Functional Analysis of an *Orientia tsutsugamushi* Ankyrin Effector Protein in a Model Eukaryote

by

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Abstract

Orientia tsutsugamushi is an understudied, gram negative, obligate intracellular bacterium that is transmitted by mites within the genus *Leptotrombidium*. This bacterium has an epidemic area called the Tsutsugamushi Triangle that spreads roughly 13 million km^2 and an estimated one billion people are at risk of contracting the bacterium. Commonly this bacterium causes a disease called Scrub Typhus. There are many strains which cause a wide range of virulence. These strains have protein domains that have been originally identified as Eukaryotic. This domain is called Ankyrin Repeating Domain (Anks). Attached to several of these Anks are another domain call F-boxes. These F-boxes are typically used in the process of attaching ubiquitin to a substrate for later degradation by the cell. *O. tsutsugamushi* actively secretes these proteins into their host cell, but little is known what pathway these Ankyrin proteins are using. Our focus for this study was attempting to identify pathways for a set of these Anks.

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me. But that spark had dwindled down to vertebrates and if it was not for Dr. Appel giving me my first cockroach colony to help feed my bearded dragon and eating lunch in front of the mason jar while watching them do their thing, I would have never taken the second entomology course and proceeded down that road. He has always been there with a warm enthusiastic drive for not only his career but the people that are in his life.

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"All that is gold does not glitter, Not all those who wander are lost;" J.R.R. Tolkien

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List of Abbreviations

Ank	Ankyrin
OTT	*Orientia tsutsugamushi*
PRANC	Pox proteins repeats of ankyrin C terminus
T1SS	Type 1 Secretion System
T4SS	Type 4 Secretion System
SCF	Skp-Cul1-F-box

Chapter 1

Literary Review

1.1 History of the disease and vector relationship

In 3rd century B.C. China, Scrub or Bush Typhus, was first described as being transmitted by small red 'insects.' In Japan, it was known as River fever and transmitted by small red mites or *mushi* from which the disease gets the name *tsutsugamushi* or mite disease [1]. The vector was later identified as mites, or chiggers in some regions, from the genus *Leptotrombidium* [2,8,9,10], that carry an obligate intracellular, gram-negative bacterium called *Rickettsia tsutsugamushi*. Scrub Typhus is also known as tropical typhus and a list of other names (chigger-borne Rickettsiosis, Scrub Typhus, Japanese River Fever, mite-borne typhus, Tropical Typhus, Tsutsugamushi disease [13, 25]), but assumed the name *tsutsugamushi* disease or scrub typhus reported by the armed forces in the time period of 1941-1945 [31]. However, the name still varies and specifically the name Scrub Typhus is misleading because it can be found outside scrub habitats [13]. This species is currently placed in its own genus, *Orientia*, within the class: Alphaproteobacteria; order: Rickettsiales; family: Anaplasmataceae.

The Allied forces of WWII first encountered the disease in the subtropical and tropical regions of the Pacific Theater. It was gave it the common name Scrub Typhus because it was originally thought to be been restricted to what is called as secondary vegetation or scrub[13]. Secondary vegetation, for example, would be areas were grasses were allowed to overgrow an area that was previously forested. This disease is not exclusive to the Pacific and is reported; from semidesert to temperate forests to mountain regions to urban environments; basically region where both rodents and chiggers share habitats [13, 55]. The region known as the

"Tsutsugamushi Triangle", Figure 1.1, extends from Southern Asia to the South Pacific to Australia encompassing 13 million km^2 where an estimated one billion people are at risk of the disease. Annually reported cases of *Orientia tsutsugamushi*, or Scrub Typhus, are close to one million people [3, 18].

Problems arose for the U.S. during WWII due to having limited to no history with this disease, unlike the British army that had previous, but limited encounters with the disease [12]. In the Pacific Theater there were close to 18,000 cases, yet mortality rate ranged depending on the region where the disease was contracted [4]. At times during WWII, there were more casualties from Scrub Typhus than Malaria [31]. In a two-month period, roughly 18% of one battalion contracted Scrub Typhus and 5% died while along the Tiddim Road near Manipur, India [31]. The high rate of infection and mortality lead to the troops becoming more afraid of Scrub Typhus than of Malaria. It was also thought that the disease was the Japanese forces using biological warfare against the U.S. [25].

To combat the disease, several orders were given to try to protect the soldiers in the field and to prevent any more losses. One order created the US Army-Navy-Public Health Service and the Typhus Commission, (early 1942) [12] and another called for troops in the Pacific Theater to always be in full uniform and wear repellant [25]. The insecticide DDT was used at first to try to control the spread of the disease but failed [31]. This is possibly due to a structural difference in mite's sodium ion channels as compared with other arthropod. The Australian military was the first to manage to control the disease by using dimethyl phthalate soap emulsion impregnated uniform [25].

Dieldrin, another insecticide, was tested against mites and resulted in a 92% drop in mite populations in field conditions [42]. After its uses through the 1970s, studies indicated many

environmental problems with Dieldrin, lead for many countries to ban its use. Cultural controls for the disease, including removing potential food sources or habitats for the rodents. However, avoiding areas where the disease is prevalent is the most reliable defense for against Scrub Typhus. In the cases where this is unavoidable, several pesticides such as, sulphur dust, dimethyl phthalate, benzyl benzoate, or permethrin are often used [46, 47] while Deet is a repellant that can be used on skin [48].

More recent studies show that Scrub Typhus occurs outside of the Tsutsugamushi Triangle. In South America, a strain of *Orientia tsutsugamushi* closely related to the strain found in Kato, Japan was identified [5]. A new species, *Orientia chuto*, has also been identified in Dubai that has homology on the 16S rRNA sequence and partial 56kDa gene with *O. tsutsugamushi* strains Ikeda, Kato, Karp, and Gilliam [6]. In Africa, individuals have antigens recognizing *Orientia tsutsugamushi*, and rats and a dog also had serological evidence of *O. tsutsugamushi* [45]. Identification of strains of *O. tsutsugamushi*, outside the Tsutsugamushi Triangle suggests that the disease is substantially underdiagnosed or have even been misdiagnosed for years. Since Scrub Typhus is prevalent in areas were both rodent hosts and the mites live together [13, 55]. Unidentified *Orientia* species and possibly nonpathogenic strains are likely in regions not known to have *O. tsutsugamushi*. If non-pathogenic strains are identified, further studies into the biology and immunology *O. tsutsugamushi* could be performed under safe experimental conditions.

1.2 Mite Biology

With the US troop's introduction to Scrub Typhus during WWII and the establishment of military bases within endemic regions, research was initiated in disease prevention. The main

hosts for the mites that typically vector *O. tsutsugamushi* are primarily within five orders of small mammals that comprise: bats (Chiroptera); rabbits (Lagomorpha); treeshrews (Scandentia); shrews and hedgehogs (Insectivora); and mice, squirrels, and rats (Rodentia) [2]. Humans are an accidental host when they enter the habitats of the main hosts and encounter the mites. During World War II, it was believed that the mites were contracting *O. tsutsugamushi* from their host rodents. Then the mites would bite humans, thus spread the disease through classical vector biology transmission mechanisms. However, transmission of *O. tsutsugamushi* occurs from mother to offspring within mites at a near 100% rate [11, 53]. This mode of transmission is maternal transovarial transmission and is not common amongst vector borne diseases. There are endemic populations of infected mites in which the vector only has to bite a single host to transmit the disease. Interestingly, *Orientia* is closely related to *Wolbachia*, which is also transmitted maternally at high frequencies. It is possible that mechanisms of maternal transmission in arthropods are conserved between *Orientia* and *Wolbachia*.

