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Selective Evolution of Lithium Ions by *Shewanella loihica*

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Honors Thesis

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Abstract

The prevalent use of lithium-ion batteries in consumer electronics poses a significant sustainability challenge to both manufacturing and source availability over the next 50 years. There are several methods to extract lithium from salt water that are commercially available but require caustic reagents and environmental hazards. However, several lithium-resistant bacteria can precipitate or mineralize lithium ions into a reduced form of lithium that can be processed readily into a lithium source material for batteries. The goal of this project was to culture, characterize, and selectively evolve *Shewanlla loihica* using lithium-ion concentration changes both aerobically and anaerobically. This strain has published metal-reducing capabilities and by selectively pressuring the organisms with higher and higher concentrations of Li we will understand the fundamental lithium tolerance of the organism. The project includes an understanding of the changes in growth rate due to the subsequent culturing of organisms with increasing concentrations of Li-ion. The hope for the outcome of the project was to find a potential biological approach to the precipitation of Li ions from salt water.

Acknowledgements

I would like to thank Dr. Biffinger for his mentorship throughout this project, the University Honors Program for funding and support, and the Department of Chemistry, College of Arts and Sciences – Dean's Summer Fellowship for funding this project.

University *of* Dayton

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Introduction

The world is in a constantly growing energy crisis. As the world becomes more reliant on technology, the need for energy to support such advancements is skyrocketing. There have been trends of the energy demand slowing due to finding renewables and "greener" energy sources **[1]**. One major "greener" source that has been utilized is lithium-ion batteries (LIBs). However, these batteries pose a threat to the environment due to their spent electrolyte components. For example, they can react in the water and pose toxicity issues **[1]**. There are currently methods for lithium extraction from spent battery materials such as solvent extraction, adsorption, electrodialysis, and a few others. However, these reclamation processes each pose weaknesses that make them difficult to use at a large enough scale. These weaknesses include cost, hazardous materials, and inefficiency time-wise **[2]**. Thus, new forms of recycling LIBs are needed to not only meet the energy demands of the future but also protect the environment. There are several biological approaches (both living cells and extracted proteins) that might meet this challenge. The goal of this project was to investigate the lithium tolerance of *Shewanella loihica* for applications in metal ion recycling for sustainability.

Today there is an increase in the usage of lithium-ion batteries **(Figure 1) [3]**. Due to the increasing demand and short lifespan of these electronic devices, lithium batteries are used in just about everything, and the world is becoming increasingly reliant on them. Unfortunately, many metals used in Li battery materials (Lithium, cobalt, manganese, etc…) are ultimately not sustainable with the current projections for Li battery use over the next 50 years **[4-5]**. The current problems being faced are the energy-intensive and environmentally unfriendly approaches to reclaim these metal ions and the impact that the inaccessible waste metals have on the environment **[6]**. Additionally, many critical metals for battery technologies rely on foreign import sources, posing a serious supply chain and security risk for the United States **[7]**. Thus, more environmentally friendly approaches to recovering the metals from lithium batteries are essential to make that technology sustainable for the next 50 years.

The microorganism selected for these experiments was *Shewanella loihica*. It is a marine bacterium originally isolated from a submarine volcano in Loihi, Hawaii with a high metabolic capacity **[8]**. The general morphology is rod-shaped with a singular flagellum **(Figure 2) [9]**. Members of the *Shewanlla* genus are found in a wide range of aquatic environments including both freshwater and saltwater environments. The most well-known characteristics of the genus are high metabolic versatility and the ability to grow in tandem with numerous electron acceptors **[10]**. The growth with electron acceptors allows for *Shewanella* that allows it to act as an electron donor and aid in the reduction of metals.

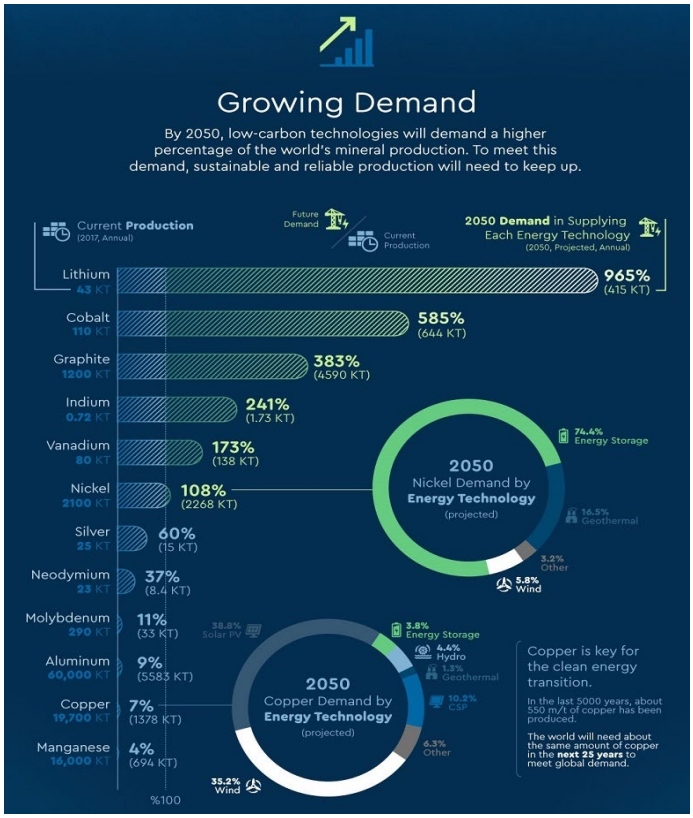


Figure 1. Increasing Usage of Lithium-Ion Batteries

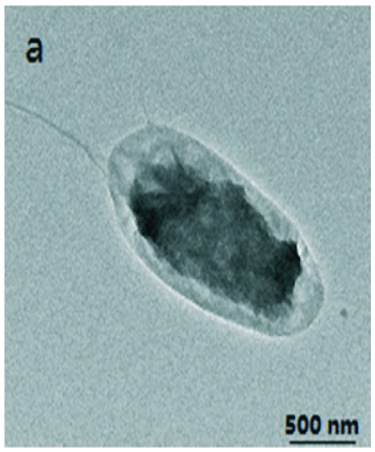


Figure 2. Morphology of S. Loihica Microorganism

Materials and Methods

Growth Experiments

Luria-Bertani Broth

Using sterilized Luria-Bertani Broth, *Shewanella loihicia* was inoculated from a frozen stock in 10mL of the broth and incubated at 37ºC in sterilized test tubes. Optical density measurements were recorded at 600nm at inoculation, after 1 hour, 20 hours, and 48 hours.

Defined Medium (DM)

The defined medium was prepared with 2.501g $NaHCO_3$, 0.086g $CaCl_2*2H_2O$, 1.04g NH_4Cl, 0.22g $MgCl_2*6H_2O$, 10.07g NaCl, and 7.30g HEPES in 1 L reverse osmosis (RO) water **[11]**. The pH was adjusted to 7. Using 1mL cells grown in the LB broth, cells were washed 3 times with 1mL PBS. Each time cells were pelleted at 12000 rpm for 1 min. The pellet was then vortexed in 1 mL of fresh PBS following the final wash. A 1% inoculation (100 µL) of the cells was added to 10mL of the defined medium along with 200 µL of pH 5 lactate to create an overall 10mM lactate solution. Optical density measurements were taken at 600nm at inoculation, after 1 hour, after 4 hours, and after 24 hours.

Anaerobic Growth

For anaerobic conditions, subcultures were established with a 1% inoculation from aerobically grown day-old *S. loihica* culture grown in LB. One of the new cultures contained LB and 20 mM fumarate. The other culture contained LB, 20 mM fumarate, and 50 mM lithium chloride. The cells were sealed into tubes and grown for 2 days measuring the optical density at 600nm at 0 hours, 1 hour, 3 hours, 24 hours, and 47 hours. At the end of the 2 days 500 µL of each culture was mixed with 500 µL of 20% glycerol and placed in the -80ºC freezer for storage.

Using the previously frozen anaerobic cultures, new anaerobic cultures were started from toothpicks. The anaerobic wild-type (WT) *S. loihica* cells were inoculated in a solution with 10mL LB and 20mM fumarate and a solution with 10mL LB and 50mM LiCl. After being grown for 3 days, each was subcultured at a 1% inoculation to the same conditions where growth was measured based on optical density at 600nm at 0 hours,1 hour, 4 hours, 24 hours, and 48 hours.

Using the previously frozen anaerobic cultures, anaerobic cultures were started from toothpicks. The WT culture anaerobically grown with no lithium before freezing was placed in LB only. The culture that had been grown in 50 mM lithium before freezing was grown in LB with 50 mM LiCl, LB with 50 mM LiCl and 20 mM fumarate, and LB with 50 mM LiCl and 20 mM sodium acetate. Cells were grown for 24 hours with optical density measurements taken 600nm at 0 hours, 1 hour, and 24 hours.

Once again, using the previously frozen anaerobic cultures, anaerobic cultures were started from toothpicks. The WT culture anaerobically grown with no lithium before freezing was placed in LB and 20 mM fumarate. The culture that had been grown in 50 mM lithium before freezing was grown in LB with 50 mM LiCl and 20 mM fumarate, LB with 100 mM LiCl and 20 mM fumarate, and LB with 150 mM LiCl and 20 mM fumarate. Cells were grown for 24 hours and then subcultured at 1% inoculation into the same conditions. Optical density measurements were taken of the subcultures at 600nm at 0 hours, 1 hour, 4 hours, 24 hours, and 48 hours.

Selective Evolution

Luria-Bertani Broth

Using 1% inoculation from a day-old culture of *S. loihica* in LB, cells were placed in 10mL LB broth with a 50mM concentration of sterile LiCl (~2mL of 10g/L). Cells were incubated at 37ºC for 24 hours while growth was monitored using 600 nm optical density measurements at 0 hours, 1 hour, 4 hours, and 24 hours. After the 24-hour incubation, a new subculture was made using 1% inoculation in a new 10 mL LB broth with a 50 mM concentration of sterile LiCl. Optical density was taken at 600 nm using the same time scale. A final subculture was made and growth was monitored using the

same steps as previously used. Following a 24-hour incubation, 500 µL of culture was frozen in 500 µL of 20% glycerol at -80ºC.

Using 1% inoculation from newly grown frozen stock wild-type *S. loihica* in LB, cells were placed in 8.5mL LB broth with an overall 150mM concentration of sterile LiCl (~1.5mL of 1 M LiCl). Cells were incubated at 37ºC for 24 hours. After the 24-hour incubation, a new subculture was made using 1% inoculation in a new 8.5mL LB broth with 150mM concentration of sterile LiCl. An additional two subcultures were made using the same steps as previously used. Following the last subculture's 24-hour incubation, 500µL of culture was frozen in 500µL of 20% glycerol at -80º.

Using 1% inoculation from a day-old culture of *S. loihica* in LB, cells were placed in 10mL LB broth with a 50mM concentration of sterile LiCl (~2mL of 10g/L). Cells were incubated at 37º for 24 hours. After the 24-hour incubation, a new subculture was made using 1% inoculation in a new 10mL LB broth with a 50mM concentration of sterile LiCl. A total of 5 subcultures were made over 5 days, each tube getting 24 hours to grow before inoculation of the next. Following a 24-hour incubation of the final subculture, 500µL of culture was frozen in 500µL of 20% glycerol at -80º.

