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Submitted in partial fulfillment of Honors

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Expression of Human Neutrophil Elastase in *K. Lactis*

By: Haley Klimecki
April 2010

Abstract

Human neutrophils are the most abundant type of white blood cell and provide the body with a line of defense against foreign, infectious microorganisms. Contained within the azurophilic granules in the cytoplasm of neutrophils are three serine proteases, Human Neutrophil Elastase, Cathepsin G, and Protease 3. Once a foreign bacterium is engulfed by white blood cells, these enzymes attack and degrade the invading body, thus killing it (Reeves et al., 2002). The focus of this research is centered on the production of one of the serine proteases, human neutrophil elastase (HNE), and while the importance of HNE can be seen, genetic mutations or improper regulation can compromise a person's immunity. Neutropenia (a low neutrophil count) is one such disease caused by a genetic mutation of HNE that results in susceptibility to infection (Li and Horwitz, 2001). Additionally, HNE is a powerful enzyme that can attack the elastin of the lung if not properly controlled. Consequently, genetic deficiencies of alpha-1 proteinase inhibitor protein in the blood can result in emphysema because active HNE released from neutrophils is free to degrade lung tissue (Laurell and Eriksson, 1965).

Recombinant HNE is not currently available, and the enzyme must be isolated from human blood cells, which has inherent hazards. Additionally, the lack of recombinant HNE has prevented studies involving site–directed mutagenesis to study the intracellular processing of HNE near its C-terminal end where mutations have been found

to result in neutropenia. Kinetic studies of the full-length HNE might shed some light on why its C-terminal region is removed before storage in cytoplasmic granules.

The HNE DNA sequence was first codon optimized for yeast and commercially synthesized. It was then fused with DNA for eGFP (enhanced green fluorescent protein) via an enterokinase cleavage site (D4K). This DNA construct (eGFP-D4K-HNE) was then inserted into the *Kluyveromyces lactis* (*K. lactis*) pKLAC1 vector, downstream of the alpha mating factor which directs proteins for secretion. Then, chemically competent GG799 cells (a strain of *K. lactis*) were transformed with the linearized pKLAC1-eGFP-D4K-HNE insert through a protocol from New England Biolabs. Theoretically, the gene integrates into the yeast genome upon transformation via sequences within the pKLAC1 vector that are homologous with the LAC4 gene promoter that allows for galactose utilization (Colussi 2005). Acetamide was used as a selectable marker because wild type *K. lactis* cells are not able to use acetamide as a nitrogen source. The pKLAC1 vector, however, contains the *Aspergillus nidulans* gene acetamidase (*amdS*) that allows only transformants to grow on plates with acetamide as the sole nitrogen source (Read 2007).

Selected colonies were transferred to both liquid and agar-based synthetic media with galactose to induce transcription and translation of the HNE gene to produce the eGFP-D4K-HNE fusion, and screened via fluorescence microscopy for production of eGFP. None of the screened colonies tested positive for the presence of the fusion protein.

Introduction

The body's first line of defense against attack by infectious microorganisms is its neutrophils, the most abundant type of white blood cell circulating in the blood. Though neutrophils have a short half-life of around seven hours, they are continually produced by the bone marrow when an infection arises. Upon arrival at sites of infection in the body, neutrophils engulf invading bacteria by phagocytosis. After ingestion, the azurophilic granules within the cytoplasm of neutrophils release certain proteases that aid in bacterial killing and digestion (Reeves, Lu et al. 2002). One such enzyme is human neutrophil elastase (HNE), a serine protease. HNE is an enzyme that is able to degrade bacterial proteins in the killing process, and it is termed an "elastase" because it will also degrade elastin which is resistant to other proteases such as trypsin. While the role of HNE is essential for the proper functioning of a person's immune system, mutations of the enzyme's DNA as well as the improper regulation of its activity can cause disease. One such disease, neutropenia, occurs when the number of white blood cells circulating in the bloodstream is too low to fight off infection (Horwitz, Duan et al. 2007). Naturally occurring HNE works without the use of its C-terminal peptide which is cleaved at some point during its activation (see Figure 1).
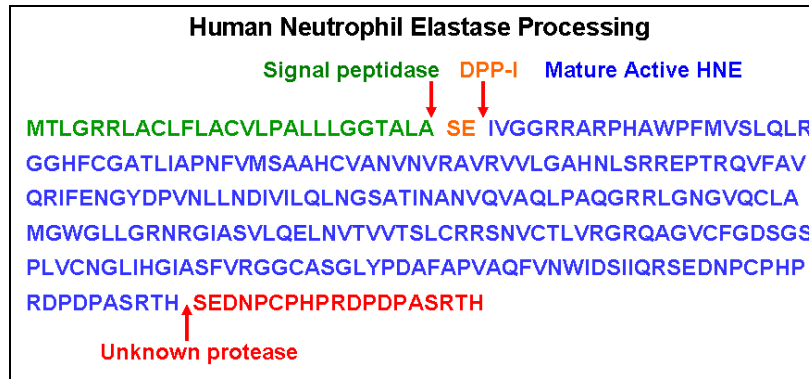
Figure 1: Active HNE sequence with full C-terminus. Signal peptidase removes the signal peptide that directs the protein to the endoplasmic reticulum. DPP-I removes the pro-peptide (SE) to activate HNE and cleavage by the unknown protease does not occur in neutropenia.