All immature arthropods molt or shed their exoskeleton to grow larger and *Leptotrombidiun* mites are no exception. Durring molting, cellular reorganization can exclude or eliminate pathogens. It is not known where in the mite *O. tsutsugamushi* is localized. In contrast, *O. tsutsugamushi* is transstadially transmitted, which allows bacterial infections to be maintained across life stages [11, 12, 53]. An additional mechanism of disease transmission within mites is horizontal transmission, whereby one uninfected mite could become infected by interactions with an infected mite. Horizontal transmission would cause the disease to spread even faster than would be the case if only transovarially transmitted from mother to offspring. Some researchers believe that there is potential for horizontal transmission (from infected mite, to host, to uninfected mite), or for transfer of the disease via co-feeding (an uninfected mite obtaining the

bacteria from feeding within close proximity to an infected mite) [8]. There is some skepticism to the co-feeding hypothesis due to the manner in which these mites feed. Mites feed not by tapping into a blood vein (hematophagy), which does produce horizontal transmission in ticks and other arthropods, but by dissolving the epidermal layers and feeding on lymph tissue by creating a stylosome or feed channel created by digestive salivary enzymes. This is in contrast to the colloquial rumors; chiggers do not borrow under the skin, like Scabies, trombiculid mites feed at the outermost layer of skin [12, 13]. When mites feed, they do so with a near painless bite, and can have differing preferences on location of attachment. Common attachments are in crevasses of the body and areas of compression, such as between the fingers, behind the knee, around the groin, and the waist of pants [13]. One possible means of horizontal transmission would be a second chigger visiting a vacant stylosome or eschar created prior from an infected chigger. Thus, these cases could occur where co-feeding occurs on hosts with multiple chigger populations.

There are multiple species of mite that are vectors for Scrub Typhus. With the endemic area of the Tsutsugamushi Triangle spreading roughly 13 million square kilometers since the 3rd Century [3], there are multiple species of mite vectors. Many studies have shown that the genus of mites that can vector Scrub Typhus is within *Leptotrombidium* which can be found worldwide [2, 8, 9, 10, 54]. In Korea, *Leptotrombidium pallidum* was the most abundant at 54.6% while *L. scutellare* (27.2%) and *L. orientale* (6.25%) were the next two most abundant species in the sampled regions [37]. While in the Shanong Province of Northern China, *L. scutellare, L. palpalis*, and *L. intermedia* were the most common mites sampled [39].

Figure 1.2 illustrates the typical life history of a *Leptotrombidium* mite. The life cycle is hemimetabolous and utilizes successive larval and nymphal stages to reach adults which mate

and lay eggs. Once the egg hatches, six-legged larvae emerge. Two stages encompass the larva stage: prelarval and larval stages. Larva feed on mammals for 2-4 days to a few weeks before they drop from the host to the soil [7, 8, 34, 53]. Following the feeding are the eight-legged stages, including the proto-, deuto-, and trionymphs and the adult stages. Adults feed on decaying vegetation or eggs of other arthropods such as Collembolans [7, 8, 34]. One study reported one species of mite laying about 350-450 eggs a month for three to five months; parthenogenesis is present in at least one species [33]. Parthenogenesis is the ability to reproduce without the fertilization of an egg. Specifics of the life cycle vary greatly depending on the species of mite and many environmental factors such as rainfall and temperature [13]. There is an increase in mites collected in the field after the rainy season [13, 34] with seasonal increases during the fall and winter months for some species, such as *L. palpalis*, while other species have greater abundance during the summer months (*L. intermedia*) [39]. In some regions multiple species of mites can harbor *O. tsutsugamushi*. Environmental factors, such as seasonality and humidity, cause some species of the mites to increase. A trade off in season abundance can be seen in Japan with *L. scutellare* being the major vector in the fall and winter, while *L. akamushi* is more prevalent during a wet summer [13].

1.3 *Orientia tsutsugamushi* Pathogenesis Symptoms and Treatment

Orientia tsutsugamushi is an obligate intracellular, gram-negative bacterium within the family *Anaplasmataceae* and is spread by mites or chiggers throughout much of Asia, or what is known as the Tsutsugamushi Triangle [3, 18]. Recent studies have shown the spread of the disease outside of its native range [5, 6]. It can be simply stated that anywhere there is a *Leptotrombidium* mite and a rodent host, Scrub Typhus can also be found [13]. Understanding

how this bacterium, *Orientia tsutsugamushi*, interacts and modifies its host system will give great insight into how it lives within both mites and humans.

When the mites feed on a human their saliva creates a stylosome or feeding channel for them to feed. The saliva introduces *Orientia tsutsugamushi* into the mite's host system. The combination of the stylosome and bacteria leaves a lesion or black scab called an eschar. Eschars occur in most, if not all, cases of Scrub Typhus and appear shortly after the first symptoms. Eschars can be used to quickly identify the presence of the disease by running a PCR from the DNA harvested from the eschars [14, 15]. Additional symptoms for Scrub Typhus historically have occurred after 7-18 days after the incubation period of the initial bite [1]. Additional symptoms include a fever, dizziness, body aches, and more severe symptoms of organ failure and neurological issues [1, 16, 17, 48]. These symptoms could vary regionally based on the genotype of the strain of Scrub Typhus [17]. Due to the wide range of symptoms and variability of strain intensity and symptoms, general symptom diagnostics is unreliable and can lead to misdiagnosis. More conclusive methods for diagnosis include Weil-Felix, indirect immunofluorescence antibody, indirect immunoperoxidase, ELISA, dot-blot immunoassays, and rapid flow assay, but these are either not sensitive enough, commercially available, or used on an extensive basis. The most common method for diagnosis is serological testing [18]. For many of the areas where Scrub Typhus is endemic these methods, for identification are either very costly, unavailable or not sensitive enough.

O. tsutsugamushi typically responds well with treatments of doxycycline, with alternatives being chloromycetin, azithromycin, and telithromycin and in some cases rifampicin is more effective than doxycycline [12, 19, 32]. Since there is no vaccine for Scrub Typhus, antibiotic resistance could develop due to the narrow list of treatments. There are cases where

treatments with doxycycline have little effect, thus a higher need to understand the mechanisms of this bacterium [12]. A proof of concept for a vaccine was found when humans showed some resistance to *O. tsutsugamushi* stn. Pescadores, but this strain is milder than other strains [26]. Other attempts to develop vaccines have been either ineffective or only provided partial protection for humans or mice [26]. Difficulty arises where one strain may be vaccinated, other strains may not be affected by the vaccine and studies have shown that mites can have multiple strains of *Orientia tsutsugamushi* [28].

1.4 *Orientia* Biology

While co-feeding has been reported for Rickettsial diseases, it is difficult to determine if chiggers can transfer the bacteria via co-feeding [56, 57]. Rickettsial diseases tend to rely on transmission via saliva to enter the blood stream by directly feeding in the blood vein. Chiggers do not directly feed on blood, but instead feed on lysed tissue from the host [12, 13].

Pathogenesis within humans occurs when the mite saliva is transmitted into humans via the stylosome [51]. While the pathogenesis has been limited, transmission from mite to human via bite and from one generation of mite to the next transovarially, suggests that the bacteria resides within the hemolymph of the mite and has incubation sites in the genitalia and the salivary glands. Once the mite bites the host, inoculation of the host has begun. The bacterium grows at the site of the bite then infects the immune response cells below the dermis [51]. Once *O. tsutsugamushi* has bound to certain receptors, it invades the host cell by utilizing the host's cell clathrin mediated endocytosis system [52] which involves the host cell taking in the bacteria via a vesical after the recognition of a clathrin receptor. Clathrin is the major component in the endocytosis and vesical formation of a cell. *O. tsutsugamushi*'s invasion of the host cell typically

involve its binding with the host's cell membrane and a vesical is created which envelops *O. tsutsugamushi*. Once inside the cell it escapes from the vesical and resides within the cytoplasm of the host cell (Figure 1.3 [30, 52]). It then proceeds to carry out its regulatory functions which will take about 9 hours for replication via binary fission [52]. Not only will *O. tsutsugamushi* secrete proteins that interact with the host cell to prevent its destruction such as with two Ankyrins (Ank01_02 and Ank06) that are released that prevent the NF-κB antimicrobial pathway [30]. It has also been seen to move via the microtubules to a location in the cell that lacks lysosomes [52]. Once inside the cell the bacterium utilizes the host cell's citric acid cycle and ATP along with vitamin B6 [61]. Replication is accomplished by binary fission and the use of the host cell to send the new bacterium to a neighboring cell [59] or the bacterium will enter the blood and lymph vessels before entering another cell [60]. Moreover, irradiation stressed mice, had an increase susceptibility to Scrub Typhus upwards of a year after infection, while humans were able to house the bacteria for 4-5 months after the initial infection [29, 51].