Lithium Toxicity

Growth in Luria-Bertani Broth

Several sets of experiments were designed to selectively evolve *S. loihica* using variable concentrations of LiCl in a nutrient broth like Luria Bertani. 25 mL aliquots were prepared of sterile pure LB broth, LB broth at 100mM LiCl, LB broth at 200mM LiCl, and LB broth at 300mM LiCl. Using the aliquots, 2 new 5mL aliquots were made of each solution. A 1% inoculation of growing wild-type (WT) cells was added to one of each solution's 5 mL aliquots. The remaining 5mL aliquots received a 1% inoculation of the 3x subcultured 50mM LiCl selectively evolved (push) cells. Prepared sterilely in the biosafety hood, 250µL of the controls (no cell aliquots), wild-type aliquots, and push aliquots were added to a 96-well plate, each having 6 replicates. The plate was covered and set on the plate reader at 27ºC and shook for 24 hours. Optical density measurements were taken at 600nm on the hour for 24 hours.

25 mL aliquots were prepared of sterile pure LB broth, LB broth at 100mM LiCl, LB broth at 200mM LiCl, and LB broth at 300 mM LiCl. Using the aliquots, 3 new 5mL aliquots were made of each solution. A 1% inoculation of growing wild-type cells was added to one of each solution's 5mL aliquots. Another group of the 5mL aliquots received a 1% inoculation of the 3x subcultured 50mM LiCl selectively evolved cells. The final group of the 5mL aliquots received a 1% inoculation of the 5x subcultured 150mM Li selectively evolved cells. Prepared sterilely in the biosafety hood, 250µL of the controls (no cell aliquots), wild-type aliquots, 3x subcultured 50 mM evolved aliquots, and 5x subcultured 150 mM evolved aliquots were added to a 96-well plate, each having 6 replicates. The plate was covered and set on the plate reader at 27ºC and agitated (slow setting) for 24 hours. Optical density measurements were taken at 600nm on the hour for 24 hours.

25 mL aliquots were prepared of sterile pure LB broth, LB broth at 100mM LiCl, LB broth at 200mM LiCl, and LB broth at 300mM LiCl. Using the aliquots, 3 new 5mL aliquots were made of each solution. A 1% inoculation of growing wild-type cells was added to 1 of each solution's 5mL aliquots. Another group of the 5mL aliquots received a 1% inoculation of the 3x subcultured 50mM Li selectively evolved cells. The final group of the 5mL aliquots received a 1% inoculation of the 5x subcultured 50mM Li selectively evolved cells. Prepared sterilely in the biosafety hood, 250uL of the controls (no cell aliquots), wild type aliquots, 3x subcultured 50mM evolved aliquots, and 5x subcultured 50mM evolved aliquots were added to a 96-well plate, each having 3 replicates. The plate was covered and set on the plate reader at 27ºC and shook for 19 hours. Optical density measurements were taken at 600nm on the hour for 19 hours.

25 mL aliquots were prepared of sterile pure LB broth, LB broth at 200mM LiCl, LB broth at 400mM LiCl, LB broth at 600mM LiCl, and LB broth at 800mM LiCl. Using the aliquots, 2 new 5mL aliquots were made of each solution. A 1% inoculation of growing wild-type cells was added to 1 of each solution's 5mL aliquots. Another group of the 5mL aliquots received a 1% inoculation of the 3x subcultured 50mM Li selectively evolved cells. Prepared sterilely in the biosafety hood, 250µL of the controls (no cell aliquots), wild type aliquots, and 3x subcultured 50mM evolved aliquots were added to a 96-well plate, each having 3 replicates. The plate was covered and set on the plate reader

at 27º and shook for 36 hours. Optical density measurements were taken at 600nm on the hour for 36 hours.

Growth in Defined Medium

Using cells frozen in LB broth, cultures were started from the frozen WT culture under the following conditions: Defined media (DM)/15mM lactate, DM/15mM lactate/10mM Li, DM/15mM lactate/20mM LiCl, DM/15mM lactate/50mM LiCl. The DM is used following the literature and discussed in the materials and methods section**[11]**. After 24 hours, the cells were subcultured at a 1% inoculation into the same conditions. Growth was then measured by taking optical density measurements at 600nm after 0 hours, 1 hour, 4 hours, 24 hours, and 48 hours following inoculation.

Using cells frozen in LB broth, cultures were started from the frozen WT culture and frozen 3x LiCl 50mM acclimated culture. The WT was grown in only LB and the 3x 50mM acclimated was grown in LB/50mM LiCl. After 24 hours, 2 tubes of each were prepared under the following conditions: DM/15mM lactate/20mM LiCl, DM/15mM lactate/50mM LiCl, DM/15mM lactate/100mM LiCl. The conditions were finished with a direct 1% inoculation of the cells growing in LB, the WT in one tube of one condition set and the 3x 50mM acclimated in the other. Growth was then measured by taking optical density measurements at 600nm after 0 hours, 1 hour, 4 hours, 24 hours, and 52 hours following inoculation.

Live/Dead Staining

Using LIVE/DEAD BacLight™ Bacterial Viability Kits, the staining suspension was prepared. Following the 24-hour toxicity read, 100uL of the cells from the 96-well plate were transferred to a fluorescence 96-well plate. To form a 2X stain solution, 100µL of the suspension was added to each well. The plate was covered and shaken for 15 min. The plate was then read for fluorescence at 530 nm and 630 nm with the excitation centered at 485 nm both times.

Results and Discussion

Growth in Luria-Bertani Broth

We established and compared changes in how S. loihica adapted to lithium-ion stress through changes in the growth of the organisms. We observed that *S. loihica* grows consistently at pH 7. As the pH was changed to be more acidic, a pH of 4, *S. loihica* was unable to grow **(Figure 3)** strongly indicating that S. loihica would not be suitable for work below pH 6. In contrast to the growth of the organism in a nutrient-rich broth, growth was observed in the defined medium at pH 7, however, at a lower optical density which would be expected in a more defined growth medium. Over 24 hours, the microorganism grew to an optical density measure of about 0.75 in LB broth and only about 0.23 in the defined medium. Additionally, in the defined medium the lactate as a carbon source was demonstrated to be a necessary component for growth (**Figure 4)**.

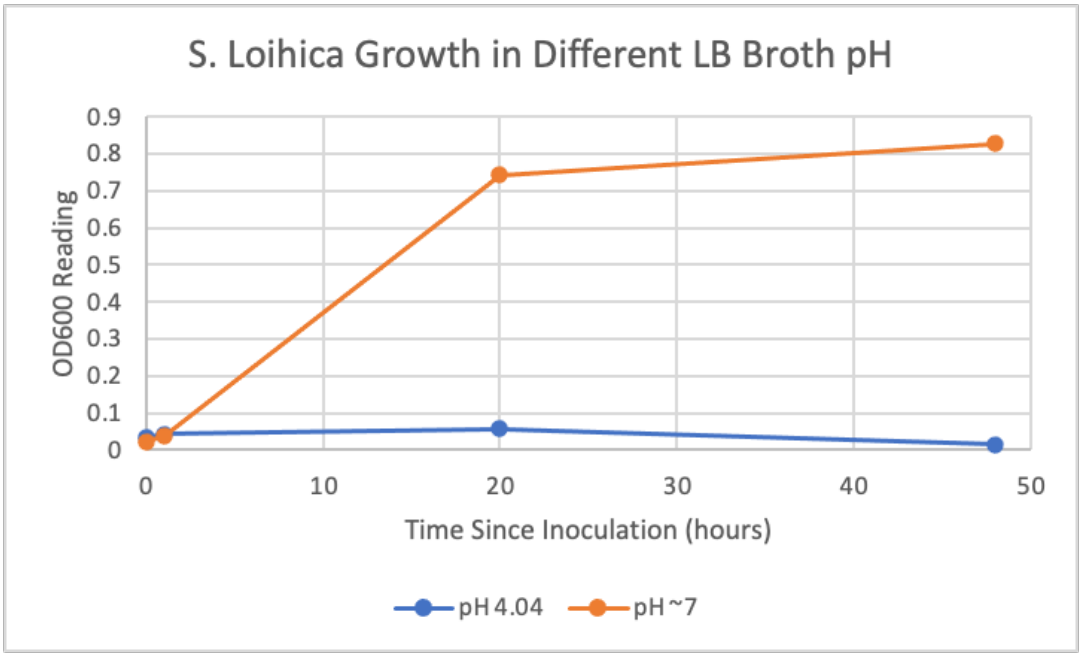


Figure 3. *S. loihica* Growth in LB with Differing pH

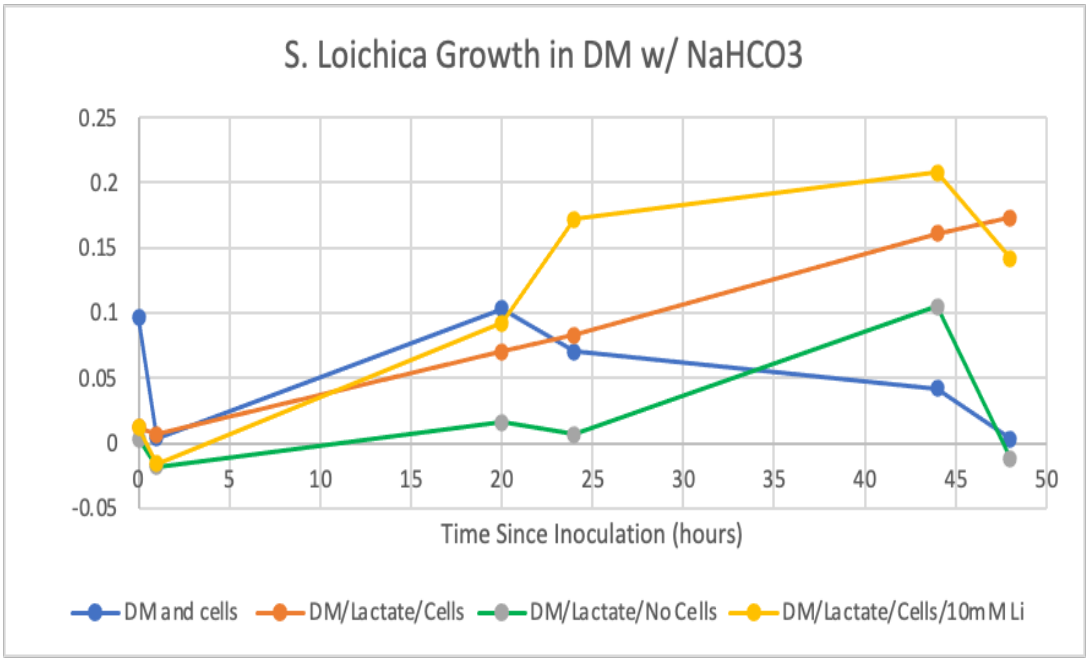


Figure 4. Growth of *S. loihica* in DM w/ $NaHCO_3$

Since our first experiments were directed toward understanding the growth of S. loihica with elevated lithium ion concentrations we pursued experiments at pH 7 in LB first. , The growth in the different concentrations of lithium ranging from 10mM Li to 100mM Li concluded that S. loihica has some natural tolerance for Li ions. There appeared to be a negative correlation between lithium concentration and growth at 100 mM LiCl. As the concentration of lithium increased the overall density of cells decreased **(Figure 5.)**

