Acid Resistance Mechanisms in Laboratory Evolved *Escherichia coli*

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Abstract

E. coli are Gram-negative neutralophiles that endure a wide variety of pH stresses on their way to colonize the gastrointestinal tract. Strains of *E. coli* that were evolved in acidic conditions for 2,000 generations were tested in a variety of ways to better understand their fitness advantage over wild type *E. coli* in pH 4.8.

Wild type *E. coli* use Lysine and Arginine decarboxylase systems to consume H^+ and release CO_2 in order to decrease the acidity of their environment. Lysine and Arginine decarboxylase assays were used to determine whether their acid regulation mechanisms remained functional after the evolution. These assays revealed that long term exposure to acid resulted in a loss in these systems.

RNA-Seq analysis was used to compare gene expression of evolved isolates to that of the ancestor. Surprisingly, this analysis showed a general down-regulation of acid stress response genes and an up-regulation of catabolism genes. We believe that inability to decrease the acidity of the media resulted in constitutive expression of acid response genes, causing useful energy to be wasted attempting to regulate a buffered environment.

Fluorescent microscopy revealed that internal pH of both wild type and evolved strains was equivalently lower in acidic conditions. Cell morphology was also only dependent on the conditions of the media present, causing filamentation of both wild type and acid-evolved strains in acidic media.

Introduction

E. coli must survive pH as low as 2 as it passes through the stomach on its way to colonize the human gastrointestinal tract (1), and can grow in pH as low as 4.6 (2). Although it can grow in these harsh conditions, *E. coli* is a neutralophile and must use acid resistance mechanisms such as proton motor force to maintain a neutral internal pH (3).

Previously, wild type *E. coli* was evolved in buffered media at pH 4.8 for 2000 generations and fitness was noticeably increased over time. Amino acid decarboxylase systems are used to help neutralize internal pH through the integration of a cytoplasmic proton with an amino acid (1). We previously reported that the acid-evolved strains showed progressive loss of lysine decarboxylase activity (4).

Additionally, mutations in the RNA polymerase subunits are involved the expression of stress dependent genes (5). Growth of *E. coli* under mildly acidic conditions (pH 6.0) has been shown to cause old pole cells to grow more slowly than under neutral conditions (6).

Methods

Microscopy:

Fluorescence microscopy was conducted at pH 4.8 and 7.0 on the wild type (W3110) and one acid-evolved clone (B11-1) with a GFP plasmid introduced. Cells were cultured in LBK 100mM HOMOPIPES, 10 g/l malic acid, 50 µg/ml ampicillin, 0.4% L-arabinose, suspended in molten agarose, and dotted onto a coverslip. The cells were viewed from 16-18 hours of growth under an 100x oil immersion objective lens on an Olympus BX61WIF-5 microscope. The cells received continuous access to nutrients in a media perfusion chamber (bioptechs).

RNASeq:

Arvind Bhagwat of United States Department of Agriculture Research Service conducted the RNA sequencing for these strains. RNA strand read counts were then analyzed with the R-package DESeq to determine the relative expression of various mRNA sequences. Fold change expression was then cropped by a four fold change up or down along with a p-value <0.001.

Decarboxylase Assays:

In a 96 well plate, *E. coli* was cultured in 200 µl Moeller decarboxylase broth (5g/l L-lysine or L-Arginine, 1 g/l glucose, 3 g/l yeast extract, and 15 mg/l bromocresol purple pH 6.8 and 5.5 respectively) by picking single colonies from growth on agar plates. The plates were sealed for anaerobiosis and incubated at 37° C. After 24 hours of growth the plate was visually inspected for changes in media color as a result of changing pH. The plate was also read in a SpectraMax Plus384 microplate reader (Molecular Devices). The ratio of absorbance between 570-590 nm and 400-450 nm to create a ratio of yellow to purple absorbance. The higher the ratio, the greater the activity of the lysine and arginine decarboxylase systems.

