Characterization of the Role of Aquaporin-7 in Nematode Acid Survival

A Major Qualifying Project Report

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ABSTRACT

There are nearly 23,000 described species of nematodes and most thrive within neutral environments. However, some nematodes are able to live under more "extreme" conditions. *Turbatrix aceti* is able to thrive within an especially acidic environment. Prior research from the Alkema lab suggests that *T. aceti* survives in part by metabolizing acetic acid taken in by Aquapoirn-7. To analyze the role of AQP-7, I cloned and characterized the gene that encodes an ortholog of AQP-7 found in *Caenorhabditis elegans*. These studies show that *C. elegans aqp-7* is expressed in several tissues and suggest that *C. elegans* AQP-7 is able to transport acetic acid across the membrane. Due to the similarity, it is likely that *T. aceti* AQP-7 performs this way as well.

ACKNOWLEDGEMENTS

Thank you to the members of the Alkema Lab at the University of Massachusetts Medical School for all of your support throughout this project. Through the help of Jeff Grant, PhD, Andrea Thackery, and Jeremy Florman I was able to accomplish all that I have. Ultimately, I would like to thank my advisors Mark Alkema and Professor Carissa Olsen for their guidance and knowledge during the course of this year and aid in constructing this report.

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1. BACKGROUND

Nematodes are one of the most abundant classes of animals. There are over 23,000 species that have been identified and described[1]. They thrive in a variety of environments and range in size from a few millimeters to 1 meter in length. This diversity suggests great potential for research using these animals, such as investigation into survival in extreme environments.

1.1 *Turbatrix Aceti* thrives in acetic acid

Turbatrix aceti (Figure 1) is a nematode with a unique ability to thrive in very acidic environments. Exactly how this organism has adapted to survive in such a low pH is still unknown[3]. However, preliminary data from the Alkema lab has shown that these organisms may utilize the glyoxylate cycle in order to metabolize acetic acid. As such, they are not only able to thrive in this rather hostile environment but are also able to metabolize the acid around them into energy. Preliminary results from the Alkema lab suggest that Aquaporin-7 (AQP-7) may be involved in the transport of acetic acid across the cellular membrane. Aquaporins are known for transporting water and sometimes other small-uncharged molecules. It is possible that AQP-7 from *T. aceti* can transport the small, uncharged molecule of acetic acid.

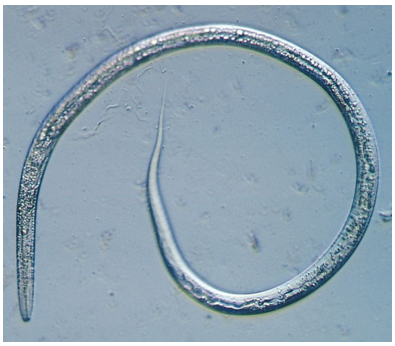

Figure 1. *Turbatrix aceti*[2]

1.2 The glyoxylate cycle aids the yeast Saccharomyces cerevisiae in thriving in acidic environments

Simpler organisms, such as certain types of yeast and bacteria, are also able to thrive in an acidic environment. Studies have shown that the yeast, *Saccharomyces cerevisiae*, utilizes the glyoxylate cycle, a modified TCA, cycle during metabolism. This cycle shares some reactions with the TCA cycle. But ultimately, acetate bypasses oxidative decarboxylation and is converted into carbon dicarboxylic acids[4]. From there, is can be used to create other molecules used during the process of metabolism. If *T. aceti* is able to survive in a similarly acidic environment to *S. cerevisiae,* then it is possible that it also employs the glyoxylate cycle.

Preliminary data from the Alkema laboratory has shown that *T. aceti* may utilize the glyoxylate cycle as well as the transmembrane protein Aquaporin-7. Figure 2 is a model of this projected metabolic pathway. Acetic acid is transported into the cell through AQP-7 and subsequently deprotonated. From there, it is used to create the metabolite Acetyl-CoA which can be used both inside and outside of the mitochondria.

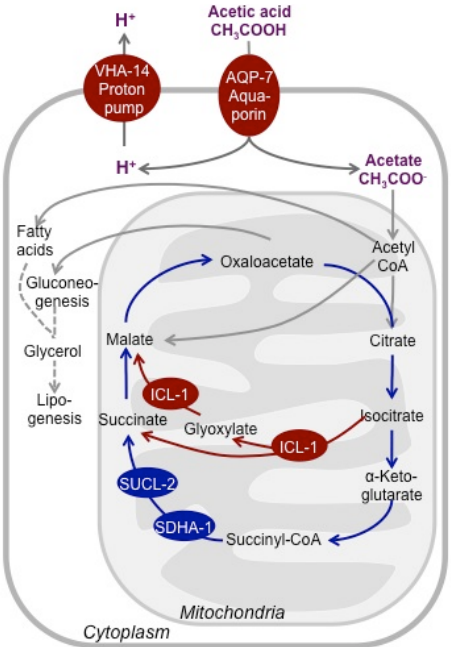

Figure 2. Model of AQP-7 and the glyoxylate cycle[5]

1.3 *Turbatrix aceti* Aquaporin-7 as an acetic acid transporter

Preliminary data has shown that *T. aceti* up-regulates a protein called Aquaporin-7 when grown in apple cider vinegar (pH 2.5) as opposed to a neutral pH. This suggests that AQP-7 could play a significant role in the survival of this species. As indicated by their name, this class of proteins typically transports water molecules. In addition, other small molecules are sometimes able to pass, such as glycerol[6]. Previous studies have also shown that aquaporins, such as aquaporin-7, can transport small, protonated organic acids such as acetic acid and lactic acid[7]. It is possible that the protonated form of acetic acid is small enough to be transported as well. Once inside, it could participate in the glyoxylate cycle, similar to that of *S. cerevisiae* as described above.

