Epitope tagging of *Tetrahymena* SIRT6 homolog, THD8, for proteomic analysis
Ross Keesling Spring 2011

**Abstract**

The sirtuin class of genes was first found in *Saccharomyces cerevisiae* as a major player in DNA repair and modification and has also been associated with aging. SIRT6 found in *Mus musculus* is a well studied sirtuin, and a homolog has been found in *Tetrahymena thermophila* called THD8. In previous experiments, THD8 has been cloned into the pENTR vector. This plasmid was made by previous researchers for our later use. The purpose of this paper is to further the prior work done with THD8 in order to learn more about the function and protein it encodes. This is accomplished by cloning THD8 into a destination vector containing a tag of either GFP or 2HA. The resulting vectors are then purified and used in proteomic analyses like western blot, and fluorescence microscopy. The results of the western blot and fluorescence microscopy have given insight into the protein interactions of THD8. The expression of THD8 was measured using qRT-PCR, which gave an indication of the possible function of THD8. It was found during qRT-PCR that the activity of THD8 increases during starvation, and that UV DNA damage treatment had no effect on expression. Western blotting gave us useful insight into the amount of expression and size of THD8. With the successful tagging of THD8 with GFP and 2HA further proteomics can be accomplished, such as localization of THD8 in the cell using fluorescence microscopy of the GFP tagged genes.

**Introduction**

Sirtuins are proteins that are involved in regulation of important biological pathways, including aging, gene transcription, apoptosis, and energy usage during starvation. The name comes from the original name of the gene that was found in yeast, Sir 2, which stands for “silent mating-type information regulation 2.” Most sirtuins are deacetylases or mono-ribosyltransferases of proteins.
Some act on histones, the chaperone proteins that fold and supercoil DNA, allowing for large amounts of genetic information in a small area (Liszt et al., 2005).

We believe the activity of the THD8 protein is from its sirtuin domain, and because it has this domain we believe it will have a similar responsibility to the other Sir2, NAD+ dependent homologs, which in this case would be DNA modification and repair. Histones are acetylated in order to provide openings for the binding of transcription factors and other gene expressing proteins. When de-acetylated, the DNA is able to wind up tighter, removing these binding sites, and thus down regulating or preventing the expression of the particular gene. In multiple species this gene has been implicated as controlling aging, such as *Drosophila* and *C. elegans*. In these cases, expressing even just one extra copy of the Sir2 homologs increased life spans by significant amounts (De Ruijter et al, 2003).

In Humans there are seven sirtuin homologues of which, SIRT6, has seemingly involved in the aging process in mice. This paper explores *Tetrahymena* and its sirtuin-6 homolog, THD8, in the hopes of learning a little bit more about sirtuins and their functions.

THD stands for *Tetrahymena* Histone Deacetylase, which has been chosen due to evidence gathered from previous experiments involving the particular gene. *Tetrahymena*, a ciliate, is a good model organism for many different reasons. It is a single celled organism that can be found in a range of environments, but the cells are large and rival the complexity of human cells. The cells are also easy and inexpensive to grow in many different types of media and conditions. *Tetrahymena* also contains many major processes that are conserved across several eukaryotic species, unlike yeasts. Their simple means of care and their cellular characteristics makes molecular biology experiments with them ideal. (CGD)

**Methods**

**Plasmid Purification- 5 Prime Manual Fast Plasmid Mini Kit**
The initial purification of the plasmids that were cloned previously was done using a purification kit from 5 prime. This kit utilized a very normal purification method, but also cut out some steps in order to make it a faster method. However, this also decreased the total yields but only minimally. Essentially the kit utilizes a single solution for lysis, resuspension and DNA trapping. The biggest difference in this procedure is in the use of a spin column assembly. The spin column acts similar to column chromatography, with the centrifugal force pushing the solution containing the sample through. The column binds the DNA or plasmid while the rest of the unwanted things go through the column and collect at the bottom of the eppendorf. This solution is discarded and the spin column washed with a wash buffer. After assuring the column has been washed and the unwanted parts discarded, the column is transferred to a fresh tube and the Elution buffer added. This will break the bonds between the column and the DNA and thus the DNA will elute out for collection.