An integral part of being an obligate intracellular bacterium is having a well-developed Type IV secretion system, which has been noted, is highly conserved amongst many of the *Rickettsia* bacteria. *Orientia tsutsugamushi* has a high rate of recombination of repeated sequences especially that of the Type IV Secretion System (T4SS) which allows for protein secretion into its host [27]. *Orientia tsutsugamushi* has more Type IV Secretion System (T4SS) genes that code for and work with the T4SS than other Rickettsial bacteria [50, 52]. It has more Type I Secretion System (T1SS) which allows it to actively communicate with its host cell [55]. *Orientia tsutsugamushi* has 79 locations on its genome that code for proteins involved with the T4SS and it has been proven that many Ankyrin repeats are involved with this pathway [50, 52]. It also has more Ankyrins than many other Rickettsial diseases, by having 38 genes [55]. These

provide a way for the bacteria to regulate its host cell since Ankyrin can be found in a wide variety of proteins. One such host manipulation with Ankyrins is with Ank9 and its interactions with between the Goli Body and the endoplasmic reticulum [44].

O. tsutsugamushi has a reduced genome making it deeply dependent on a host cell. *O. tsutsugamushi* strain Ikeda has a single chromosome comprised of 1967 protein coding sequences, many of which have been theoretically adapted from sources foreign to itself. Furthermore, the *Orientia* genome, when compared with other Rickettsial bacteria, appears to be scrambled and lack collinearity [50]. *Orientia tsutsugamushi* has lost the pathway to create its own vitamin B6 and ATP [61].

1.5 Ankyrin Repeat Domain

The ankyrin (Ank) domain is a protein motif that is important for protein-protein interactions. Ank repeat proteins frequently combine Ank repeats with other functional protein motifs and therefore have diverse functions. Anks are cell cycle regulators, inhibitors, transcriptional factors, structural scaffolding, protein organizers, and toxins [20]. This domain was originally thought to be a eukaryotic sequence but has been found abundantly in obligate, intracellular and facultative host associated bacteria long with some Archaea and free living bacteria [21, 50].

The structure of an Ankyrin domain is an antiparallel α-helices connected by a β- turn. Proteins often string these together repeats of these domains with a loop leading to the Ank or into the next structural component of the protein, hence *Akyrin repeats* [21]. It is also known as a β-hairpin-helix-loop-helix [20] (Figure 1.4). Early investigations of Anks highlighted a role in human erythrocytes [35, 36]. In erythrocytes, Anks were identified as cytoplasmic proteins that interact with microtubules [35]. Anks have interactions with band 3 [36] which are integral in the

exchange of Cl^-/HCO_3^-, a vital exchange that is key in transferring oxygen from erythrocytes to tissues [38].

Oientia tsutsugamushi genome encodes for one of the largest known bacterial genome for Anks, T1SS, and T4SS components [55]. Many of the Anks in *O. tsutsugamushi* have a convergence amino acid sequence of LDAVTSIF which is conserved on the C-terminal end between 37-63% for the Ankyrins. Of *O. tsutsugamushi*'s genome, 19 out of the 20 Anks interact with the T1SS secreting chimeric Ank proteins in an HlyBD-dependent manner using *E. coli* BL21 [55]. This shows that these Anks are potentially utilizing the T1SS instead of the T4SS [55, 58]. *O. tsutsugamushi* shows its need to express these Ankyrins but expending energy to secrete these Anks via the T1SS in order to manipulate the host cell. The T1SS main structural component for the ATP-binding cassette (ABC) portion of the T1SS is shown in Figure 1.5.

Many Anks used by *O. tsutsugamushi* have C-terminal sequences that code for either Pox proteins of Ankyrin C-terminal (PRANC) which is a domain that has a poxvirus F-box sequence, or a F-box alone at the C-terminal region of the protein [22]. The F-box aids in regulating the binding of the E3 ligase [23,24]. The E1 enzyme actives the ubiquitin, the E2 enzyme carries the activated ubiquitin moves it to the substrate where the E3 ligates the ubiquitin to the substrate. This process, called ubiquitinylation, is based on which lysine of the ubiquitin is bound to the substrate and denotes what fate awaits the bound substrate. The most well-known of the lysine to be bound is K48, which signals for the attached substrate to be broken down. This method utilizes a pathway by which bacteria can modulate their host cell is via the SCF complex or the Skp1-Cullin-F-box (SCF1) protein. This structure is key in mediating a diverse group of regulatory and signaling proteins which can affect the interaction of the E3 or the ubiquitin-protein ligase [43] which binds ubiquitin to the substrate.

The F-box recognizes and binds to the substrate, which allows for the other region of the SCF1 protein complex to bind ubiquitin to the substrate. Most well-known is the binding of K48, or the 48th Lysine of the ubiquitin. After a chain of ubiquitin has been built on the substrate, the substrate is then sent to be degraded [24]. F-boxes are the key component in recognizing and holding the substrate to be degraded. In the *O. tsutsugamushi* strn. Ikeda genome, 16 of the 20 Anks have sequences that are homologous with known eukaryotic/poxvirus F-boxes are known to interact with the SCF [44, 55]. This leads to the hypothesis that *O. tsutsugamushi* modifies the host cell regulation by utilizing Ankyrin Repeats and F-boxes to control its host cellular function.

As previously mentioned, *O. tsutsugamushi* potentially adapted to and modified the host cell to survive intracellularly by using Ank01_02 (OTT_0753) and Ank6_02 (OTT_1149), which inhibit a key transcription factor, NF-κB. This transcriptional factor is used in the antimicrobial response pathway [30]. NF-κB is involved with both the innate and adaptive immunity [40]. The adaptive immunity pathway primarily occurs within the lymphoid organs and a decrease in the expression of NF-κB can cause B cells and lymph nodes and other secondary lymph organs to not fully develop [41]. By using Ank01_02 and Ank6_02 to weaken the host cell's defense system, *O. tsutsugamushi* has a greater chance of survival and can persist within the lymphatic system for months or years [26].

O. tsutsugamushi modifies its host by disrupting interactions between the Golgi bodies and the endoplasmic reticulum protein secretion. It manages this with the aid of Ank9, and the already manipulated SKP1 and CUL1 [44, 49]. It is important to note that *Orientia tsutsugamushi* uses a single protein for multiple functions. By hijacking the host cell's Golgi bodies and endoplasmic reticulum and can take over the construction of molecules.

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Figure 1.1. Map depicts regions where Scrub Typhus has become an epidemic occurrence but may not list the regions were antibodies have been found during serological testing. The blue triangle represents the “Tsutsugamushi Triangle”.

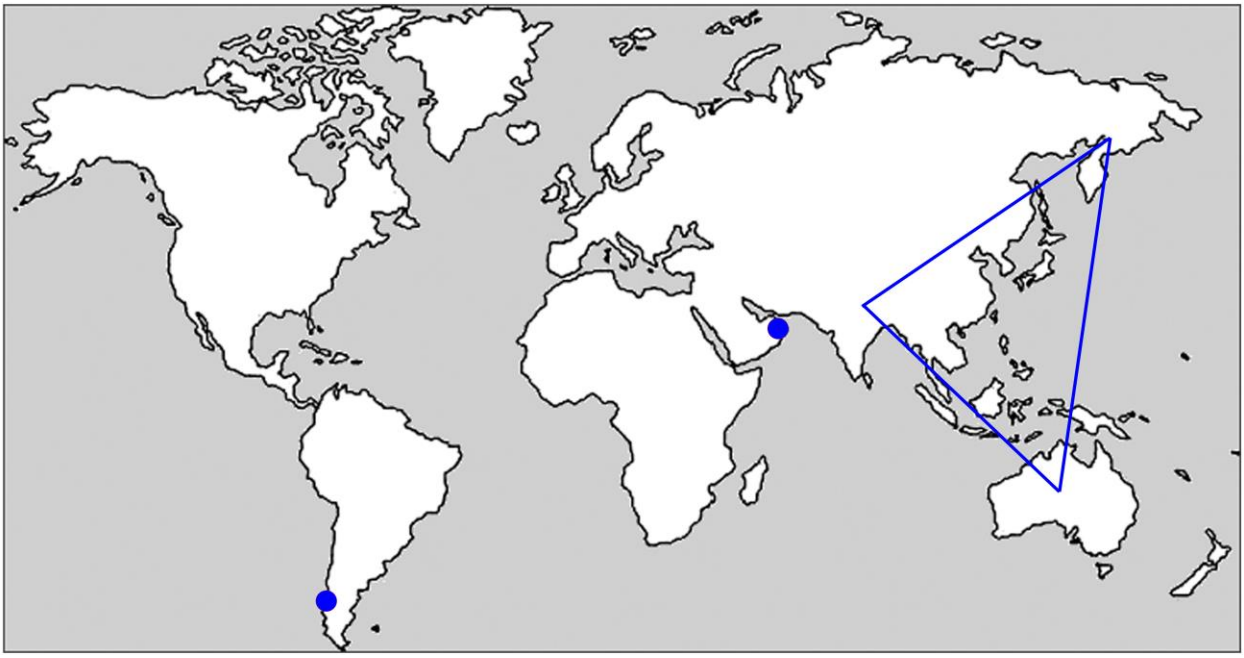


Figure 1.2: Generalized life cycle of a mite or chigger in the family Trombiculidae. There is a high rate for transmission of *O. tsutsugamushi* being passed from one life stage to the next and a near 100% transmission from mother to offspring.