1.4 Phylogenetic analysis reveals homolog in *Caenorhabditis elegans*, a model organism

A knockout variant of *T. aceti* or RNA*i* sequence against Aquaporin-7 has not yet been developed. In order to investigate this protein further, a phylogenetic analysis was performed to determine homologs in other organisms. *T. aceti* Aquaporin-7 was compared with *Caenorhabditis elegans* aquaporins 1-12, as well as to human aquaporins, Drosophila proteins, and the yeast *Saccharomyces cerevisiae*. The results of this analysis show that overall aquaporins are generally genetically conserved. Moreover, *T. aceti* Aquaporin-7 and *C. elegans* Aquaporin-7 are found to be very similar genetically. Because of this, it can be hypothesized that the two perform similar functions. Because *C. elegans* is within the same phylum as *T. aceti*, is easy to maintain, and has been studied extensively, experimentation with this organism was performed. Lastly, it is important to observe that aquaporins found in *Homo sapiens* are relatively close to aquaporin 7 of *T. aceti*. This could yield a potential application in humans. For example, *H. sapiens* AQP-9 is relatively close to AQP-7 of *T. aceti* and *C. elegans*. Prior research has shown that human AQP-9 is permeable to lactic acid[7]. This could suggest a similar function to that of AQP-7 as described in Section 1.3 above. It is possible that there are proteins found in humans that have similar abilities to that of *T. aceti* AQP-7.

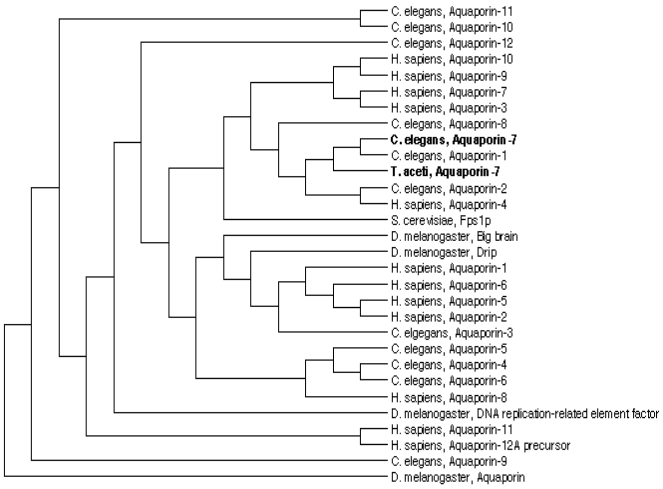

Figure 3. Phylogenetic analysis of various aquaporins

1.5 Caenorhabditis elegans Aquaporin-7

Caenorhabditis elegans is a "model organism" due to its easy genetic manipulability and relatively fast life cycle[1]. The genome of this animal has been sequenced and studied in detail by past researchers. Techniques such as RNAi knockdown and transgenic expression have been optimized for *C. elegans*, making it extremely useful for genetic studies. With all of this knowledge, there is much room for innovation. Through the phylogenetic analysis described in

the Section 1.4 above, *C. elegans* Aquaporin-7 has been found to be genetically similar to *T. aceti* Aquaporin-7. Overall, it is a protein with a molecular weight of 31.9 kDa and has an isoelectric point of 8.43[8]. From the amino acid sequence, there are six transmembrane portions (green), which is consistent with other aquaporins (Figure 4). In addition, the sequence is consistent with the superfamily domain "Aquaporin-like" (blue). Overall, Aquaporin-7 has been known to transport both water and glycerol. A study placed AQP-7 within *Xenopus* oocytes and observed an increase in water and glycerol permability[9]. A similar trend might be seen with acetic acid if AQP-7 also transports this molecule.

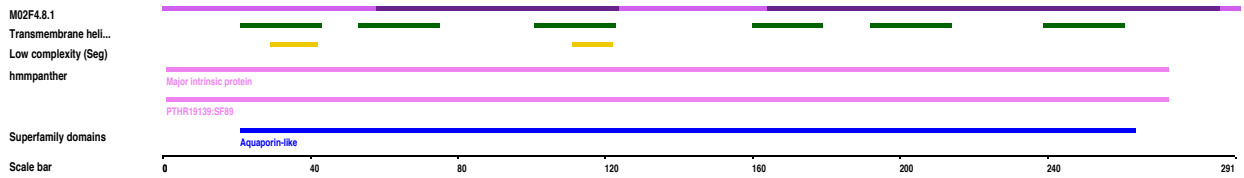

Figure 4. Predicted structure of *C. elegans* AQP-7; transmembrane segments in green

2. METHODOLOGY

Investigation of Aquaporin-7 first began with the creation of both a transcriptional and translational reporter tagged with GFP for analysis under a florescent microscope. Techniques for amplification, incorporation into a bacterial vector, and culturing of *E. coli* injected cells were all performed to create a transcriptional reporter and translational reporter. The resulting DNA was injected into embryos of *C. elegans* in order to observe where this protein is expressed in the organism. The translational reporter resulted in the creation of two successful lines. One of the lines appeared to express GFP more than the other, but the two are fundamentally the same. These two lines were subjected to three assays and tested against wild type N2 animals.

2.1. Cloning and analysis of *C. elegans* aqp-7 expression patterns

To investigate Aquaporin 7, the gene sequence was first identified along with the cut sites for enzymes and primers (Figure 5 below). Preliminary experimentation involved cloning of just the promoter (about 4,000 bp), creating a transcriptional reporter (aqp-7 promoter::GFP). This reporter highlights the tissues that the protein is expressed in. Second, a translational reporter (aqp-7 promoter + gene::GFP) was amplified including the 4kb promoter and the gene itself (about 5,000 base pairs). This reporter is the properly folded protein and highlights where it localizes in the cell. Overall, this process was performed to create a line of animals to be used for experimentation.

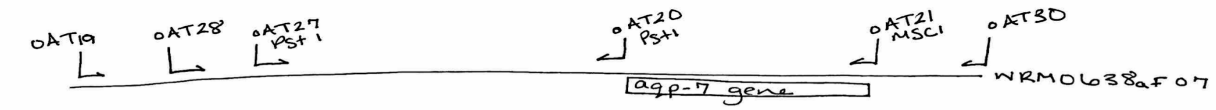

Figure 5. Aquaporin-7 DNA template and cut sites

For both reporters, polymerase chain reaction (PCR), purification, digestion, ligation, and transformation were performed. The general protocols for these processes are described in Appendices A-E. The transcriptional reporter PCR was performed utilizing the primers oAT27 and oAT20 to gain just the 4kb promoter. The translational reporter was created utilizing oAT27 and the oAT21 to obtain both the promoter and the gene. Each construct was purified and then run through gel electrophoresis to ensure a correctly sized product. The product was then digested using either PstI and BamHI (transcriptional reporter) or PstI and MscI (translational reporter). The vector pPD95.75 was digested with the respective enzymes as well. The vector was ligated with the digested translational and transcriptional reporters separately. The translational reporter plasmid can be seen in Figure 6. The transcriptional reporter did not have the *aqp-7* gene piece in red but instead had just the promoter (gray) ligated to GFP.