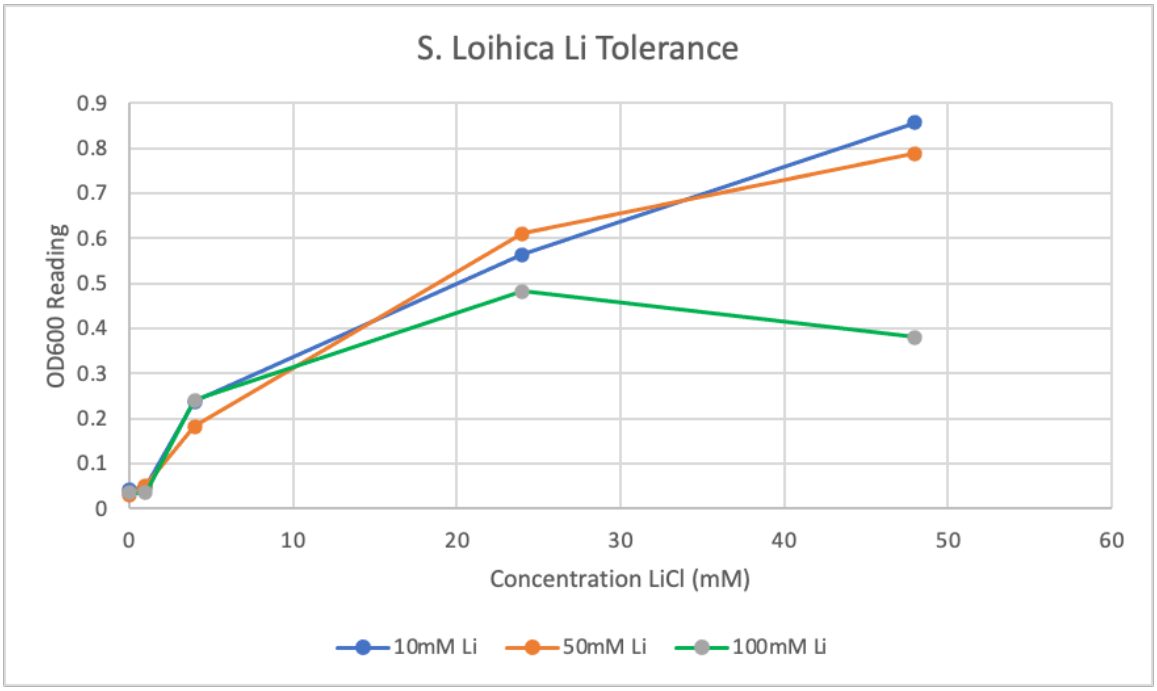


Figure 5. Preliminary *S. loihica* Growth In increasing concentrations of Lithium

Lithium Acclimation for Directed Evolution in Luria-Bertani Broth

We use subculturing to establish lithium tolerance in ever-increasing concentrations of LiCl. We selectively evolved S. loihica cultures three times into the 50 mM lithium LB medium. We measured the peak optical density reading for both the wild type and evolved culture was roughly the same for growth in pure LB, LB and 50mM Li, LB and 100mM Li, and LB and 150mM Li **(Figure 6)**. After performing a Student paired t-test for each condition comparing the means of the peak optical density reading for the WT and 3x 50mM Li acclimate at a 95% confidence interval, we found that there was no statistically significant difference between the readings suggesting that at 50mL LiCl the cells were not impacted. Additionally, the growth curves were nearly identical for the wild type and evolved culture for each growth medium tested **(Figure 7)**. This result led to increasing the LiCl concentrations in the cultures.

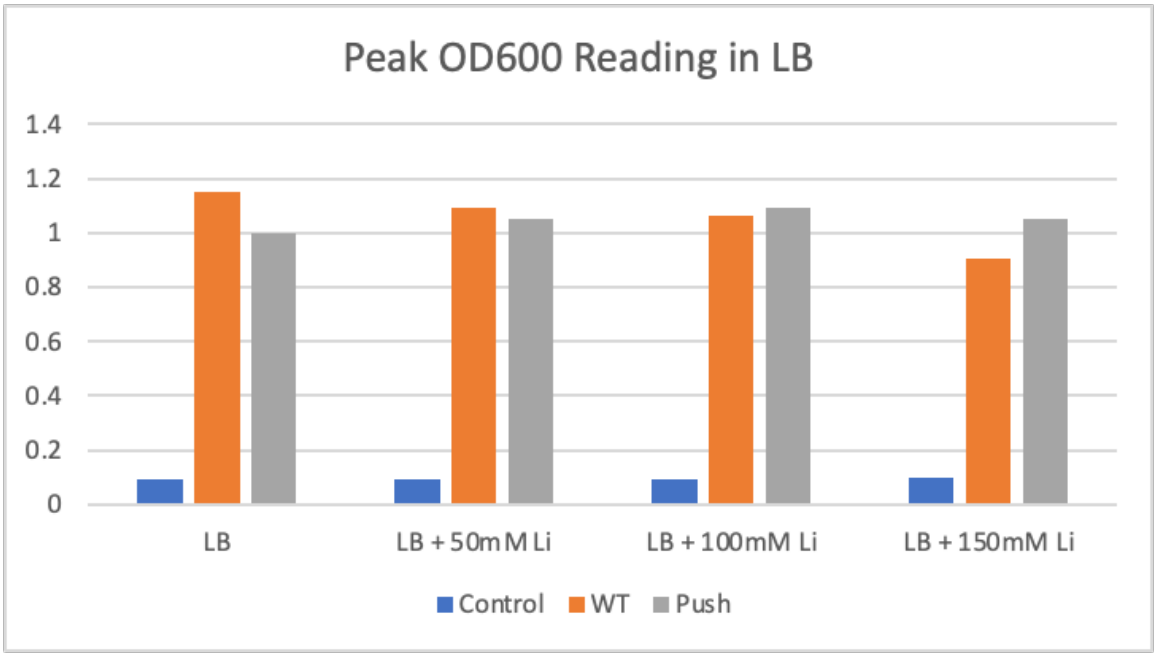
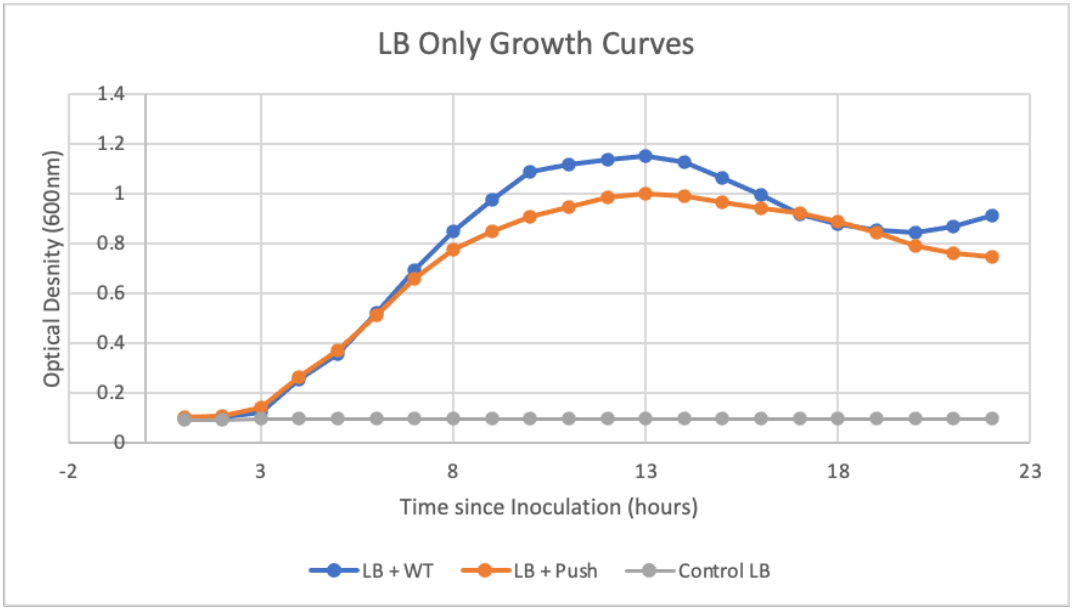
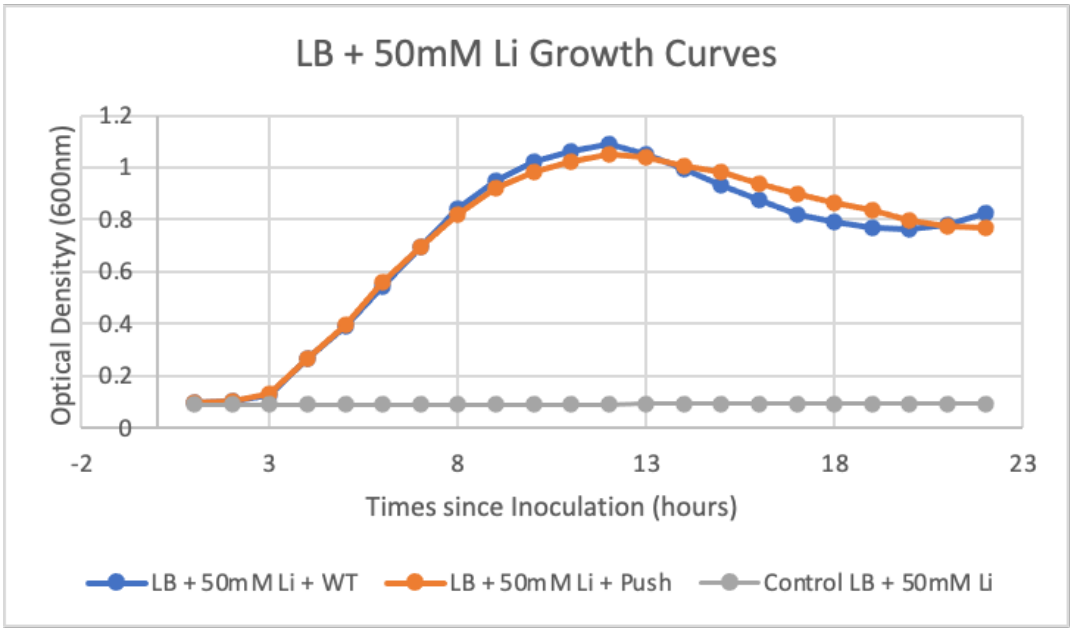
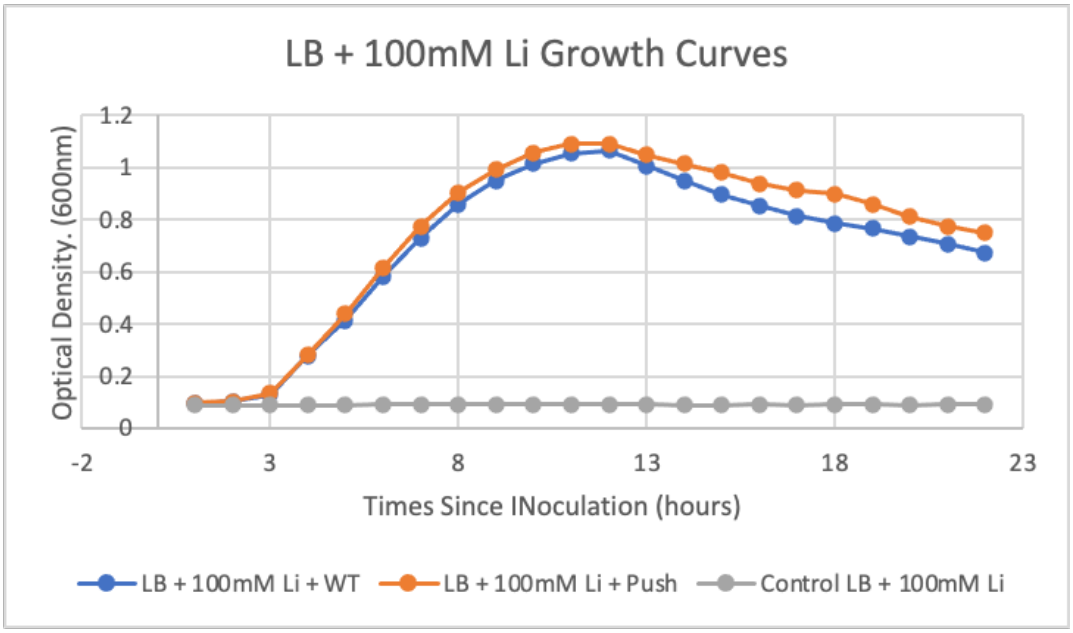