It is thought that if HNE is mutated at a specific position on its C-terminus then the HNE is kept from moving out of the neutrophils. Some research has shown that several types of neutropenia are caused by the mutation of neutrophil elastase found in the cytoplasmic granules of neutrophils. Whether the protease becomes membrane bound or not affects the type of neutropenia that affects the body (Benson, 2003). For one type of neutropenia, cyclical hematopoiesis, the mutation of ELA2 acts to "prevent membrane localization of neutrophil elastase" (Benson, 2003). Furthermore, the chromosome that has been isolated to be the most likely location of mutation of ELA2 is the 19p13.3 chromosome (Ancliff, 2001). For mutations of ELA2 (which encodes neutrophil elastase) that cause severe congenital neutropenia (SCN), however, the protein becomes membrane bound and unable to move outside of the neutrophil (Ancliff, 2001). While the degree to which membrane bound HNE effects the life of the neutrophil and the mechanism by which the neutrophil dies is unknown, the presence of membrane bound

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HNE has been seen in specific variations of the disease. Additionally, failure to control the activity of HNE released from short-lived neutrophils can prove destructive to the body's tissues, causing disease. Pulmonary emphysema is thought to be caused when components in cigarette smoke, called oxidants, inactivate the blood protein called alpha-1 antitrypsin, which is the primary inhibitor of HNE in the blood. This protein acts as an inhibitor of HNE, therefore when the inhibitor itself is genetically deficient or damaged by oxidation, active HNE is free to wreak havoc on healthy tissues (Laurell and Eriksson 1965).

The study of HNE would be greatly facilitated by stable expression of the recombinant enzyme, which would eliminate the hazards associated with isolating the enzyme from human tissues and allow for site-directed mutagenesis studies. For example, natural HNE is missing a C-terminal peptide that, if not removed, results in neutropenia. Recombinant HNE would allow production of HNE with this C-terminal extension so its role in neutropenia could be studied further, and full-length HNE could be used to study this processing event.

The yeast *K. lactis* was first studied as a medium for heterologous protein expression (Swinkels 1993) and has been used to express a number of recombinant proteins (van Ooyen, Dekker et al. 2006). In addition to being easily manipulated genetically and having a "fully sequenced genome" (Dujon 2004), another advantage of *K. lactis* is that correctly folded proteins are secreted into the culture media when fused with the alpha mating factor (Colussi 2005). Yeast, however, have a different preference

for codon usage than do mammals (see Figure 2), so the HNE gene was commercially

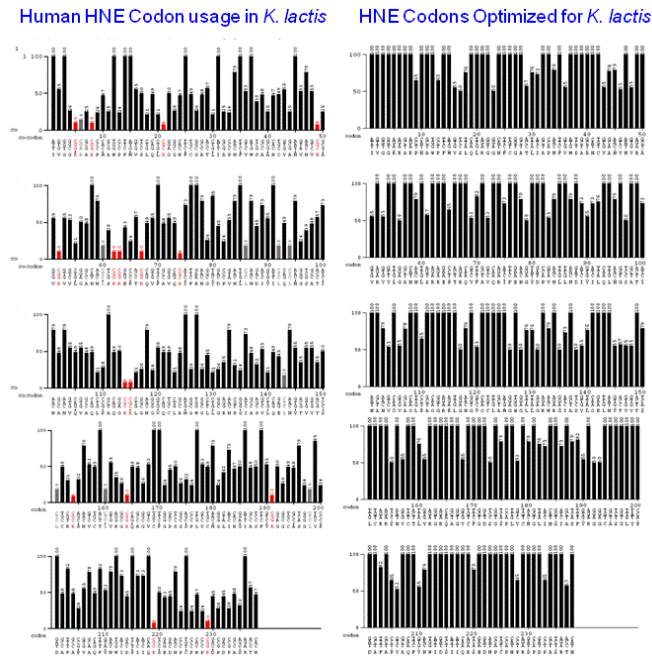synthesized (MrGene) to optimize codon usage for expression in yeasts.

