Antibiotic Resistance in Aromatic Acid Evolved *E. coli*

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Abstract

After adapting 24 populations of *E. coli* for 2000 generations in potassium benzoate, a permeant acid, we sequenced genomes to gain a better understanding of the adaptive process. Sequence data revealed that two isolates contained full *Mar* operon deletions in their genomes. This was a significant finding as *Mar* plays a central role in antibiotic resistance. The role of permeant aromatic acids such as benzoate and salicylate involved in inducing a number of antibiotic resistance genes, including Mar and Rob is well understood. However, this research demonstrates that aromatic acids can bind to the repressor protein MarR and induce the *Mar* Operon. We predicted that benzoate actually induces the *Mar* operon and/or other antibiotic resistance regulons creating a translational strain on the *E. coli* cell. This would explain evolutionary selection towards reduced expression of antibiotic resistance genes, and result in, after 2,000 generations, an overall increase in antibiotic susceptibility. In this study, Antibiotic sensitivity levels were obtained for 24 of the benzoate evolved strains using MIC assay tests. 24 of the low pH evolved strains obtained through the ALE experiment were also analyzed using MIC tests. I used the MIC values to identify specific strain’s to study for possible significant mutations and interesting phenotypes. Identified mutations and Phenotypes are confirmed through PCR and phage transduction.

Introduction

***E. coli* and Antibiotic Resistance**

- A number of systems and phenotypic changes are required to survive antibiotics. (2)
- Many of the proteins that cause these changes are regulated through the *Mar, Rob* and *Sox* regulons. (6)

Benzoate Induction of Antibiotic Resistance

- The *Mar* operon is regulated by the repressor protein MarR
- MarR can be deactivated and the Mar operon induced through aromatic acids such as Benzoate.
- *marA*, a gene in the Mar operon, transcribes a protein that functions as a transcription factor for at least 60 other genes.
- A number of other antibiotic resistance systems are believed to be modulated by aromatic acids as well.
- A5-1 and A5-2 contain full Mar deletions (MarA, MarB, MarC, and MarR)

Benzoate Evolution (lo

- In benzoate, in its acid form it is able to permeate the cell membrane.
- Within a cell, due to the higher cellular pH, some of the benzoate molecules deprotonate lowering the internal pH of the cell.

Minimum Inhibitory Concentration (MIC)

- MIC is a clinical term used to describe the minimum amount of an antibiotic in μg/ml that results in a significant growth reduction in a target strain.
- Typically this is done using test tubes with a single endpoint however to get more quantitative results these were done in a 96 well plate over a period of 22 hours.

Methods

MIC Assay: Selected evolved strains were grown in pH 7 5mM KB LBK rotating overnights for 18 hours at 37ºC. The overnights were serially diluted 1:100 then 1:20 into a 96 well plate containing experimental concentrations of antibiotic dissolved in 5mM KB. The 96 well plates were run kinetically in a spectrophotometer over a span of 22 hours at 37ºC with readings every 15 minutes. Data was analyzed using SoftMax Pro 6.4 and Microsoft Excel. Variations of this experiment included assays without benzoate in an attempt to determine if the aromatic acid induces antibiotic resistance.

Phage Transduction Procedure: Keio knockout strains with a kanamycin markers were grown in LBK overnights for 18 hours. Overnights were infected with a non specific phage. The phage was collected and introduced into both an ancestral D13 strain and a benzoate evolved G5-2 strain. The infected strains were kept at 30ºC to prevent the phage from replicating and lycing the cell. The infected D13 and G5-2 cells were plated on 25μg/ml kanamycin plates. After 24 hours the colonies that grew on the 25μg/ml plates were transferred to 50μg/ml kanamycin plates. Colonies that survived the 50μg/ml kanamycin plate were then analyzed using PCR.

