The Influence of *slp-gad*X Mutation in Benzoate Resistance of *Escherichia coli*

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Abstract

Long-term microplate dilution of *Escherichia coli* was conducted by the Slonczewski lab. *E. coli* evolved in the increasing concentration of potassium benzoate for 2000 generation. Compared to the ancestor strain W3110 (D13), we found that five out of six benzoate-evolved *E. coli* clades showed the mutation related to *slp-gad*X.

Previously, the Slonczewski lab found that evolving strain A1-3 started to have higher benzoate and salicylate resistance compared to the wild-type D13 at generation 897. 2000 generation-evolved strain A1-1 showed a decreased in chloramphenicol (CHL) resistance compared to D13. Both A1-3 and A1-1 showed a large deletion in *slp-gad*X. Consequently, we hypothesized that the *slp-gad*X deletion might be related to the benzoate and salicylate, but not CHL, resistance.

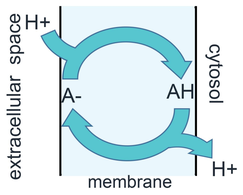
We found that the *slp-gad*X deletant strain showed a significant increase in 15 mM benzoate, while it is sensitive to 20 mM benzoate compared to both A1-3 and A1-1. At the same time, the *slp-gad*X deletant showed an increased resistance in 7 mM salicylate compared to the D13. However, the *slp-gad*X deletant showed no difference compared to D13 in 4 μg/mL CHL, suggesting that it might not be the reason for the trade-off between benzoate resistance and CHL sensitivity.

We also conducted the single gene knock-out strains of each gene in the acid fitness island to study if there is one single gene contribute more to the resistance of benzoate and salicylate. Our results suggested *gad*EF might contribute to the resistance. Also, no single gene mutation in the acid fitness island influenced the sensitivity of CHL compared to A1-1.

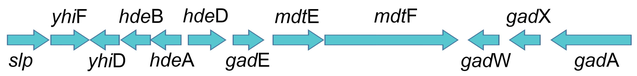
Introduction

☆ Benzoic acid (benzoate) is a permeant weak acid, which can decrease the proton motive force of the bacteria (1). Thus, the benzoic acid can weaken bacterial ability to pump proton out across the membrane and dynamic balance the charge between the extracellular space and cytoplasm (2).

☆ Both benzoate and salicylate have the structure of weakly acidic uncouplers. Though protonophoric activity, they can transport proton into the cell membrane and decrease the intracellular pH. The negative charge ion of the uncoupler (A-) catches the proton (H+) from the environment, transports to the reverse side of the membrane, releases the proton into the cell, and travel back to catch the proton again (4).



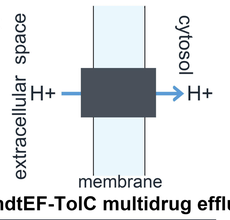
☆ Many genes, located in the acid fitness island, are associated with the acid-resistance phenotype (5).



***E. coli* acid fitness island.** According to whole genome sequence, the benzoate evolving strain only showed mutation in *slp-gad*X. Consequently, in our experiment, Jeremey used recombineering and deleted *slp-gad*X.

☆ *mdt*E and *mdt*F code for mdtEF-TolC multidrug efflux transport system. Some of the membrane protein in this system can pump proton into the cytosol from the outer membrane space.

☆The acid fitness island related genes, such as *gad*X, *gad*W, and *gad*E, are related to the acid resistance system. This system can utilize intracellular protons and increase the intracellular pH through the decarboxylation of glutamic acid.



mdtEF-TolC multidrug efflux

Methods

Bacterial strain: *Escherichia coli* W3110 (D13) was the ancestral strain of benzoate-evolved strain A1-3 (897 generation) and A1-1 (2000 generation). In previous experimental evolution, Slonczewski lab diluted D13 in the increasing concentration of potassium benzoate for 2000 generations. The *slp-gad*X deletion strain is conducted by Jeremey Moore by recombineering. The background strain of *slp-gad*X deletion is the D13.

P1 phage transduction: The unmuted gene in *E. coli* W3110 was inserted by A1-1 mutation according to the standard P1 phage transduction procedure. Constructs were amplified and checked by colony PCR according to the procedure of CloneID 1X Colony PCR Master Mix (Lucigen).