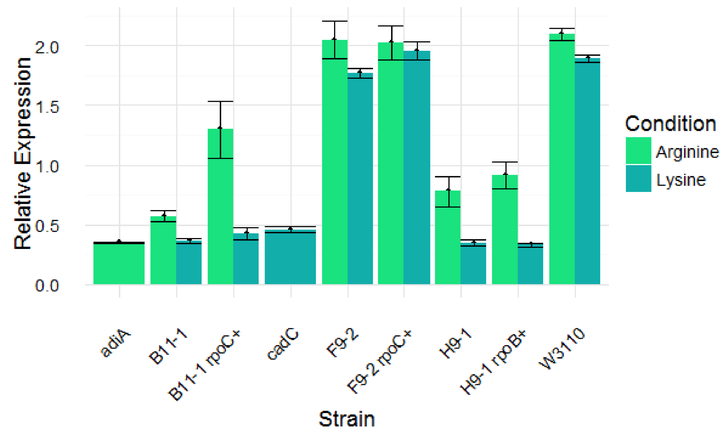
Lysine and Arginine Decarboxylase Systems Less Active

Figure 1: Expression of acid stress resistance mechanisms lysine and arginine decarboxylases measured through the ratio of purple absorbance to yellow absorbance. Error bars=SEM

Both F9-2 and F9-2 *rpoC*+ have lysine and arginine expression levels equal to those of the ancestor W3110.

B11-1 and H9-1 have similarly low levels of lysine and arginine decarboxylase systems compared to strains with known mutations in *adi* and *cad* operons, though each have more active arginine systems than lysine.

B11-1 *rpoC*+ has a restoration in arginine decarboxylase systems to a certain degree, although not equal to the ancestor’s activity.



Gene	Position (bp)	Mutation	H9-1	H9-2	B11-1	B11-2	F11-1	F11-2	F9-2	F9-3
[gadA]-slp	3,972,854	D14,321bp					X	X		
[gadX]-slp	3,975,089	D12,084bp							X	X
adiY	4,342,252	C⟶G (missense)			X	X				
adiY⟵/⟵adiA	4,342,646	Δ4bp::insH(_)+4bp::Δ6bp	X	X						
[cadA]-cadC	4,362,296	Δ4,171bp				X				
cadC⟵	4,365,903	Δ4bp::insH(+)+9bp::Δ1bp					X			
cadC⟵	4,365,918	Δ6bp::insH(_)+4bp::4bp	X	X						
cadC⟵	4,366,467	Δ6bp::insH(_)+4bp::Δ6bp			X					
rpoD⟶	3,212,521	G⟶A (missense)					X	X		
rpoC⟵	3,449,011	A⟶C (missense)							X	X
rpoC⟵	3,449,813	C⟶A (missense)			X	X				
rpoB⟵	3,453,401	G⟶A (missense)	X	X						

Table 1: Mutations to the amino acid decarboxylase systems and RNA polymerase subunits in the acid-evolved *E. coli* strains. Whole genome sequences were matched to the *E. coli* K-12 W3110 reference genome using *breseq* version 0.27.1. Mutation table compiled by Stephanie Penix ‘16 and Amanda He ‘16.

Conclusions

Transcriptomic Analysis

- Acid-evolved strains upregulate catabolism and downregulate acid stress genes compared to the ancestor.
- In F11 and F9, deletion of the *gad* regulon causes these genes to appear highly downregulated.
- Catabolism genes upregulated in the acid-evolved strains include glycerol catabolism, sugar alcohol breakdown, and anaerobic respiration.

Fluorescence Microscopy

- B11-1 does not significantly show a difference in its ability to maintain a higher internal pH when compared to the ancestor.
- When exposed to pH 4.8, both the ancestor and the B11 strain begin to filament and have an increased cell length compared to cells in the neutral condition.
- Both B11- 1 and the ancestor maintain a similarly lower internal pH in the acidic condition, and do not differ in the regulation of internal pH.

Lysine and Arginine Decarboxylase Activity

- Acid-evolved *E. coli* tend to select against the acid resistance mechanisms of amino acid decarboxylases when exposed to low pH for many generations.
- F9-2 maintained its amino acid decarboxylase activity after 2,000 generations in acid.
- Reversion of the RNA polymerase of B11-1 to the wild type polymerase revealed a restoration in arginine decarboxylase activity, although not to wild type levels.

Catabolism is Upregulated while Acid Stress is Downregulated in Acid-Evolved Strains

Acid Stress Genes: highlighted in red. General Stress/ Starvation Genes: highlighted in green. Catabolism Genes: highlighted in yellow