Results

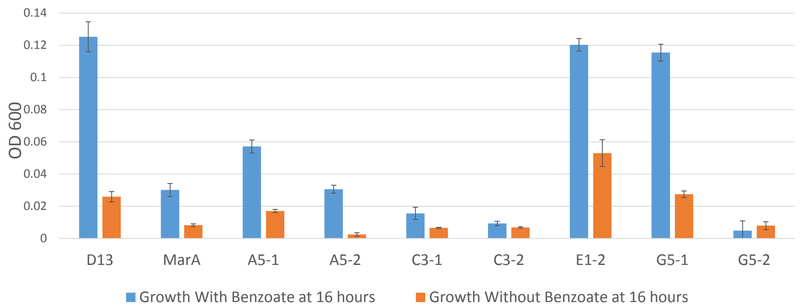

Figure 1 MIC assay of 8 sequenced benzoate strains with and without Benzoate. Benzoate evolved strains were grown in 5mM KB LBK and plain LBK overnights. These strains were then analyzed using the MIC assay procedure at 64μg/ml chloramphenicol. Strains represented by blue bars were grown with benzoate while the red bars were grown without benzoate. Error bars= SEM, n=8.

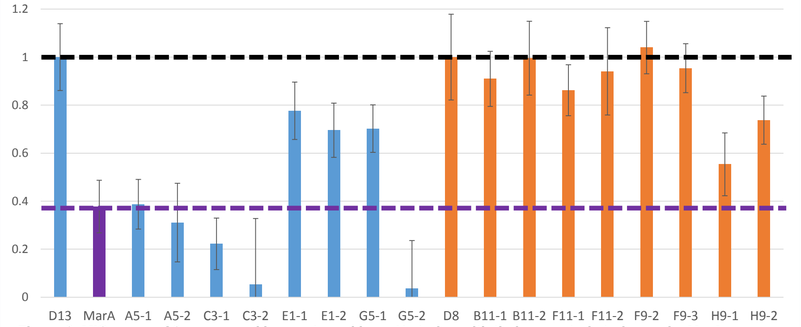

Figure 2. MIC assay of 8 sequenced benzoate and low pH strains with their ancestral strains and a MarA knockout. Strains were grown in 5mM KB LBK solution and analyzed using the MIC assay procedure at 64μg/ml chloramphenicol. All were normalized to their ancestral strain. Error bars= error propagation. Black dotted line= ancestor strain, purple dotted line= MarA knockout. Blue bars are benzoate evolved and ancestor, orange bars are low pH evolved and their ancestor. Purple is a MarA Knockout strain. n=3

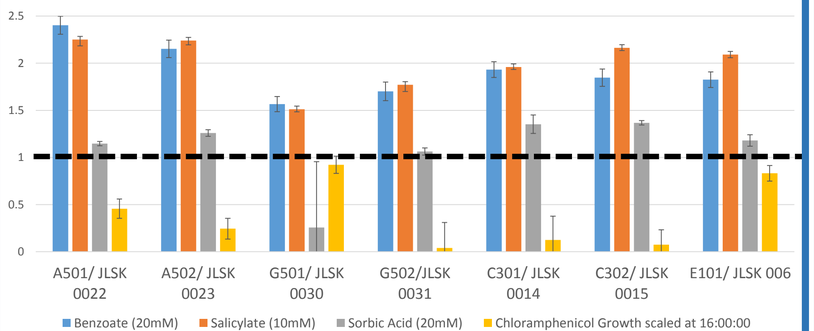

Figure 3. Profiles of 7 sequenced benzoate strains normalized to ancestral D13. Relative growth rate was analyzed through growth curves for benzoate, salicylate and sorbic acid. An MIC assay was used to determine chloramphenicol Growth. Chloramphenicol reading was taken at 16 hours. All were normalized to W3110. Error bars= error propagation of SEM, dotted line= W3110, n=3