Growth assay and media: A 2 mL overnight culture was diluted 1:200 into an aliquot of different growth media. The OD_{600} values were measured in spectramax spectrophotometer at 37℃ every 15 minutes for 22 hours. All our strains were cultured in Luria-Bertani Potassium (LBK) media (10 g/L tryptone, 5 g/L yeast extract, 7.45 g/L KCl). Depend on the desired pH, the media was buffered with either with 100 mM 1,4-Piperazinediethanesulfonic acid (PIPES, pKa 6.66) or 100 mM 3-Morpholinopropane-1-sulfonic acid (MOPS, pKa 7.01).

Statistical analysis: We used RStudio to analyze data. We used ANOVA and TUKEY to test the significant differences between OD_{600} values at 16-hour endpoints.

Results

A1-3 and A1-1 mutation

Table 1:The mutations founded in A1-3 (897 generation) and A1-1 (2000 generation) compared to the genome of *E. coli* W3110. Red represents the changed base pairs; green represents synonymous mutation; blue represents missense mutations; purple represents IS-mediated deletion. The blue shadow indicates the mutation focused in this experiment.

A1-3	A1-1	Position	Mutation	Annotation	Gene	Description
		56,273	G→A	L279L (CTC→CTT)	*lpt*D (imp)←	exported protein required for envelope biosynthesis and integrity, outer membrane lipopolysaccharide transport and assembly complex
		556,778	A→C	F63V (TTC→GTC)	*fol*D ←	bifunctional 5,10methylenetetrahydrofolate dehydrogenase/5,10methylene tetrahydrofolate cyclohydrolase
		1,213,665	(C)8→9	intergenic (85/+615)	*elb*A ← / ← *ycg*X	hypothetical protein/hypothetical protein
		1,218,024	IS5 (+) +4 bp	coding (7982/267 nt)	*ari*R (*ymg*B) →	acid stress activator of Gad; associated with biofilm
		1,349,606	IS5 +5 bp	coding (1021/1935 nt)	rnb ←	exoribonuclease 2
		2,646,569	C→A	E1459* (GAG→TAG)	*yfh*M ←	hypothetical protein
		2,931,775	C→A	P190P (CCG→CCT)	*fuc*A ←	Lfuculose1phosphate aldolase
		3,454,320	C→T	G373S (GGC→AGC)	*rpo*B ←	RNA polymerase subunit beta
		3,532,025	A→G	N107S (AAC→AGC)	*cpx*A →	sensory histidine kinase in twocomponent regulatory system with CpxR
		3,948,766	G→A	R320H (CGT→CAT)	*bcs*B →	regulator of cellulose synthase, cyclic diGMP binding
		3,967,563	C→A	A171S (GCG→TCG)	*yhj*C ←	DNAbinding transcriptional regulator
		3,976,435	Δ10,738 bp	insHmediated	[*gad*W]–*slp*	[*gad*W], *mdt*F, *mdt*E, *gad*E, *hde*D, *hde*A, *hde*B, *yhi*D, *yhi*F, *slp*
		4,200,197	A→C	K271Q (AAA→CAA)	*rpo*A →	RNA polymerase subunit alpha

*slp-gad*X mutation strain showed different effect on differnt concentration of benzoate

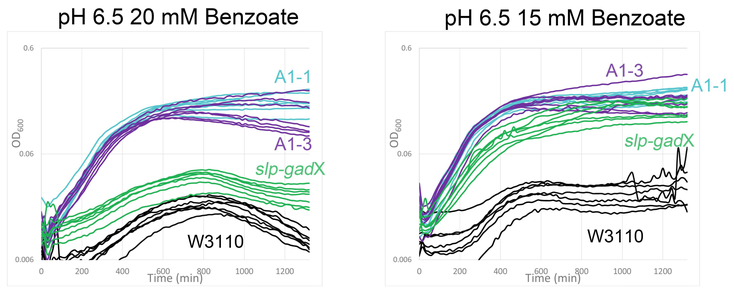


Figure 1: (a) W3110, A1-1, A1-3, and *slp-gad*X deletant were grown in 15 mM benzoate with 100 mM PIPES at pH 6.5. The 16-hour data was collected and analyzed (F=81.8, p=5.8e-14).
(b) W3110, A1-1, A1-3, and *slp-gad*X deletant was grown in 20 mM benzoate with 100 mM PIPES at pH 6.5. The 16-hour endpoint data was collected and analyzed (F=89.8, p=1.8e-14).

