Analysis of *Acanthamoeba's* Adhesion to Host Cells Via Mannose Binding Protein

A Senior Honors Thesis

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By

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Abstract

Acanthamoeba is a ubiquitous opportunistically pathogenic organism that can cause a severe ocular infection (*Acanthamoeba* keratitis) and a fatal infection of the central nervous system (granulomatous amoebic encephalitis). *Acanthamoeba* is thought to bind to host cells via the mannose binding protein (MBP) and produce a potent cytopathic effect (CPE) which leads to targeted cell death. Recent studies have shown that *Acanthamoeba's* pathogenic potential directly correlates with MBP expression and that MBP has the potential to serve as a marker for pathogenicity, however the mechanism of variable pathogenicity in *Acanthamoeba* isolates is still unknown. We hypothesize that the differences in pathogenicity of *Acanthamoeba* in humans is due to differences in MBP sequence between isolates of *Acanthamoeba* and that the capacity of *Acanthamoeba* to bind to human cells may be the main factor in its ability to initiate infection. Different isolates of *Acanthamoeba* were cultured, the DNA extracted, and partial MBP gene was amplified using PCR. The MBP amplimers were then sequenced. Analysis of the DNA sequences of the MBP of *Acanthamoeba* revealed differences between the DNA sequences of MBP between different isolates of *Acanthamoeba*. The results suggest that differences within the mannose binding protein may lead to different levels of pathogenicity within *Acanthamoeba*. Future research will be geared towards understanding how MBP is related to the cytotoxic effect as well as determining if MBP is the sole contributor to *Acanthamoeba's* ability to bind to cells.

Introduction

Free-living amoebae of the Genus *Acanthamoeba* are eukaryotic organisms that are ubiquitous and are considered opportunistic pathogens (11). *Acanthamoeba* has been found in nearly every natural environment including water reservoirs and storage tanks, beaches, body surfaces and tissues, fruits and vegetables, drinking water, soil, bottled water, tap water and factory discharges (11). It has also been found in the cold climate of Antarctica. This is significant because it demonstrates that *Acanthamoeba* can survive at frigid temperatures and in a very dry atmosphere (4). *Acanthamoeba* has also been found in man-made and clinical environments like air-conditioning units, Jacuzzi tubs, and in hospitals (13).

Amoebae were first discovered in the 1870's and were classified as a new genus based on their crawling-like movement, now termed amoeboid motion (17, 19, 20). Amoebae are a group of protozoa that moves by means of pseudopods (17, 19, 20). Amoebae are classified into many different groups including both potent parasites and opportunistic pathogens (17, 19, 20). Some examples are *Entamoeba histolytica*, which is a potent parasitic organism that was discovered in 1873 from a patient suffering from dysentery; *Naegleria fowleri,* which is the causative agent of fatal central nervous system infections; and *Sappinia diploidea,* which is a free-living amoeba that was discovered in lizard feces and found to cause granulomatous amoebic encephalitis (GAE). Others include *Acanthamoeba,* which was discovered as a eukaryotic cell culture contaminant in the 1930's and found to cause GAE in the 1970's, and *Balamuthia mandrillaris,* which was discovered in the brain of a baboon that died from meningoencephalitis in 1986 and given a new genus name a few years later (13, 17, 19, 20).

Acanthamoeba has two stages to its life cycle, the reproductive trophozoite (troph) phase and the dormant cyst phase (Fig. 1 & 2). The troph phase dominates when growth conditions are

optimal like an abundant supply of food, neutral pH, cultured at approximately 30˚C and at 50-80 mOsmol (2, 12). In the troph phase, *Acanthamoeba* undergoes asexual reproduction via binary fission (2, 12). *Acanthamoeba* usually encysts, or forms cysts, when it is in harsh environmental conditions such as starvation or variable pH (2, 12). Many morphological changes occur from troph to cyst form including the termination of cell growth and decreased metabolic activity. Cysts have been shown to have decreased levels of RNA, proteins, triacylglycerides, and glycogen compared with trophs (2, 12). When *Acanthamoeba* are encysted, they contain a bilayered cell wall that supports survival in harsh conditions. The outside wall is a laminar, fibrous layer termed the ectocyst, while the inner wall is a layer of fine fibrils termed the endocyst (2, 12, 13).

Acanthamoeba preys on unicellular filamentous algae, bacteria, and yeast, feeding by either pinocytosis or phagocytosis in the troph stage (13). Pinocytosis is an energy-dependent process that is used in the uptake of large volumes of solutes. Phagocytosis is also an energy-dependent process that is a specialized form of endocytosis (13). *Acanthamoeba* phagocytizes microorganisms such as *Enterobacter aerogenes* and other organic molecules for nutrients. The phagocytized microbes and organic molecules are then broken down by lysosomes located in vacuoles within the *Acanthamoeba* (13).

Acanthamoeba was traditionally classified into three groups based only on two characteristics, the cyst size and the number of arms within a single cyst (13). Group one has four species which exhibit large trophs, while group two has eleven species of the most commonly isolated and widespread *Acanthamoeba*. Group three has five species that have a thin ectocyst and a larger endocyst (13). Now, *Acanthamoeba* is classified based on its 18s ribosomal DNA sequence into 15 different genotypes (T1-T15) because the rDNA genes are highly

conserved, precise and reliable (3, 18). Each genotype exhibits 5% or more divergence between different genotypes. However, this is an arbitrary number and can be subject to change based upon morphology and complementarity of certain genes. Within these fifteen genotypes, there are at least twenty-four named species as well (13, 18).

Acanthamoeba is very important to humans for many reasons, one being that it is a relatively newly-discovered organism. *Acanthamoeba* was discovered in 1930 but was not shown to be infectious until 1954 (13). It was found to cause GAE in 1972 and *Acanthamoeba* keratitis (AK) in 1973, two very serious diseases of the central nervous system and eye respectively (13). AK is a severe ocular infection caused by *Acanthamoeba* (12). A key risk factor to AK infection is the use of contact lenses that are exposed to contaminated water (16). In AK, the eye's cornea becomes inflamed and is characterized by intense pain and usually leads to impaired sight (12, 15, 16).

Acanthamoeba expresses a mannose-binding protein (MBP) on its surface, which specifically binds to mannose-containing glycoproteins on the surface of the eye (6). When the eye becomes injured, as can occur through contact lens use, the number of mannose-containing glycoproteins increases (11). *Acanthamoeba* can then enter the damaged eye and bind to the mannose-containing glycoproteins and induce a cytopathic effect (CPE) due to the production of serine proteases by *Acanthamoeba* that will destroy corneal epithelial cells (16). If infected with AK, one will most likely go blind without treatment (9, 12). If detected early, AK can be treated with polyhexamethylene biguanide and propamidine isethionate (11, 13). This, in conjunction with antibiotics like neomycin or chloramphenicol, has been shown to be a highly effective treatment against AK (13).