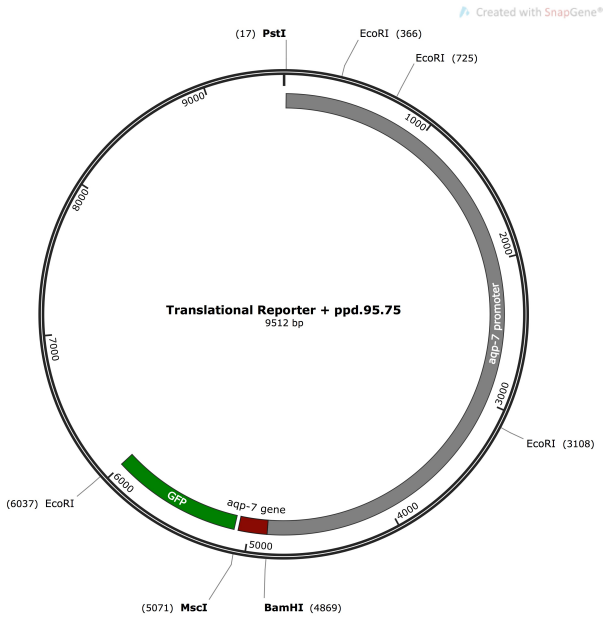

Figure 6. Model of translational reporter ligated to GFP within the plasmid ppd.95.75 along with enzymatic cut sites

Next, these plasmids were transformed into DH5α cells. Following transformation, the E. coli colonies were cultured at 37ºC overnight in hopes of further amplifying each insert. The next day, the colonies were mini-prepped and the resulting DNA was test-digested to determine which inserts were correct. The protocol for this step can be seen in Appendix F.

In order to ensure a correct construct, a test digest was performed utilizing the EcoRI enzyme. As seen in Figure 6 above, a correct construct would display 4 bands following digestion. Once a correct construct was identified for both the transcriptional and translational clones, the construct was injected into gravid adults with the *lin-15* mutation (multi-vulva). The construct was co-injected with the *lin-15* rescue construct. Therefore, worms containing the reporter would not display this mutation. These worms were used instead of wild type so that the correct transgenic worms could be identified under a regular microscope, making for easier maintenance and experimentation. The mutation is sometimes visible at the L4 stage but usually does not appear until the worm is a young adult.

For the transcriptional reporter, twenty-five singled *lin-15* worms were injected. A few days later, these plates were analyzed for transgenic offspring. In total, twelve of the original animals created a transgenic line. From this, the three exhibiting the most expressive lines were chosen and named QW1880, QW1881, and QW1882. A similar procedure was followed for the translation reporter. Sixteen *lin-15* animals were injected and 6 translational lines were created as a result. The two best expressing lines were chosen and named QW1889 and QW1890. Both the transcriptional and translational lines were viewed under a confocal imaging microscope. The results of this procedure are outlined in Section 3.1. The translational reporter lines were used for further experimentation

2.2 Starvation assay to identify whether *C. elegans* can metabolize acetic acid

A starvation assay was carried out in order to observe if *C.* elegans has the potential to metabolize acetic acid as *T. aceti* does. Both wild type N2 worms and the translational reporter worms were utilized for this experiment. The eggs of gravid worms were placed into varying M9 solutions (pH 5, pH5 + acetate, pH6, pH6 + acetate, pH7, pH7 + acetate). At a lower pH, the non-ionic, protonated form of acetic acid is more present. Presumably, there would be increased movement of acetic acid through AQP-7 at this pH. If *C. elegans* were able to metabolize acetic acid, the worms in solution containing acetate would live longer than those in solution lacking acetate. This would be especially true within a lower pH. In addition, the worms with the translational reporter and suspected overexpression would survive longer than the N2 worms.

First, animals of each type were grown on large NGM plates for several days in order to gain a sufficient supply of adult animals. Second, the animals were washed and bleached according to standard protocol. Following the final M9 wash after bleaching, the egg pellet was re-suspended in M9 to determine the number of eggs in the test tube. Finally, the eggs were placed in test tubes with 2mL of varying M9 solution. At regular intervals, 50μL of each solution were taken and deposited onto regular NGM plates. These plates were observed two to three days later and the number of surviving worms was counted. This experiment was conducted in a 25°C incubator with the test tubes placed in a shaker for a total of 15 days. A more detailed procedure is outlined in Appendix G. The results are outlined in Section 3.2.

2.3 Acidic survival assay to determine the effect of AQP-7 overexpression on worm acid response

An assay was performed to test the response of AQP-7::GFP fusion expressing translational reporter worms to acidic environments. If AQP-7 can transport acetic acid, the translational reporters would be more sensitive to acidity due to overexpression of AQP-7. Thirty worms were placed in wells with varying amounts of apple cider vinegar (0%, 10%, 15%, 20%, and 25%). The number of animals alive was recorded at 30-minute intervals over the course of three hours. "Alive" was defined as any animal exhibiting movement, even if slowed. This process was repeated and an average of the two was taken. A more detailed procedure is outlined in Appendix H. The results are outlined in Section 3.3.

2.4 Expression assay to determine whether AQP-7 is regulated by environmental acidity

An induced expression experiment was carried out in order to understand whether decreases in pH could increase the expression of AQP-7. First, worms were grown on NGM plates of varying acidities, with and without acetate. The media was created by using different KPO_4 solutions and otherwise pouring the plates according to protocol. The resulting plates were:

(1) pH5 + Acetate
(2) pH6
(3) pH6 + Acetate
(4) pH7
(5) pH7 + Acetate

Three L4 worms of each strain (N2, QW1889, QW1890) were placed on each of the different plates. After about 5 days of recovery and growth, six young adult animals were selected and placed on a microscope slide for analysis. Computational software, FIJI (ImageJ), was used to quantify florescence. A more detailed procedure is outlined in Appendix I. The results are outlined in Section 3.4.

3. RESULTS

3.1. Cloning and analysis of *C. elegans* aqp-7 expression patterns

Confocal imaging of the transcriptional reporter shows expression in the hypodermis and some nerve cells of the worm. Figures 7 and 8 below are images of the same transcriptional reporter worm.

