Escherichia coli Lose Antibiotic Tolerance During Experimental Laboratory Evolution in Benzoate Stress

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Abstract

The model organism *Escherichia coli* is an important inhabitant of the human gut microbiome, and it encounters molecules that we consume such as benzoate and salicylate. These aromatic permeant acids are present in the human diet as a common food preservative and as the main active metabolite of aspirin (acetylsalicylate), respectively. Both molecules depress cytoplasmic pH and proton motive force while inducing multidrug efflux systems. We previously evolved *E. coli* in the presence of 5-20 mM benzoate for nearly 3000 generations, and over 100 mutations were found in the genomes of 16 strains sequenced at 2000 generations. Strains from four populations A1, C3, A5, and G5 were isolated and tested for improvements in benzoate and salicylate tolerance as well as correlations with loss of antibiotic tolerance at eight different generations from the evolution. We found that tolerance in benzoate and salicylate increases over the course of the benzoate evolution while chloramphenicol tolerance decreases. We determined minimum inhibitory concentrations (MICs) of chloramphenicol and tetracycline for selected strains from populations A1, C3, A5, E1, and G5 at generation 2000. All strains but E1-1 showed a reduction in MIC for both antibiotics as compared to the ancestral strain W3110-D13; most showed resistance comparable to or lower than that of a *marA* deletion. This suggests that the gut environment of a human with a diet rich in salicylates or on an aspirin regimen is continually evolving *E. coli* that are more drug-sensitive. Our data also provides evidence that there are other previously undocumented multidrug resistance mechanisms outside of the *Mar* operon.

Introduction

- Humans consume benzoate and salicylate on a regular basis; benzoate (or benzoic acid) is a common food preservative (1), and salicylate regulates plant defense (2).
- Salicylate is also the main metabolite of acetylsalicylate (or aspirin) (3), and an aspirin regimen can result in circulating salicylate levels of up to 0.5 mM (4).
- Aromatic permeant acids such as these depress cytoplasmic pH and cause energy stress by running down proton motive force (pmf) (5). They are also inducers of the *Mar* operon (*marRAB*), which is responsible for several multidrug efflux mechanisms (6).
- Previously, we evolved 24 populations of *Escherichia coli* in benzoate stress using serial dilution for ~2900 generations. At 2000 generations, we chose 16 strains representing 12 different populations for DNA sequencing. **(Figure 1)**.
- Benzoate concentrations were increased from 5-20 mM throughout the course of the evolution in order to cause persistent energy stress as the *E. coli* evolved to cope with previously inhibitory concentrations of benzoate **(Figure 2)**.
- Evolutionary adaptation led to the accumulation of 110 different mutations total in the *E. coli* genomes between the 16 strains at 2000 generations (7), presumably for the purpose of increasing fitness in benzoate stress.

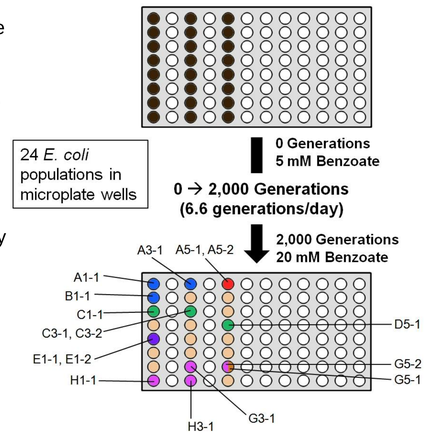


Figure 1. Explanation of experimental laboratory evolution (7).

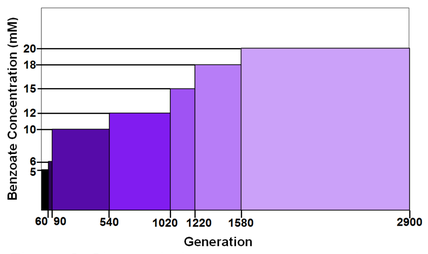


Figure 2. Change in benzoate concentration over benzoate evolution timeline.