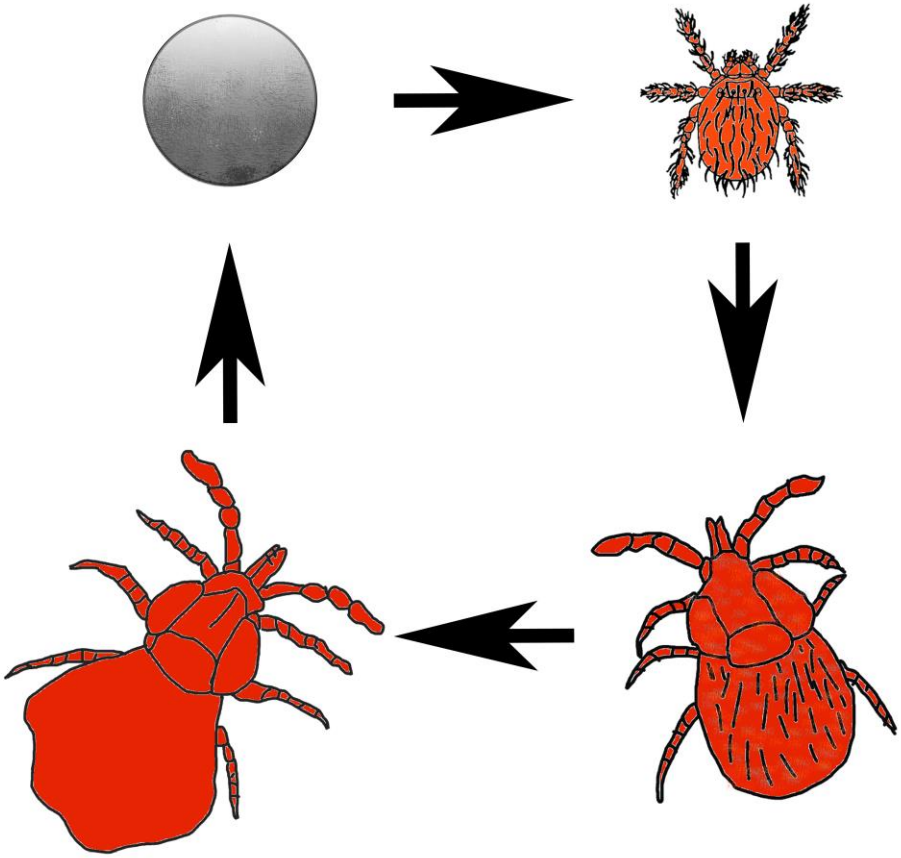


Figure 1.3: *O. tsutsugamushi* will bind with the host cells receptor Syndecan-4 and other host cell receptors. The host cell then utilizes Clathrin mediated endocytosis. Once inside the host *O. tsutsugamushi* will escape from the phagosome, but how it is able to go about this to prevent being broken down by the host cell lysosomes is unknown [52].

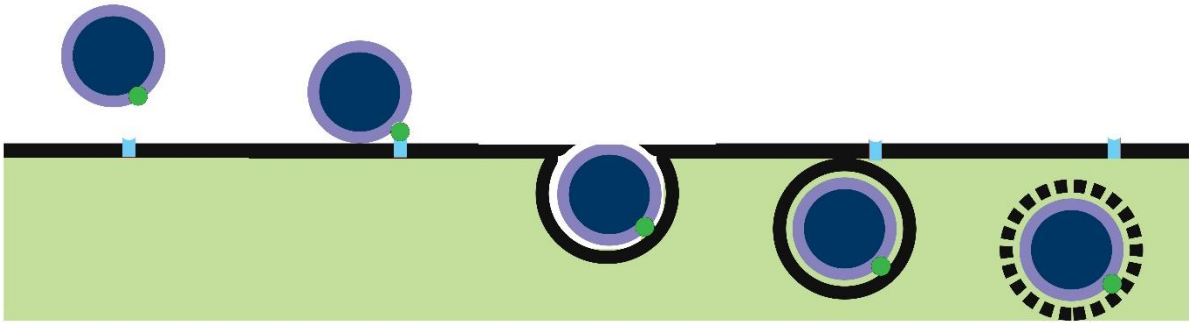


Figure 1.4: The protein structure to the left indicates a complex of Ankyrins Repeats bound together. The protein structure to the right indicates a single Ankyrins Repeat, where the green segment shows the α-helices and the purple indicates the β- turn. This easily shows the β-hairpin-helix-loop-helix. PDB: 2BKG. [62].

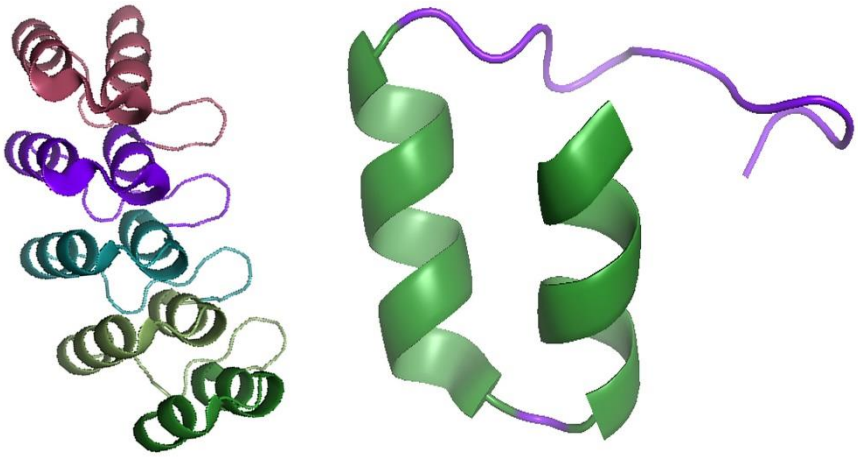
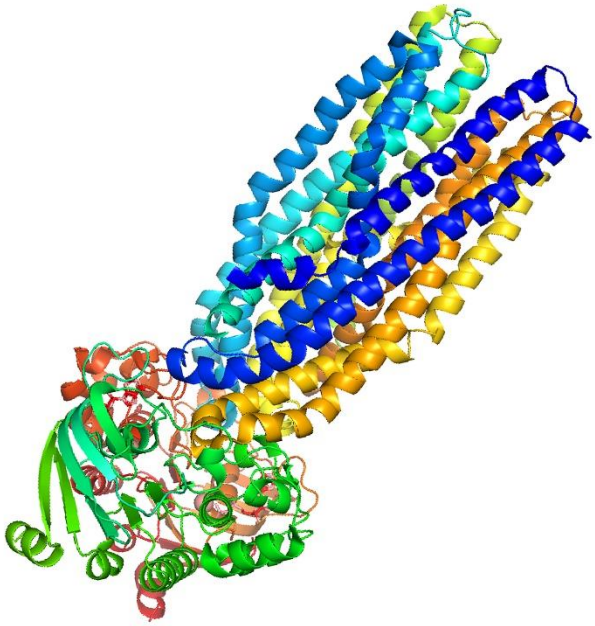


Figure 1.5: Structure of the Type I Secretion System inner membrane protein in which is used for channeling substrates with ATP activating the protein. PDB: 5L22. [63].