Figure 2: Yeast prefer a different codon usage then do mammals. The HNE gene was thus codon optimized for its use in *K. lactis* cells.

PCR was used to engineer the HNE gene downstream of the gene for eGFP

(enhanced green fluorescent protein) with an intervening enterokinase (D4K) cleavage

site. This DNA construct (eGFP-D4K-HNE) was cloned into the *Kluyveromyces lactis*

(K. lactis) pKLAC1 vector, downstream of the alpha mating factor. Sequencing

confirmed the correct construction of the pKLAC1-eGFP-D4K-HNE vector (see Figure
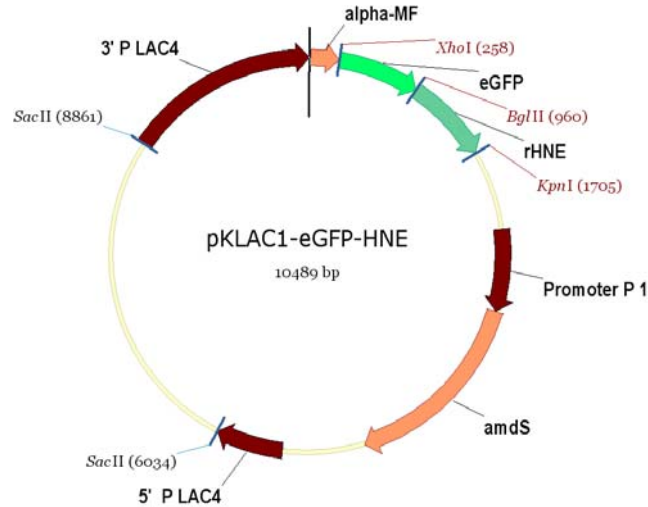
3).

Figure 3: The vector map of pKLAC1-eGFP-D4K-HNE.

Chemically competent GG799 cells (a strain of *K. lactis*) were transformed with the

linearized pKLAC1-eGFP-D4K-HNE vector. The GG799 cell line was chosen because

of its availability in a commercial kit from New England Biolabs (van Ooyen 2006).

Theoretically, the gene of interest integrates into the yeast genome via sequences in

pKLAC1 that are homologous with the LAC4 gene under control of the LAC4 promotor,

which is activated by galactose utilization. The Kex2 protease cleaves the alpha mating

factor from the fusion protein as it passes through the Golgi prior to secretion of the

eGFP-D4K-HNE fusion. Additionally, acetamide selection was chosen because "*K.

lactis* cells have a limited ability to process acetamide" (van Ooyen 2006) as a nitrogen

source. The pKLAC1 vector, however, contains the *Aspergillus nidulans* gene

acetamidase (amdS) that codes for a protein that allows only transformants to grow on

plates with acetamide as the sole nitrogen source. Thus, when acetamide is incorporated

into nitrogen-free growth plates, non-transformed cells will die (Read 2007).

Objective

Once a sequenced DNA vector resembling pKLAC1-eGFP-D4K-HNE was constructed and verified through sequencing, the vector was linearized and inserted into the *K. lactis* yeast system GG799. The vector is "targeted to insert into the promoter region of the *LAC4* chromosomal locus" (Read 2007). Transformed cells were grown on agar media containing only acetamide as a nitrogen source. This selection method is not as ideal as antibiotic selection because the non-transformed *K. lactis* has low levels of endogenous acetamidase. However positive transformants grow much better on acetamide plates and after about five days the transformed colonies are large enough to pick and re-plate.

Materials & Methods

pKLAC1 Vector and eGFP-D4K-HNE Insert.