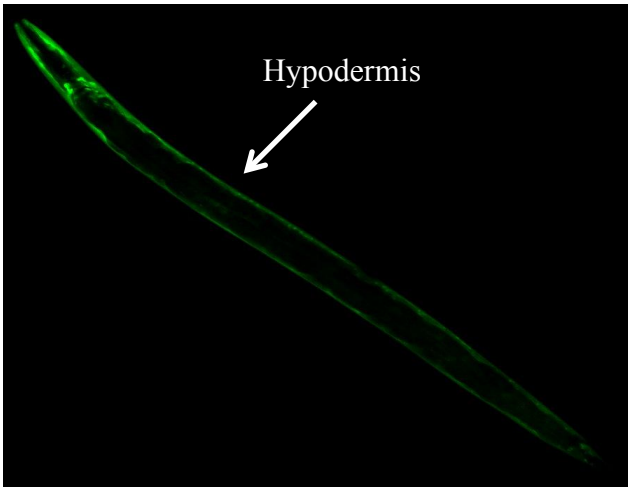

Figure 7. Transcriptional reporter, highlighting expression in the hypodermis, 10x

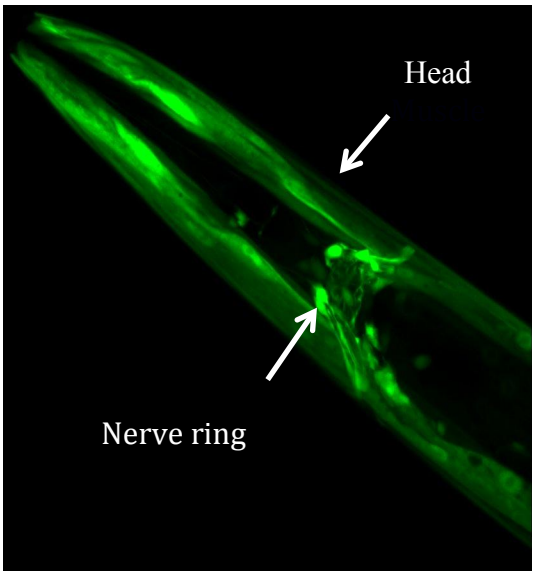

Figure 8. Transcriptional reporter, highlighting expression in the head and nerve ring, 40x

Confocal imaging of the translational reporter shows expression in nervous and muscular tissue. Figure 9 on the following page is the body of a young adult worm. The head of the worm was left overexposed in order to highlight the illuminated muscular and nervous tissues in the body. Figures 10 and 11 display the mid-body and tail of the worm. There, more body wall muscle as well as neuronal connections can be seen.

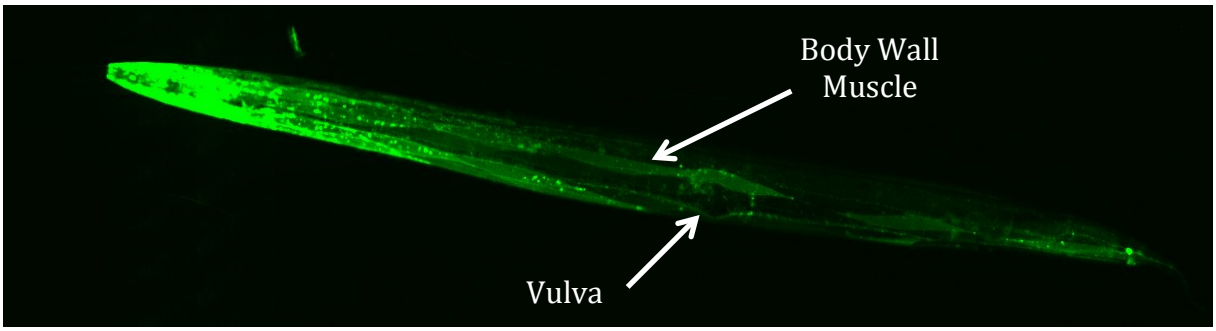

Figure 9. Translational reporter, highlighting the body wall muscle, 20x

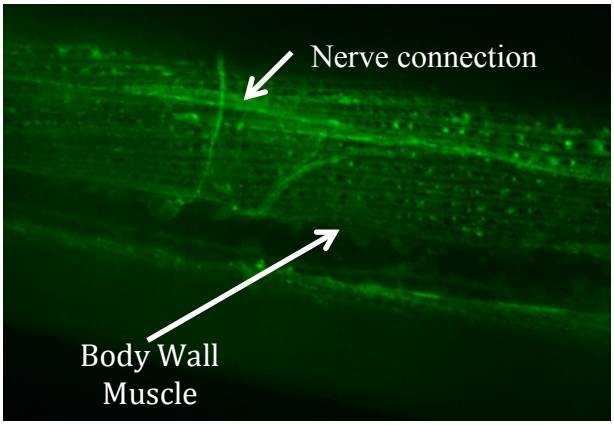

Figure 10. Mid-body image of translational reporter, highlighting body wall muscles and a neuronal connection, 63x

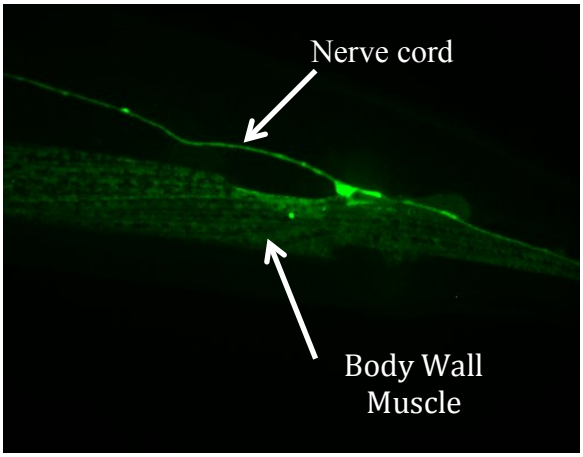

Figure 11. Tail image of translational reporter, highlighting body wall muscle and nerve cord, 63x

3.2 Starvation assay to identify whether *C. elegans* can metabolize acetic acid

During this experiment, six different M9 solutions were used and the time taken for the L1 worms to lose viability was measured. Aliquots were taken at regular intervals and placed on regular NGM plates. Two days later, the number of eggs that recovered into growing worms was counted. Overall, no apparent difference was found between the eggs that had been subject to solutions with and without acetate. However, a slight difference was observed between the translational reporter worms and the wild type worms. The translational worms died more quickly than the wild type, especially in lower pH solutions. The graphical results of this experiment can be seen on the next page. Overall, this assay suggests that *C. elegans* is not able to metabolize acetic acid.