Figure 6. Peak Optical Density Measures of WT and 3x Acclimated *S. loihica* in Lithium




LB + 50mM Li Growth Curves
Optical Densityy (600nm)
1.2
1
0.8
0.6
0.4
0.2
0
-2
3
8
13
18
23
Times since Inoculation (hours)
LB + 50mM Li + WT
LB + 50mM Li + Push
Control LB + 50mM Li


LB + 100mM Li Growth Curves
Optical Density. (600nm)
1.2
1
0.8
0.6
0.4
0.2
0
-2
3
8
13
18
23
Times Since INoculation (hours)
LB + 100mM Li + WT
LB + 100mM Li + Push
Control LB + 100mM Li

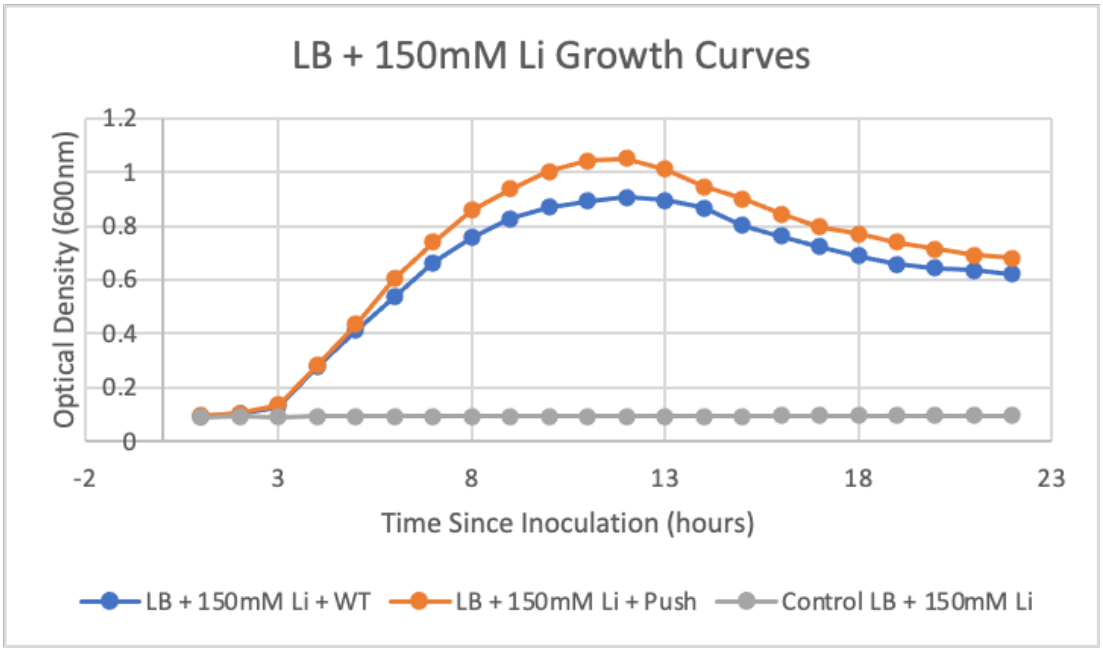


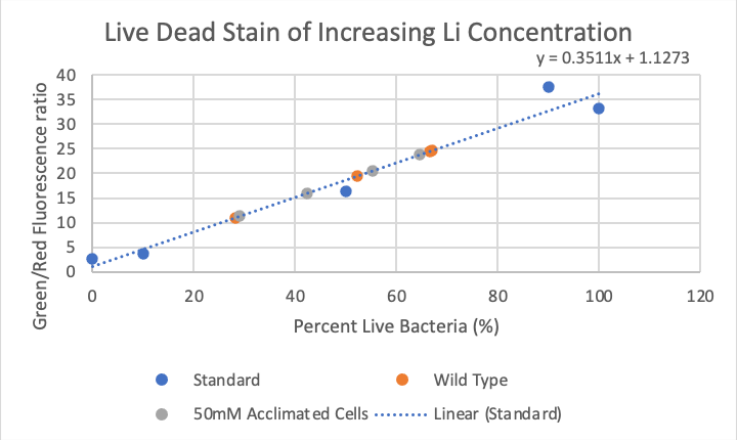
Figure 7. Preliminary *S. loihica* Growth Curves in Increasing Lithium Concentrations

Moving into higher concentrations of lithium, toxicity experiments were performed on the wild type and 3x 50mM Li acclimated strains. The growth of each organism was evaluated at 0mM Li, 100mM Li, 200mM Li, and 300mM Li, after 24 hours of growth. These data suggested that the 200mM LiCl had the highest percentage of living cells and at 300mM the cells started to be less viable. **(Figure 8)**

There was no large difference between viable cell percentages for the WT and 3x 50mM acclimated so the organism was "pushed harder" to 3x 150mM Li acclimated. The toxicity experiment was repeated this time including the 3x 150mM Li acclimated cells. Similar results were shown with the 200 mM concentration leading to the largest number of living cells **(Figure 9)** Data analysis was performed using Student t-tests at a 95% confidence interval; comparing the different cells together in the different conditions. The only optical densities that showed statistically significant differences were the 3x 50mM acclimate and the 3x 150mM acclimate in 100mM Li. Thus, the cells were still showing no advantage after being acclimated.

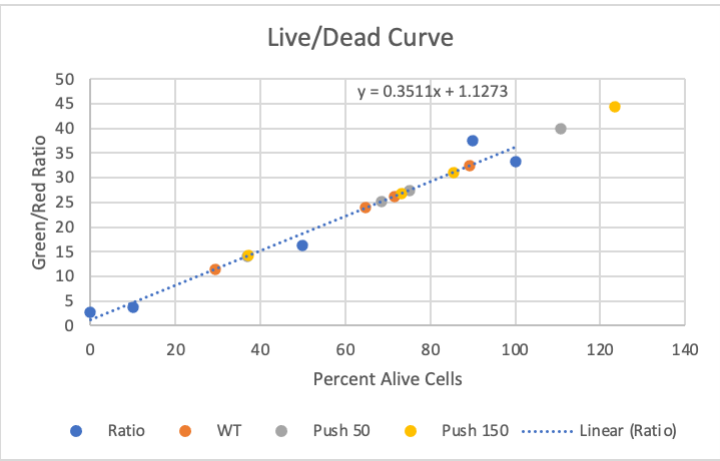
To test the growth of the cells in the 300mM Li concentration, a 20-hour growth curve was performed. These data confirmed in replicate experiments that the 150 mM

acclimated cells were potentially compromised by the high lithium concentrations to have consistent and good results. So we did not pursue culturing at 150mM LiCl further. In addition, these data suggested that WT were more tolerant to Li in the 3x 50mM Li acclimated cells, yet there was no statistical difference at a 95% confidence interval. **(Figure 10.)**



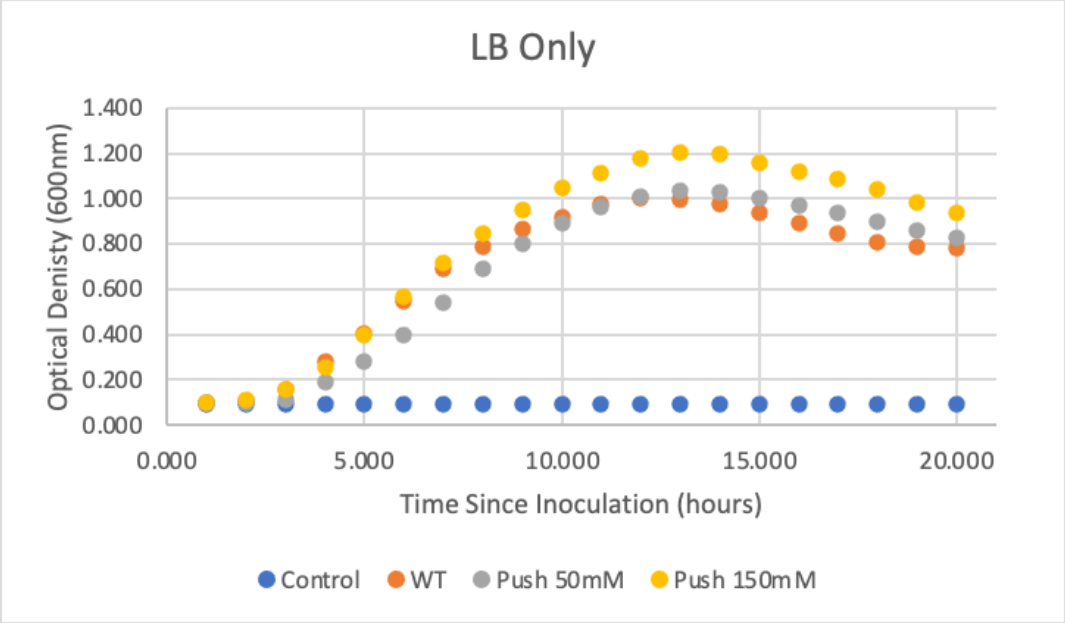
Conc. Li (mM)	WT	% Alive Cells	Push	% Alive Cells
0	24.7	67.1	23.8	64.6
100	19.5	52.2	16.0	42.3
200	24.5	66.6	20.6	55.4
300	11.0	28.2	11.3	29.0

Figure 8. Live/Dead Staining of *S. loihica* in Increasing Li Concentrations of WT and 3x Acclimated to 50mM Li



Conc. Li (mM)	WT	% Alive Cells	Push 50mM	% Alive Cells	Push 150mM	% Alive Cells
0	24.7	67.1	23.8	64.6	44.4	123.4
100	19.5	52.2	16.0	42.3	31.1	85.4
200	24.5	66.6	20.6	55.4	26.9	73.3
300	11.0	28.2	11.3	29.0	14.2	37.1

Figure 9. Live/Dead Staining of *S. loihica* in Increasing Li Concentrations of WT, 3x Acclimated to 50mM Li, and 3x Acclimated to 150mM Li