The pKLAC1 vector and the eGFP PCR DNA were cut with BglII and XhoI and gel purified. The eGFP was then ligated into the pKLAC1 vector. The D4K-HNE gene was synthesized commercially with the proper restriction sites on either side of the gene. The pKLAC1-eGFP vector was then cut at the BgIII and the KpnI sites, and the D4K-HNE was ligated into the vector. The full vector (eGFP-D4K-HNE) was then transformed into *E. coli*, and transformants were selected on LB agar plates with Carbenicillin. Positive growth colonies were detected via colony PCR using primers specific for the PCR insert. Sequencing confirmed that the eGFP-D4K-HNE DNA sequence was correct and in frame with the vector's Alpha mating factor start codon (see Figure 3).
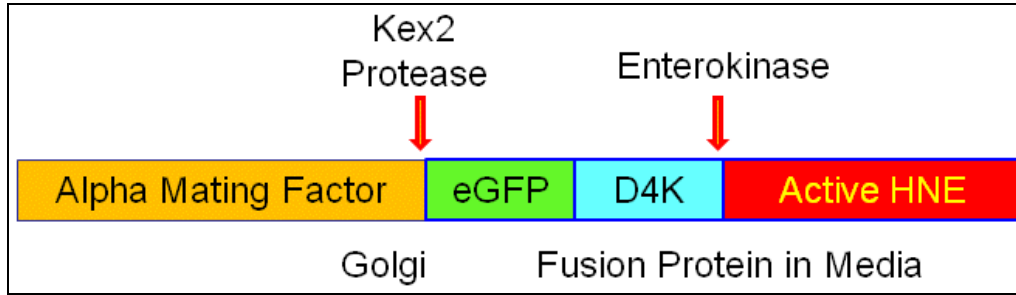
Figure 3: HNE vector construct downstream of the Alpha Mating Factor.

Linearization of pKLAC1 vector

Following a restriction digest protocol from Fermentas, the restriction enzyme SacII was chosen to linearize the pKLAC1 vector for transformation. The SacII restriction sites are situated on either side of the insert of interest. This positioning allows for the full insert to remain intact upon linearization. Both the pKLAC1-eGFP and the pKLAC1-eGFP-HNE vectors were linearized. The concentration of the pKLAC1-eGFP-HNE vector to begin with was 957 µg/ml, and about 10 µg were cut in a total volume of 20 µl. Added to 10 µl of DNA was 1 µl of SacII enzyme, 2 µl of buffer, and 7 µl of sterile water. The pKLAC1-eGFP vector was at a starting concentration of 348.5 µg /ml. Again 10 µg of DNA was cut. Added to 28 µl of DNA was 1 µl of SacII enzyme, 2 µl of buffer, and 9 µl of sterile water to bring the reaction volume up to 40 µl. Each reaction mixture was incubated at 37°C for one hour followed by a heat inactivation at 65°C for twenty minutes. Cut samples were then placed in the refrigerator for storage.

Competency and Transformation of YAP-3 and YPS-7 cells

When research was begun, one goal was to find or create a protocol that made protease deficient YAP-3 and YPS-7 *K. lactis* cells competent and able to take up the target DNA. Several months were spent trying different procedures from various papers and publications in order to make some progress on this path. In the end, however, none of the attempts successfully achieved positive transformants. Briefly, a few of the most promising procedures follow.

Zymo Method

A competency/transformation kit was purchased from Zymo Research. This kit is one type of universal transformation kit in which Zymo provides certain solutions, numbered one through three, that aid in both the chemical competency and the chemical transformation of certain yeast cells. The protocol included in the kit was followed. To start, a 10 ml YPD culture of both YAP-3 and YPS-7 cells was shaken overnight. A 1:10 dilution was performed the next morning, and the cultures were allowed to continue shaking at 30°C for four hours. Once the O.D. of each culture reached between 0.8-1.0, the cells were centrifuged at 5000 x g for four minutes. The cells were resuspended in 10 ml of EZ 1 solution, and the centrifugation was repeated. Finally, the cells were resuspended in 1 ml of EZ 2 solution to complete the competency procedure. Aliquots of 500 μl were made, and the cells were stored at -80°C for future use.

To begin the transformation procedure, frozen competent cells were thawed on ice. Next, 50 μl of competent cells was mixed with 2 μg of DNA. Two cell mixtures

were made for each cell line. One mixture of YPS-7 cells was mixed with the pKLAC1-eGFP-HNE vector and another with the pKLAC1-eGFP vector. The same procedure was performed on the YAP-3 cells. Added to each mixture was 500 μl of EZ 3 solution. Each mixture was then incubated at 30°C for 45 minutes while vortexing each cell mixture every 15 minutes. After the incubation, 100 μl of each transformed cell mixture was spread on YCB + 5mM Acetamide + 50 μg/ml Ampicillin plates and placed at 30°C for growth. Also plated was 50 μl of competent cells that had not been transformed. This plate served as a negative control. After several days no growth was seen on any plates.