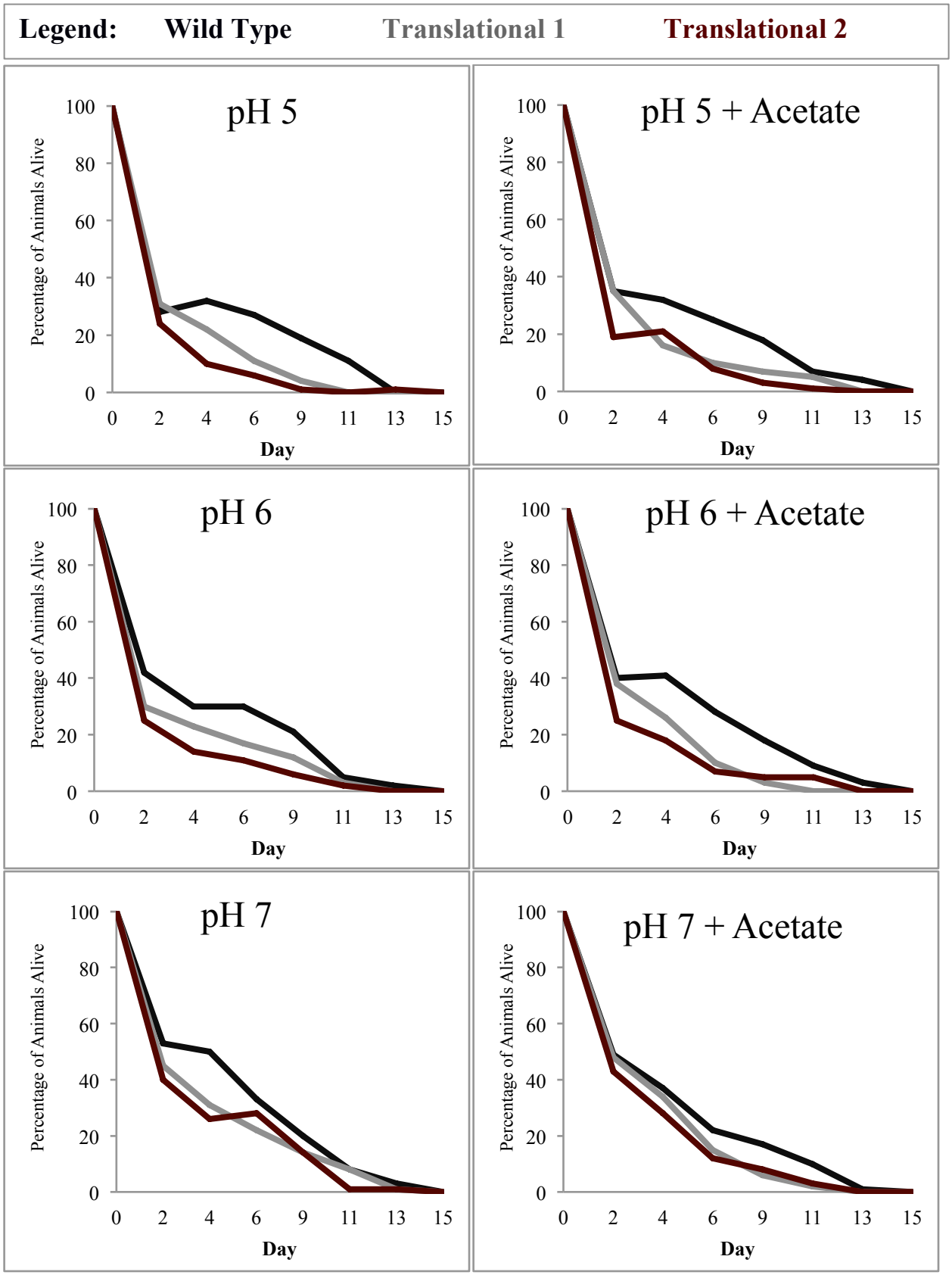

Figure 12. Survival assay results, displaying survival of each strain over time

3.3 Acidic survival assay to determine the effect of AQP-7 overexpression on worm acid response

In this experiment, thirty worms of each of the three strains were subjected to wells with varying amounts of apple cider vinegar (ACV). As depicted in the graph below, nearly all thirty of the three types of worms remained alive in the 0% ACV solution. Inversely, all three strains died off quickly in the 20% and 25% ACV. The most notable difference between the strains is at 15% ACV, the translational worms appear to die much faster than the N2 worms.

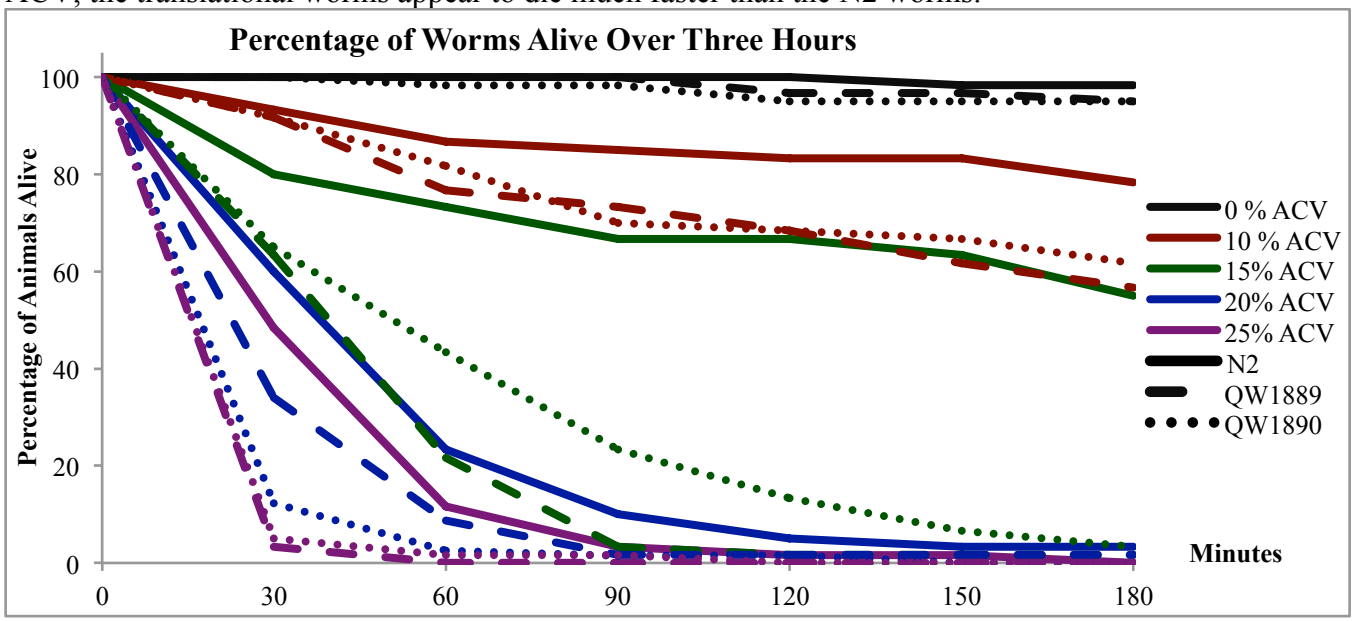

Figure 13. Survival assay results, displaying varying concentrations of apple cider vinegar as well as the survival of the three worm strains over time