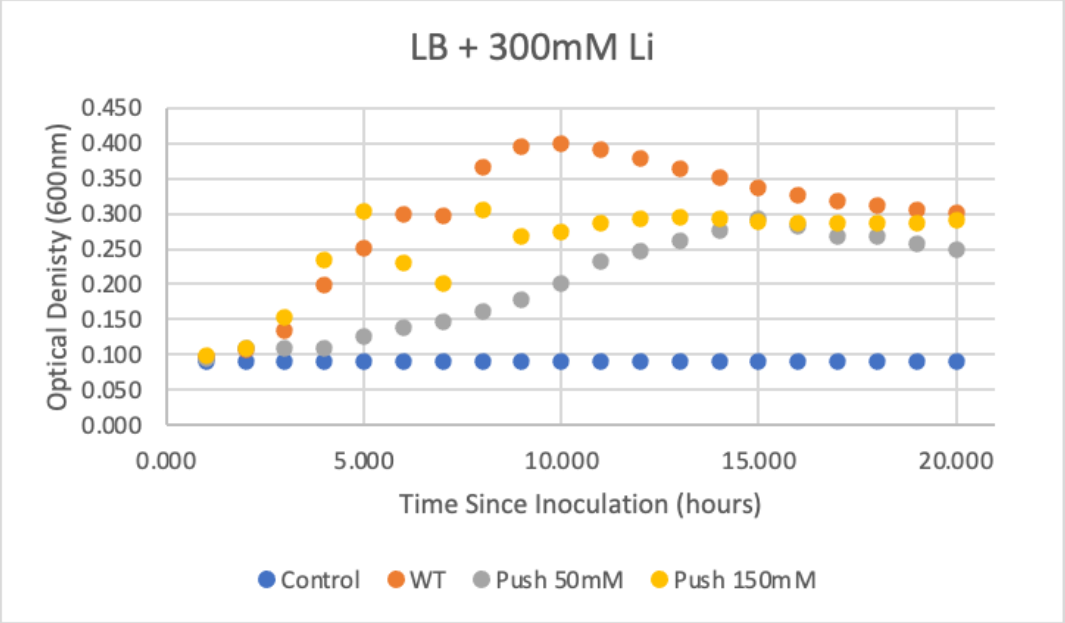
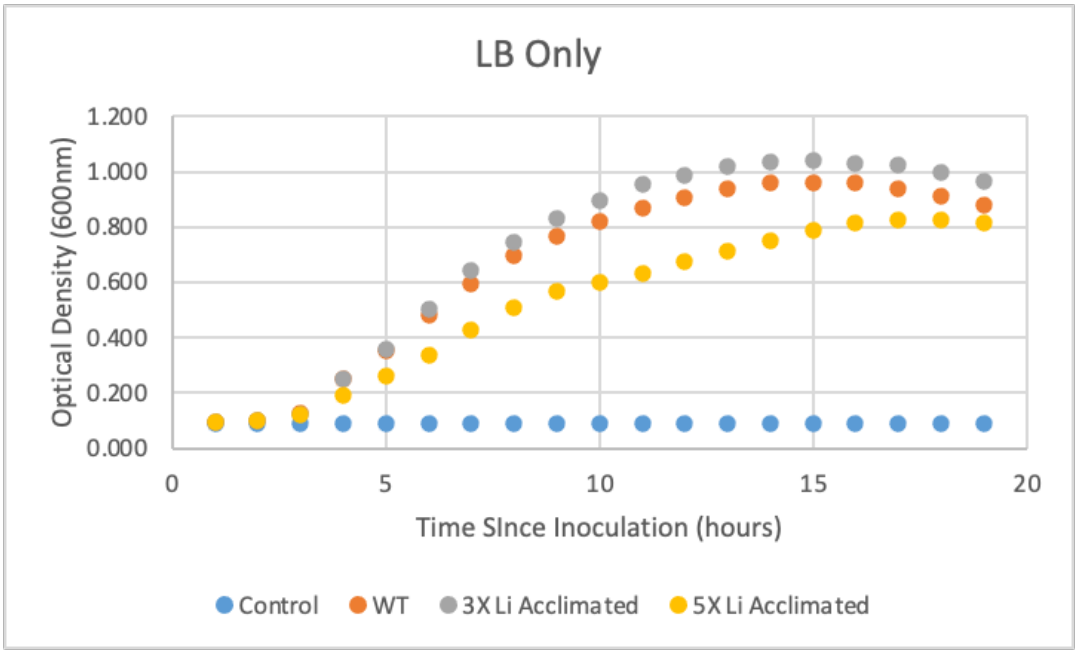


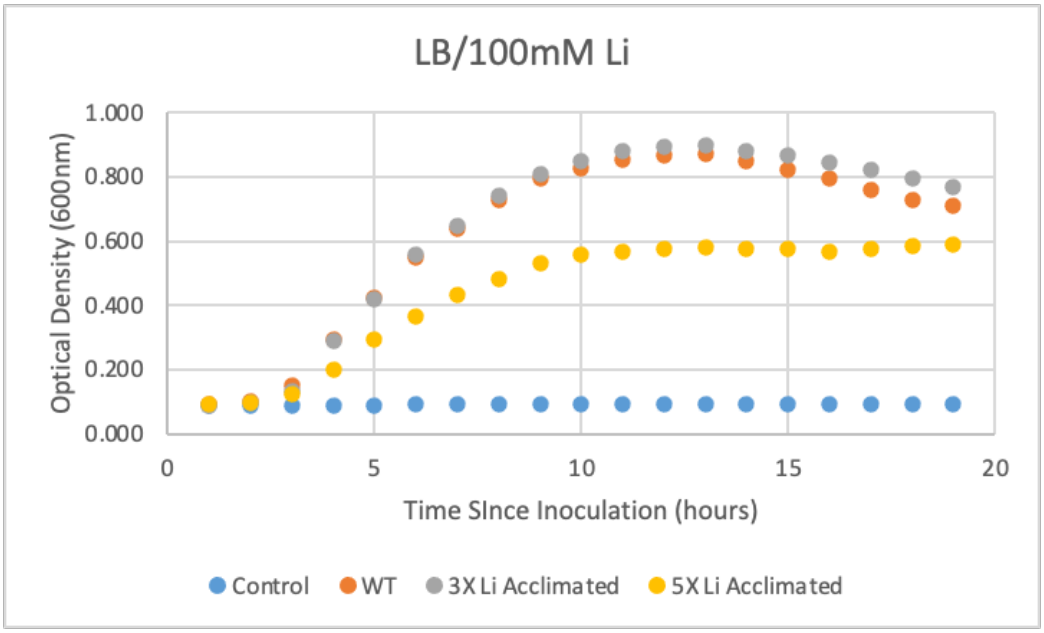
Figure 10. *S. Loihica* Growth Curves in LB and LB/300mM Li of WT, 3x 50mM acclimated, and 3x 150mM acclimated

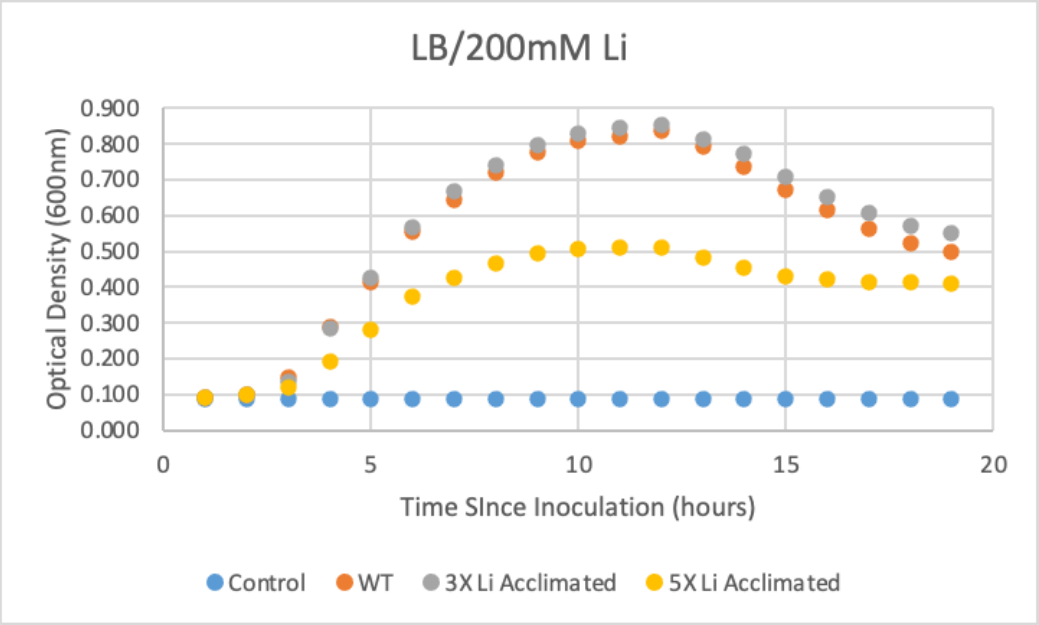
Since there was no statistical difference in the 3x 50mM acclimated cells and the WT cells, a 5x 50mM Li acclimate was pursued. A growth curve was collected with concentrations of 0mM Li, 100mM Li, 200mM Li, and 300mM Li added to the medium. The cell types run are WT, 3x 50mM Li acclimated, and 5x 50mM Li acclimated. After performing a t-test on the WT versus the 5x acclimate in different conditions and a t-test on the 3x acclimate versus the 5x acclimate, both at a 95% confidence interval, the 5x acclimate did statistically significantly worse than the other cells. **(Figure 11)** Thus, the 5x 50mM Li acclimate was not used for further studies.

With the previous growth curve, however, it was found that the WT and 3x 50mM Li acclimated were both performing comparably and statistically the same at a 95% confidence interval in growth up to the 300mM LiCl mark. Thus, the organism was acclimated into conditions of 0mM Li, 200mM Li, 400mM Li, 600mM Li, and 800mM Li. The growth curve was collected for 36 hours and still showed no statistically significant difference at a 95% confidence interval between the two cell types. **(Figure 12)** However, the WT strain was exhibiting lithium tolerance without any acclimation as it was surviving in concentrations up to about 400mM Li.

Despite the findings of the lithium tolerance in LB of WT alone, since no statistically significant difference was ever found between the WT and acclimated cells, the actual occurrence of acclimation in LB broth is unlikely. The reason for this could be that there are so many other secondary nutrients in a nutrient broth that the cells are healthier and thus not sensitive to even the higher concentrations of LiCl. Thus, we are planning on switching back to DM to try to acclimate the organism with Li without the additional secondary nutrients.


LB Only
Optical Density (600nm)
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
20
Time SInce Inoculation (hours)
Control
WT
3X Li Acclimated
5X Li Acclimated


LB/100mM Li
Optical Density (600nm)
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
20
Time SInce Inoculation (hours)
Control
WT
3X Li Acclimated
5X Li Acclimated