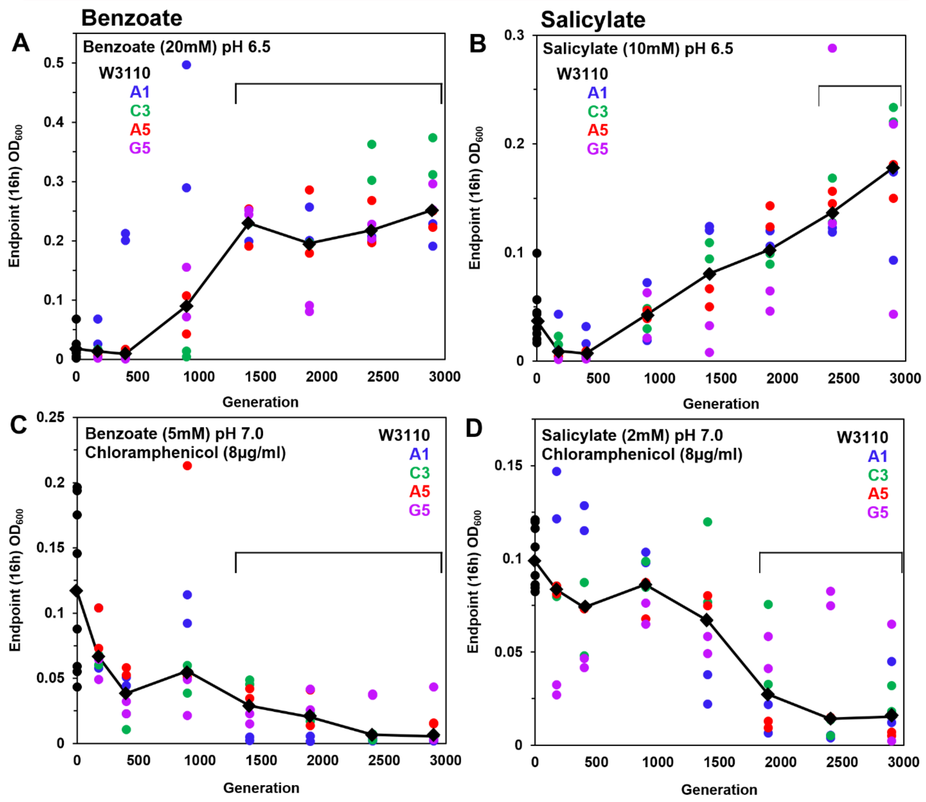
Methods

Cross-Generational Growth Analysis: Benzoate-evolved strains were obtained from frozen microplates. Samples from wells A1, C3, A5, and G5 from generations 173, 399, 897, 1402, 1897, 2402, and 2900 were streaked onto LBK agar. Two individual colonies from each agar plate were used to inoculate two microplate wells (with eight colonies from ancestral strain W3110 used to inoculate eight wells) filled with 200 µl LBK 100mM PIPES pH 6.5, then grown at 37ºC at OD_{600} in a spectrophotometer for 22 hours with shaking and readings every 15 minutes. This freezer stock plate was treated with 100 µL 50% glycerol 100mM PIPES pH 6.5 and frozen. Per trial, two overnight plates were created by filling appropriate wells with 200 µl LBK with either 100 mM PIPES 5 mM KB pH 6.5 or 100mM MOPS 5 mM KB pH 7. Wells of both overnight plates were inoculated in a 1:200 dilution from the freezer stock plate and then grown with the same spectrophotometer parameters as with the freezer stock plate. Overnight plate wells were diluted 1:200 into the two condition plates: LBK 100 mM PIPES 20 mM KB pH 6.5 and LBK 100 mM MOPS 5mM KB pH 7 with 8 µg/mL chloramphenicol. Condition plates were grown with the same spectrophotometer parameters as with frozen stock and overnight plates. Raw and exported csv files were saved for analysis of 16 h OD_{600} endpoint cell density “E” values. The same procedure was repeated, replacing 5 and 20 mM benzoate with 2 and 10 mM salicylate.

Minimum Inhibitory Concentration (MIC) Assay: Ten microplate well columns in eight microplates were filled with 200 µl LBK 100 mM MOPS 2 mM salicylate pH 7.0 with increasing concentrations of chloramphenicol ranging from 1-24 µg/ml. 0.285% ethanol was used as a control. Each plate had wells inoculated in a 1:200 dilution from a single overnight of A1-1, A5-1, C3-1, E1-1, G5-1, G5-2, marA, or W3110 grown in LBK 100 mM MOPS 2 mM salicylate pH 7.0. All plates were put in an incubator at 37ºC, and OD_{600} readings were taken at 22 h for analysis. The procedure was repeated for tetracycline concentrations ranging from 1-12 µg/ml with 0.171% ethanol as a control.

Results

During benzoate evolution, benzoate/salicylate tolerance increases while antibiotic tolerance decreases