3.4 Expression assay to determine whether AQP-7 is regulated by environmental acidity

During this experiment, both translational reporter worm lines were grown on plates of varying acidity, with and without acetate. Six young adult animals from each environment were selected for expression analysis. Utilizing FIJI software, the images were inverted and the integrated density of each of the worm was calculated. A higher integrated density indicates a higher degree of GFP expression. This data was normalized against the control. The results below indicate that exposure to acidic conditions, with or without acetate, does not affect AQP-7 expression.

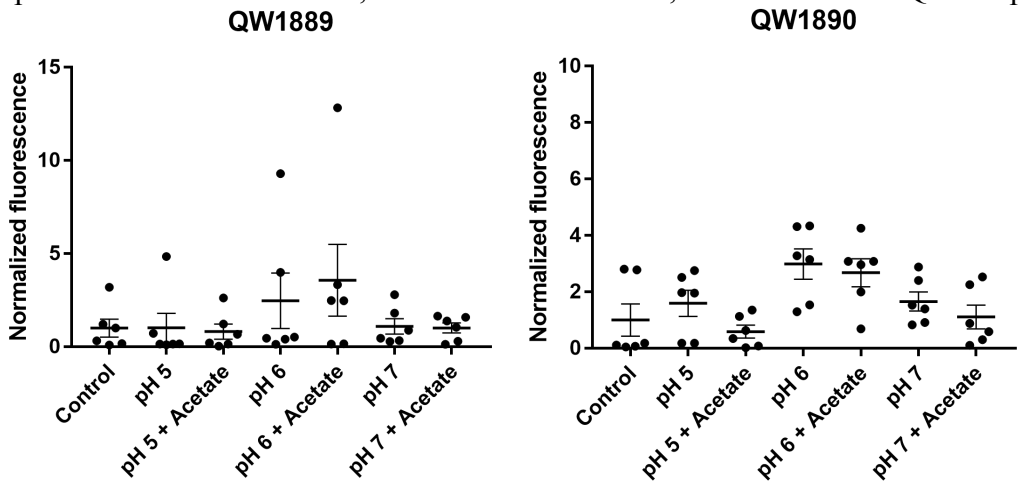

Figure 14. Expression assay results, displaying the florescence of two translational worm strains normalized to the control

4. DISCUSSION

4.1 Conclusions

The results of these experiments lead to four major conclusions:

- Increased mortality of transgenic animals supports AQP- 7 as an acetic acid transporter
- Aquaporin-7 is expressed in muscular tissue, hypodermis and nervous tissue
- *C. elegans* likely cannot metabolize acetic acid as *T. aceti* does
- Further expression of Aquaporin-7 is likely not induced by the environment

We have predicted that AQP-7 in T. *aceti* is involved in acetic acid metabolism. Based on this, we expected that expression would mainly be found in the intestine of the animal. However, the homolog AQP-7 in *C. elegans* appears in the hypodermis, nerve cells, and muscular tissue. But, this does not completely negate the possibility that AQP-7 performs a similar function. *C. elegans* thrives mainly under neutral conditions, unlike *T. aceti*. Therefore, *C. elegans* lacks evolutionary drive to adapt to metabolize acid as shown by the survival assay. AQP-7 may incidentally be able to transport acetic acid if it is presented with it, though its primary function is to transport water and glycerol.

Experimentally, the acid survival assay is consistent with acetic acid transportation by AQP-7. Almost all of the worms survive within more neutral conditions. As acidity increases, the over-expressing worms begin to die more readily than the wild type. This data suggests that AQP-7 may be able to transport acetic acid. Since the over-expressing animals have higher levels of AQP-7, their cells can be acidified more rapidly. Acetic acid may be pulled into the cell.

4.2 Further Investigations and Applications

There are several ways in which this protein can be further investigated. First, investigation into *C. elegans* AQP-1 may provide more information. This protein is also genetically similar to that of *T. aceti* AQP-7 (see Figure 3) and may have a similar ability to transport acetic acid. In addition, direct measurements of acetic acid transport through AQP-7 could be examined through the use of heterologous expression systems, such as yeast, bacteria, or mammalian cell lines such as HEK cells. HEK cells are embryonic kidney cells, which can be used to display proteins from many other organisms. This technique has been widely used due to its reliability and efficiency[10]. Expression within these cells would help to display trends in acidification. Ultimately, the most descriptive investigation would be through the manipulation of *T. aceti* itself. The creation of a knockout using CRISPR would produce a mutant worm that could be directly tested. This could also be accomplished through inactivation of AQP-7 with RNAi.

Overall, it is important to understand that aquaporins are highly conserved (Section 1.4). Many aquaporins have remained closely related despite major evolutionary change between different organisms. It is possible that humans contain an aquaporin with a similar ability to that of *T. aceti* Aquaporin-7. A similar protein could suggest a role in acetic acid metabolism or maybe even further fatty acid metabolism. In mice, it has been found that acetic acid can reduce the accumulation of body fat, despite being given a high fat diet[11]. This has been observed in addition to the up-regulation of proteins involved in fatty acid oxidation as well as acetyl-CoA[11]. If this effect is seen in mice, it is possible that it can also be induced in humans. A protein similar to Aquaporin-7 could be a transporter that could affect this pathway in humans.