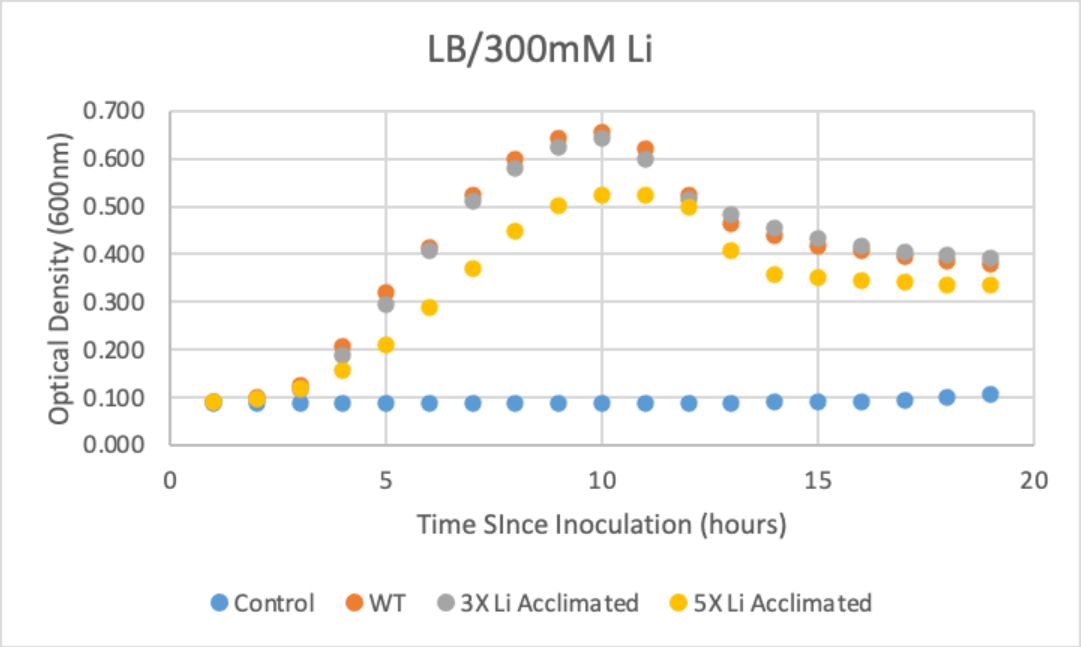
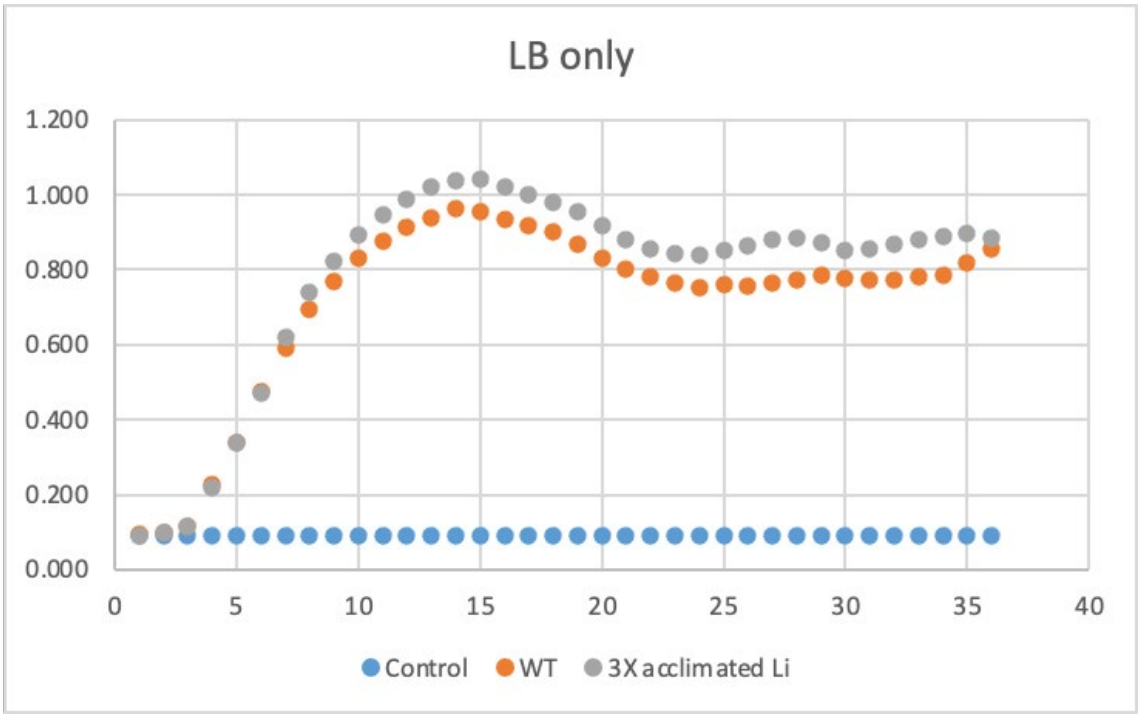
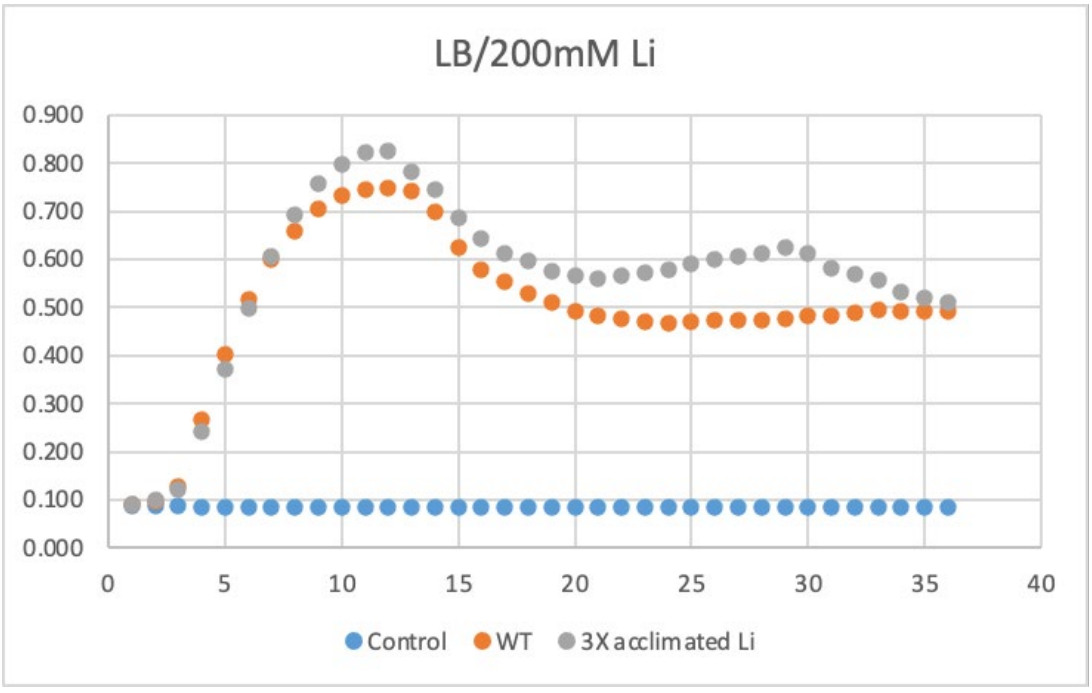
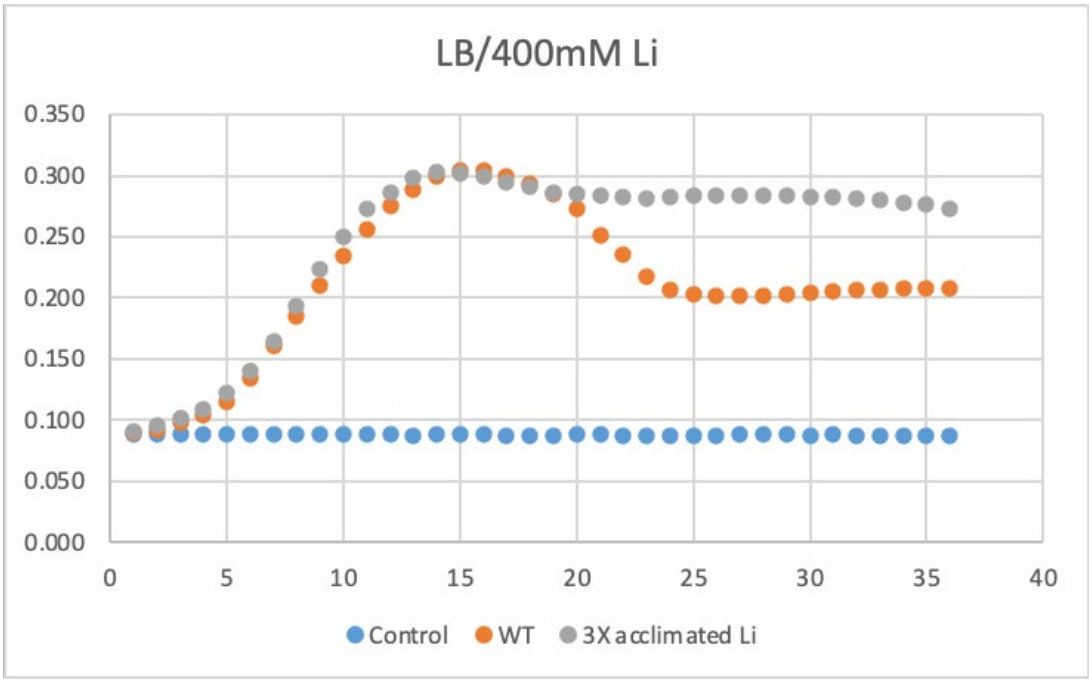
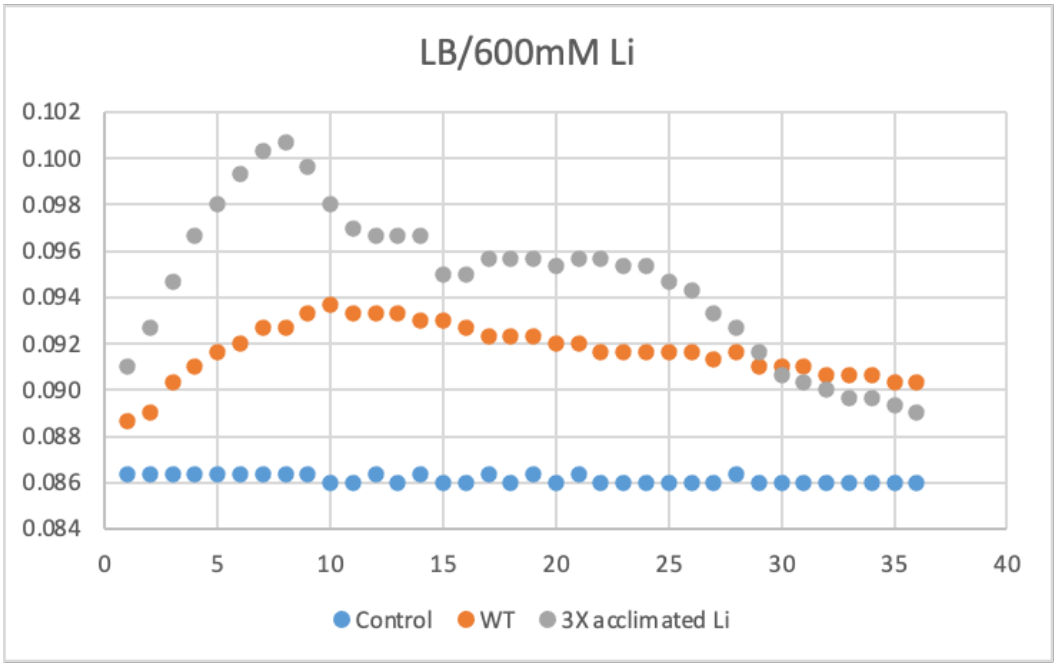


Figure 11. Growth of *S. loihica* in Increasing Lithium Concentrations up to 300mM Li WT, 3x 50mM Li Acclimated, and 5x 50mM Li Acclimated


LB only
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
5
10
15
20
25
30
35
40
Control
WT
3X acclimated Li


LB/200mM Li
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
5
10
15
20
25
30
35
40
Control
WT
3X acclimated Li


LB/400mM Li
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0
5
10
15
20
25
30
35
40
Control
WT
3X acclimated Li


LB/600mM Li
0.102
0.100
0.098
0.096
0.094
0.092
0.090
0.088
0.086
0.084
0
5
10
15
20
25
30
35
40
Control
WT
3X acclimated Li

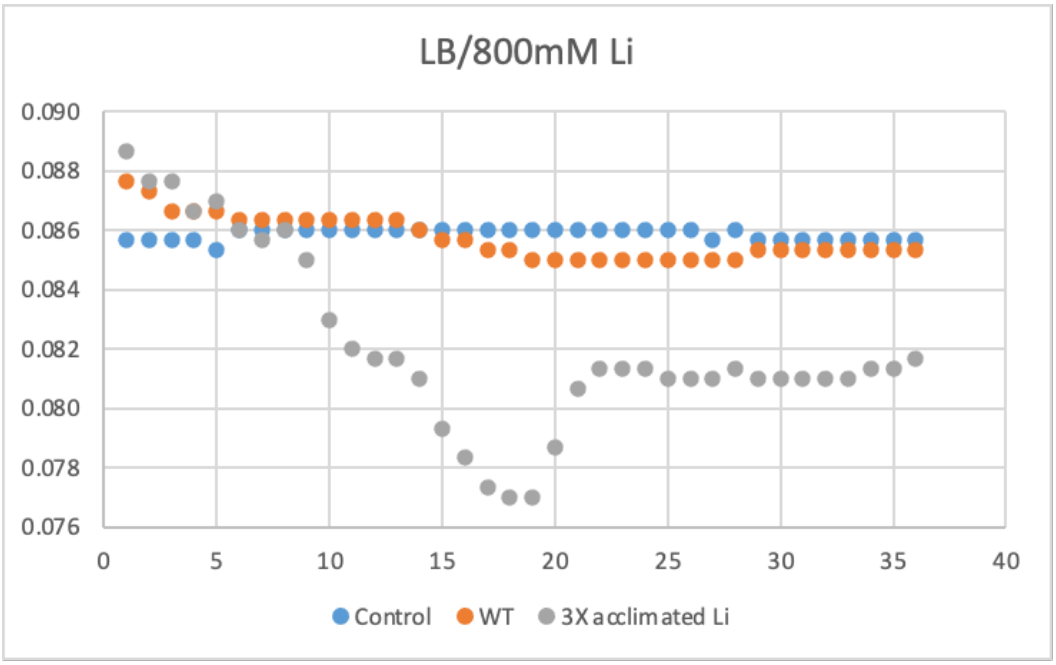


Figure 12. Growth of *S. loihica* in Increasing Lithium Concentrations up to 800mM Li WT and 3x 50mM Li Acclimated

Anaerobic Growth in Luria-Bertani Broth

In the first experiment with the frozen WT cells in 20mM fumarate and 50mM LiCl, it was found that the cells grew to a high cellular density in the fumarate but not with Li as the terminal electron acceptor. This suggests that *S. loihica* is unable to use lithium as a terminal electron acceptor and fumarate could be added to these growth experiments with Li to generate viable cells anaerobically. **(Figure 13)** With this information, we evaluated if lithium had an impact on growth when combined with the fumarate.