Electroporation

Several papers were studied in order to come up with a suitable method for electroporation. The main concern was the machine used and the parameters that it allowed. Starting with a concentration of about 4 μg of DNA per 10 ml, combined with 50 µl of cells was 1 µl of DNA. Several different mixtures using different media were carried out at once:

YAP-3 cells in YED:

1. Cells only

2. pKLAC1-eGFP

3. pKLAC1-eGFP-HNE

YAP-3 cells in YPD:

1. Cells only

2. pKLAC1-eGFP

3. pKLAC1-eGFP-HNE

YPS-7 cells in WM9:

1. Cells only

2. pKLAC1-eGFP

3. pKLAC1-eGFP-HNE

YPS-7 cells in YPD:

1. Cells only

2. pKLAC1-eGFP

3. pKLAC1-eGFP-HNE

The DNA and cells were first combined in PCR tubes and incubated on ice for 15 minutes. Each reaction mixture was then transferred to 0.2 cm cuvettes. The reaction conditions were as follows: R=129 ohms, C=40 µF (fixed by machine), and V=1.1 kV. In some electroporation procedures the voltage was kept at 1 kV. The 0.1 increase was in the hopes of getting more effective transformations. The time constant for all mixtures ranged from 4.5 to 4.8 ms. After each sample was electroporated, 500 µl of YPD and 500 µl of 1M Sorbitol were added to each tube. The entire 1 ml sample was then transferred to an Eppendorf tube, and all samples were incubated on ice for 15 minutes and then shaken at 225 r.p.m. and 30°C for one hour. Possible transformants were then plated on YCB + 5 mM Acetamide plates + 50 µg/ml Carbenicillin and left at room temperature to grow. YCB stands for yeast carbonbase and is essentially media that contains glucose, vitamins, and minerals but no viable nitrogen source. No transformants were seen after several days of growth (Sanchez 1993).

Single Stranded Carrier DNA

A culture of wild type YAP-3 and a culture of YPS-7 cells were begun by inoculating 25ml of 2X-YPD with a sample of each cell type. These samples were

shaken overnight at 30°C. The following morning, the density of each culture was

checked, and a 1:100 dilution was performed to bring the O.D. of the cultures to around

0.1. The cultures were again placed in the incubator at 30°C with shaking for several

hours. The O.D. of each culture was continually checked throughout the day, and the

cells were harvested when they reached an O.D. of between 0.8 – 1.0.

Then, the carrier DNA was placed in a boiling water bath for five minutes to

allow for the DNA to denature. The sample was then immediately placed in an ice water

bath. Calf Thymus double stranded carrier DNA at a concentration of 1.6mg/ml was

obtained from Dr. Musich. Additionally, 2ml of each culture sample was harvested and

pelleted at 13,000 r.p.m. for 30 seconds. The supernatant was discarded and a total

volume of Transformation Mix (T Mix) was then added. The T mix included the

following, added in this order: 240μl of polyethylene glycol 3350 (50%), 36μl

Lithium acetate (1M), 50μl of boiled salmon sperm carrier DNA, 2μl of pKLAC1-GFP-

HNE vector, and finally 32μl of sterile water. For the control, the T mix included: 240 μl

of polyethylene glycol 3350 (50%), 36 μl lithium acetate (1M), 50 μl of boiled carrier

DNA, 4 μl of pKLAC1-GFP vector, and finally 30 μl of sterile water. Each reaction

mixture ended with a total volume of 360 μl. After the T Mix was added, each mixture

was placed in a 42°C water bath for two hours. Once the two hour incubation was

complete, the cells were pelleted at 13,000 r.p.m. for 30 seconds, and then resuspended in

1ml sterile water. From this, 100 μl of cells was plated onto YCB + 5mM Acetamide +

50mg/ml Ampicillin plates and placed at 30°C to induce growth. No growth was seen

(Geitz 2007).

Transformation of GG799 K. lactis cells

To begin the transformation process, the pKLAC1-eGFP-HNE vector had to be linearized. A multiple cloning site was also present within the pKLAC1 vector in a position that allowed our target gene to be sandwiched within the LAC4 promoter upon transformation. This "linear expression cassette…integrates into the promoter region of the *LAC4* locus of the *K. lactis* chromosome upon its introduction into *K. lactis* cells" (Colussi 2005).

After the pKLAC1 vector was linearized, a tube of competent GG799 cells from New England Biolabs was thawed on ice. Aliquoted out into three tubes was 50 μl of cells to which was added 155 μl of NEB Yeast Transformation Reagent. Each tube was shaken. Next, 1 μg of DNA was added to each tube. To the first tube, 1 μg of pKLAC1-eGFP was added and to the second tube, 1 μg of pKLAC1-eGFP-HNE was added. The third tube of cells was used as a negative control to which no DNA was added.

