Effects of *hepA*, *rfaY*, and *cspC* Genes on Benzoate Tolerance and Antibiotic Resistance in Benzoate Evolved *Escherichia coli*

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Abstract

Escherichia coli underwent evolution for 2000 generations in potassium benzoate conditions that increased in concentration over time. Strains were isolated from this experiment and testing them using growth curves revealed that strains had begun to show a resistance to benzoate yet began to be more sensitive to antibiotics (Creamer et al., 2016). After sequencing their genomes, many mutations were found that could be causing this phenotype.I focused on the mutations in the genes *hepA*, *rfaY*, and *cspC*. These genes were knocked out in the original background strain, W3110, and tested in several environments to observe growth differences. Growth curves revealed that the *hepA* knockout showed decreased growth in benzoate conditions, the *rfaY* knockout showed decreased growth in chloramphenicol (CHL), and the *cspC* knockout showed increased growth in benzoate conditions. For *hepA* and *rfaY*, these results raise more questions than they answer. Why would the bacteria select for mutations that cause decreased growth? The mutation in *hepA* is a late one, therefore it could be a corrective mutation, however results are not clear. With further testing, the genes responsible for the phenotype seen in the evolution experiment will be found and will hopefully explain why the bacteria selected to lose antibiotic resistance and if there is a possible tradeoff for benzoate tolerance.

Introduction

For 2000 generations, *E. coli* underwent evolution in the presence of increasing concentrations of benzoate by the Slonczewski lab at Kenyon College. This environment created an acid stress that the bacteria had to grow and adapt in. Strains collected from this experiment have been phenotyped as having increased fitness in 15mM benzoate and having a lowered resistance to several antibiotics such as chloramphenicol (CHL) (Cremer et al., 2016). After these strains had been sequenced and compared to the wild type (D13) many mutations were found in the evolved generations. A few of these mutations included those in the genes of *hepA*, *rfaY*, and the intergenic region near *cspC*. These mutations were selected during evolution, therefore most are thought to have positive effects on the growth of the bacteria in the benzoate environment. For this reason, an important question has been asked of this experiment: why are the bacteria selecting for mutations that are causing decreased growth in antibiotics?

In order to begin to answer this question, mutations in genes within the evolved strains must be studied to determine their effect on growth. For my project, I chose to study the affects of many of these genes and I have found that growth in certain environments differs when a particular gene is knocked out, or deleted. The three genes I have found results with include *hepA*, *rfaY*, and *cspC*. The *hepA* gene is known to produce an RNA polymerase-associated protein and its knockout shows increased sensitivity to UV light (Muzzin et al., 1998) The gene *rfaY* (*waaY*) produces a protein involved in the making of the cell membrane and mutations in this gene can result in a "leaky" outer membrane (Raetz, 1996). *rfaY* mutants have also been seen to be more sensitive to substances such as menadione and plumbagin however they were more resistant to paraquat (Lee et al., 2009). The *cspC* gene codes for a cold-shock protein, and creating a knockout of this gene has been shown to confer an overall gain of fitness in normal broth cultures (Rath and Jawali, 2006).

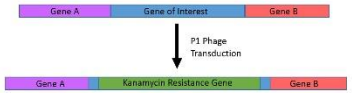

Figure 1: P1 phage transduction consist of replacing the gene of interest with a kanamycin resistance gene, creating a knockout. The use of the resistance gene allows for selection of transduced cells versus the original recipient cells.

Mutations in *hepA*, *rfaY*, and *cspC*

Strain	A5-3	A5-4	A5-5	A5-1	C3-3	C3-4	C3-5			
Position								Mutation	Annotation	Gene
62,682								+G	coding (583/2907 nt)	**hepA** ←
1,908,877								C→A	intergenic (-103/+63)	**rrnA** ← / ← **cspC**
3,840,032								Δ4 :: insH I55(-) +4 bp :: Δ4	coding (585 - 586/699 nt)	**rfaY**

Table 1: Mutations in *hepA*, *cspC*, and *rfaY* occur in strains of benzoate evolved *E. coli*. The whole genome sequences were matched to the reference genome of *E. coli* K-12 W3110. Mutations were found using *breseq* analysis.

hepA knockout showed decreased growth in benzoate compared to the background strain