Shifting to look at the effects of combining lithium and fumarate anaerobically, it was first noticed that lithium might be aiding growth. In an experiment testing the combination of 50mM lithium with 20mM fumarate, it grew to an OD_{600nm} of 0.743 at 24 hours. **(Figure 14)** On the other hand, the previous experiment with only 20mM fumarate demonstrated growth to only OD_{600nm} 0.514 at 24 hours.

To continue to test the idea of lithium aiding growth, the organism was anaerobically grown in LB, 20 mM fumarate, and differing concentrations of lithium (50 mM Li, 100 mM Li, and 150 mM Li). Grown for 48 hours, the results show that Li is still not used as a terminal electron acceptor or aiding growth as the cultures with increasing lithium grew no better than the culture containing only 20mM fumarate as an electron acceptor. Additionally, there seems to be a greater sensitivity to lithium anaerobically as there was slower growth seen at as low of a concentration of 150mM Li **(Figure 15)**. Compared to aerobically where the growth slowed around 400mM Li. **(Figure 12)** Overall, when grown anaerobically, *S. loihica* cannot use lithium as its terminal electron acceptor and thrives when grown with fumarate as the terminal electron acceptor. Finally, the sensitivity to Li is slightly higher than when grown aerobically.

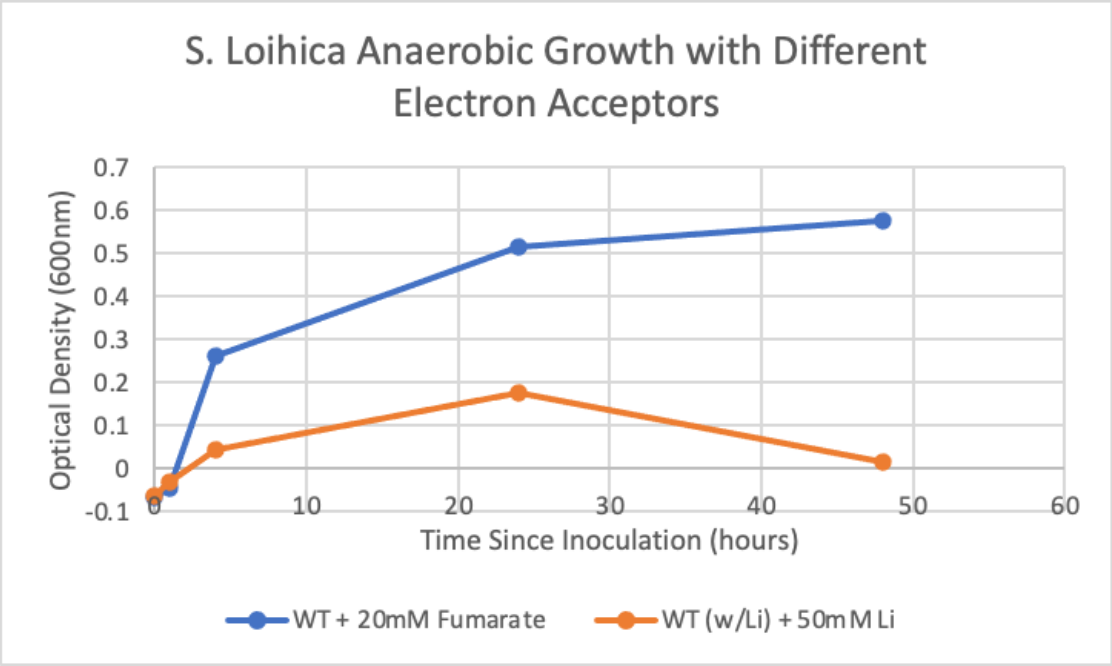


Figure 13. Growth Curve for Anaerobically Grown S. loihica Given Different Electron Acceptors

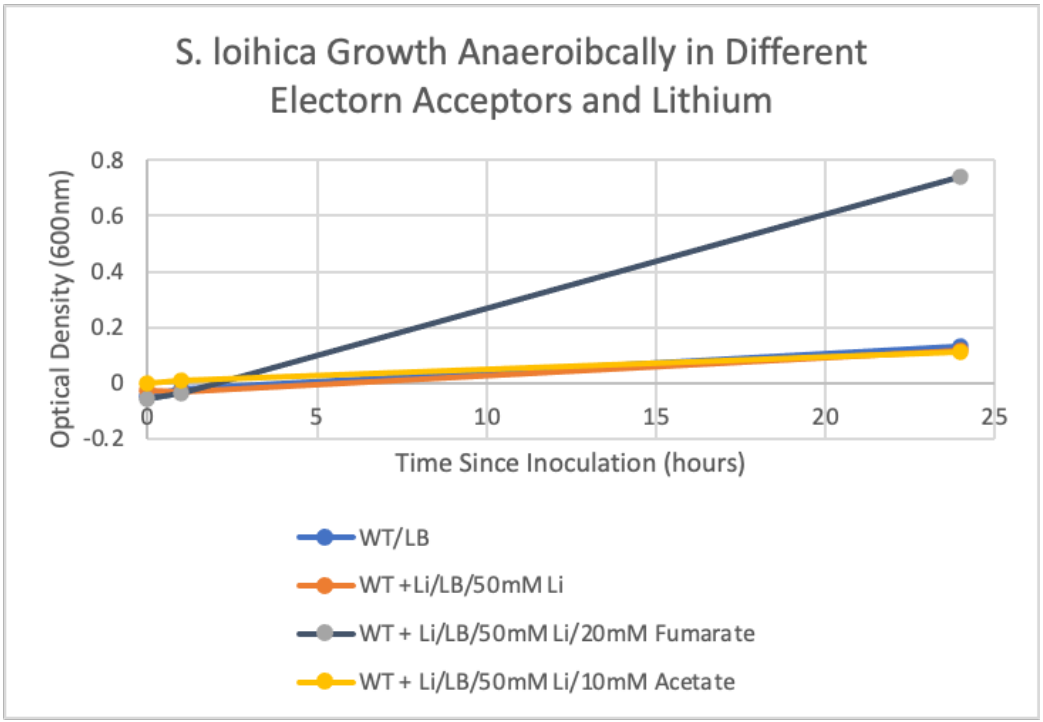


Figure 14. Growth Curve for Anaerobically Grown S. loihica Given Different Electron Acceptors Combined with Lithium

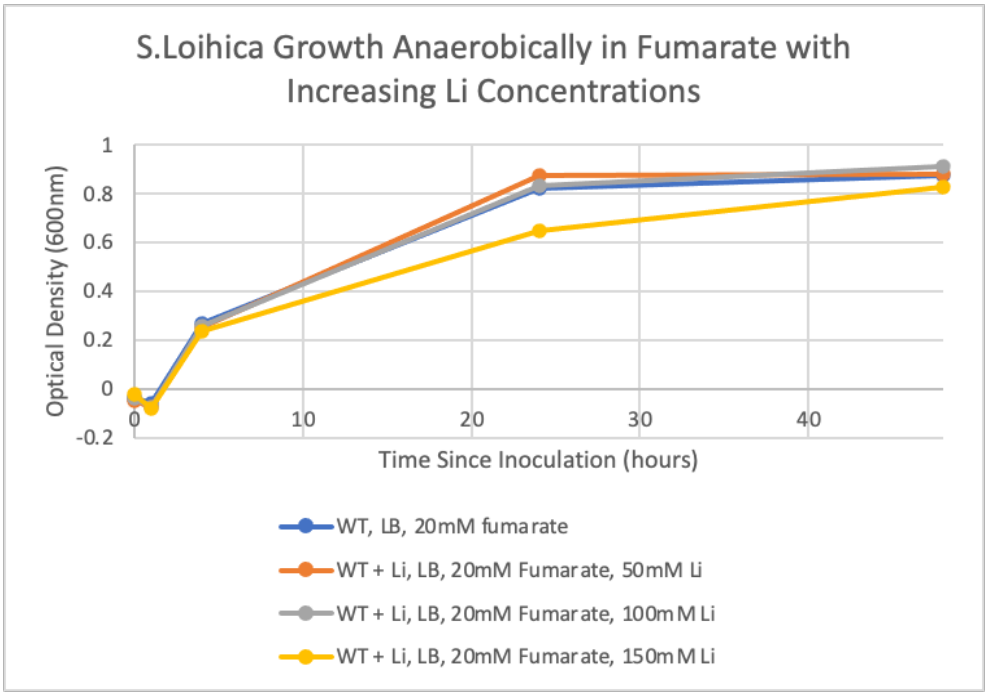


Figure 15. Growth Curve for Anaerobically Grown S. loihica in Fumarate with Increasing Lithium Concentrations

Growth in Defined Medium

The first growth experiment in the defined media demonstrated that the medium in literature containing $NaHCO_3$, while slow, allowed the growth of *S. loihica* **[11]**. Thus, when switching over from LB to a less nutrient-rich medium, we modified the composition of the DM to include carbonate ions. The preliminary growth of frozen WT inoculated directly into the modified DM was measured over 48 hours and confirmed that *S. loihica* is not able to grow in DM with the additional carbonate ions directly from the -80C stock. **(Figure 16.)**