Objectives and Approaches

Since *O. tsutsugamushi* uses well-developed means to alter the host cell, my aim is to identify a potential Ankyrin Repeat protein that explains why this bacterium has a range of virulence across the different strains. The Ankyrins of the study was selected based on the presence of a F-box and the potential to mediate with the SCF1 [44] This means these proteins are being actively secreted for means of substrate manipulation. The selected Anks included: Ank01_02 (OTT_0753), Ank2 (OTT_0049), Ank3_08 (OTT_1112), Ank4_01 (OTT_0210), Ank05_01 (OTT_0214), Ank8 (OTT_0257), Ank9 (OTT_0298), and Ank16 (OTT_1271). To accomplish this aim, we will clone select set of Anks that contain F-box proteins into yeast. These transformed yeasts can be used to identify any potential of host cell death and then delve into what pathway the Ank is using. Anks will also cloned into the Multi Cloning Site of a 2 micron vector, pYes2-gal1, and a CEN vector, pRS316 and pRS416. This will be done to test if the genes cause cellular death when placed into a model Eukaryotic cell, yeast W303. A suppressor screen will identify the pathway the Ankyrin phenotype that was used. Chimeras and deletions will also be used to identify regions of the protein that produce the toxicity.

Chapter 2

Functional Analysis of an *O. tsutsugamushi* Ankyrin Effector Protein in a Model Eukaryote

Abstract

Orientia tsutsugamushi has a wide variation among strains which produces mortality rates ranging from 0% to 70%. While some of this variation is based on location, patient history, and preexisting medical conditions, the important to the virulence is variations in the genetic code of *O. tsutsugamushi*. Notably, *O. tsutsugamushi* has multiple open reading frames (ORFs) with a homology to an Ankyrin Repeats (Anks). The Ankyrin domain is present in proteins having diverse roles and cellular functions. Some of these Anks have a C-terminal F-box, which works in substrate recognition for ubiquitylation. Ubiquitin is a small post-translational modification that often leads to cellular degradation of the substrate by the proteasome. In this study I cloned several *O. tsutsugamushi* Anks repeats genes containing C-terminal F-boxes domains to investigate their cellular phenotypes in yeast. I identified gene, Ank01_02 which induces toxicity to yeast cells. In contrast, multiple Ank homologs of Ank01_02 did not induce toxicity. The closest homolog of Ank01_02 was Ank05_01, which was nontoxic. To identify regions necessary for toxicity I performed deletion analysis and produced chimeras with respect to Ank01_02 and Ank05_01. Deletion of the Ankyrin region and F-box domains produced cellular death, suggesting that toxicity of Ank01_02 can be attributed to both the Ankyrin and F-box domain.

Introduction

Orientia tsutsugamushi (*Anaplasmataceae*) is an obligate intracellular, gram-negative bacterium that is transmitted by mites in the genus: *Leptotrombidium*. It causes Scrub Typhus, a

disease that has been recognized as the world's most underdiagnosed disease [6]. Scrub Typhus symptoms include a fever and chills, headache, enlarged and tender lymph nodes, nausea, vomiting, diarrhea, insomnia, convulsions, and if gone untreated for long enough, organ failure [7, 8, 9, 10]. The most notable sign of infection is the eschar that typically occurs a few days after the mite feeding on the human [9, 10]. Mortality can range from 0% to 70%, based on the strain of *O. tsutsugamushi* [1], the area in which the disease is contracted, and access to proper diagnosis and antibiotics. The historical distribution of this disease is known as the "Tsutsugamushi Triangle", which is a 13 million km^2 area in Southern to Eastern Asia. As of 2003, an estimated 1 billion people are affected by the disease. An estimated one million people die annually [11, 12].

One biological key of *O. tsutsugamushi* is its adaptation to the host cell and its ability to manipulate the cell for survival. *Orientia tsutsugamushi* parasitizes its host cell and must control cellular responses in order to replicated. A common theme of intracellular bacteria is to use horizontally acquired eukaryotic gene motifs against the eukaryotic host. The Ankyrin and F-box motifs, studied here, are exemplary host hijackers. The Ank motif promotes protein to protein interactions and Anks actively secreted by the Type 1 Secretion System of *O. tsutsugamushi* [2, 5, 15].

The F-box domain is a part of the ubiquitin proteasome System (UPS) which regulates cellular degradation amongst other functions. F-Box proteins complex is a core constituent of Skp-Cullin-F-box (SCF) complexes. The role of the SCFs is to act as an E3 ligase adding ubiquitin to substrates targeted for degradation. The ubiquitin pathway utilizes a series of peptides to flag a substrate with chains of ubiquitin. The better-known attachment site of the ubiquitin is on K48, or the 48^{th} Lysine residue of the ubiquitin protein. The specific role of the F-

box is to specify which substrates are targeted for ubiquitylation and subsequent degradation. By controlling F-box proteins, bacteria can redirect protein degradation in cells to suit their needs [3, 4].

In the *O. tustusgamushi* genome 16 of the 20 Ankyrins have sequences that are homologous to F-boxes [4]. Eight of these Anks were cloned into the Multi Cloning Site of a galactose inducible high copy 2-micron yeast vector, pYes2-gal1, and later low copy Centromeric (CEN) plasmids, pRS316 and pRS416. These heterologous constructs were used to test toxicity of *Orientia* Ank and F-box proteins in a model eukaryotic cell (baker's yeast, strain W303). Deletion and chimeric analysis of the constructs was performed to identify which region of the protein produced toxicity. A future aim will be to continue this work with a high copy suppressor screen capable of identifying which pathways hijacked by the Ank and F-box motifs.

Materials and Methods

Primer Design

PCR oligonucleotides were designed for cloning individual Ank ORFS into the Multi Cloning Site of the pYes2-gal1 plasmid and allow for a restriction enzyme digest. Primers (Table 2.1) recognize *O. tsutsugamushi* strain Ikeda as the template for amplification. Primers created for Site Directed Ligase Independent Mutagenesis SLIM [13] PCR reaction (Table 2.3) were designed to use pYes2-gal1_Ank01_02 and pYes2-gal1_Ank05_01 plasmids as templates for insertion of a Flag epitope tag on the N-terminal of the protein Primers for a SLIM PCR (Table 2.4) added a C-terminal tag to Ank01_02.

Bacterial and Yeast Cultures

pYes2-gal1 and pYes2-gal1-Ankyrin were transformed into Top10 *E.coli* and stored at -80°C. Ampicillin LB agar plates were used to grow the transformed bacteria, and the colonies were selected for sequencing and liquid LB with ampicillin was used to grow the selected colonies for plasmid production. The recipes for these plates are found in Table 2.2. pYes2-gal1 was selected as the plasmid vector due to its gal1 promotor which induces protein expression in the presence of galactose and repression in the presence of glucose [17].

Yeast, W303, were grown in YDP media. After adding the constructs into the yeast, the cells were grown on either Synthetic Defined (SD) glucose media lacking uracil or the equivalent with galactose substituting as carbon source (Table 2.2).

Cloning and Transformation

Polymerase chain reaction (PCR) reaction was used to produce amplicons and was purified with a PCR purification kit (Omega). Amplicons and plasmids were digested with restriction enzymes (New England Biolabs) and purified by subsequent agarose gel (0.8%) electrophoresis, visualization with using ethidium bromide, and purified with a gel purification kit (Omega). Restriction digest reaction recipes and conditions followed NEB recommendations.

Ligations were performed according to NEB guidelines. Briefly, 1 µl of the purified cut plasmid and 16 µl of the purified PCR digest, 1 µl of T4 DNA ligase (NEB) and 2 µl of T4 ligase buffer were added and incubated overnight at 18°C. Assembled plasmids were chemically transformed into Top10 F' *E.coli*. 5 µl of the ligation was added to chemically competent cells that had been thawed from -80°C on ice for 5 minutes. After the ligation was added, it was set on ice for 30 min and then heat shocked at 42°C for 45 sec and then put back on ice for 2 min. One ml of Luria Broth (LB) was added and incubated at 37°C for 1-3 hours. The cells were then

centrifuged at 13,000 RPM, and 900 µl of the supernatant was removed and the pellet resuspended in the remaining 100 µl of LB. This solution was then plated on Ampicillin agar plates.

Colonies were picked and grown overnight in Ampicillin LB. To make laboratory stocks, 800 µl of the cells were mixed with 300 µl of 75% glycerol and stored at -80°C. The plasmids were extracted and sent to MC Lab for Sanger sequencing to confirm sequence fidelity. Once the accuracy was confirmed, the plasmid was transformed into yeast strain, W303 [13].

