computational pipeline *breseq* (version 0.27.1). Δ represents deletions. (6)

Acknowledgements

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Conclusions and Future Directions

- ***hfq* and *add* may play a role in chloramphenicol resistance, but not in benzoate tolerance**
  → in benzoate, Δ*hfq* and Δ*add* showed the same or slightly increased growth as the ancestor
  → in chloramphenicol, Δ*hfq* and Δ*add* had a similar phenotype to G5-2

- **Deletion of the *pqqL gene* does not affect chloramphenicol or benzoate tolerance**
  → Δ*pqqL* had similar growth to the ancestor in both conditions

- **G5-2 showed greater sensitivity to chloramphenicol in the absence of benzoate**
  → G5-2 has an MIC of 8 µg/mL chloramphenicol in benzoate and 4 µg/mL chloramphenicol when no inducer is present
  → Our evolved strain may have mutations in antibiotic resistance genes that are not inducible by benzoate

- **Future research will:**
  → construct single and multiple knockout strains of the genes listed in Table 1 and test their growth in chloramphenicol with and without benzoate as an inducer
  → sequence intermediate generations of benzoate-evolved strains to determine mutation timeline
  → test growth of our benzoate evolved strains in other types of antibiotics to check for loss of other drug resistance mechanisms

Methods

Strain Preparation

Escherichia coli strains with kanamycin resistance cassettes in place of the genes studied in this experiment (*hfq, add, pqqL*) were obtained from the Keio collection (4) and were transduced into the *E. coli* K-12 W3110D13 background strain via P1 phage transduction.

Growth Curves

Strains were grown overnight in either LBK 100 mM MOPS 5 mM potassium benzoate pH 7.0 or LBK 100 mM PIPES 5 mM potassium benzoate pH 6.5. Overnight cultures were diluted 1:200 in LBK 100 mM MOPS 8 µg/mL chloramphenicol 5mM benzoate pH 7.0 or LBK 100 mM PIPES 20 mM benzoate pH 6.5 respectively. For the no inducer assay, overnight cultures were grown in LBK 100 mM MOPS pH 7.0 and tested in the same media with 1, 2, 3 or 4 µg/mL chloramphenicol. Growth was recorded in a Spectramax over a 22 hour period where reads were taken every 15 minutes at OD_{600}. Growth rate (doublings per hour) was measured between 1-3 hours and endpoint values were taken at 16 hours.

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