GAE is a very rare infection of the central nervous system that has a fatality rate of well over 90% (13). Although the precise mechanisms of pathogenicity are unclear, GAE is mostly seen in immunocompromised individuals (1). It is hypothesized to enter the body through skin lesions or the nasal passages and enter the brain at the blood-brain barrier (1). GAE is usually not diagnosed until post-mortem due to its low sensitivity and prevalence, and it has very limited treatment, which has little effect (1, 13).

Interest in *Acanthamoeba* has been increasing recently within the scientific and medical communities. This is partially due to the fact that the number of *Acanthamoeba* infections is increasing (13). It is also due to the fact that *Acanthamoeba* serve as a host or reservoir for microbial pathogens including viruses, prokaryotes, other protozoa and fungi (13). The role of *Acanthamoeba* within the ecosystem is also a heavily researched topic and all of this interest that has been generated has led to discoveries about its function and mechanisms of pathogenicity.

Genetically, there is a lot of uncertainty surrounding *Acanthamoeba*. The genome project to sequence the entire genome of an *Acanthamoeba* isolate is well underway, but not yet completed. While *Acanthamoeba* has been shown to have ample amounts of DNA, its ploidy level is still unknown as is the number of chromosomes *Acanthamoeba* carries (13).

As mentioned above, *Acanthamoeba* expresses a mannose binding protein on its plasma membrane. The MBP is thought to play an important role in the pathogenesis of the infection by mediating the adhesion of *Acanthamoeba* to the host cells (6). After adhesion, *Acanthamoeba* produces a CPE through the signaling of serine proteases leading to apoptosis of infected cells (6). It has been shown that the adhesion of *Acanthamoeba* to corneal epithelial cells can be inhibited by free methyl-α-mannopyranoside but not by a number of other sugars (5, 10, 11, 14,

20, 21). The mannose binding protein is approximately a 400-kDa protein composed of multiple 130-kDa subunits (6). The MBP gene itself is composed of six exons and five introns that span 3.6 kb of the amoeba's genome which codes for a precursor protein of 833 amino acids in length (Fig. 4 & 5). The protein is thought to contain a signal sequence (residues 1-21 aa), an *N*-terminal extracellular domain (residues 22-733 aa), a transmembrane domain (residues 734-755 aa), and a *C*-terminal cytoplasmic domain (residues 756-833 aa) (6).

Previous research has shown that *Acanthamoeba* MBP is a novel protein with little homology to any known protein and that it is a transmembrane protein with characteristics of a typical cell surface receptor (6). *Acanthamoeba* MBP adheres to host cells by binding to mannose-containing glycoproteins located on the surface of host cells and is itself a mannose-containing glycoprotein (6, 7). However, MBP is only expressed during the infective troph phase and not the dormant cyst phase, which might explain why cysts lack the ability to bind to host cells (6, 7). The pathogenic potential of *Acanthamoeba* directly correlates with the expression level of MBP and MBP has the potential to serve as a marker for pathogenicity (8). We hypothesize that the differences in pathogenicity of *Acanthamoeba* in humans is due to differences in MBP sequence between isolates of *Acanthamoeba* and that the capacity of *Acanthamoeba* to bind to human cells may be the main factor in its ability to cause disease and death. In this project, we examine the differences in the MBP sequence between pathogenic and nonpathogenic *Acanthamoeba* isolates to determine if differences in the MBP, which could lead to some *Acanthamoeba* being better adapted to bind to host cells, is correlated with the relative pathogenicity of these isolates. Understanding the method with which *Acanthamoeba* attaches to host cells is the first step in learning how the binding of *Acanthamoeba* to human cells could be inhibited.

Methods

Culturing of *Acanthamoeba*:

Five different genotypes of *Acanthamoeba* were cultured which included nine different isolates (Table 1). The first two numbers of each isolate correspond to the year that they were obtained and the last three correspond to the order in which they were obtained.

OSU Isolate Name	Source	Genotype
03-001 (OSU Neff)	ATCC 30010 Environ	T4
03-027	Skin biopsy	T4
07-072	AK case	T4
08-008	AK case	T4
07-069	AK case	T4
08-001	Dog Kidney	T1
07-029	Corneal Scraping	T10
03-028	Environ	T7
07-101	Unknown	T4

Table 1: *Acanthamoeba* isolates cultured

Non-nutrient Amoeba Saline (NNAS) was used as our culture medium. Preparation of NNAS is as follows:

Compound	Stock Solution (mol/L)	Final Concentration (mol/L)
NaCl	0.2	0.002
$CaCl_2$	0.0036	0.000036
$MgSO_4$	0.0033	0.000033
Na_2HPO_4	0.1	0.001
KH_2PO_4	0.1	0.001

Table 2: Stock Solutions used in the preparation of NNAS

10 mL of each stock solution was added to 950 mL di-H_2O and autoclaved. Plates were also prepared using NNAS and 18 grams of agar per liter of NNAS. *Enterobacter aerogenes* was used as the *Acanthamoeba* prey. Preparation of *Enterobacter aerogenes* solution is as follows:

Enterobacter aerogenes was grown on Luria agar plates and resuspended in 1 mL of NNAS.

The nine isolates were each added to a flask for growth: 20 mL of NNAS was added to the flask along with 200 µL of *Enterobacter aerogenes* solution and 25 µL of *Acanthamoeba* in NNAS. All of the *Acanthamoeba* isolates were incubated at 30˚C until *Acanthamoeba* reached a density of 1 x 10^7 cells/mL and were ready for DNA extraction as determined by microscopic inspection of the flasks.

Extraction of *Acanthamoeba* DNA:

Each *Acanthamoeba* isolate's DNA was extracted using the Qiagen DNeasy© Blood and Tissue Kit.

Amplification of the MBP gene using PCR:

Each *Acanthamoeba* isolate's MBP DNA was amplified using the Polymerase Chain Reaction (PCR) from genomic DNA. The reaction for each PCR is listed in table 3.

Component	Volume (microliters)
DNA Template	5.00
MBP Primer 5'	1.00
MBP Primer 3'	1.00
Standard PCR Reaction Buffer	2.50
dNTPs	4.00
Taq DNA Polymerase	0.25
Water	11.25
Total	**25.00**

Table 3: Standard PCR Reaction Components

MBP primer sets that were used are listed in table 4.

Primer Set	Direction	Sequence	Annealing Temperature
Set 1	Forward	ATGCGGAGCCTTCCCGTCTT	71.1˚C
	Reverse	TACCCGGAGGACGGCGATCAT	73.5˚C
Set 2	Forward	GGCTTCTACCTCAAGCAGGA	63.4˚C
	Reverse	ACCTTGTTGCCTGGGCAGGT	70.1˚C
Set 3	Forward	AACTACAACGGCAAGTGCGA	65.6˚C
	Reverse	TCTTGTTGTGCGCTTAGTTT	60.1˚C
Set A	Forward	CCTCCAATCCCCACGATACG	68.4˚C
	Reverse	CCACGGCCACAACGAACGCG	72.3˚C
Set B	Forward	AGGGCGAGACCTACGATAGC	66.9˚C
	Reverse	GGGCAGACGCAGTCGCACTT	72.1˚C
Set C	Forward	GGAGGTGTGGGACACGGCCA	70.7˚C
	Reverse	AGGATTAGAGGGTGTGCAGG	63.0˚C

Table 4: MBP primer sets and annealing temperatures

The PCR conditions for each reaction are listed in table 5.