**Restriction Enzyme Digestion**

Restriction endonucleases cut DNA in specific recognition sites based on sequence. This is extremely useful in the molecular bio lab to cut our plasmids at known sites. If the plasmid contains a multiple cloning site, a sequence can be inserted here. However, in this particular case the enzymes are used to verify the proper insertion of THD8 into the pENTR plasmid. Since the enzymes cut by particular sequence, the sizes of the fragments indicate proper insertion. Verification was done using two digests: EcoRV in NEB buffer #3 and BSA, and a double digest of NheI and AvrII in NEB buffer #4 without BSA. The digests were done in preps of 0.5 ul of enzyme, 0.2 ul of BSA, 2 ul of buffer, 3 ul of plasmid DNA, and water to the final volume of 20 ul. The second digests we did were to confirm the LR clonase reaction was successful, and EcoRI and SpeI were used to confirm this, they were also done for a shorter time with incubation at a higher temperature to speed the reaction.
**Agarose Gel Electrophoresis**

Running gels is an extremely valuable technique in molecular biology. The specifics of the technique can be altered in order to gather different results, such as the type of agarose used, the % used, and the voltage run at. In the first confirmation of restriction digests to determine the correct orientation of the plasmids, it was a 1.5% gel run for 35 minutes at 80V and 20 ul of sample and 2 ul of loading dye were added to the wells. TE buffer was used as the running buffer and the agarose gel was prepared with Ethidium Bromide already in the gel, Bromophenol blue was also used to determine the movement of the bands. The second gel was nearly identical to the previous, with the purpose of confirming the orientation of the plasmid after the LR Clonase reaction.

**Making Electrocompetent Cells**

The purpose of this exercise was to prepare a culture of cells capable of transformation using electroporation. This is a difficult process because the cells are very fragile and must be handled with a lot of care, including keeping them chilled at all times. The two cell lines made were strains of *E. Coli* DB3.1 and DH10B. The cells were grown in a 10 mL culture culture overnight in SOB and MgCl₂ and a 1 L SOB culture was then inoculated with the overnight culture. They were grown to O.D.₅₅₀ of 0.8-1.0 and washed with water and 10% glycerol. The competency of these cells was then tested in a set of controlled situations in order to determine if they were capable of electroporation and transformation. This was set up in three separate controls for each cell line: one with no DNA, one with a pUC plasmid and one with a GTW plasmid. The cells were then grown and counted in order to test the efficiency of transformation of these different controls. From here an efficiency was found for each.

**Bioinformatics**
An exercise in bioinformatics was done in order to research a little more on the THD8 gene, to get more information on the protein sequence and domains as well as find homologs in other species. Several sites and programs were used, such as Expasy PROSite, Mega 4.0, T-COFFEE and a primer generation program.

**L-R Clonase Recombination**

The clonase recombination reaction is a technique that is very similar to using a multiple cloning site in conjunction with restriction enzymes, but with an enzyme called a clonase. It was developed by Invitrogen as an alternative to the typical cloning process, but is more expensive. The upside is that it is extremely efficient, more so than normal cloning. This process involves the plasmid containing the gene of interest, THD8, and pairing it with a plasmid called a gateway vector. The pair is treated with clonase, and the gene of interest is then inserted into the gateway vector. There are two sites in the plasmids, L and R, which are used as indicators for where excision and insertion of sequences occurs. The gateway plasmids contain epitope tags, either GFP or 2HA. After the reaction the epitope tag will then be at the beginning of the sequence and will be important later when we do our proteomic analysis. The GFP gateway plasmid has an RPL coding region (ribosomal proteins) and is cyclohexamide resistant. The 2HA gateway plasmid contains a BTUI-K350M site that when removed has paclitaxel resistance. Each plasmid has an inducible by cadmium promoter called MTT for metallothionine. These characteristics are important when it comes time for selection of cells and for the induction of THD8 expression.