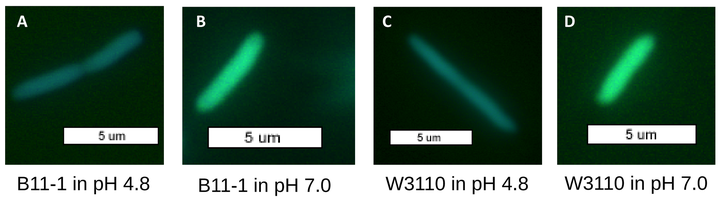
F11 upregulated			B11 upregulated			F9 upregulated		
Gene	Log2 fold change	p-value	Gene	Log2 fold change	p-value	Gene	Log2 fold change	p-value
glpA	6.86	2.98E-20	tdcA	3.99	5.17E-06	yeaR	7.41	2.35E-34
glpB	6.23	1.11E-19	dcuA	3.38	6.93E-61	yoaG	6.28	1.14E-10
glpT	5.64	6.35E-38	srlB	3.33	2.02E-09	ytfE	6.08	6.03E-19
yeaR	5.19	1.62E-20	srlA	3.29	8.61E-08	glpA	5.92	1.29E-16
glpC	5.14	4.07E-16	ydhY	3.12	3.54E-09	hmp	5.25	4.17E-30
yoaG	4.81	2.50E-07	ykgE	3.07	4.80E-51	glpF	5.09	2.60E-113
glpD	4.64	8.91E-100	ttdB	2.99	7.35E-06	glpB	4.83	8.12E-14
glpK	4.49	7.58E-95	srlD	2.95	2.81E-08	glpK	4.71	1.16E-101
glpF	4.44	4.84E-92	ttdA	2.94	6.34E-08	glpD	4.7	9.16E-102
glpQ	4.02	1.99E-27	srlE	2.84	2.57E-07	norV	4.64	8.32E-06
ytfE	3.86	9.96E-10	citC	2.59	1.32E-06	glpT	4.59	8.74E-28
yzgL	3.73	9.27E-67	dcuC	2.44	2.6E-06	norW	4.02	3.43E-08
nirD	3.6	2.31E-07	adiY	2.43	2.10E-09	ykgE	3.83	1.38E-73
nirB	3.44	4.01E-06	citD	2.4	0.000212	ygbA	3.79	2.16E-08
pgrR	3.19	5.89E-33	ybcW	2.39	6.07E-21	yzgL	3.65	2.90E-64
yfaH	2.81	1.27E-10	ykgF	2.38	3.10E-33	glpC	3.58	1.58E-09
hmp	2.77	4.70E-11	ybcV	2.36	4.56E-10	ykgF	3.17	5.79E-54
insH-8	2.61	1.70E-08	ydhV	2.26	1.50E-11	glpQ	2.87	9.37E-16
ygbA	2.35	0.000264	dcuB	2.22	8.82E-09	yhjX	2.85	6.92E-18
ykgE	2.06	4.99E-25	yhaO	2.17	7.91E-24	ykgG	2.61	6.61E-23

Table 2: (left) Top 20 upregulated genes in strains F11, B11, and F9 as compared to the ancestral transcriptome. Genes encoding catabolic pathways, sugar alcohol pathways, glycerol degradation, and anaerobic respiration tend to be upregulated (yellow). Also upregulated are genes that fight oxidative stress (green).

Table 3: (right) Top 20 downregulated genes in strains F11, B11, and F9 as compared to the ancestral transcriptome. Genes expressed in order to help maintain a higher internal pH in the presence of acid, such as those for the amino acid decarboxylase systems are down regulated. Other acid stress are also downregulated (red). Additionally genes to fight starvation stress are downregulated (green). Deletion of the *gad* regulon in F11 and F9 (shown in table 1) causes the apparent downregulation of *gad* regulon genes due to their loss through mutation.