Step	Temperature (˚C)	Time (minutes)
1	95	7
2	95	1
3	58	1
4*	72	2
5	72	15
6	10	pause

Table 5: PCR conditions

*After step 4, the reaction cycled back to step two 39 times before moving on to step 5.

Figure 6 displays the location of the primers within the MBP gene.

Gel electrophoresis:

Agarose gel electrophoresis was used to determine if the correct amplimers were present in the PCR reactions (Fig. 3). The 1% gel was analyzed under ultraviolet light for the presence of 1 kb bands.

Gel Extraction and Purification:

The correctly sized amplimers were then excised using a razor blade over an ultraviolet light box used to visualize the bands. The excised bands were then purified using the Qiagen QIAquick© Gel Extraction Kit. A sequencing PCR reaction was performed on the MBP amplimer. The components and conditions are listed in table 6.

Component	Volume (microliters)
PCR Product	4.00
MBP Primer 5' or 3'	1.00
Big Dye	0.50
Sequencing Buffer	2.00
Water	2.50
Total	**10.00**

Step	Temperature (˚C)	Time
1	94	30 sec
2	50	15 sec
3*	60	4 min
4	10	pause

Table 6: Sequencing PCR Reaction and Conditions

*After step 3, the reaction cycled back to step two 25 times before moving on to step 4.

DNA Sequencing:

The sequencing PCR product was then purified for DNA sequencing using ethanol precipitation and placed into the ABI Prism™ 310 Genetic Analyzer for direct sequencing.

Data Analysis:

The generated sequences were first examined using the electropherograms of each sequencing reaction. The clean sequences were then used in a BLAST search using the National Center for Biotechnology (NCBI) database that incorporates data from the *Acanthamoeba castellanii* isolate sequenced in 2004 (6). The sequences were aligned using MEGA© 4.0 and the number of single nucleotide polymorphisms (SNPs) was determined per isolate along with the number of synonymous and non-synonymous changes to the DNA that led to changes within the translated amino acid. The changes in the type of amino acid (i.e. polar, charged etc.) were quantified and analyzed by hand.

Figures

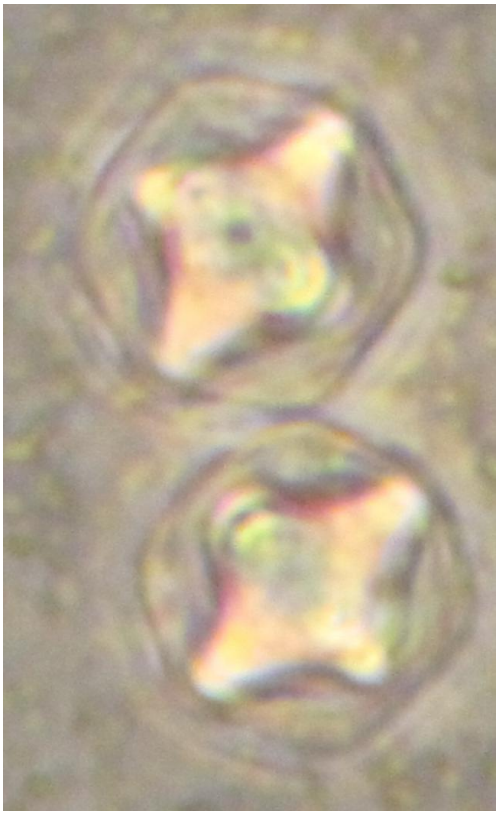

Fig. 1: *Acanthamoeba* Cyst

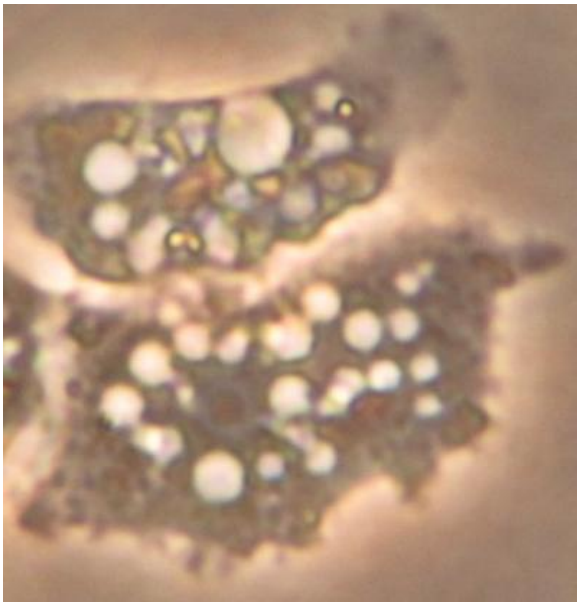

Fig. 2: *Acanthamoeba* Troph

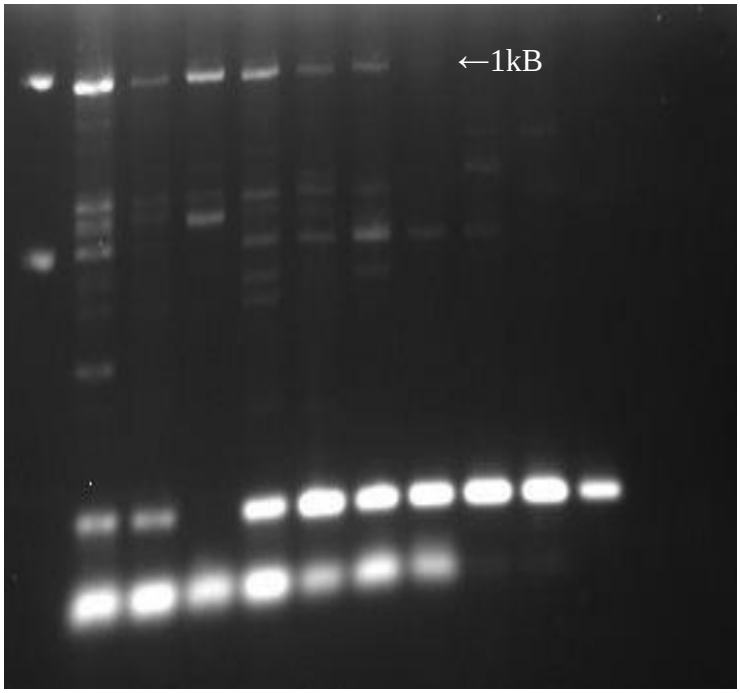

Fig. 3: MBP gel

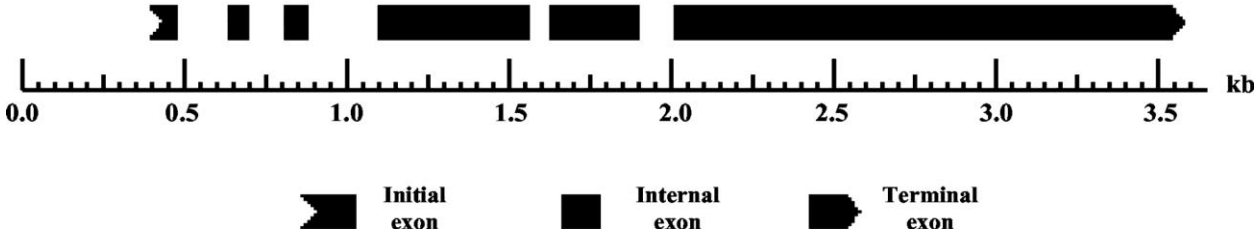

Fig. 4: *Acanthamoeba* Mannose Binding Protein Gene (6)