**Transformation of Electrocompetent E. coli**

The DH10B cells were transformed with the pENTR-THD8 plasmids via electroporation. An electric shock opens the pores in the cells membrane, allowing the plasmids into the cell. The cells are suspended in SOC media (containing tryptone, yeast, etc.) and pulsed for about a second. The cells are allowed to incubate for an hour and then plated on AMP plates and the number of colonies recorded.
**Plasmid Purification/Digest of GFP/THD8 and 2HA/THD8**

To purify the transformed plasmids from the *E. coli* cells, the method of boiling was used. This utilizes a sucrose lysis buffer and lysozyme with the addition of NaOAc and Isopropanol for precipitation of the DNA. The boiling portion is to denature the proteins and DNA and make them easier to retrieve. The purified plasmid was then digested with restriction enzymes (SpeI/EcoRI). These enzymes are chosen using the plasmid map on the Gene Construction Kit. For this reaction buffer 4 was used along with 2.2 ul of BSA. Once the plasmid was confirmed the best sample was maintained on plates containing Ampicillin and Kanamycin.

**Lysosome-Boil Midiprep**

The boiling method was used once again to purify the DNA and washed with ethanol and then put in the vacuum to dry the samples. They were re-suspended in 1X TE and RNase treated with a 30 minute incubation at 50*C. The DNA was then precipitated using Phenol Chloroform at an equal volume to the sample. This was centrifuged for 5 minutes and the top layer removed/moved to a new tube, where NaOAc was added at 1/10th the sample amount and then 2.5 times the sample of 100% ethanol was added and saved in the freezer.

**Biolistic Transformation of Tetrahymena**

*Tetrahymena* cells are grown in 25 mL cultures of 2% PPYS+PSF and 1-3X10^5 cells/mL are transferred to a 100 mL culture. The cells are starved the next day using 10mM Tris at pH 7.5. Gold beads are prepared in using 30mg in a 1.5 mL tube with 1 mL of 70% ETOH. This is washed and resuspended in 1 mL water three times whence 0.5 mL 50% glycerol is added. The DNA is digest to linearize it (SacI and KpnI for 2HA and BlpI and Earl for GFP) at 80 ug of DNA in 200ul reaction with 5 ul
of enzyme. The mixture is precipitated using phenol: chloroform, chloroform and ethanol and washed with 70% ethanol. This is resuspended in TE and the concentration adjusted to 1.0 ug/ul. 5 ul of DNA is coated onto 25 ul of beads along with 25 ul Calcium Chloride and 10 ul of 100mM spermidine. After 30 minutes of vortexing the samples are washed with ETOH. The gene gun is prepared and the cells spun down. 1 mL of cells is placed on the filter paper in the petri dishes. The cells are transformed and placed in 50 mL of 2% PPYS+PSF overnight. The cells are then plated in 96 well plates at 100 ul each along with 1.0 mg/mL paclitaxel for 2HA and cyclohexamide for GFP.

**GoTaq RT-PCR**

While the cells are growing up, a reverse transcriptase PCR reaction is run. Forward and reverse primers for the reaction were prepared by Integrated DNA Technologies according to our preferred sequence. Two reactions were run, one with genomic DNA, and one with Tetrahymena cDNA. These primers were diluted to 20 pmol/ul and added to the PCR mix. This includes 1X GoTaq, the two primers at 0.4 pmol/ul, and 14 ng/ul gDNA or cDNA, and water to 25 ul. The thermocycler was run for 2 minutes at 95*C, 45 seconds at 95*C, and then another 45 seconds between 50-58*C. Step four 1 minute 45 seconds at 72*C, then the second step 32 times. Next step 6 for 5 minutes at 72*C and 4*C forever. Four different temperatures were used for the annealing temperature, 52.2, 54.6, 57.4, and 61*C. The resulting reactions were then run on a gel to determine the purity and success of the PCR reaction.

**qRT-PCR with SsoFast**

Real time or quantitative reverse transcriptase PCR was run as a test initially using three concentrations of gDNA, 0.1, 0.01, and 0.001 ug/ul and a sample of cDNA standard. This was done using BioRad’s SsoFast EvaGreen supermix at 10 ul, the two primers at 0.5 ul, RNase free water at 8 ul and the gDNA at 1 ul. This reaction was then run on a gel to determine its success. After determining
the test was successful, the real reaction was run. This reaction had the same contents, only the samples were as follows: Log cells, Starved cells, NO UV, 0 hours UV, 1 hour UV, 2 hours UV, 3 hours UV and 4 hours UV.