The mixture was incubated at 30°C for 30 minutes. After this, the cell mixtures were heat shocked by incubation at 37°C for one hour. The cells were then pelleted by microcentrifugation at 7000 r.p.m. for two minutes, and the supernatant was discarded. The pellet was resuspended in 1 ml of sterile deionized water, and the cells were then repelleted by microcentrifugation at 7000 r.p.m. for two minutes. The supernatant was again discarded. Again the pellet was resuspended, but this time in 1 ml of YPD, and the

entire cell mixture was transferred to a sterile culture tube. The culture tubes were incubated with shaking at 30°C for 30 minutes.

After the incubation period, the mixture was transferred to 1.5 ml microcentrifuge tubes, and the mixture was again repelleted by microcentrifugation at 7000 r.p.m. for two minutes. Once pelleted, the cells were washed twice in 1 ml sterile deionized water followed by microcentrifugation. Finally, the cell pellet was resuspended in 100 μl of sterile deionized water. From this, 80 μl of solution was plated on YCB + 5mM Acetamide + 50 μg/ml Cipro and Ampicillin. The remaining 20 μl was plated onto YPD plates as a positive control. Each plate was placed at 30°C for growth. Growth took several days. Once adequate growth had occurred, colonies were picked and transferred to YPGal plates. These plates contained galactose which theoretically activates the HNE. Each colony was then subjugated to whole-cell PCR to see if the cells did, in fact, contain the insert of interest.

Whole-Cell PCR

From each colony on the YPGal plates, a small sample was taken and resuspended in 25 μl of 1 M Sorbitol containing 2 mg/ml Lyticase. The Lyticase simply breaks the cells apart so that the DNA inside can be reached. Each sample was mixed by vortexing and placed in a 37°C water bath for one hour. Next, the samples were lysed in a thermocycler at 96°C for 10 minutes. Then, a colony PCR was conducted. In addition to the 25 μl DNA template, which is genomic DNA obtained from the Lyticase treated cells, 10 μl of Primers 1 and 2 were added, along with 5 μl of Protease free water, and 50

μl of reaction mix (GoTaq). The samples were placed in a standard PCR thermocycle heat block, and a colony PCR reaction was run.

Once the PCR reaction was complete, a FlashGel was run on the samples. The FlashGel is an easy and fast way of telling whether the target DNA was isolated and replicated by the PCR. In the wells, 4 μl of sample were combined with 1 μl of the FlashGel loading dye. The gel was then run for seven minutes. No bands corresponding to an insert were seen in any of the samples.

Results

In the past, the yeast *Kluyveromyces lactis* was used to express many recombinant proteins because of its ability to secrete correctly folded proteins into media (Swaim 2008). In order to bioengineer the correct DNA sequence for the HNE construct, however, the HNE sequence had to be commercially synthesized because yeast prefer different codon usages than do mammals. This technique is known as codon optimization. The company Mr. Gene was used to codon optimize the gene for human neutrophil elastase. The optimized DNA is known as cDNA because it is an artificial gene that contains no introns that native genes contain. These introns are removed via mRNA splicing in native genes. Therefore, the cDNA transcribes directly into the mature mRNA. Once the correct sequence was made, it was fused with the sequence for enhanced green fluorescent protein (eGFP). Linking the eGFP and HNE sequences is an enterokinase (D4K) cleavage site that once activated could be targeted to activate HNE into the media. This DNA construct (eGFP-D4K-HNE) was then fused downstream of

the alpha mating factor in the *K. lactis* pKLAC1 vector, because when secreted, the alpha mating factor carries the attached proteins into the media. This vector was then sequenced in order to confirm the correct DNA sequence.

Chemically competent GG799 cell strains of *K. lactis* were then transformed with the pKLAC1-eGFP-D4K-HNE vector using a procedure from NEB. Theoretically, the gene is able to integrate into the yeast's genome via homologous regions within the LAC4 promoter which is a transcription promoter for beta-galactosidase (Colussi 2005). The sugar galactose activates the HNE. For selection, acetamide was incorporated into the YCB agar plates. Untransformed *K. lactis* cells are unable to use acetamide and die, however, the pKLAC1 vector also contains the gene *Aspergillus nidulans* acetamidase (amdS) which codes for a protein that allows transformants to convert the acetamide into a viable nitrogen source, so only transformed cells live. After attempting the NEB transformation multiple times, no transformants were seen. Communication between this lab and experts at NEB who specialized in the *K. lactis* system was helpful in that the experts provided invaluable advice on the transformation protocol and the mistakes that may have been made. Despite the help, no positive transformants were ever grown.