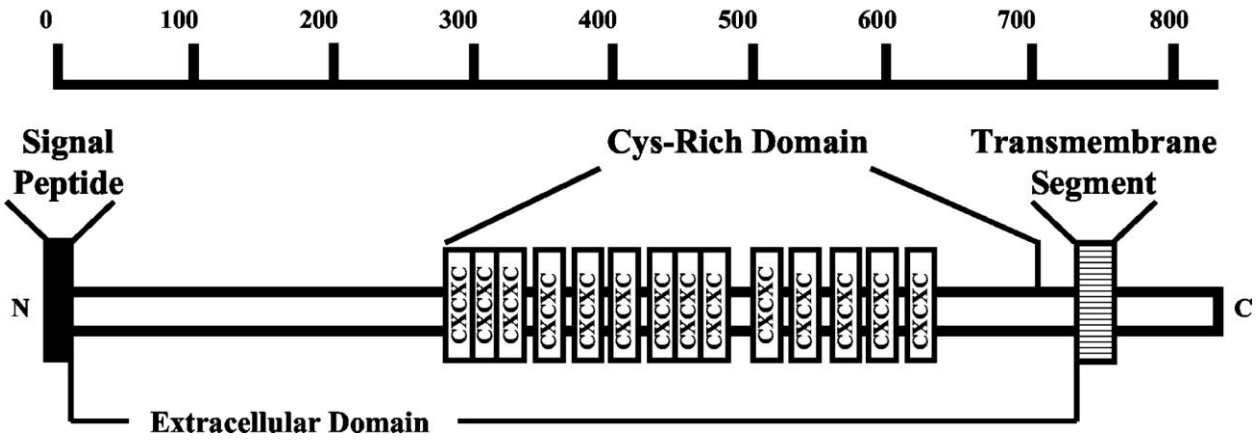

Fig. 5: *Acanthamoeba* Mannose Binding Protein (6)

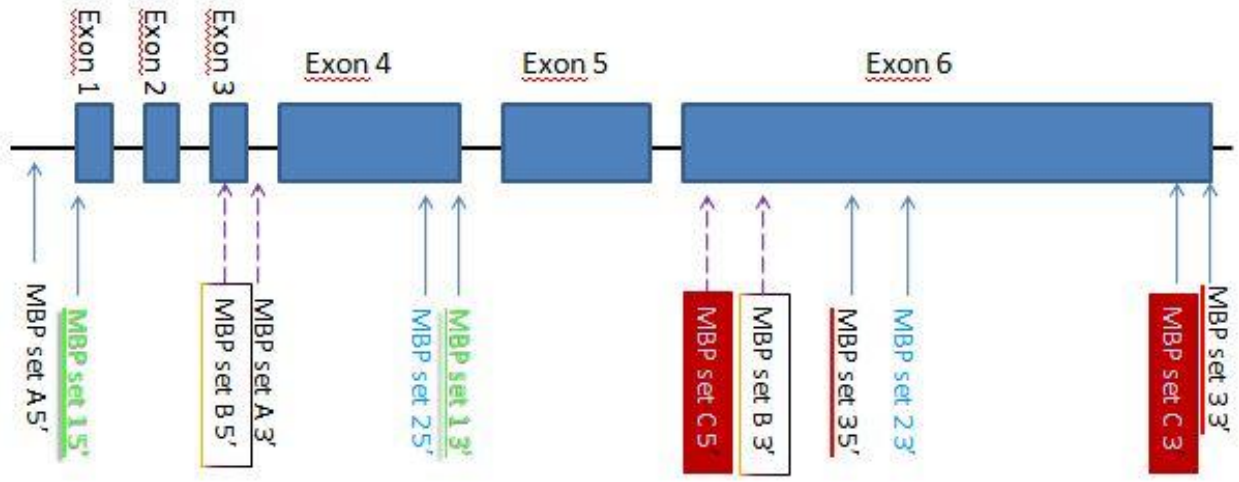

Fig. 6: *Acanthamoeba* Mannose Binding Protein Gene with Primer Sets

Results

Sequence data was obtained from seven different *Acanthamoeba* isolates utilizing MBP primer set 1. In addition, two reference strains were used to evaluate the data: *Acanthamoeba castellanii* which was sequenced in 2004 (6) and the *Acanthamoeba* genome project Neff Reference sequence. The results shown in Figure 7 compare 212 total bases of the 2,502 base pair DNA of MBP from the isolates *A. castellanii,* genome project Neff Reference sequence, OSU Neff, 03-027, 07-101, 07-072, 08-001 and 07-069. Figure 8 compares 312 total bases between *A. castellanii* and 08-008C from base 330 to base 641 of the MBP DNA.

A single nucleotide polymorphism (SNP) is a variation at a specific single site in the genome. The total number of SNPs was calculated for each isolate in relation to the *A. castellanii* reference strain and is shown in table 7.

OSU Isolate Name	# SNPs	Total # Bases Compared	% SNPs
Genome Project Neff	20	212	9.43
03-001 (OSU Neff)	27	180	15.00
03-027 T4	9	190	4.74
07-101 T4	17	182	9.34
07-072 T4	11	95	11.58
08-001 T1	1	58	1.72
07-069 T4	7	101	6.93
08-008C T4	18	312	5.77
Total	**110**	**1330**	**8.27**

Table 7: Single nucleotide polymorphisms (SNPs) between *A. castellanii* and isolates

Based on the MBP DNA sequences that were obtained, the amino acid (AA) sequence was obtained by translating the mRNA based on known splice sites from known reference sequences (Fig. 9 and 10). Table 8 lists the number of amino acid changes when each isolate was compared with *A. castellanii's* MBP amino acid sequence.

OSU Isolate Name	# AA changes	Total # AA's compared	% change in AA sequence
Genome Project Neff	6	70	8.57
03-001 (OSU Neff)	11	57	19.30
03-027 T4	5	62	8.06
07-101 T4	10	60	16.67
07-072 T4	5	31	16.13
08-001 T1	1	19	5.26
07-069 T4	3	30	10.00
08-008C T4	11	97	11.34
Total	**52**	**426**	**12.21**

Table 8: Number of Amino Acid changes between *A. castellanii* and isolates

Table 9 lists details of all the amino acid changes in figure 9 and their significance based upon the structures of the changed amino acids versus the conserved amino acids in *A. castellanii*.