5. REFERENCES

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APPENDIX A. POLYMERASE CHAIN REACTION

Step 1. Preparing the solution

*Keep the solution on ice at all times

- Add the ingredients listed in Table A below into a master mix (use of 4.4 times the solution accounts for pipetting error).
- If using different templates for each tube, do not add the template into the master mix but, instead into the individual PCR tubes specifically

Table A1. Example master mix and amount of each component in individual tubes

	1x Solution (µL)	**4.4x Master Mix (µL)**
(1) Water	31.5	126
(2) 5xQ5 Buffer	10	44
(3) 2mM dNTPs	5	22
(4) 25µM Forward Polymerase*	1	4.4
(5) 25µM Reverse Polymerase*	1	4.4
(6) Template	1	4.4
(7) Q5 Polymerase	0.5	2.2
TOTAL VOLUME (µL)	**50**	**200**

*order in which these are added does not affect reaction

Step 2. Divide master mix evenly among tubes

Step 3. Place tubes into PCR cycler and set for specific conditions

Table A2. Conditions set into PCR cycler

Temperature (°C)	**Length of time conducted**	
98	30 seconds	
98	10 seconds	
Annealing temperature or gradient	30 seconds	35 cycles
72	2 minutes	
72	2 minutes	
10	∞ (until retrieval)	

Step 4. Pool reactions accordingly and run through gel electrophoresis

- Useful to confirm the proliferation of the correct segments
- This gel can be cut and purified for use in further experiments (Appendix B).

APPENDIX B. GEL PURIFICATION

Part A: Extracting gel piece

Step 1: Place on UV protection (glasses, shield, lab coat)

Step 2: Image gel piece using the UV trans illuminator, camera, and computer program

Step 3: Once proper UV precautions have been taken (glasses, shield, lab coat), cut gel piece utilizing the trans illuminator as a guide

Step 4: Place gel pieces into pre-labeled microfuge tubes (may need to cut gel into smaller pieces in order to fit into the tubes)

- It may be useful to have double-gloved prior to this procedure. This way, an uncontaminated glove can be used to hold the microfuge tubes which will be traveling back to the workstation

Step 5: Remove safety equipment and properly dispose of excess gel + gloves

Part B: Purifying the gel piece

Step 1: Fill the microfuge tubes with OG buffer to dissolve the extracted agarose gel

- Place on 55°C block in order to fully dissolve gel piece
- Make sure that the gel isn't creating an air pocket at the bottom of the tube. It is beneficial to get as much QG buffer into the tube as possible

Step 2: Split the dissolved solution(s) into 2 microfuge tubes and fill with QG to 1mL mark

Step 3: Add 300µL of isopropanol to each of the tubes, let sit for 2 minutes

Step 4: Apply the mixture to a labeled spin column 800µL at a time and spin at 13000 rpm for 30 seconds, discard flow through

Step 5: Once the entire volume has been added to the spin column, wash with 500µL of OG buffer and spin at the same conditions listed above

Step 6: Wash the column with 750µL of PE buffer and let sit for 2 minutes

Step 7: Spin column with same conditions to remove all PE buffer, discard flow through and spin for 1 more minute in order to make sure all of the PE is removed

Step 8: Remove the outer tube and place the spin column into a labeled microfuge tube

Step 9: Elute the DNA off of the column by pipetting 25µL of warmed EB onto the center of the column

Step 10: Spin the columns for 1 minute at 13000 rpm, measure concentration, can store at -20°C until needed

APPENDIX C. DIGESTION AND LIGATION

Part A: Digestion of purified insert and vector

Step 1: Place all components into labeled microfuge tube(s)

Table B1. Components for digestion of purified DNA

	Insert DNA Tube (µL)	**Vector DNA Tube (µL)**
Milli-Q water	100-(13 + $V_{Insert\ DNA}$)	100-(13 + $V_{Vector\ DNA}$)
DNA	*	*
10x Buffer	10	10
Restriction Enzyme 1	1.5	1.5
Restriction Enzyme 2	1.5	1.5
Total Volume	**100**	**100**

* This procedure will vary based on desired insert as well as concentration of purified DNA

Step 2: Flick mix all ingredients and do a quick spin to bring all components back together

Step 3: Place microfuge tubes into 37°C incubator for 120 minutes

- At the 90 minute mark, add 1µL CIP (calf intestinal phosphatase) to the vector DNA in order to prevent ligation

Step 4: Column purify (Appendix D)

Step 5: Place at -20°C until ready for ligation

Part B: Ligation

Step 1: Place all components into labeled microfuge tube(s)

- Empty vector ligation serves as a control

Table B2. Components for ligation of digested DNA

	Ligation (µL)	**Empty vector (µL)**
Milli-Q water	10-(3 + $V_{Insert\ DNA}$)	7
10x Ligase Buffer	1	1
Digested Vector**	1	1
Digested Insert	*	0
T4 Ligase	1	1
Total Volume	**10**	**10**

* This procedure will vary based on desired insert as well as concentration of purified DNA

** May dilute

Step 2: Leave samples at room temperature for about 2 hours or place at 15 °C overnight until ready for transformation. If expecting a more difficult ligation, may place in 37°C incubator

APPENDIX D. COLUMN PURIFICATION

Step 1: Pool reactions into microfuge tube to determine total volume, record

Step 2: Add 5 volumes of PA buffer

Step 3: Add 10µL of 3M NaOAc, pH 5.2

Step 4: Apply mixture to ONE spin column, 800µL at a time, spin 30 seconds @ 13000 rpm, discard flow through

Step 5: Wash with 750µL PE buffer, let sit for 2 minutes

Step 6: Spin at 13000 rpm for 30 seconds, perform second spin for 1 minute to remove all PA buffer

Step 7: Elute with 25µL of warmed (55°C) EB buffer

Step 8: Measure concentration via gel comparison
- 1µL of DNA vs. 1µL ladder
- 1µL DNA vs. 3µL ladder

Step 9: Measure concentration via Nano-drop reader

Step 10: Send for sequencing if considered correct

APPENDIX E. TRANSFORMATION OF DH5α CELLS

Step 1: Thaw homemade DH5α cells on ice (~15 minutes)

Step 2: Add DNA (approximately 200ng) to cells and incubate on ice for 30 minutes

Step 3: Heat shock at 42°C for 90 seconds

Step 4: Recover on ice for 2 minutes

Step 5: Add 900µL of LB (no selection)

Step 6: Incubate (shaking) at 37°C for about 1 hour

Step 7: Plate onto LB Amp plate with a sterilized spreader in small amounts, allowing the bacteria to dry between each application

Step 8: Incubate plates overnight at 37°C (≤ 18 hours)

Step 9: Grow 6-10 colonies, each in 3mL liquid LB Amp overnight in 37°C shaker

APPENDIX F. PLASMID MINI-PREP

Step 1: Grow transformed bacteria

- Once transformed colonies have been confirmed on bacterial plate, transfer colonies to labeled test-tube containing LBAmp
- Incubate in 37°C shaker for 16-18 hours

Step 2: Pellet bacteria

- Transfer 1.5 mL of culture to labeled microfuge tube
- Spin for 30 seconds at 13000 rpm
- Using a pipette, remove all of the LB (LB interferes with lysis)

* May freeze at -80°C and continue another day if needed

Step 3: Re-suspend Bacteria

- Add cold 250µL P1 tris buffer with RNAse to the pellet
- Thoroughly mix using a vortexer, make sure to fully resuspend

* Can hold on ice temporarily if a break is needed

Step 4: Alkali Lysis

- Add 250µL of P2 alkali lysis buffer (SDS, NaOH)
- Gently invert 5-6 times.