**Protein Isolation and Immunoprecipitation**

THD8/2HA transformed *Tetrahymena* are induced with 1.5 ug/mL CdCl₂ for 2 hours. The cells are then spun down and transferred to eppendorf tubes and 600 ul of Breaking buffer including protease inhibitors are added and vortexed/then spun for 15 minutes. The protein total is quantitated using the Bradford protein assay Reagent (795 ul water, 200 ul reagent, 5 ul sample) and the standard curve made using BSA. The absorbance is measured allowing the immunoprecipitation to be set up. The immunoprecipitation is carried out using the IP dilution buffer II and IP wash buffer II. The immunoprecipitation is then run in a SDS PAGE gel. The gel then is transferred using western blotting techniques and imaged.

**Cryopreservation of Bacteria**

The bacteria that were transformed with the pENTR-THD8 plasmid are transferred into LB liquid media tubes containing 50 ug/mL of Kanamycin. They are allowed to grow overnight. 700 ul of the cells are transferred to cryopreservation tubes containing 700 ul of 50% sterile glycerol and then frozen using powdered dry ice and kept in the -80*C freezer for later use.

**Results**

The original plasmid was purified using the FAST protocol and the purity and concentration measured using the nanodrop spectrophotometer (Table 1). The restriction digest and successive gel were crucial in deciding which strain of the plasmid to use for the rest of the research. The
concentration readings provide valuable information regarding the best strain. The gel that was used to determine proper insertion of the gene into the plasmid is in Figure 1. Well 1 is the NEB 1 kB standard. The best strain was in well 3 and is strain 1 of THD8. This strain provided the best evidence of the proper insertion of the THD8. This is evidenced by the sizes and positions of the bands. The predicted sizes for our gel using the AvrII/NheI cut were 2.4 kB, 1.3 kB and 0.1 kB. At first wells 2 and 5 looked most promising, but on further investigation, it seems as though 3 was the best digest. The bottom band has traveled farther than the two bands in wells 2 and 5. This means that it is slightly smaller, and after looking at the predicted gel sizes, it indicates that the smallest piece indeed has been cut, and run off of the gel. Wells 2 and 5 have no such cut, and therefore the small piece has been retained, allowing for less travel. Wells 7-10 however were unsuccessful in digestion, and a hypothesis for this is given in the discussion section.

The bioinformatics portion of the experiment was in order to learn more about THD8 using computer programs like ExPaSy PROsite, T-COFFEE, and Mega 4.0. Figure 2 shows the amino acid sequence of THD8 which was used in some of the programs. The nucleic acid sequence was also used for many of the programs. The top three homologs in comparison to THD8 were found using the TGD website. They are THD9, THD11, and THD12. The human homolog for these four genes is SIRT6, while the yeast homolog is SIRT 2. THD8 also has homologs in other species, also obtained from the TGD: TAIR, FlyBase, ToxoDB, WormBase, PlasmoDB, DictyBase (two), and ParameciumDB.

ExPaSy PROSITE analysis of several different amino acid sequences were done in order to determine the similarities in the protein sequences. The sequences chosen were from: *Drosophila melanogaster, Homo sapiens, Saccharomyces, Schizosaccharomyces, Arabidopsis, and Bifidus longus*. The sequences are compared to each other in order to find commonalities, especially in finding the domains that are formed by the sequences. A notable fact was that
THD11 had no hits during the search, and therefore must not have a SIRT domain as the others do. Also included are THD8, THD9, THD11 and all the human sirtuins. Each domain is a green sirtuin domain, and they all contain a histidine residue.

Next was a multiple sequence alignment of all the sequences, with each line of the sequence being compared to the lines of the other sequences to look for similarities. This was done using T-COFFEE in order to visualize the similarities in the sequences. Next phylogenetic trees were created by entering the sequences of THD8 and the homologs into Mega 4.0. This creates the UPMGA tree, the neighbor-joining tree, and the bootstrap tree. The next step was to come up with primers for PCR. This was done using a primer generator, and the results were sent out in order to have an independent company (IDT) generate them for us.

The results of the cell competency check are in Table 2. All of the DH10B cells were determined to be competent. We also made sure that the cells were properly transformed with our plasmids along with the controls containing only 2HA and GFP. GFP tagged THD8 had approximately 400 colonies while 2HA tagged THD8 had approximately 6 colonies. The 2HA control only had 2 colonies. We also were able to find the efficiency of the cells using the formula below Table 2.