F11 downregulated			B11 downregulated			F9 downregulated		
Gene	Log2 fold change	p-value	Gene	Log2 fold change	p-value	Gene	Log2 fold change	p-value
yhiD	-13.26	4.00E-32	cysJ	-4.72	1.30E-100	ynaI	-12.28	1.56E-59
hdeB	-12.52	0	cysI	-4.6	1.16E-96	mdtF	-9.64	5.60E-24
mdtE	-12.36	2.37E-16	cysD	-4.6	1.65E-94	yhiD	-9.61	8.74E-27
slp	-12.35	6.20E-37	cysH	-4.51	3.74E-92	mdtE	-9.54	8.08E-13
hdeA	-12.25	6.83E-43	yeeE	-4.39	8.31E-44	hdeB	-9.52	5.08E-262
hdeD	-12.12	2.78E-73	bhsA	-4.35	5.92E-44	gadW	-9.51	5.05E-25
mdtF	-12.04	1.29E-29	mdtJ	-4.19	2.80E-70	ynaJ	-9.5	3.02E-62
gadX	-12.02	3.28E-100	cysN	-3.93	3.77E-75	gadE	-9.46	2.72E-11
gadE	-11.99	2.28E-14	cysP	-3.92	3.55E-74	slp	-9.45	1.05E-28
gadA	-11.72	5.41E-07	cysU	-3.88	2.48E-70	hdeA	-9.34	1.03E-31
gadW	-11.55	1.35E-29	cysA	-3.71	4.42E-67	hdeD	-9.28	4.59E-58
mppA	-10.46	9.51E-178	cysW	-3.71	8.43E-63	fnr	-9.27	1.82E-212
gadB	-8.14	3.27E-07	yghF	-3.62	3.05E-29	uspE	-9.07	2.71E-218
gadC	-6.86	9.78E-06	entC	-3.59	1.63E-17	dctR	-8.61	7.96E-36
ybaS	-5.48	2.34E-05	argO	-3.57	1.59E-61	ompW	-7.31	3.74E-35
flu	-4.57	2.54E-15	cysC	-3.56	1.45E-23	gadB	-7	4.33E-06
glcD	-4.06	2.93E-07	flu	-3.48	2.17E-10	gadA	-6.72	0.000488
tnaA	-4.01	8.06E-18	ydjN	-3.38	1.01E-59	gadC	-6.26	3.55E-05
rmf	-3.93	2.12E-09	yeeD	-3.36	1.08E-57	ynfO	-5.71	2.16E-30
tnaC	-3.85	6.77E-09	lysA	-3.32	1.14E-26	yehD	-5.56	3.68E-39

Depressed Internal pH and Filaments in Acid Stress



B11-1 in pH 4.8 | B11-1 in pH 7.0 | W3110 in pH 4.8 | W3110 in pH 7.0

Figure 2: Fluorescent images of *E. coli.* Cells in pH 4.8 appear more blue, confirming the lower internal pH as a result of stressful media. Using the calibration from a standard curve, pH can be calculated from the ratio of two fluorescence intensities. Ratios show that green correlates to neutral pH and blue correlates to acidic pH.

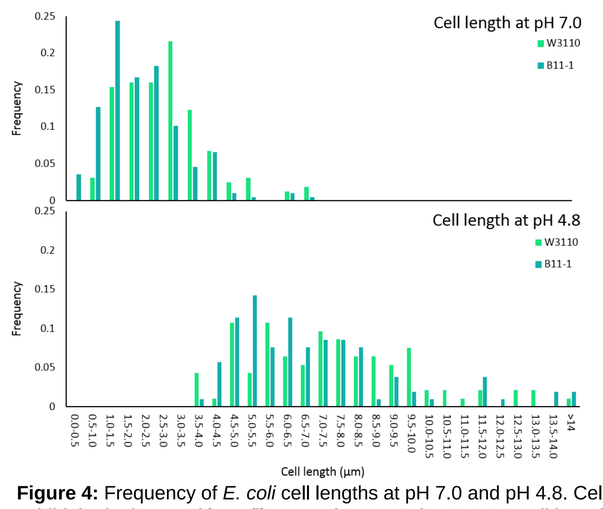
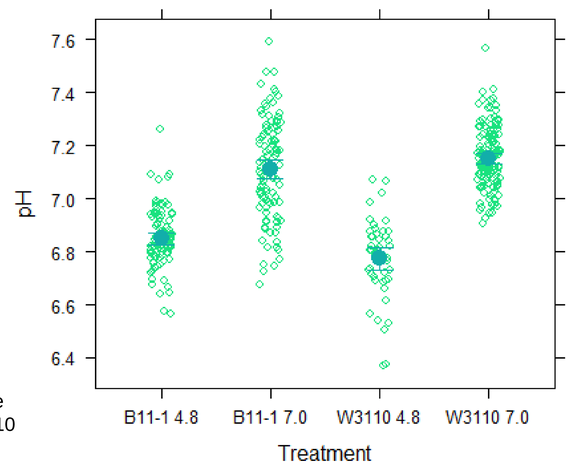


Figure 4: Frequency of *E. coli* cell lengths at pH 7.0 and pH 4.8. Cells exhibit both short and long filament phenotype in pH 4.8 condition while both strains are shorter in pH 7.0 condition. n(W3110 7.0)=162, n(W3110 4.8)=93, n(B11-1 7.0)=197, n(B11-1 4.8)=105.

Figure 3: Difference in cellular internal pH between B11-1 and W3110 in pH 7.0 and 4.8 media observed through fluorescence microscopy. Cells maintain lower internal pH when exposed to the pH 4.8 media. Error bars= 95% CI



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