Chimeras and Deletions

Amino acid sequences for Ank01_02 and Ank05_01 were compared using the PubMed BLAST. After designing the chimeras and deletions, constructs were synthesized by Genscript within the pYes2-gal1 vector. The plasmids were then chemically transformed into Top10 F' *E.coli*.

Serial Dilutions

Growth of yeast was assessed by spotting 5 fold dilutions. We used a synthetically defined media that lacked uracil since W303 lacks the ability to produce its own uracil [14]. This forces the yeast cell to keep the plasmid due to the Ura3 cassette that is a part of pYes2-gal1 plasmid has the ability to produce its own uracil. The experimental groups were run on plates with galactose and stored at either 30°C, 32°C, 34°C, or 36°C and the control group was run at 36°C. This variation in the temperature confirmed if the added gene had greater effects while the yeast was stressed. Any temperature above 31°C the yeast will be stressed and temperatures between 37-38°C stops cellular replication. The control was run at 36°C and could grow well. The experimental groups at or below this temperature should grow uninhibited.

Results and Discussion

Heterologous expression of Ank01_02 showed toxicity in yeast (Figure 2.1). The selected Ankyrins: Ank2 (OTT_0049), Ank3_08 (OTT_1112), Ank4_01 (OTT_0210), Ank05_01 (OTT_0214), Ank8 (OTT_0257), Ank9 (OTT_0298), and Ank16 (OTT_1271) did not cause cellular death. When compared structurally with the other Ankyrins tested, Ank01_02 is most similar to Ank05_01, which was non-toxic. BLASTP analysis between Ank05_01 and Ank01_02, showed 52% similarity on the N-terminus and 92% similarity on the C-terminus.

Do to the similarity between Ank01_02 and Ank05_01 we created chimeras and deletions that took the first half of Ank01_02 and combined it with the second half of Ank05_01 (Figure 2.4). The chimeras would show whether the Ankyrin repeat domain region of the gene that is causing the cellular death. Deletions of the F-box and PRANC regions were created to test the toxicity of these regions. The serial dilutions (Figure 2.5) for these showed that deleting out the F-box did not cause cell death, but the full PRANC was deleted there was complete cell death. The chimeras of An01_02 and Ank05_01 both caused host cell death.

When Ank01_02 and Ank05_01 (Figure 2.2**)** were subcloned into the CEN vector, Ank01_02 produced the same levels of toxicity as the 2-micron vector while Ank05_01 still showed to be nontoxic.

A serial dilution was run to make sure the toxicity of Ank01_02 was still being produced when an N-terminal Flag tag was added. Ank05_01 showed no toxicity but Ank01_02 showed a reduction in toxicity (Figure 2.3). Due to this a C-terminal tag was added to see if it reduced the change in toxicity in Ank01_02. The streak plate analysis for the C-terminals (Figure 2.6) showed both the Flag-tag and Ha-tag had a similar toxicity as the wild type Ank01_02.

Conclusion

Orientia tsutsugamushi is an obligate intracellular bacterium that is transmitted by the bite of mites within the genus, *Leptotrombidium*. This bacterium causes a disease commonly known as Scrub Typhus. This disease has been historically understudied. *Orientia* has ORFs encoding Ank and C-terminal F-box protein motifs [3, 4]. These F-boxes are a part of the ubiquitin pathway and aid in substrate recognition. When a set of these *O. tsutsugamushi* Anks were cloned into a high copy plasmid and studied in yeast, Ank01_02 induced toxicity to the eukaryotic host cell. Toxicity was strong because when tested in a lower copy CEN plasmid complete toxicity was still exhibited.

To test our control (Ank05_01), a N-termial Flag tag was added to the gene utilizing a Site-Directed, Ligase-Independent Mutagenesis PCR. With this addition there appeared to be no change with Ank05_01, but a decrease in toxicity. A C-terminal tag was added and had similar toxicity as the wild type Ank01_02, thus showing these would be needed for a Western Blot.

The Deletion Analysis showed that with the deletion of the F-box there was survival, thus is a region that aids in the toxicity of Ank01_01. The chimera showed death in both the Ank01/05 and Ank05/01 thus this means both the Ankyrin domain and F-box contribute to the toxicity of the protein.

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Table 2.1: Primers designed for the template of *Orientia tsutsugamushi* strain Ikeda to be cloned into the yeast plasmid pYes2-gal1. The plasmids are the vector the genes were cloned into and the stars indicate the Multi Cloning Site.

Name	Gene Size	Annealing Temperature	Restriction Enzymes	Sequence
Ank9	1269 bp	59.5°C	BAMHI	F' - CCCCGGATCCATGGGACGTTTTACTAGGCTG
(OTT_0298)		59.2°C	Xhol	R' - CCCCCTCGAGCTAGTCACATATAGCATATCCTC
Ank4_01	1181 bp	59.2°C	BAMHI	F'- GGGGGGATCCATGAATAACGGTAATTTATTACATGAC
(OTT_0210)		55.4°C	Xhol	R'- GGGGCTCGAGTCATGAATTAGTGTTCTCTCC
Ank2_01	1035 bp	56.4°C	BAMHI	F'- GGGGGGATCCATGGGTAATACTGCTTTACATG
(OTT_0049)		57.5°C	Xhol	R'- GGGGCTCGAGCTATTCTCCCTCATATAGAGC
Ank16	801 bp	59.2°C	BAMHI	F'- GGGGGGATCCATGAATAATAATGATTTATTGCGTGAG
(OTT_1271)		56.6°C	Xhol	R'- GGGGCTCGAGTTAAATCATTCCATGAATACTGTC
Ank03_08	708 bp	59.2°C	BAMHI	F'- GGGGGGATCCATGATATTAAAATCTAAGACTAAGTATAC
(OTT_1112)		57.5°C	Xhol	R'- GGGGCTCGAGTTAGCAACTGGAGAACAGTTG
Ank05_01	1038 bp	55.5°C	BAMHI	F'- GGGGGGATCCATGATGACTGTTTTACATAAAGC
(OTT_0214)		57.5°C	Xhol	R'- GGGGCTCGAGCTACTCTCCCTCATATATAGC
Ank08	1170 bp	58.4°C	BAMHI	F'- CCCCGGATCCATGTATAATACTGATTTACATGATGC
(OTT_0257)		55.4°C	Xhol	R'- CCCCCTCGAGCTACGATTCCTTATGCATAAC
Ank01_02	990	58.3°C	Kpnl-HF	F'- GGGGGGTACCATGAATAATTATTTACGGCTTCGC
(OTT_0753)		60.1°C	EcoRI	R'- GGGGGAATTCCTATTCTTCCTCATATATAGCATATG

Table 2.2: Bacterial/Yeast plate recipes

Plate	Recipe	
YPD Plate	Bacto yeast extract	6g
	Bacto peptone	12g
	Bacto agar	12g
	dH_2O	570mL
	--Autoclave, allow to cool to touch, then add:	
	1% Adenine	1.2mL
	40% Glucose	30mL
Synthetic Agar –ura Glucose	Sterile 3% agar	300mL
	--Autoclave	
	20x SD	25mL
	40% Glucose	25mL
	100x AA	5mL
	1% adenine	1mL
	Sterile diH_2O	150mL
Synthetic Agar –ura Galactose	Sterile 3% agar	300mL
	--Autoclave	
	20x SD	25mL
	20% Galactose	50mL
	100x AA	5mL
	1% adenine	1mL
	Sterile diH_2O	150mL
Luria Broth (LB)	Yeast extract	5g
	Tryptone	10g
	NaCl	5g
	dH_2O	to 1L
	--Autoclave	

Table 2.3: Primers designed for a SLIM PCR to add a N-terminal Flag-tag to plasmid pYes2-gal1; the process for the insertion follows the process laid out in Chiu, J., et al. 2004.

Name	Sequence
Ank05_01 (OTT_0214)	F' 5' ATGGACTACAAAGACGATGACGACAAGATGATGACTGTTTTACATAAAGC F''5' ATGATGACTGTTTTACATAAAGC R' 5' CTTGTCGTCATCGTCTTTGTAGTCCATGGATCCGAGCTCGGTAC R''5' CATGGATCCGAGCTCGGTAC
Ank01_02 (OTT_0753)	F' 5' ATGGACTACAAAGACGATGACGACAAGATGAATAATTATTTACGGCTTCGC F''5' ATGAATAATTATTTACGGCTTCGC R' 5' CTTGTCGTCATCGTCTTTGTAGTCCATGGTACCAAGCTTAATATTCCC R''5' CATGGTACCAAGCTTAATATTCCC

Table 2.4: Primers were designed for a SLIM PCR to add a C-terminal Flag-tag to plasmid pYes2-gal1; the process for the insertion follows the process laid out in Chiu, J., et al. 2004.