The image in Figure 6 shows the image of the gel used to confirm the L-R clonase reaction was a success. The gel includes four GFP tagged THD8 plasmids and three 2HA tagged THD8 plasmids. Also controls of 2HA and GFP gateway vectors alone. By looking at the predicted band sizes for the GFP tagged THD8 plasmid in Table 3, the best GFP clones were chosen, which were clones 1 and 2. All three of the 2HA clones were successful, even according to the predicted band sizes. Therefore clones 1 and 2 of GFP were used, and all of the 2HA
clones were used. Figure 7 and 8 are the images of the gel that was prepared prior to the transformation of *Tetrahymena*. Figure 7 is the 2HA plasmids, and Figure 8 the GFP plasmids. The 2HA plasmids were cut with Sacl and KpnI and GFP with Blp1 and Earl, in order to linearize them, easing the transformation process. The gels were run in order to confirm the linearization, and the separation of the bands shows that they were correct. This allowed for proceeding to the transformation with the gene gun.

The gel image obtained for confirmation of the RT-PCR reaction is in Figure 9. The gDNA bands are all much more prominent than the cDNA bands, but in the 61°C wells (wells 7 and 8) the product was not as pure and didn’t fluoresce as much. However the results in the rest of the wells were promising and showed a very pure product.

The graph of the amplification during the test qRT-PCR is shown in Figure 10. This shows the amplification of the samples (STD1, STD2, Neg Control, and cDNA). Figure 11 shows the melt temperatures of the test run samples during qRT-PCR. The graph shows that the samples all melted at the same temperature, right around 78°C. This is good, and shows the samples were pure. Figure 12 was to confirm the qRT-PCR products. The test run was done so that when it came time for the real run, it was known it would work for sure. As seen the products were pure, as the bands are all very solid.

Figure 13 is the amplification graph of the real qRT-PCR run where the samples all were under different conditions of stress, from starved to UV exposure for varying time periods. All of the cells aside from the Standards are amplifying at the same level. The melt graph is in Figure 14, and it shows that all the samples were melting at the same temperature. The only differences were with STD1 which melted at 77.5°C and STD3 which melted at 78.5°C. Figure 15 shows the relative expression of the protein product of THD8 during the different cell conditions. There was no major change in activity of the gene/protein during log phase and any of the UV stressors, but during starvation the activity
increased 15 fold. This is an extremely important development when it comes to the understanding of the function of THD8. Because it has such a strong response to the levels of stress starvation induced, it may indicate that the function of THD8 is in stress response, or energy conservation, or a number of other related processes. Regardless it’s obvious that it is important in starvation conditions.

The western blot of 2HA plasmids at five minutes of expression is in Figure 16. Wells 4 and 5 are the THD8 samples. The blot shows the size of the proteins expressed. THD8 shows two different sizes of proteins, which may be due to the fact that the protein is modified to be smaller or larger or that it is degraded quickly. Figure 17 is the western blot of the 2HA plasmids at 20 minutes of expression. The blot is separate quite a bit more as the protein is degraded, maybe even much quicker than the other samples. The last thing run was a sequence of our gene. Unfortunately the sequence didn’t align with the predicted THD8 sequence but rather THD13.

**Tables and Figures**

**Table 1**: Represents the nanodrop results of the purified plasmids containing THD8 extracted from *E. coli*. The strain chosen was #1, which had the lowest concentration, but the best digest.

<table>
<thead>
<tr>
<th>THD8 Strain</th>
<th>260 O.D.</th>
<th>260/280</th>
<th>Concentration (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>1.22</td>
<td>1.92</td>
<td>0.0611</td>
</tr>
<tr>
<td>#3</td>
<td>2.43</td>
<td>1.89</td>
<td>0.1216</td>
</tr>
<tr>
<td>#5</td>
<td>2.45</td>
<td>1.89</td>
<td>0.1225</td>
</tr>
<tr>
<td>#6</td>
<td>2.375</td>
<td>1.82</td>
<td>0.1187</td>
</tr>
</tbody>
</table>

**Figure 1**: Gel image of two restriction digests of original plasmid containing THD8. Wells 1 and 6 are the NEB 1kB standards. Wells 2-5 were done with AvrII and NheI. Well 2 is strain 3, well 3 is strain 1, well 4 is strain 5, and well 5 contains strain 6. Wells 7-10 are the failed digest with EcoRV.
Figure 2: Amino acid sequence of translated THD8. Also the TTHERM number found on the Tetrahymena Genome Database.