Discussion

The goal of this research was to be able to produce stable, active human neutrophil elastase using the *K. lactis* yeast system. To begin, protease deficient YAP-3 and YPS-7 cell lines were used in an attempt to transform them with our target DNA sequence (alpha mating factor-eGFP-D4K-HNE). Protease deficient cell lines were used

because it was initially thought that there would be less of a chance that the target enzyme (HNE) would be severed and thus degraded by proteases present in the secretory pathway. This process proved difficult for two reasons. One, no method attempted was able to provide competent YAP-3 and YPS-7 cells to start which meant that no protocol was successful in producing positive transformants, our second problem. Everything from electroporation techniques to simple chemical techniques were attempted, however none produced positive results. Also, it was shown that if casamino acids were added to the growth media for these cells, proteases present in the secretory pathway had enough amino acids to consume and would presumably not affect the enzyme in question. In addition to this revelation was the knowledge that the selection on acetamide plates was not as stringent as antibiotic selection.

For selection, our vector contained a gene for acetamidase which in theory allowed only true transformants to be able to grow on the YCB + Acetamide plates. To grow, the acetamidase gene allows transformants to utilize the acetamide present in the plates and convert it into a viable nitrogen source for growth. All other cells would theoretically die as they had no way to obtain the necessary nitrogen. Growth was seen on the plates, however, even when the cells that had not undergone transformation were plated on them. Upon speaking with an expert on the *K. lactis* system, however, it was discovered that wild type *K. lactis* strains contain a native acetamidase gene which allows even untransformed cells to utilize the acetamide in the agar plates. These cells, however, have a low basal level of expression so that growth is not seen until several days after plating. Positive transformants grow faster too because they can metabolize

acetamide much more quickly. A background lawn of cells was expected but not

individual colonies on these negative controls (see Figure 4).
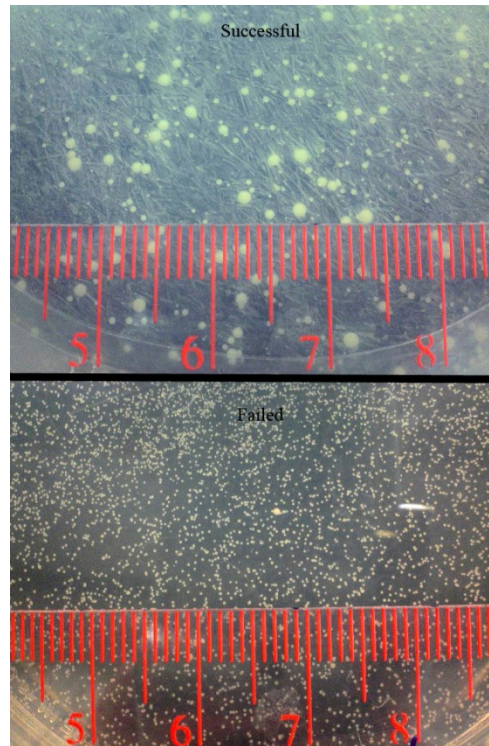

Figure 4: The top plate shows what the colonies from a successful transformation look like. The bottom plate shows a lawn of cells which means the transformation failed.

Furthermore, cells did grow on the plates where transformants should have been

seen. Colonies selected on acetamide plates were transferred to YCB galactose plates to

induce expression. Once viewed under the fluorescent microscope, however, the cells

did not glow green meaning that the cells were not positive transformants. In effect, the

big question became how are the cells able to grow on the acetamide plates even without

the acetamidase gene? One possibility was the fact that in many of the protocols

attempted, the cells were incubated in YPD media directly before the plating step. Without a wash step in sterile deionized water, it was thought that perhaps the media had provided enough nitrogen for cells to begin to grow on the plates even without the ability to convert acetamide into nitrogen. While the use of YAP-3 and YPS-7 cells was ultimately abandoned, the issue of non-transformants growing on the acetamide plates persisted. Because the YAP-3 and the YPS-7 cell lines proved difficult to make competent as well as to transform, another *K. lactis* cell line was chosen, GG799. These cells were not protease deficient but were purchased chemically competent. Following the NEB procedure for transformation, however, proved fruitless as the supposed transformed cells did not, in fact, contain our DNA of interest. To determine this, however, a new method of identification had to be found. The use of the fluorescent microscope had proved subjective in the past because the shade of green of positive transformants varied so much. Therefore, human error was a great factor in determining whether or not the cells contained the vector of interest. Instead PCR was used to amplify the insert, but no insert was seen after the PCR results were run on an agarose gel (1.2% Lonza FlashGel).