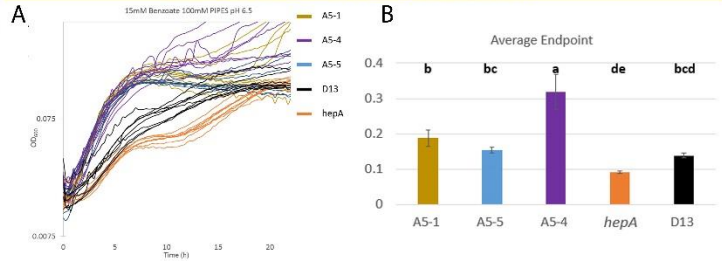

Figure 2. A+B: The *hepA* knockout strain was grown in 15mM potassium benzoate 100mM PIPES pH 6.5 along with benzoate evolved strains and its background strain (D13). Endpoint data from t=16h was analyzed (ANOVA w/Tukey, F=26.94 p= 2e-16, n=8 for each strain).

rfaY knockout showed decreased growth in CHL compared to the background strain

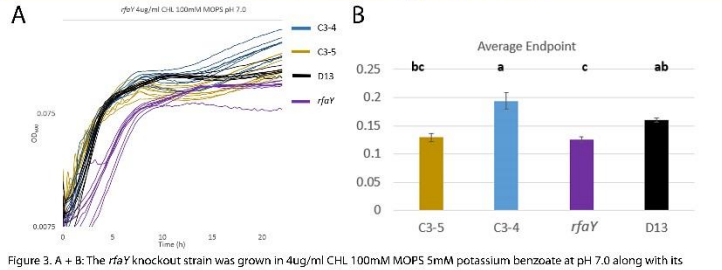

Figure 3. A + B: The *rfaY* knockout strain was grown in 4ug/ml CHL 100mM MOPS 5mM potassium benzoate at pH 7.0 along with its background strain (D13) and benzoate evolved strains. Endpoint data from t=16h was analyzed (ANOVA w/ Tukey, F=11.08 p= 1.06e-08, n=8 for each strain)

cspC knockout showed increased growth in benzoate compared to the background strain

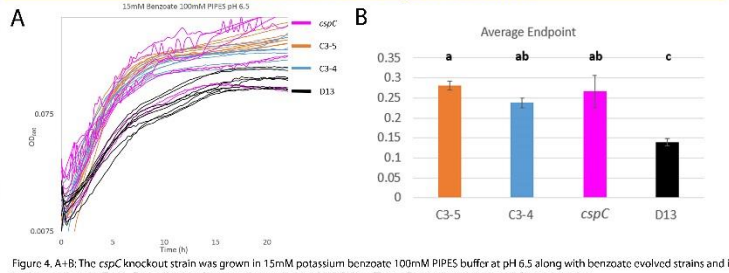

Figure 4. A+B: The *cspC* knockout strain was grown in 15mM potassium benzoate 100mM PIPES buffer at pH 6.5 along with benzoate evolved strains and its background strain (D13). Endpoint data from t=16h was analyzed (ANOVA w/Tukey, F=76.16, p= 2e-16, n= 8 for each strain)

Methods

Construction of Knockouts: The targeted gene was interupted by a kanamycin resistance gene through P1 phage transduction. See figure 1.

Colony PCR: To check if the transduction worked, PCR was used to identify changes in length of the targeted region. Primers were designed using NCBI Blast

Growth Curves: Using a spectramax spectrophotometer, the growth of the *E. coli* was measured using OD values that were measured every 15 minutes for 22 hours. The growth curves were read in sterile 96 well plates that contained 200µl of LB based growth medium innoculated with 1µl of *E. coli*.

Conclusions

~ The *hepA* knockout shows decreased growth rate yet same endpoint in 15mM benzoate compared to D13 (Figure 2). The mutation in the evolved strain may be a corrective mutation.

~ The *rfaY* knockout shows decreased growth in 4ug/ml of CHL in both growth rate and endpoint (Figure 3). The growth is less than that of the evolved strains. The *rfaY* knockout does not show sensitivity to benzoate, salicylate, or ampicillin.

~ The *cspC* knockout shows increased growth rate and endpoint in 15mM benzoate compared to D13 (Figure 4). The knockout grew similarly to C3-5 and C3-4 despite the mutation only being present in C3-5.

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