*mdt*F and *mdt*E deletion increased benzoate resistance

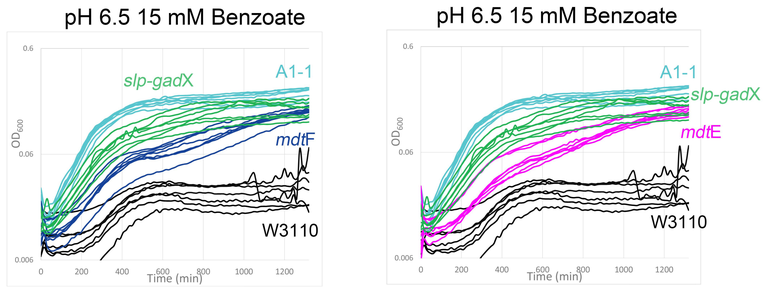


Figure 2: (a) W3110, A1-1, *slp-gad*X, and *mdt*F deletant were grown in 15 mM benzoate with 100 mM PIPES at pH 6.5. The 16-hour endpoint data was collected and analyzed (F=87.9 p=2.3e-14).
(b) W3110, A1-1, *slp-gad*X, and *mdt*E deletant was grown in 15 mM benzoate with 100 mM PIPES at pH 6.5. The 16-hour endpoint data was collected and analyzed (F=93.8, p=1.0e-14).

*slp-gad*X mutation showed a increased CHL resistance

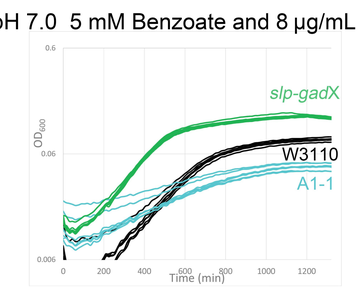


Figure 3: W3110, A1-1, and *slp-gad*X were grown in 5 mM benzoate and 8 ug/mL CHL with 100 mM MOPS at pH 7.0. The 16-hour endpoint data was collected and analyzed (F=1200, p<2e-16).

Conclusions

☆ In 15 mM benzoate, *slp-gad*X showed a significant increase in benzoate resistance compared to the ancestor. However, in 20 mM benzoate, it showed no statistical difference in resistance. Consequently, other genes help benzoate resistance in 20 mM benzoate. We might use *slp-gad*X deletant as the background strain and transduce other mutations that we found in the whole genome sequencing (table 1).

☆ Benzoate and salicylate, two uncouplers, might decrease the proton motive force of the bacterial membrane. The A1-1 might increase the benzoate-resistance through *mdt*E and *mdt*F resistance. In this way, they might lose the ability to get proton from the outside environment, and thus increase the intracellular pH and decrease the negative influence of both the benzoate and salicylate.

☆ *gad*X, *gad*W, and *gad*E may play a role in the resistance of 15 mM benzoate, while not in that of 20 mM benzoate. According to Mates et al. (2007), those genes might help the acid resistance system by consuming intracellular proton through the decarboxylation of glutamic acid. For example, *gad*E contributes to the acid-resistance through limiting the glutamate that bacteria utilized in the acid resistance. However, in our experiment, we did not find that the glutamic acid decreases the survival of bacteria in the 15 mM benzoate environment.

☆ Both the whole *slp-gad*X deletant and any single gene deletion in the acid fitness island showed no effect on the decrease of CHL resistance. Thus, the CHL resistance showed in A1-1 might not related to *slp-gad*X.

☆ The *hde*A, *hde*B, *ybi*D, *hde*D deletions did not show significant differences in resistance to both 15 mM benzoate and the 7 mM salicylate. Consequently, each gene by itself might not contribute to the benzoate and salicylate resistance. In the future, we might try some combination of genes to see if they help or not.

Future studies

☆ mdtEF-TolC multidrug efflux transport system is a resistance nodulation division type multidrug efflux system. The overexpression of the pump can make the bacteria sensitive to some types of antibiotic, such as β-lactams, macrolide antibiotic erythromycin, doxorubicin, crystal violet, ethidium bromide, rhodamine 6G, tetraphenylphosphonium bromide (TPP), benzalkonium, SDS, deoxycholate. It is interesting for us to test some similar structure antibiotic in the future experiment.

☆ The *slp-gad*X deletant showed a significant resistance of 15 mM benzoate resistance to compared to D13, while it showed no difference in the 20 mM benzoate resistance. At the same time, only four mutations were found, such as *fol*D, *ari*R, *bcs*B, and *yhj*C. Consequently, in the future study, it might be interesting to transduce or recombineer the single A1-3 mutation into the *slp-gad*X deletion strain. In this way, we can study which mutation or mutations might contribute to the differences between the benzoate resistance in different concentrations.

☆ According to Mates et al., the benefit of acid fitness island might relate to the presence of glutamate, Glutamic acid is present in the LBK medium that we used in all experiment. It might be interesting if we run the experiment in other kinds of medium.

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