TTHERM_01018420 = THD8
MNTAHKTDDEKKEFFDSPKELEEKVNILVDMIKRSEHFAFTGAGISTSTGIPDFRSGIN
PTPGAWKEKLAKGSGSKSNKVSMKASPSPTHMLSLVEQLQRQGYLKFLISQNVGDGLHRR
GFSTYHLAEHLGNTNLKCGKCGKYMRDFVQVHDHTGKCDNNQCNQDLYD
SIINFGENLPEKDQDDGFVHSQLADLHLVGLSSRLVTAPADMPETTAKLGKNLVIVNLQKTP
LDSLAILRINAMCDDVMKMKKLKIEPEFILERRIVLQDKDSLLVSADSNESPYEIIK
KVTIEYGKANEAKQPKNFPTQKNQFTVSLGYEYGEQEFKLQDMIAALPLNKVVQR
QYQVSLQKWISCKQI

Figure 3: ExPaSy PROsite analysis of all homologs. The numbers in parentheses indicate the amino acid sequence length that forms the sirtuin domain. The red diamond is a histamine residue. These show proof that each contains the sirtuin domain.

D-M-SIRT6

35 - 275:  score = 40.718

H-S-SIRT6

1 - 175:  score = 27.884
S-C-SIR2

245 - 529: score = 49.049

S-P-SIR2

147 - 432: score = 46.504

B-L-SIR2

1 - 241: score = 42.320

A-T-SIRT2

35 - 269: score = 45.278

T-T-THD8

26 - 273: score = 40.859

T-T-THD9

59 - 310: score = 38.302

T-T-THD12

26 - 273: score = 42.462
SIRT3
(257 aa)

1 - 240: score = 49.320

SIRT2
(389 aa)

65 - 340: score = 50.817

SIRT1
(747 aa)

244 - 498: score = 52.455

SIRT4
(314 aa)

45 - 314: score = 44.913

SIRT5
(310 aa)

41 - 309: score = 47.906

SIRT6
(355 aa)

35 - 274: score = 46.645

SIRT7
(400 aa)

90 - 331: score = 40.930
SIR2

245 - 529: score = 49.049

Figure 4: Bootstrap Tree created using Mega 4.0. The different branches stand for the following species respectively: Arabidopsis thalia, Homo sapiens, Drosophila melanogaster, Tetrahymena thermophila, Bifidus longus, Saccharomyces cerevisiae, Schizosaccharomyces pombe.

Figure 5: The results of our query for the optimal primer.

Pair 1:

Left Primer 1: Primer_F

Sequence: GTGCAGGTATTTCTACTTCTACTG

Start: 128 Length: 24 bp Tm: 54.4 °C GC: 41.7 % ANY: 4.0 SELF: 1.0

Right Primer 1: Primer_R
Sequence: ACATATGAGTAGGAGATGGTATTG

Start: 295  Length: 24 bp  Tm: 54.2 °C  GC: 37.5 %  ANY: 6.0  SELF: 0.0

Product Size: 168 bp  Pair Any: 6.0  Pair End: 3.0

1  ATGAATACAG CTCATAAAAC AGACGATGAA AAAAAAGGAAT TCTTTGATTC
51  ACCTAAAGAA TTAGAAGAAA AAGTAAATAT TTTAGTGAT ATGATAAAAC
101 GTTCTGAGCA CTTGTGGCT TTTACAGGGT CAGGTATTTT TACCTCTACT
151 GGAATTCCAG ATTTTAGAAG TGGTATCAAT ACAGTACTTC CAACCTGGTCC
201 AGGTGCATGG GAAAAATTAG CTAAAAGAGC AGGCAAGCCT TAAAATATG
251 TAAAAGTAAG CATGTCTAAG GCAATACCAT CTCTACTCA TATGTCTCTT
301 GTTGAAATTAC AGAGATAAGG ATATCTTAAA TTTTGATAA GTTGAATGT
351 AGATGGTCTA CACAGAAAGA GTGGATTTTC TACTTACCA TTAGCCGAAT
401 TGCATGGCAA CACAAATCTA GAAAAATGCT AGAAGTGTGG CAAAAGATAC
451 ATGAGAGATT TTAGAGTCAG AACAGCCCAA TAACTGACTG ATCATAAAAC
501 TGGTAGAAG AGTAGAATTAC TAAATGTTA TGGAGATTTA TATGACTCTA
551 TTATAAAATT TGGTGAACAC CTCCCTGAAA AAGACTAAGA TGATGGATTC
601 GTTCATTCTT AGTTGGCTGA TTTGCATTATA GTTTTAGGA GCAGTTTAAG
651 AGTAACTCCT GCAGCTGATA TGCCTGAAAC TACAGCAAAA TTAGGAAAAA
701 ATTTAGTTAT TGTTAATTGT TAAAATGCTA TGAGGAAAAAC TACAGCTACT
751 CTCAGAATAA ATGCCATGTG TGGATGATTT ATGAAAAATGG TTATGAAAAA
801 GCTTTAAAAATA GAAATCCCAAG AATTATCTTT AGAAGAAGGG ATAGTCTTTT
Table 2: These are the results of the cell competency portion of the experiment. The numbers indicate the number of colonies.

<table>
<thead>
<tr>
<th></th>
<th>No DNA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amp</td>
<td>Kan</td>
<td>Amp</td>
</tr>
<tr>
<td>DH10B #1</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>DH10B #2</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>DB3.1</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

Efficiency:

\[ 1 \text{ul of 39 ng (pUC) or 1.4 ng (GTW)/ 500 ul} = 0.078 \text{ ng/ul x 100ul} = 7.8 \text{ ng/ plate} \]

\[ \text{CFU/0.0078 ug} = \text{efficiency} \]

Table 3: Predicted band sizes for the confirmation gel of L-R clonase reaction.

<table>
<thead>
<tr>
<th></th>
<th>Fragment Sizes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENTR-THD8-2HA</td>
<td>3211</td>
</tr>
<tr>
<td></td>
<td>1795</td>
</tr>
<tr>
<td></td>
<td>1667</td>
</tr>
<tr>
<td></td>
<td>890</td>
</tr>
<tr>
<td>pENTR-THD8-GFP</td>
<td>4625</td>
</tr>
<tr>
<td></td>
<td>2875</td>
</tr>
<tr>
<td></td>
<td>1582</td>
</tr>
<tr>
<td></td>
<td>890</td>
</tr>
</tbody>
</table>
Figure 6: Gel image of the restriction cuts of control GFP and 2HA (lane 1 and 4) respectively, as well as the four different THD8+GFP plasmid cuts (lanes 2,3,6,7) and the THD8+2HA (8,10,11). The 1kB ladder is in lanes 5 and 9.

Figure 7: Gel image prior to transformation of *Tetrahymena* by the gene gun. The plasmids were linearized by digestion using SacI and KpnI. The gel was to confirm this linearization. This is the gel of the 2HA and Flag plasmids. The 2HA-THD8 sample is in well 5.

Figure 8: Gel image prior to transformation of *Tetrahymena* by the gene gun. The plasmids were linearized by digestion using SacI and KpnI. The gel was to confirm this linearization. This is the gel of the GFP and RFP plasmids. The GFP-THD8 sample is in well 4.
Figure 9: Gel image for confirmation of RT-PCR products. Well 1 contains gDNA, 2 cDNA at 52.2°C. Well 3 and 4 at 54.6°C, well 5 and 6 at 57.4°C, and wells 7 and 8 at 61°C. Alternating g and cDNA.

Figure 10: Amplification graph of test run of qRT-PCR. STD1 is blue STD2 is RED, Negative control (-TEMPLATE) is BLACK, and cDNA is GREEN.
Figure 11: Melt graph of test run of qRT-PCR. STD1 is blue and STD2 is RED, Negative control (-TEMPLATE) is BLACK, and cDNA is GREEN.