AA Site #	Changed AA	Conserved AA	Isolate	Significance
9	Isoleucine	Valine	08-001	Not significant, hydrophobic AA replaces hydrophobic AA
16	Valine	Alanine	OSU Neff	Not significant, hydrophobic AA replaces hydrophobic AA
18	Alanine	Valine	Genome Project Neff	Not significant, hydrophobic AA replaces hydrophobic AA
			OSU Neff	
22	Aspartic Acid	Glycine	OSU Neff	Significant, acidic AA replaces neutral AA
			07-072	
	Arginine		07-101	Significant, basic AA replaces neutral AA
25	Lysine	Asparagine	OSU Neff	Significant, basic AA replaces neutral, polar AA
29	Threonine	Alanine	Genome Project Neff	Significant, polar AA replaces hydrophobic AA
			OSU Neff	
			07-072	
31	Alanine	Lysine	Genome Project Neff	Very significant, six different AA's coded by seven isolates
	Glutamic Acid		OSU Neff	
	Aspartic Acid		03-027	
	Threonine		07-072	
	Methionine		07-069	
32	Glutamic Acid	Glutamine	07-101	Significant, hydroxide replaces amide in R group
36	Alanine	Aspartic Acid	Genome Project Neff	Significant, hydrophobic AA replaces acidic AA
			OSU Neff	
			07-072	
37	Tryptophan	Cysteine	07-101	Very significant, aromatic AA replaces sulfur containing AA
38	Aspartic Acid	Serine	OSU Neff	Significant, acidic AA replaces neutral AA
39	Lysine	Serine	OSU Neff	Significant, basic AA replaces neutral AA
40	Asparagine	Threonine	07-101	Very significant, amide replaces hydroxide R group
41	Asparagine	Serine	07-072	Very significant, amide replaces hydroxide R group
42	Tryptophan	Cysteine	07-101	Very significant, aromatic AA replaces sulfur containing AA
44	Isoleucine	Serine	Genome Project Neff	Significant, hydrophobic, non-polar AA replaces polar AA
			OSU Neff	
			03-027	
46	Arginine	Serine	07-101	Significant, basic AA replaces neutral AA
49	Lysine	Phenylalanine	Genome Project Neff	Significant, basic AA replaces hydrophobic AA
55	Histidine	Glutamine	03-027	Significant, basic AA replaces neutral AA
57	Lysine	Glutamic Acid	07-101	Significant, basic AA replaces acidic AA
	Aspartic Acid		07-069	Not significant, acidic AA replaces acidic AA
59	Asparagine	Tyrosine	07-069	Significant, amide replaces aromatic hydroxide in R group of AA
60	Asparagine	Aspartic Acid	07-101	Significant, neutral AA replaces acidic AA
			03-027	
61	Isoleucine	Serine	03-027	Significant, hydrophobic AA replaces polar AA
65	Glycine	Tryptophan	07-101	Significant, hydrophobic AA replaces polar AA
67	Arginine	Leucine	07-101	Significant, basic, polar AA replaces neutral, hydrophobic AA
69	Isoleucine	Valine	OSU Neff	Not significant, hydrophobic AA replaces hydrophobic AA

Table 9: Significance of Amino Acid changes in Fig. 9

Table 10 lists details of all the amino acid changes in figure 10 and their significance based upon the structures of the changed amino acids versus the conserved amino acids in *A. castellanii*.

AA Site #	Changed AA	Conserved AA	Significance
110	Glycine	Arginine	Significant, hydrophobic AA replaces polar AA
114	Glycine	Aspartic Acid	Significant, hydrophobic AA replaces polar AA
125	Alanine	Threonine	Significant, hydrophobic AA replaces polar AA
141	Arginine	Serine	Significant, basic AA replaces neutral AA
142	Valine	Glutamic Acid	Significant, neutral AA replaces acidic AA
143	Glycine	Threonine	Significant, hydrophobic AA replaces polar AA
150	Lysine	Threonine	Significant, basic AA replaces neutral AA
152	Asparagine	Serine	Very significant, amide replaces hydroxide R group
177	Glycine	Alanine	Not significant, hydrophobic AA replaces hydrophobic AA
194	Glycine	Phenylalanine	Not significant, hydrophobic AA replaces hydrophobic AA
201	Histidine	Arginine	Not significant, basic AA replaces basic AA

Table 10: Significance of Amino Acid changes in Fig. 10

Table 11 lists the number of SNPs that were synonymous (did not change the amino acid) and non-synonymous (changed the amino acid).

OSU Isolate Name	# Synonymous Changes	# Non-synonymous changes	% Non-synonymous changes
Genome Project Neff	10	10	50.00
03-001 (OSU Neff)	12	15	55.56
03-027 T4	3	6	66.67
07-101 T4	6	11	64.71
07-072 T4	5	6	54.55
08-001 T1	0	1	100.00
07-069 T4	4	3	42.86
08-008C T4	3	15	83.33
Total	**43**	**67**	**60.91**