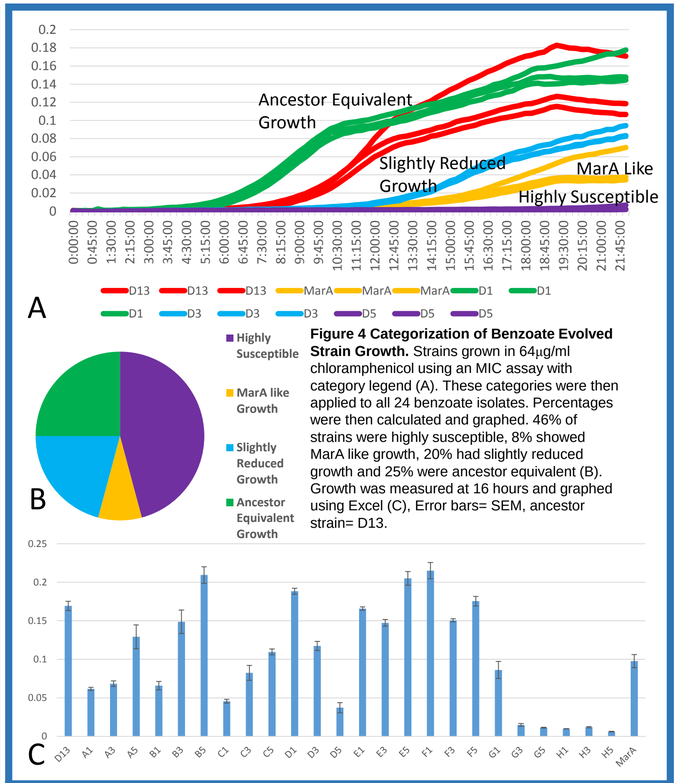

Figure 4 Categorization of Benzoate Evolved Strain Growth. Strains grown in 64μg/ml chloramphenicol using an MIC assay with category legend (A). These categories were then applied to all 24 benzoate isolates. Percentages were then calculated and graphed. 46% of strains were highly susceptible, 8% showed MarA like growth, 20% had slightly reduced growth and 25% were ancestor equivalent (B). Growth was measured at 16 hours and graphed using Excel (C), Error bars= SEM, ancestor strain= D13.

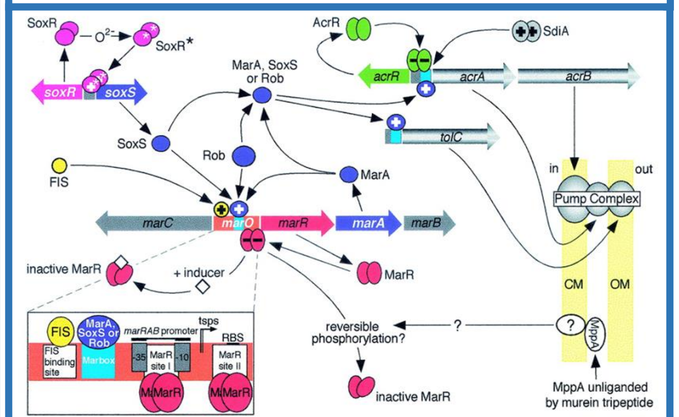

Figure 5. A part of the Mar, Rob Sox transcriptome that regulates antibiotic resistance. This figure shows how we understand how the Mar operon interacts with a number of other genes to ultimately induce the production of a pump complex. (6)

Conclusions

Mar Induction by Benzoate :

- *E. coli*’s antibiotic resistance increases significantly in the presence of Benzoate. This suggests that benzoate is not only to induce some form of antibiotic resistance but it does this more efficiently than the antibiotic itself. This supports the hypothesis put forward by Rao (1) that aromatic acids are able to induce the Mar operon. (Fig. 1)
- A5-1 and A5-2 (Fig 1), both of which have full Mar deletions, not only had higher fitness compared to other benzoate strains but also had a significant decrease in growth when benzoate was removed from solution; Implying that benzoate does induce antibiotic resistance genes that are not modulated directly by the Mar regulon.

Benzoate Induced Loss of Antibiotic Resistance:

- Our data suggests that the loss of antibiotic resistance in *E. coli* is benzoate or aromatic acid specific, and not due to general acid stress. (Fig 2)
- We hypothesize that this is because the constant induction of the Mar operon actually puts stress on the cells that, without the presence of antibiotics, reduces its fitness.
- Our hypothesis is further supported by the loss of antibiotic resistance seen in the majority of strains evolved in benzoate (Fig 4),
- Antibiotic resistance seems to be negatively correlated with growth in benzoate and salicylate. Additionally growth in benzoate and salicylate seem to be very similar.

Looking to the future

- A number of interesting mutations were found in low growing strains such as C3-1 and G5-2 that were previously not linked to antibiotic resistance.
- We hope to use phage transduction to place the evolved gene into an ancestral *E. coli* in order to observe its function
- We also hope to use these strains to find new gene targets for antibiotics and eventually model intestinal conditions with constant low dose aspirin.

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