Figure 12: Gel image of qRT-PCR test run. Well 2 and 7 are the 1KB NEB ladder. Well 3 is gDNA at 0.1 ug/ul, well 4 is gDNA at 0.01 ug/ul, well 5 is 0.001 ug/ul, and well 6 is cDNA.
**Figure 13:** Amplification graph of qRT-PCR of real UV run. STD1 (RED), STD2 (YELLOW), STD3 (BRIGHT BLUE), Log growing cells (DARK GREEN), Starved cells (TEAL), No UV damage (BRIGHT GREEN), 0hr after UV (ORANGE), 1hr after UV (GREY), 2hr after UV (PINK), 3hr after UV (DARK BLUE), and 4hr after UV (PURPLE).
**Figure 14:** Melt graph of qRT-PCR of real UV run. STD1 (RED), STD2 (YELLOW), STD3 (BRIGHT BLUE), Log growing cells (DARK GREEN), Starved cells (TEAL), No UV damage (BRIGHT GREEN), 0hr after UV (ORANGE), 1hr after UV (GREY), 2hr after UV (PINK), 3hr after UV (DARK BLUE), and 4hr after UV (PURPLE).

![Melt graph of qRT-PCR](image)

**Figure 15:** Graphical analysis of the qRT-PCR data. The data is normalized to HHP1. This graph shows the expression of THD8 during different levels of cell stress and condition.

![Graphical analysis of qRT-PCR data](image)
**Figure 16:** Western blot image of all the 2HA clones at 5 minutes. THD8 is in position 4 and 5.

![Image](image1)

**Figure 17:** Western blot image of all the 2HA clones at 20 minutes. THD8 is in position 4 and 5.

![Image](image2)

**Discussion:**

The ultimate goal of this research involved characterization of THD8. The first indication of what it may do was found in our Bioinformatics research, with the most beneficial data coming from the
ExPaSy PROsite analysis. The rest of these analyses really only provided information about similarities between the homologues of THD8. PROsite found that THD8 contains a sirtuin domain, which indicates that it will have similar activity to the rest of the sirtuins. It may therefore be involved in processes like aging and stress response. The most beneficial experiment in characterizing the function of THD8 was the quantitative RT-PCR. The data from this experiment showed that the protein product was expressed fifteen times as much as baseline during starvation. This may indicate that the protein plays an important role during starvation like energy conservation. The western blots showed the protein product is around 39 kB but there also was a larger protein product shown. This may be degradation or modification of the protein product.

From all the experiments run, it seemed obvious that THD8 had been successfully cloned into the bacteria, that it was properly inserted into the gateway plasmid. Even after transformation of *Tetrahymena* it seemed as if the research was following the right path. The expression of the gene was looking as predicted and the qRT-PCR showed the anticipated reaction to starvation. However, not until the sequencing of the cloned gene did we notice that the sequences don’t align (THD8 predicted versus the cloned gene sequence). This also occurred with THD13, and when the predicted sequence of THD8 was aligned with the sequenced THD13 clone, the sequences were identical. This happened vice versa with the predicted sequence of THD13 and the cloned sequence of THD8, they aligned perfectly. Therefore this brings up questions as to whether the right gene has been cloned into *Tetrahymena*. THD13 and THD8 have similar sizes and predicted functions. They also have similar cut sites with restriction enzymes which also made it difficult to tell where along the way they were mixed up. It’s possible that initially the genes were switched up before even being cloned into the plasmids and that all the research has been done using the wrong plasmids. It’s also possible that this happened when we started the experimentation. The last two possibilities are that Mizzou got the samples mixed up or that the samples were mixed up before being sent for sequencing.
Ultimately we learned a lot about the characterization of the gene that was cloned, whether it be THD8 or THD13 is unsure. To determine which of these was cloned, we must go back and use different restriction enzymes in order to determine which plasmid has been inserted and tagged. The future directions of this research is to further study the proteins function and location using the GFP tag and viewing it under the microscope. This will show where in the cell the gene is being expressed. One of the most important things learned from this experiment was that sometimes results aren’t always positive or negative, but rather can be inconclusive or even just plain mixed up like this situation. It is obvious that evidence has been found about the function of at least one sirtuin like histone deacetylase, but which of these it is unknown.

References

Ciliates Genome Consortium. http://tet.jsd.claremont.edu/about.php


Tetrahymena Genome Database. Ciliate.org