Table 11: Synonymous vs. non-synonymous SNPs

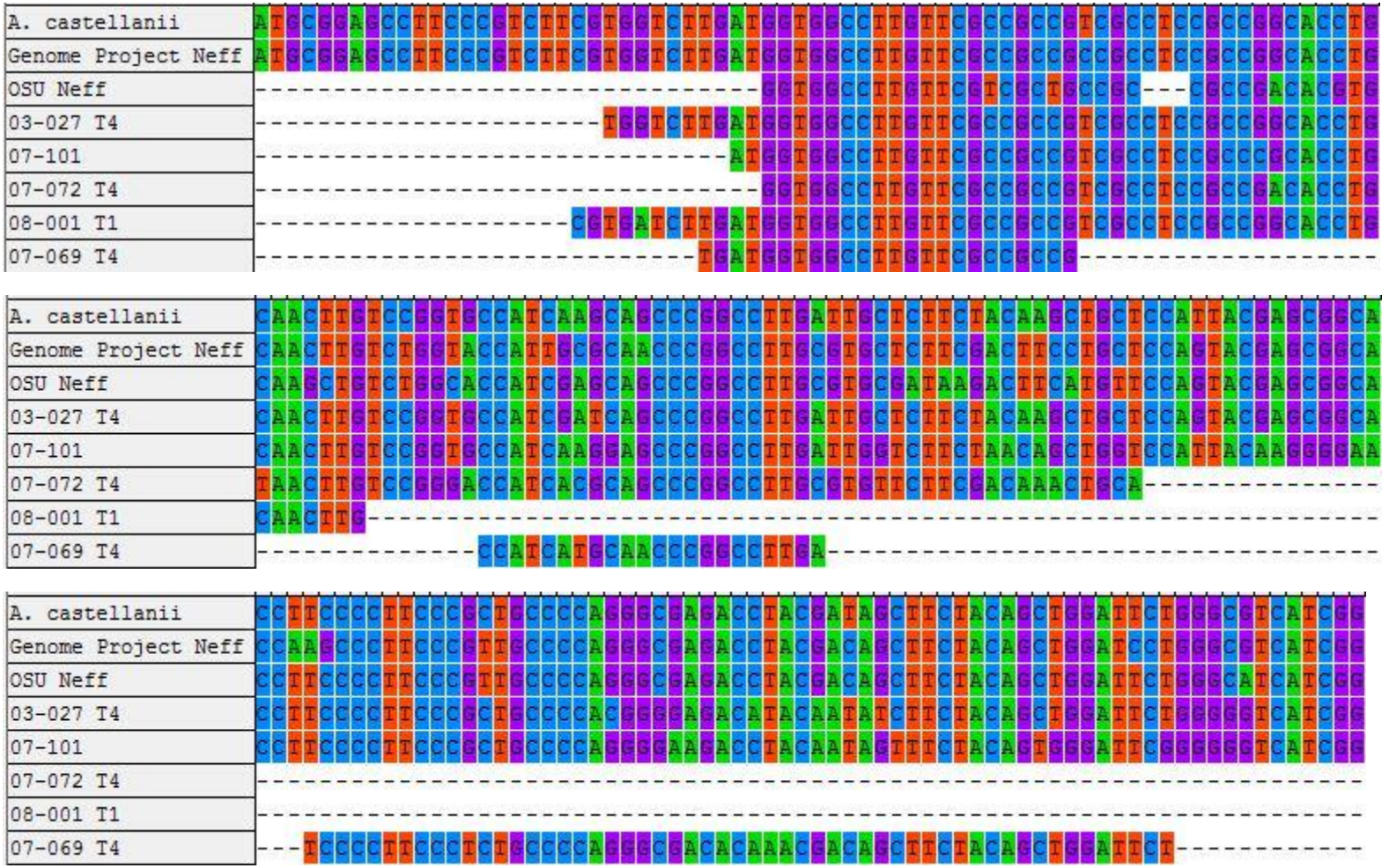

Fig. 7: *Acanthamoeba* MBP DNA Sequences of isolates

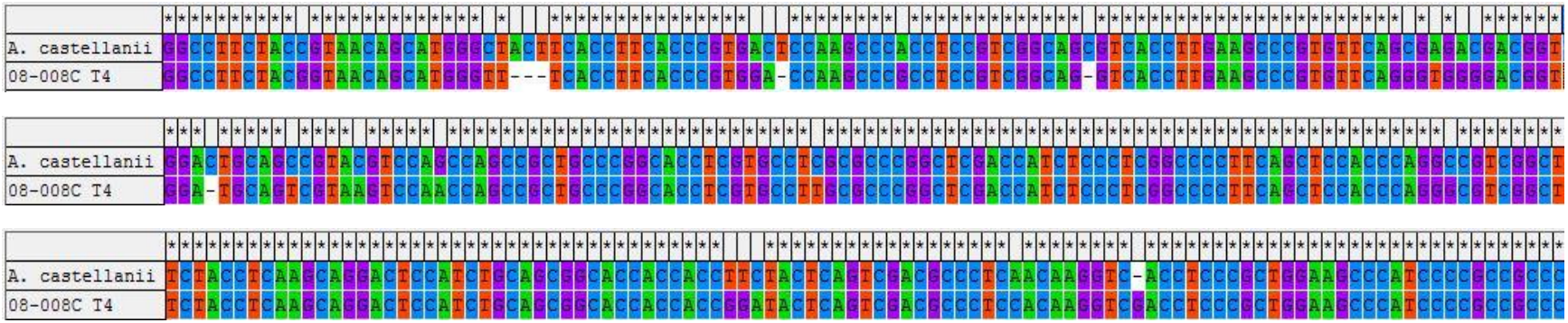

Fig. 8: *Acanthamoeba* MBP DNA Sequence of 08-008C

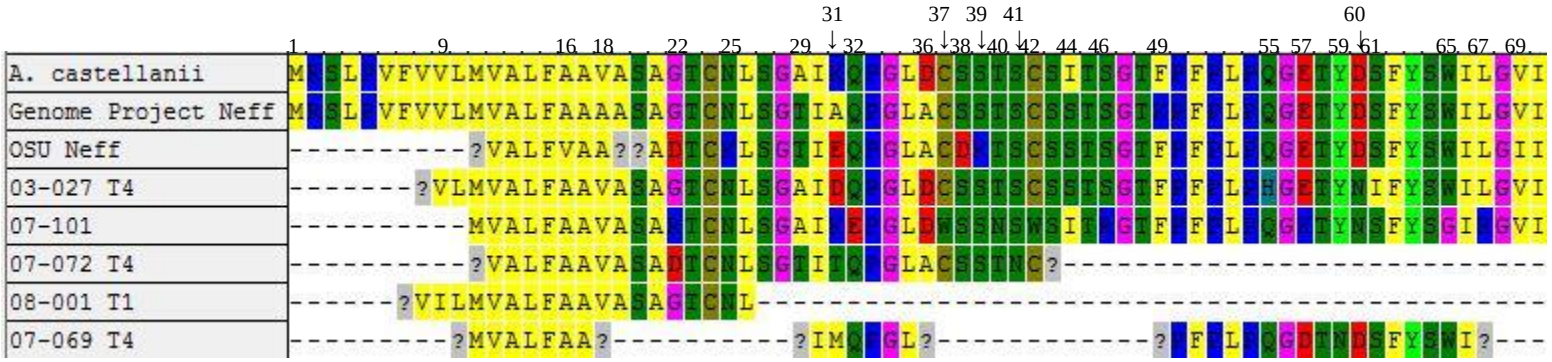

Fig. 9: *Acanthamoeba* MBP Amino Acid Sequences of isolates

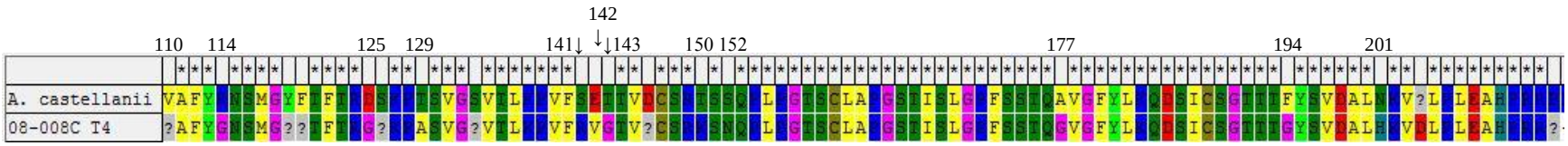

Fig. 10: *Acanthamoeba* MBP Amino Acid Sequence of 08-008C

Conclusion and Discussion

Based on the data displayed in figures 7-10, we can conclude that there are differences in the amino acid sequences of the mannose binding protein obtained from each *Acanthamoeba* isolate. This could mean that different *Acanthamoeba* isolates have variable adherence to host cell surfaces and potentially have variable pathogenicity in humans. Specific regions of the protein could be responsible for variable levels of pathogenicity and those will be discussed in the upcoming sections.