Name	Tag	Sequence
Ank05_01 (OTT_0214)	Flag	F' 5' CATATGCTATATATGAGGGAGAGGACTACAAAGACGATGACGACAAGTAGC F''5' TAGCTCGAGCATGCATCTAGAGGGCC R' 5' GCTACTTGTCGTCATCGTCTTTGTAGTCCTCTCCCTCATATATAGC R''5' CTCTCCCTCATATATAGC
	Ha	F' 5' CATATGCTATATATGAGGGAGAGTATCCGTATGATGTTCCGGATTATGCATAGC F''5' TAGCTCGAGCATGCATCTAGAGGGCC R' 5' GCTATGCATAATCCGGAACATCATACGGATCTCTCCCTCATATATAGC R''5' CTCTCCCTCATATATAGC
	V5	F' 5' CATATGCTATATATGAGGGAGAGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGTAGC F''5' TAGCTCGAGCATGCATCTAGAGGGCC R' 5' GCTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCCTCTCCCTCATATATAGC R''5' CTCTCCCTCATATATAGC
Ank01_02 (OTT_0753)	Flag	F' 5' GAAGAAGACTACAAAGACGATGACGACAAGTAGGAATTCTGCAGATATCC F''5' CATATGCTATATATGAGGAAGAAG F''5' TAGGAATTCTGCAGATATCCATCAC R' 5' CTACTTGTCGTCATCGTCTTTGTAGTCTTCTTCCTCATATATAGCATATG
	Ha	F' 5' GAAGAATATCCGTATGATGTTCCGGATTATGCATAGGAATTCTGCAGATATCC F''5' CATATGCTATATATGAGGAAGAAG F''5' TAGGAATTCTGCAGATATCCATCAC R' 5' CTATGCATAATCCGGAACATCATACGGATATTCTTCCTCATATATAGCATATG
	V5	F' 5' GAAGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGTAGGAATTCTGCAGATATCC F''5' CATATGCTATATATGAGGAAGAAG F''5' TAGGAATTCTGCAGATATCCATCAC R' 5' CTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTTCTTCCTCATATATAGCATATG

Figure 2.1: Serial dilution comparing the gene inserts within the vector pYes2-gal1 shows that with the gene sequence turned on that Ank01_02 causes cellular death, while the other inserts show no toxicity. This was run with four biological replicates.

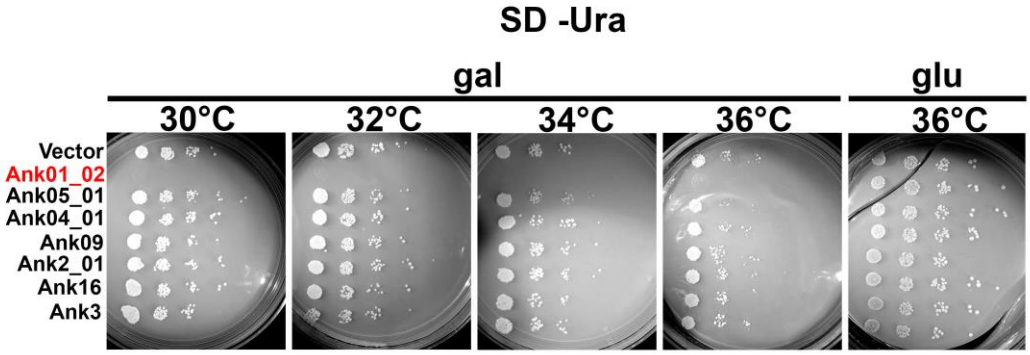

Figure 2.2: Serial dilution (A) comparing the gene inserts between the 2 micron vector, pYes2-gal1, and the CEN vector, pRS316 and pRS416, shows that the toxicity is still killing the host cell at the same levels between the high and lower expression levels. (B) A streak plate analysis was preformed to reconfirm the serial dilutions. Both A and B were run in triplicate.

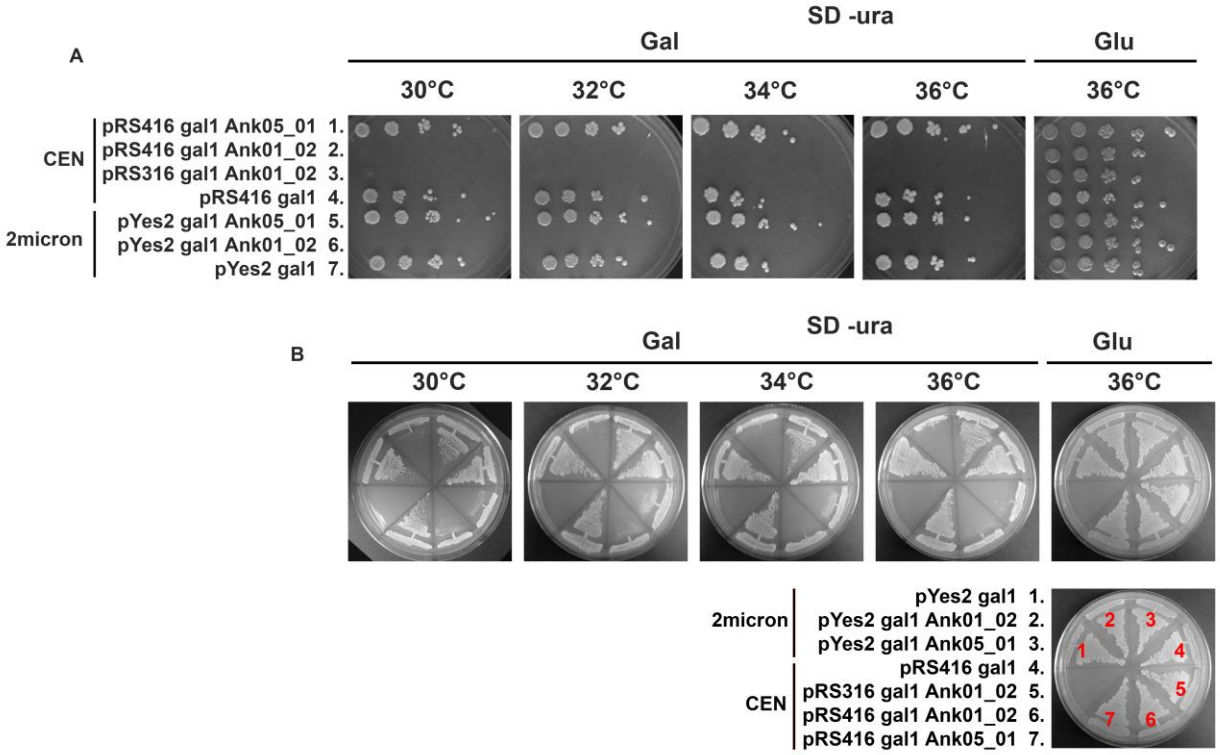

Figure 2.3: Once the Flag-tag had been added to the N-terminus of both Ank01_02 and Ank05_01 a serial dilution (A) shows there is a decrease in toxicity of the Ank01_02. This was reconfirmed with a streak plate analysis (B) and both were run with biological triplicates.