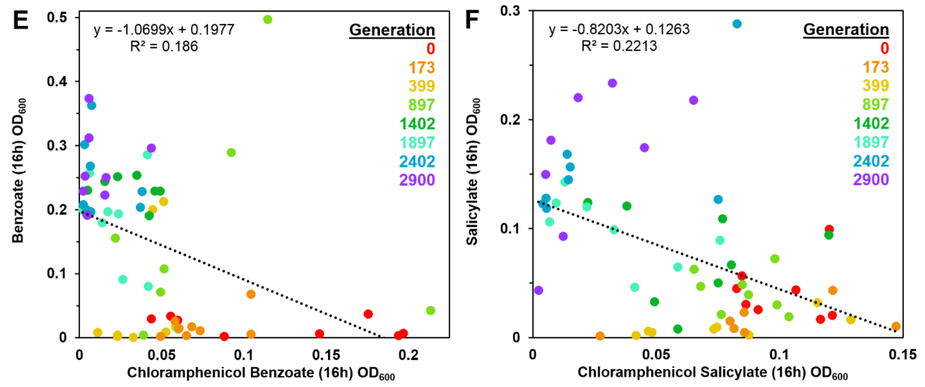


Figure 3. Growth in benzoate, salicylate, and chloramphenicol stress through generations of experimental laboratory evolution in 5-20 mM benzoate. 16 h OD_{600} endpoint cell density “E” values were obtained (N=2 wells for each population/generation). Black diamonds indicate median cell density for each generation tested. Bracket indicates generations for which the 16-h cell density differed significantly from that of the ancestral strain W3110, in 2 of 3 trials (Friedman test; post-hoc Conover pairwise comparisons with Holm-Bonferroni adjusted p-values). LBK media contained: **A.** 100 mM PIPES pH 6.5 with 20 mM benzoate (diluted 1:200 from overnight cultures with 5 mM benzoate). **B.** 100 mM PIPES pH 6.5 with 10 mM salicylate (diluted 1:200 from overnight cultures in 2 mM salicylate). **C.** 100 mM MOPS pH 7.0 with 5 mM benzoate, 8 µg/ml chloramphenicol (diluted 1:200 from overnight cultures without chloramphenicol). **D.** 100 mM MOPS pH 7.0 with 2 mM salicylate, 8 µg/ml chloramphenicol (diluted 1:200 from overnight cultures without chloramphenicol). **E.** Plot with linear regression of 16-h cell-density values for 20 mM benzoate and for 5 mM benzoate, 8 µg/ml chloramphenicol exposures. **F.** Plot with linear regression of 16-h cell-density values for 10 mM salicylate and for 2 mM salicylate, 8 µg/ml chloramphenicol exposures.

Antibiotic minimum inhibitory concentrations decrease in benzoate-evolved strains

	Chloramphenicol MIC (µg/ml)	Tetracycline MIC (µg/ml)
W3110	16	4
marA	8*	4
A1-1	8*	2*
A5-1	8*	1*
C3-1	8*	3*
E1-1	16	4
G5-1	12*	2*
G5-2	8*	2*

Table 1. Chloramphenicol and tetracycline MICs for of strains evolved 2000 generations in 5-20 mM benzoate. Median values of eight variable replicates shown. Strains cultured overnight in LBK 100 mM MOPS pH 7.0, 2 mM salicylate for 22 h. Positive for growth defined as cell density at 22 h (OD_{600} ≥ 0.05). *MIC significantly lower than that of the ancestor W3110 (ANOVA w/ Tukey, p ≤ 0.05).

Conclusions

Benzoate and salicylate tolerance increases across generations of experimental laboratory evolution in benzoate.

- Generations 1402-2900 experienced higher growth in benzoate than the ancestor.
- Generations 2402-2900 experienced higher growth in salicylate than the ancestor.

Antibiotic tolerance decreases across generations of experimental laboratory evolution in benzoate.

- Generations 1402-2900 experienced lower growth in benzoate and chloramphenicol than the ancestor.
- Generations 1897-2900 experienced lower growth in salicylate and chloramphenicol than the ancestor.
- There is a negative correlation between benzoate and salicylate tolerance and chloramphenicol tolerance over the course of the evolution from 0-2900 generations, ranging from low benzoate tolerance with variable chloramphenicol tolerance of the ancestor to high benzoate tolerance and low chloramphenicol tolerance of generation 2900.
- Mutations accumulated in benzoate evolution ultimately lead to lower antibiotic resistance, possibly due to loss of function in energetically-expensive documented and undocumented antibiotic resistance mechanisms.

At 2000 generations, several benzoate-evolved strains exhibit lower minimum inhibitory concentrations as compared to the ancestor.

- In chloramphenicol, strains A1-1, A5-1, C3-1, G5-1, and G5-2, have lower MICs than the ancestor, and A1-1, A5-1, C3-1, and G5-2 have MICs comparable to a *marA* deletion.
- In tetracycline, strains A1-1, A5-1, C3-1, G5-1, and G5-2, have MICs lower than the ancestor and a *marA* deletion.

Future Directions

Cross-generational experimentation:

- All eight isolates from generation 897, as well as one of two isolates for each strain from generation 1402 will have their genomes sequenced for the purpose of identifying mutations occurring early in benzoate evolution. DNA sequences will be used for future genetic analysis and to help put in context the order in which mutations occurred.
- Checkerboard assays will be used to determine whether the inducer salicylate acts synergistically or antagonistically with the antibiotics tested.

MIC assays:

- MIC assays will be continued, testing the same main strains at generation 2000 along with *marA* and W3110 in the remaining classes of antibiotics: fluoroquinolones, macrolides, and aminoglycosides. All future MICs will also be assessed without the inducer (salicylate).

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