The mannose binding protein of *Acanthamoeba* is a transmembrane protein with features of a typical cell surface receptor. A classic transmembrane protein contains four different domains, a signal peptide, an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The signal peptide is involved in directing the protein to bind to the extracellular membrane. The extracellular domain of the protein functions to recognize the specific host cell receptors and bind to them. The extracellular domain is located outside of the plasma membrane and can also be looped in and out of the membrane (Fig. 11).

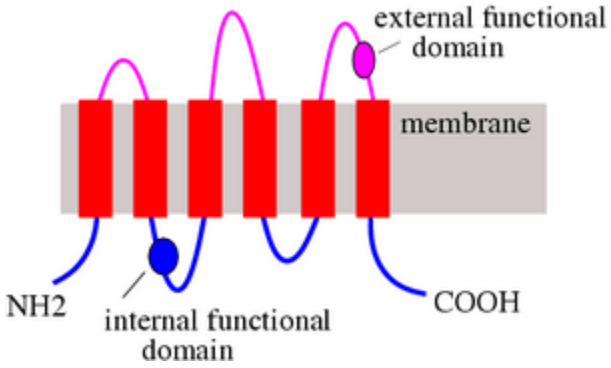

Figure 11: Structure of Transmembrane Protein

The transmembrane domain anchors the protein to the plasma membrane. This part of the protein may change its shape upon extracellular domain binding to host cell receptors. The

cytoplasmic domain of a transmembrane protein lies inside the cell. It has been known to act as a relay for the message that is sent once the extracellular domain binds to the host cell receptors. Transmembrane proteins are known to act in signal transduction. The first step in signal transduction begins with the signal peptide targeting the protein to the extracellular membrane. Once this occurs, the signal peptide is cleaved off. Then, the extracellular domain of the protein will bind the extracellular host cell receptors and intracellular signaling proteins of the host cell will distribute a signal throughout the cell that may change the metabolic functions of the cell.

The mannose binding protein in *Acanthamoeba* is an 833 amino acid peptide with residues 1-21 making up the signal peptide, residues 22-733 making up the extracellular domain, residues 734-755 making up the transmembrane domain, and residues 756-833 making up the cytoplasmic domain (6). After analyzing the data, it is very clear that the signal peptide in the eight isolates examined is highly conserved. There are only a few changes within the signaling peptide (Fig. 9) and the changes that do occur are insignificant. This means that there is probably not much variation in the way the MBP of *Acanthamoeba* reaches the extracellular membrane. Since there is not much variation between isolates in the signal peptide, it is plausible that all isolates' MBP reaches the extracellular membrane in the same fashion. Though amino acid changes may not play an important role in the signaling peptide, they may play a very significant role elsewhere.

Cysteine is a non-essential amino acid in humans with a $-CH_2SH$ R group (Fig. 12). It is a very important amino acid because it can create disulfide bonds between other cysteines in a protein and disulfide bonds play an extremely important role in the structure and function of a protein. In the data presented in figure 9, cysteine is shown to be a highly conserved amino acid in every isolate except 07-101. At positions 37 and 42 of the polypeptide, cysteine is replaced

with the aromatic, amine-containing amino acid tryptophan. Upon closer inspection of the DNA sequence, the same SNP takes place causing the amino acid changes. At both positions, 07-101 codes for TGG whereas the other isolates code for TGC or TGT. Since tryptophan is substituted for cysteine at both positions 37 and 42, two possible sites of disulfide bonding are taken away from the polypeptide. This means that this region of the extracellular domain may have a significant conformational change that could play a very significant role in the binding of the protein to the host cell. This could ultimately lead to differential serine protease secretion from the *Acanthamoeba*.

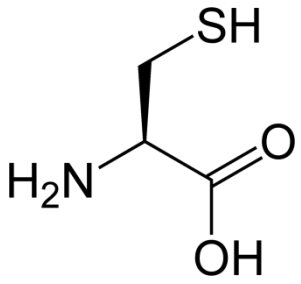

Figure 12: Structure of Cysteine

Another amino acid that could play an important role in the function of the mannose binding protein is arginine. Arginine is a basic amino acid with a positively charged amine in its R group. This positive charge gives arginine the ability to form hydrogen bonds with other amino acids or different compounds. Arginine prefers to be located in the extracellular environment of a protein and it can be ideal for binding negatively charged ions or molecules as well. After examining the data, it can be seen that the 07-101 isolate has three arginine molecules that are not present in other isolates. In fact, arginine is only present in two other isolates at all, and that is at position 2 of the signaling peptide in *A. castellanii* and the Genome Project Neff Reference strain. In 07-101, arginine is present at sites 22, 46, and 67. Since the

extracellular domain starts at position 22 of the amino acid sequence, all of the arginines are located outside the cell. These molecules could play a very specific, undetermined, role in the binding of mannose via the MBP.

This next amino acid change discussed is arguably one of the most important amino acid changes within the MBP of *Acanthamoeba*. Asparagine (Fig. 13) is a non-essential amino acid that contains an amide molecule in its R group.

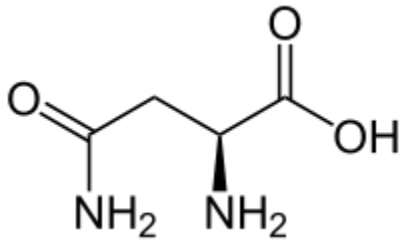

Fig. 13: Structure of Asparagine

Asparagine has been shown to play a very significant role in N-glycosylation of glycoproteins. Glycoproteins are proteins that contain carbohydrate moieties attached at an asparagine, serine or threonine residue. When carbohydrates are attached to an asparagine, it is called N-glycosylation because the carbohydrate moiety binds to the nitrogen in the R group of the amino acid. When they are attached to serine or threonine residues, it is called O-glycosylation because the carbohydrate moiety binds to the oxygen in the R group of the amino acid. When carbohydrates attach to an asparagine, an amino acid other than proline must follow asparagine, followed by either a serine or threonine molecule (i.e. Asn-X-Ser or Asn-X-Thr). Glycoproteins are synthesized in the endoplasmic reticulum (ER) of cells and processed in the Golgi apparatus. N-glycosylation occurs via the attachment of N-acetyl glucosamine (Glc-NAc), which contains three glucose and nine mannose molecules prior to processing, to an asparagine residue (Fig. 14).