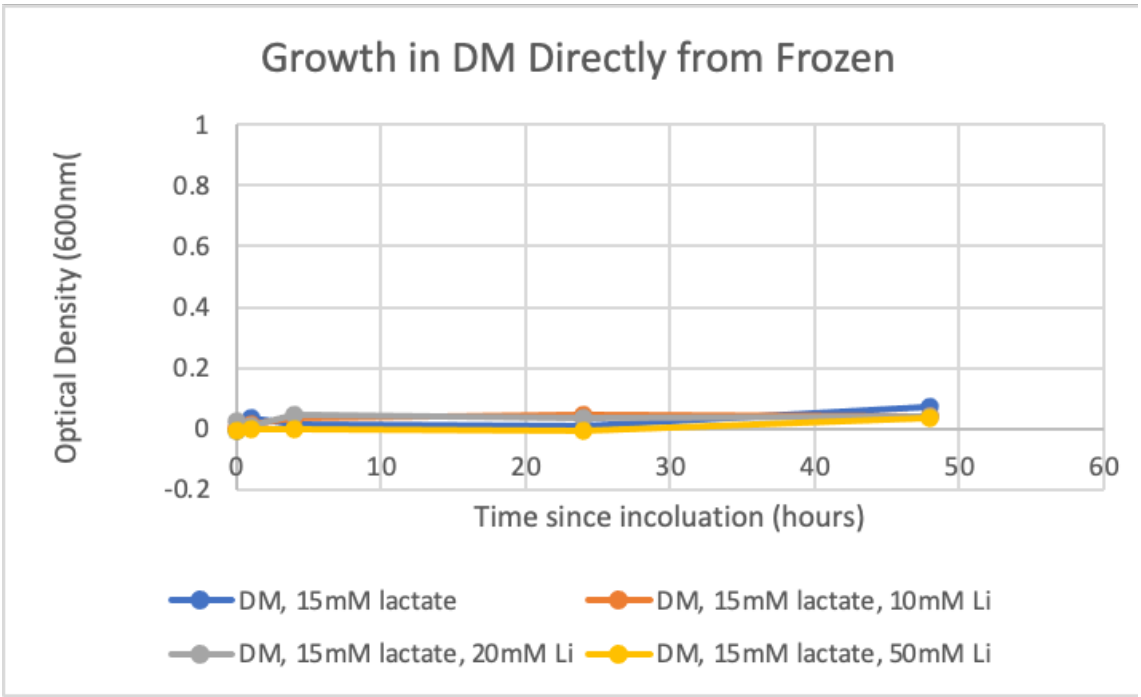
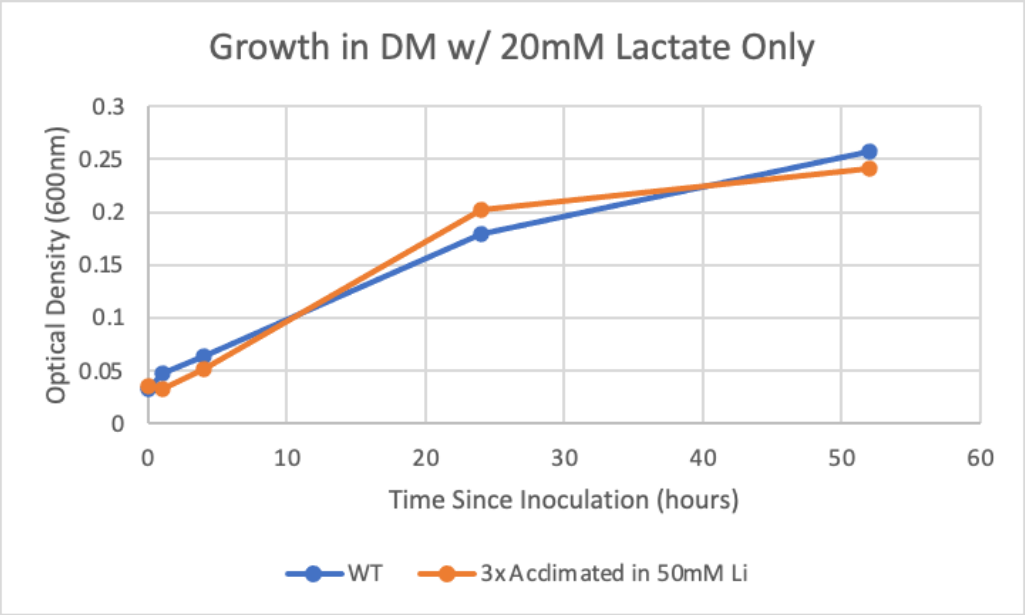
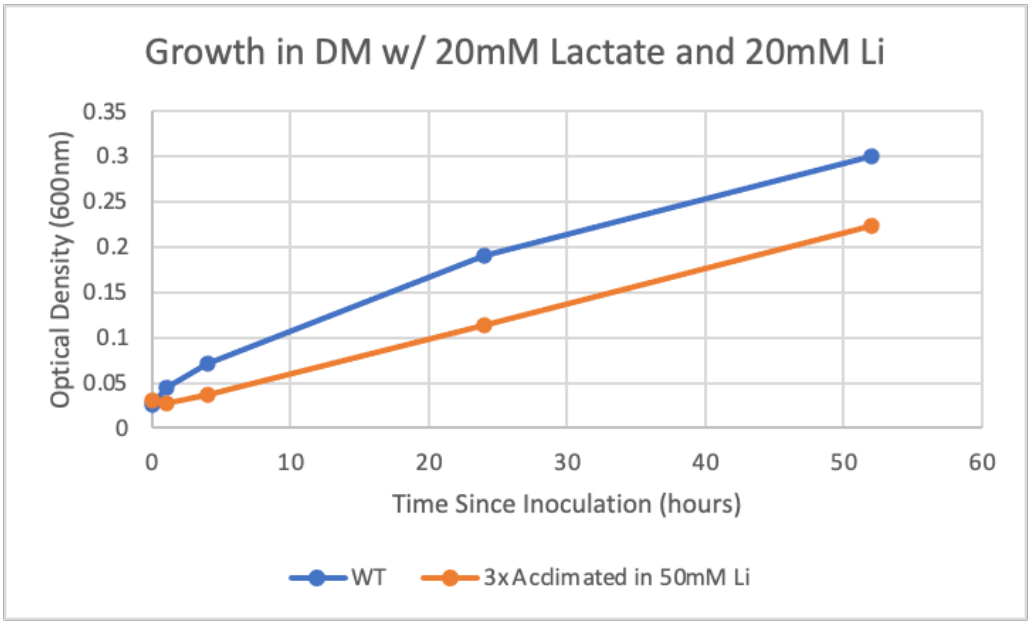


Figure 16. DM Growth Started from Frozen Directly into DM conditions

When we attempted to grow S. loihica in DM using cells initially grown in LB for 24 hours we confirmed that growth in the DM was possible. The WT cells and 3x acclimate cells were placed in increasing Li concentrations of 0mM, 20mM, 50mM, and 100mM to examine the toxicity differences in the DM. Looking at the growth curves over about 50 hours of the WT and the 3x 50mM acclimate in differing Li conditions in DM, there is no difference in growth between the WT and acclimate. Additionally, at the peak optical density, there is no statistically significant difference at a 95% confidence level. **(Figure 17.)**


Growth in DM w/ 20mM Lactate Only
Optical Density (600nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
Time Since Inoculation (hours)
WT
3xAcdimated in 50mM Li


Growth in DM w/ 20mM Lactate and 20mM Li
Optical Density (600nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
Time Since Inoculation (hours)
WT
3xAcdimated in 50mM Li

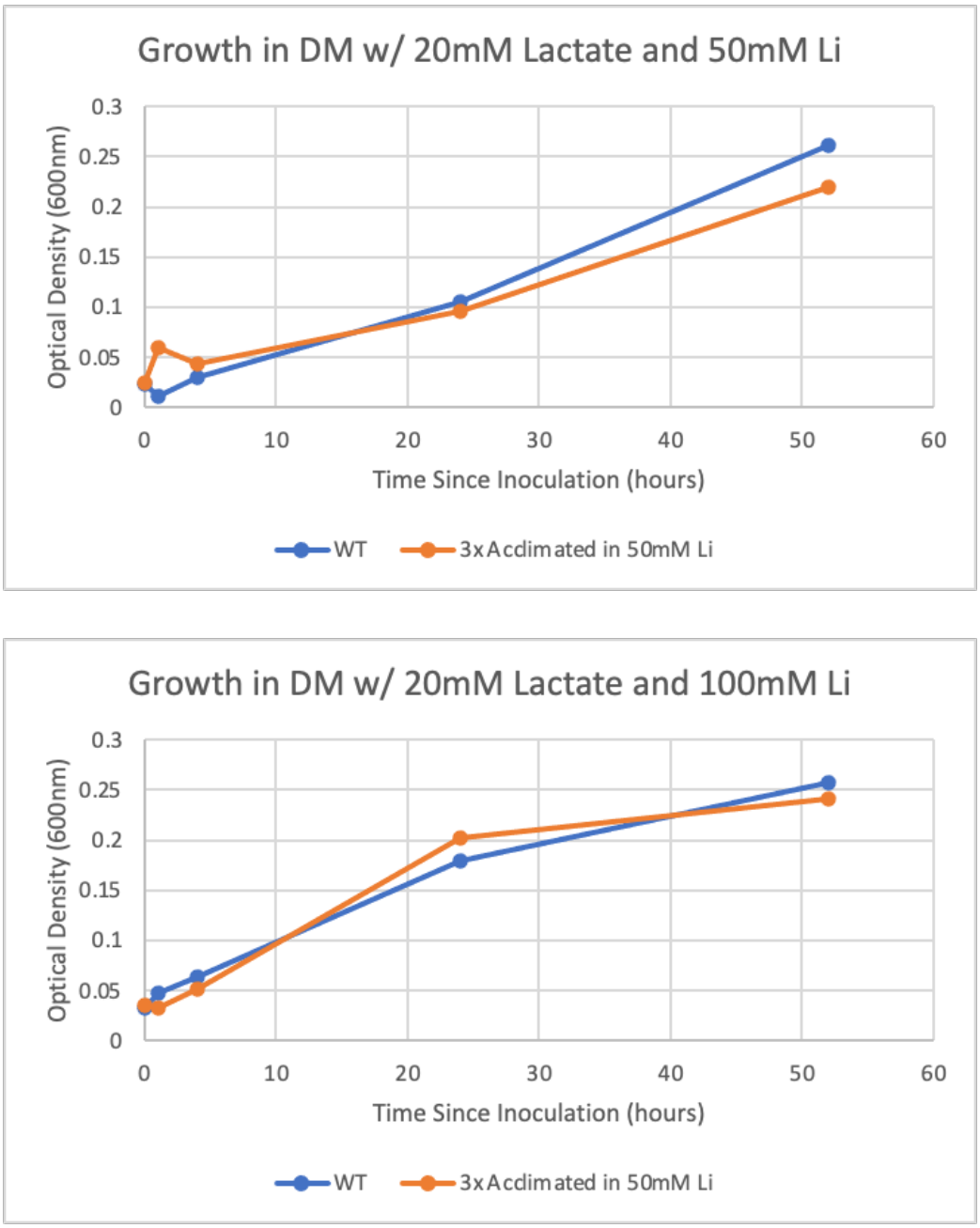


Figure 17. Growth in DM and Li Concentrations Starting from 1% Inoculation from LB Cultures

Overall, compared to the growth in LB broth, the growth in DM is much slower and the cultures maintained a lower cell density. However, as we expected, the Li

tolerance of the cells was decreased in the DM media compared to the more nutrient-rich media.

Conclusion

Overall, my results demonstrate that *Shewanella loihica* wild type has lithium tolerance capabilities. When attempting to evolve the organism to lithium selectively, the organism shows no differentiation from the wild type. Regardless of medium, LB, or DM, there is no differentiation in the growth of the acclimated cells and wild type. However, overall growth in DM is slower and less prolific than in LB. When comparing aerobic and anaerobic growth, there is also no difference between the acclimated and wild type. However, while growth speed is similar, growth seems to be less prolific anaerobically and requires fumarate as a terminal electron acceptor, Li could not be used.

Further research needs to be performed before using *Shewanella loihica* for lithium-ion recycling. The possible selective evolution in DM could show different results than the selective evolution in LB. Additionally, ion chromatography to understand where the Li is used or goes in the organism is important. It is vital to understand the mechanism of usage of Li for the organism. Additionally, research into the genetic part of lithium tolerance. Finding the specific genes that could be involved in this tolerance would lead to the possibility of amplification of these genes. With the amplification of genes, the reclamation capabilities of the biological system could be increased for increasing LIB reuse. Overall, the use of a biological system to sequester metal ions for reuse and environmental protection is present.

Resources

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