* Do not vortex because it will liberate the genomic DNA

Step 5: Neutralization

- Add 350µL N3 buffer
- Gently invert 5-6 times
- Spin 10 minutes at 13000 rpm

Step 6: Bind plasmid DNA to membrane

- Transfer supernatant (800µL) onto mini spin column (Epoch Econospin 1910-250)
- Spin 1 minute at 13000 rpm. Discard flow through

Step 7: Washing the column and cleaning bound plasmid DNA

- Add 750µL of PE buffer (ethanol and buffer)
- Spin 30 seconds, discard flow through
- Spin 1 minute to ensure all PE is gone

Step 8: Elution of Plasmid DNA off of column

- Transfer column to pre-labeled microfuge tube
- Add 25-50µL of warmed EB buffer (55°C) directly onto membrane

(Amount of EB buffer depends on expected yield, lower yields should use less buffer)

* Can elute with pH 7 H_2O if preferred

- Let membrane sit at room temperature for 1 minute or warm on 55°C block
- Spin 1 minute at 13000 rpm
- Measure concentration with Nano-drop reader

APPENDIX G. STARVATION ASSAY

Step 1: Create 3 x 200mL of each of the following M9 solutions (6 x 200mL total)

M9 Solution		
Component	**Amount per 200mL (g)**	**Concentration (mM)**
Na_2HPO_4	1.16	40.85
KH_2PO_4	0.6	17.2
NaCl	0.1	8.6
NH_4Cl	0.2	18.7

M9 + Acetate Solution		
Component	**Amount per 200mL (g)**	**Concentration (mM)**
Na_2HPO_4	1.16	40.85
CH_2CO_2K	0.34	17.2
NaCl	0.1	8.6
NH_4Cl	0.2	18.7

Step 2: Adjust each solution with HCl until there is one of each (regular M9 and M9 + acetate) at pH 5, pH 6, and pH 7.

Step 3: Autoclave the buffers

Step 4: Bleaching adult worms (repeat for N2 as well as both translational reporter lines)

- Wash adult animals off of NGM plate with ~1mL M9 solution into a microfuge tube
- Spin for 5 minutes at 3000 RPM (Eppendorf bench top centrifuge)
- Remove M9 without disturbing the worm pellet
- Add 1mL worm bleach (concentrated bleach (8.75%) 745µL; 10N NaOH 250µL; ddH_2O 3.75mL)
- Invert and incubate for about 6 minutes at room temperature
- Top off tube with M9 solution and spin again at 3000 RPM for 5 minutes
- Carefully remove bleach without disturbing egg pellet
- Wash eggs with M9 to remove bleach, spin as before, repeat once more

Step 5: Re-suspend eggs in 100µL M9, place 5µL on a slide, count number of eggs to calculate eggs/µL in the tube

Step 6: Incubate eggs at 25°C a density of 1 egg/µL within 2mL of each of the M9 solutions (repeat for N2 as well as both translational reporter lines = 18 tubes total)

Step 7: At regular time points, take 50µL from each of the tubes and pipette onto regular NGM plates

Step 8: Allow two days recovery, then count number of worms beyond L1 stage

APPENDIX H. ACIDIC SURVIVAL ASSAY

Step 1: Label a well plate

		Percentage Apple Cider Vinegar (ACV)				
Strain	N2	0%	10%	15%	20%	25%
	Trans 1	0%	10%	15%	20%	25%
	Trans 2	0%	10%	15%	20%	25%

Step 2: Fill the wells as follows (M9 solution prepared in the usual way)

Desired %	0%	10%	15%	20%	25%
ACV (µL)	0	60	90	120	150
M9 (µL)	600	540	510	480	450

Step 3: Place 30 young adult worms in the first well and record the time

*It is best to pick plenty of L4 worms the day prior so that the experimental worms are about the same age

Step 4: Repeat step 3 until all of the wells are filled with the appropriate worm type

Step 5: Record the number of worms alive at 30-minute intervals over the course of 3 hours

- An "alive" worm is defined as any worm exhibiting movement, even if it is slow

APPENDIX I. EXPRESSION ASSAY

Step 1: Create potassium phosphate buffers (100mL), then autoclave

- Create each solution so that there are solutions of pH 5, pH 5 + acetate, pH 6, pH 6 + acetate, pH 7, and pH 7 + acetate
- For the solutions with acetate, utilize potassium acetate instead of K_2HPO_4

Step 2. Create 1L solution of the following, auto clave, and cool to about 55°C

- H_2O - 975 mL
- NaCl - 3 grams
- Bacto-peptone - 2.5 grams
- Bacto-agar – 17 grams

Step 3: Add the following to the entire flask

- Cholesterol (5mg/mL) - 1mL
- 1M $MgSO_4$ - 1 mL
- 1M $CaCl_2$ - 1 mL

Step 4: Separate 160mL into 6 different flasks labeled pH 5, pH 5 + acetate, pH 6, pH 6 + acetate, pH 7, and pH 7 + acetate

Step 5: Add 4mL of each potassium phosphate buffer to the respective flasks

Step 6: Pour onto plates of desired size and allow 1 day to dry

Step 7: Seed plates with OP50 (*E. coli*) and allow another day recovery

Step 8: Place 3 adult translational reporting worms onto each type of plate and allow 2 hours for a limited lay. Then, burn off adult worms leaving only the eggs on the plate

Step 9: Allow 2-3 days recovery until there are at least 6 young adult worms on each plate to image

Step 10: Take 6 young adult worms from each plate and place on a slide for imaging

Step 11: Image worms using a florescent scope and FIJI imaging software

Step 12: Analyze each worm for average integrated density