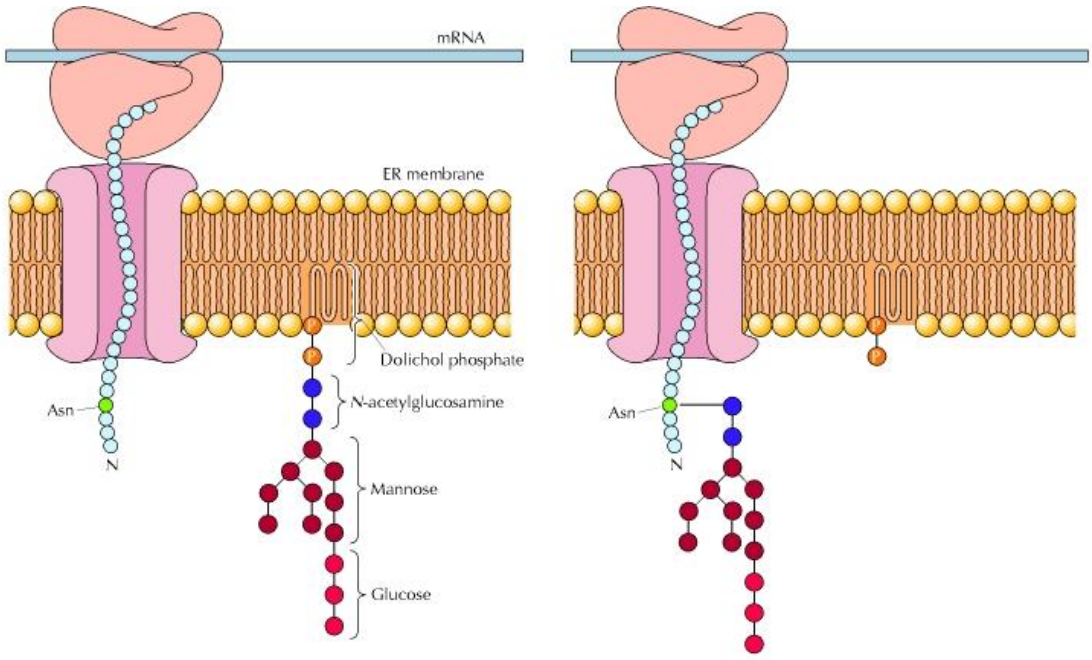

Fig. 14: N-glycosylation in the ER

O-glycosylation occurs when N-acetyl galactosamine (GalNAc) attaches to a serine or threonine residue. Glycoproteins are very important because they may influence the folding, conformation and secondary structure of a protein (7).

In the mannose binding protein of *Acanthamoeba*, approximately 15% of the mass is carbohydrate (7). However, it has been shown that *Acanthamoeba* MBP only contains significant amounts of Glc-NAc and virtually no GalNAc (7). Therefore, *Acanthamoeba* MBP only contains N-glycosylation sites and an insignificant number of O-glycosylation sites. Upon examination of the data in figure 9, the isolate 07-069 has a possible added glycosylation site in the protein at amino acid 59. At this location, asparagine is found in this isolate whereas tyrosine is found in the other five isolates. The asparagine is added before an aspartic acid and serine respectively, creating an Asn-X-Ser moiety. This is very significant because this potential N-glycosylation site in this isolate could add to the number of glycosylation sites, and presumably,

enhanced binding of mannose. The possible addition of these carbohydrates could also play a very significant role in the folding, conformation, and secondary structure of the protein as well. This could lead to differential host cell interaction and differential serine protease secretion.

The last amino acid change discussed is in the isolate 08-008. Upon examination of the data, the neutral amino acid glycine (Fig. 15) was found in five different sites in 08-008 that it was not found in the *A. castellanii* isolate. These include sites 110, 114, 143, 177, and 194. Glycine is the smallest amino acid found and is the only amino acid with hydrogen acting as its R group. This also means that it is the only achiral amino acid. These changes to the 08-008 isolate's MBP could play a very significant role in receptor recognition since it is located in the middle of the extracellular domain.

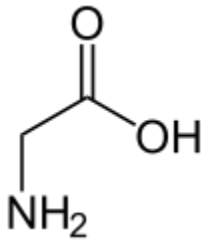

Figure 15: Structure of Glycine

Through the success and failure of doing research, there are always difficulties that present themselves and this experiment was not an exception. Some of the major difficulties were culturing the *Acanthamoeba*, extracting their DNA, and amplifying the MBP gene in the isolates. It was very difficult to culture some of the non-pathogenic *Acanthamoeba* isolates for unknown reasons. When they were cultured, it was very difficult to get a sufficient amount of DNA extracted because so few *Acanthamoeba* were present in the cell culture. Amplifying the MBP gene proved quite difficult in all of the isolates as well. Upon examination of our gels, we would find multiple bands that were amplified (Fig. 3). This could be due to homology between

the primers and another gene, but a more plausible hypothesis is that more than one MBP gene could be present in *Acanthamoeba*. Another explanation for the lack of amplification is that some of the isolates' MBP gene may be so different, especially the T7 isolate, that the primers would not effectively anneal to the gene to permit amplification.

The mannose binding protein of *Acanthamoeba* was discovered only six years ago and very little is actually known about the protein and its role in pathogenicity of *Acanthamoeba*. There are many avenues that can be taken for future research of the MBP of *Acanthamoeba.* One of them would be to continue sequencing of the MBP gene in all fifteen genotypes of *Acanthamoeba*. Once that is finished, the results could be compared using synonymous and non-synonymous SNPs. Then, it could actually be determined if similarity in this protein-coding gene correlates to pathogenicity. If the isolates that cause the same diseases have the most identical MBP amino acid sequences to each other, and the isolates that do not cause disease have very different MBP amino acid sequences, then a correlation between the MBP and pathogenicity in humans could be further examined.

Another area for future research would involve the 18S rDNA sequences. After sequencing the MBP gene in the fifteen different genotypes, a correlation could be made to the 18S rDNA sequences. If the MBP amino acid sequences that are the most similar also have the most similar 18S rDNA sequences, and the MBP amino acid sequences that are the most different also have very different 18S rDNA sequences, then this protein-coding gene can be used to further test evolutionary relationships between species of Acanthamoeba specifically in a protein-coding gene.

One of the more interesting avenues for future research could be in the evolution of the MBP gene in *Acanthamoeba*. *Acanthamoeba* are normally found in soil and bind to mannose-

containing glycoproteins on the surface of their food so that they can ingest it. If a correlation can be made between the MBP gene and the 18S rDNA gene in *Acanthamoeba* such that the more pathogenic isolates in humans are the ones that diverged later and the less pathogenic isolates are the ones that diverged earlier, then it could be hypothesized that *Acanthamoeba's* MBP is actually evolving to better adhere to its food, which is actually making it more pathogenic in humans by making it more capable of binding to mannose-containing glycoproteins on the surface of human corneal epithelium.

Other paths of research into the *Acanthamoeba* MBP involve testing whether MBP is the only protein in *Acanthamoeba* that is used to bind to host cells and understanding how MBP is related to the CPE. If MBP is not the only protein that is involved in adhesion to host cells, then research would need to be conducted to determine the pathogenic potential of these other proteins. Further investigation should be conducted into how MBP is related to the CPE and the presumable cell signaling cascade that happens subsequent to adhesion of *Acanthamoeba* to host cells. This signaling cascade is presumed to signal the secretion of serine proteases that kill host cells via apoptosis. More research should be done on how the signaling cascade functions and if different serine proteases are released upon propagation of different cell signals via different mannose binding proteins of *Acanthamoeba*.

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