Recently, a paper was found that discussed the secretion results of different proteins all fused with the α-mating factor. After much experimentation, these researchers discovered that the α-factor was unable to secrete when attached to green fluorescent protein (Kjaerulff 2005). Furthermore, with more investigation it was also found that the Kex2 site, which was placed between the alpha mating factor and the eGFP, showed a prevalence for cutting at locations within an amino acid sequence that

contained either the amino acids lysine and arginine beside each other or two arginines

side by side (Bevan et al., 1998). Because the original amino acid sequence of HNE

contained approximately four of these double arginine sites, it was reasonably thought

that the Kex2 might be fragmenting the HNE as it traffics through the Golgi.

To correct for these measures, new inserts were codon optimized and

commercially synthesized (Genscript). To begin, the pKLAC1 vector was modified by

replacing the alpha mating factor it originally contained with an optimized insert

containing alpha mating factor linked with rubredoxin and six histidines (Rub-6His). The

codon optimized insert D4K-HNE was then inserted into the multiple cloning site of this

new vector (named pKaR). Before being commercially synthesized, however, the three

dimensional figure of HNE was studied and the double arginine sites were found and

modified. The goal was to substitute other amino acids in for an arginine when two

arginines appeared together. The hydrogen bonding of the protein had to be taken into

consideration, however, so only those residues that did not participate extensively in the

hydrogen bonding of the protein were changed. Furthermore, similar amino acids

replaced the arginines such as asparagine which has a neutral charge but can still

participate in hydrogen bonding to some degree. The replacement of these arginines with

amino acids such as asparagine also lowered the isoelectric point of the protein, which

will most likely aid in its production.

With the new construct (pKaR-HNE), *K. lactis* competent GG799 cells were

transformed by the protocol from New England Biolabs. Frozen cells were thawed on ice

and mixed with DNA and NEB Yeast Transformation Reagent as per NEB's protocol. At the end of the protocol, the cells were plated onto YCB + 5mM Acetamide plates, and after a few days of growing at 30°C, positive colonies were seen. A few selected colonies were used to inoculate 2ml WM9Gal media. Theses cultures were used to extract the genomic DNA.

Acid glass beads (0.5mm diameter) were used along with solutions of Phenol/Chloroform/Isoamyl alcohol to extract the genomic DNA from the transformed cells. Once the DNA was extracted, agarose gels were run on the samples to test the integrity of the DNA. While there was some fragmentation, there was in-tact genomic DNA. Now a colony PCR will be run on the genomic DNA to ensure that the transformed cells do in fact contain the gene of interest.

Acknowledgments

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Brief Biography of Haley Klimecki

Born in Canton, Ohio on January 22, 1988 and raised in Chattanooga, Tennessee, Haley Marie Klimecki is the daughter of Kerry and Kelly Klimecki. Early on in her high school career, she knew she wanted to become a physician, and the work she performed for her senior project solidified this desire in her. Working in a pediatric oncology clinic at T.C. Thompson Children's Hospital, Haley was exposed to a number of doctors, patients, and families who became like family to her. Also, the experience proved to her that doctors could be more than "healers" as she witnessed these oncologists take an active role in their patients' fights, and this inspired her to work hard toward a career in medicine. Graduating in May 2006, Haley was the salutatorian of Red Bank's class of 2006. In the fall of that year, she began classes at East Tennessee State University as an Honors Scholar, and for the next four years spent a great amount of time studying hard and shadowing and volunteering in various hospitals. The summer after her freshmen year she was accepted into the Premedical-Medical Program at ETSU and during her junior year she began work at Quillen College of Medicine under Dr. David Johnson in the Biochemistry and Molecular Biology Department. Here she experienced one of the most challenging yet rewarding times in all of her college career. While things did not always go as planned as she and other students attempted to produce and express recombinant proteins, she learned the value of working hard, admitting mistakes, and coping with disappointment for the people who came to be mentors to her. Yes the work was frustrating at times, but she never wanted to give up because she knew the people she worked with depended on her to pull her own weight just as much as she depended on them for guidance and support. Furthermore, she feels that this team approach will

greatly impact her not only in the next stage of her education, medical school, but also in her career as a physician. And despite the struggles, her time in the lab remains one of her most shaping experiences during her college years. Being a chemistry major, Haley was especially excited to learn about the chemistry of the body and feels as if the Biochemistry of Metabolism class she took was one of the most valuable classes she took as an undergraduate. Science is not her only interest, however, and one of the best decisions she made was to become a history major in college. This program of study brought some interesting people into her life, and she enjoyed learning about the histories and cultures of various people around the world. And looking back over the past four years, Haley has realized how much college and the people she has met have shaped her into a new and more genuine version of herself.