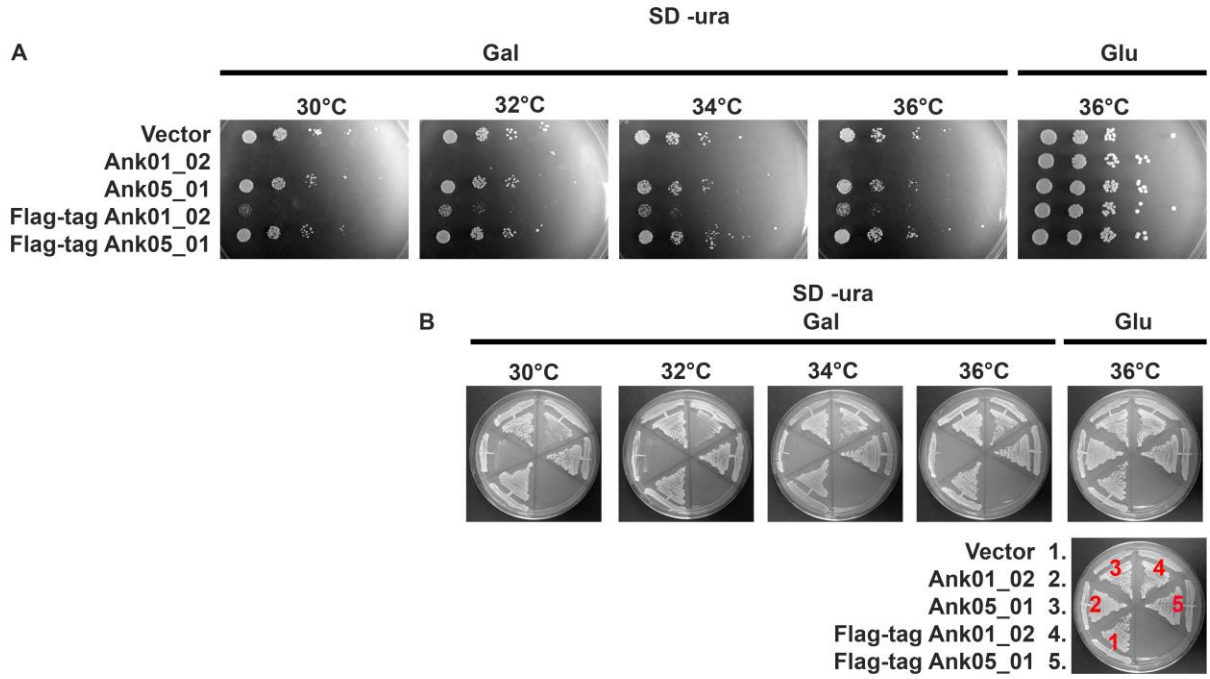

Figure 2.4: Amino acid sequence for the creation of the chimeras and deletions. The first region of Ank01_02 (shown in green) shows a 52% positive with the first region Ank05_01 (shown in purple). The second half of An01_02 (shown in grey) shows a 92% positive with the second region of Ank05_01 (shown in blue). This first region of Ank01_02 was combined with the second region of Ank05_01 and the reciprocal was done with Ank05_01. A deletion of the F-box and PRANC was created to check with toxicity of the Ankyrin Repeats.

>Ank01_02
MNNYLRLRAHLLYVTTRSIFSHTNSNPLLYAAKHNYLDVVKHLIEHGVDINTQNLRGSTALHIAAYNGNIQMGMFLLANHAEVDTQ
NVYSNTALYYAVEQSNIEMASILLSYGANPNFQCHFNVTPFEIAWVKYIDNPELHFEMMKLLVTNIVKTEHCNAVDTNLPGFLHNK
GLINESEQLKELEQQCHNEIEEMMSISVGENGKSFFDIFVLQKDINTLARCANNPDIVLCQDKFFMYSSFIEKSIKEGKARAKMLQ
GAVESIDEIFESNQDANQKSQISWLHLPLEVRMMILENLSNTDLTKLQHNDTAEADAEAELEGAYAIYEEE

>Ank05_01
MMTVLHKAAKRGDIQAVKLILIEQHGVDYINLGDMTGTTALHYAIRAESLEVTELLLIHGANPNIQNLYGHTASHYAVNVKKRIEF
IKLLLAHGANPNIQDKYCSTPLHCAAHYCDIVTIKLLLKKGANLNVLDVTHSTPFADAYRMFMVDQETNKGVMQLLVTEIVKLEHL
GIKISGSDLEGVNLNKQLINESKQLKELEQQCHKEIKKMKSICVSKNDSSFFDIFVLQKDINTLARCANNSDIVKYQNKFSMYSSF
IEKSIKEGKARAKMLQGAVESIDEIFESNQDANQESQISWLHLPPEVRMMILENLSNTDLTKLQHNDTAEAEAEAELEGAYAIYEG
E

>Ank01_02Δ175-329
MNNYLRLRAHLLYVTTRSIFSHTNSNPLLYAAKHNYLDVVKHLIEHGVDINTQNLRGSTALHIAAYNGNIQMGMFLLANHAEVDTQ
NVYSNTALYYAVEQSNIEMASILLSYGANPNFQCHFNVTPFEIAWVKYIDNPELHFEMMKLLVTNIVKTEHCNAVDTNLPGFLHNK
GLINESKQLKELEQQCHKEIKKMKSICVSKNDSSFFDIFVLQKDINTLARCANNSDIVKYQNKFSMYSSFIEKSIKEGKARAKMLQ
GAVESIDEIFESNQDANQESQISWLHLPPEVRMMILENLSNTDLTKLQHNDTAEAEAEAELEGAYAIYEGE

>Ank05_01Δ171-345
MMTVLHKAAKRGDIQAVKLILIEQHGVDYINLGDMTGTTALHYAIRAESLEVTELLLIHGANPNIQNLYGHTASHYAVNVKKRIEF
IKLLLAHGANPNIQDKYCSTPLHCAAHYCDIVTIKLLLKKGANLNVLDVTHSTPFADAYRMFMVDQETNKGVMQLLVTEIVKLEHL
GIKISGSDLEGVNLNKQLINESEQLKELEQQCHNEIEEMMSISVGENGKSFFDIFVLQKDINTLARCANNPDIVLCQDKFFMYSSF
IEKSIKEGKARAKMLQGAVESIDEIFESNQDANQKSQISWLHLPLEVRMMILENLSNTDLTKLQHNDTAEADAEAELEGAYAIYEE
E

>Ank01_02ΔPRANC(198-329)
MNNYLRLRAHLLYVTTRSIFSHTNSNPLLYAAKHNYLDVVKHLIEHGVDINTQNLRGSTALHIAAYNGNIQMGMFLLANHAEVDTQ
NVYSNTALYYAVEQSNIEMASILLSYGANPNFQCHFNVTPFEIAWVKYIDNPELHFEMMKLLVTNIVKTEHCNAVDTNLPGFLHNK
GLINESEQLKELEQQCHNEIEEMMS

>Ank01_02ΔF-box(285-329)
MNNYLRLRAHLLYVTTRSIFSHTNSNPLLYAAKHNYLDVVKHLIEHGVDINTQNLRGSTALHIAAYNGNIQMGMFLLANHAEVDTQ
NVYSNTALYYAVEQSNIEMASILLSYGANPNFQCHFNVTPFEIAWVKYIDNPELHFEMMKLLVTNIVKTEHCNAVDTNLPGFLHNK
GLINESEQLKELEQQCHNEIEEMMSISVGENGKSFFDIFVLQKDINTLARCANNPDIVLCQDKFFMYSSFIEKSIKEGKARAKMLQ
GAVESIDEIFESNQDANQKSQISWLH

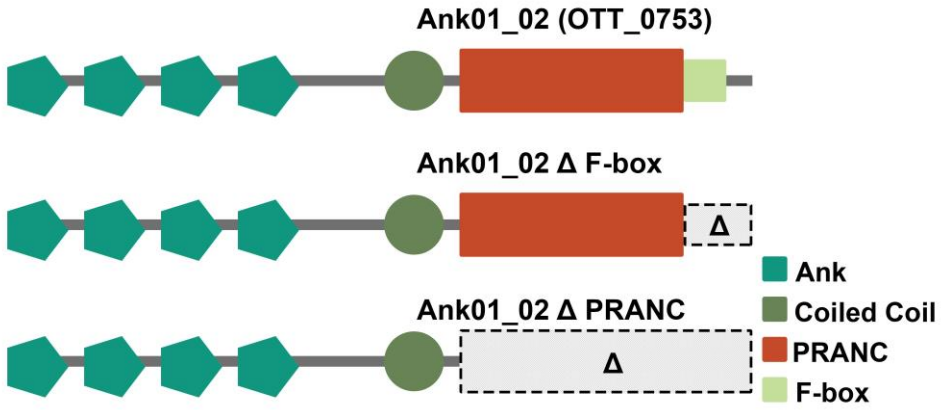

Figure 2.5: Serial dilutions (A) and Streak plate analysis (B) were run with the chimeras and deletions of Ank01_02. The serial dilution showed that Ank01/05 and Ank05/01 both kill the host cell. The deletion of the F-box proved to the nontoxic, but the deletion of the PRANC did cause host cell death.

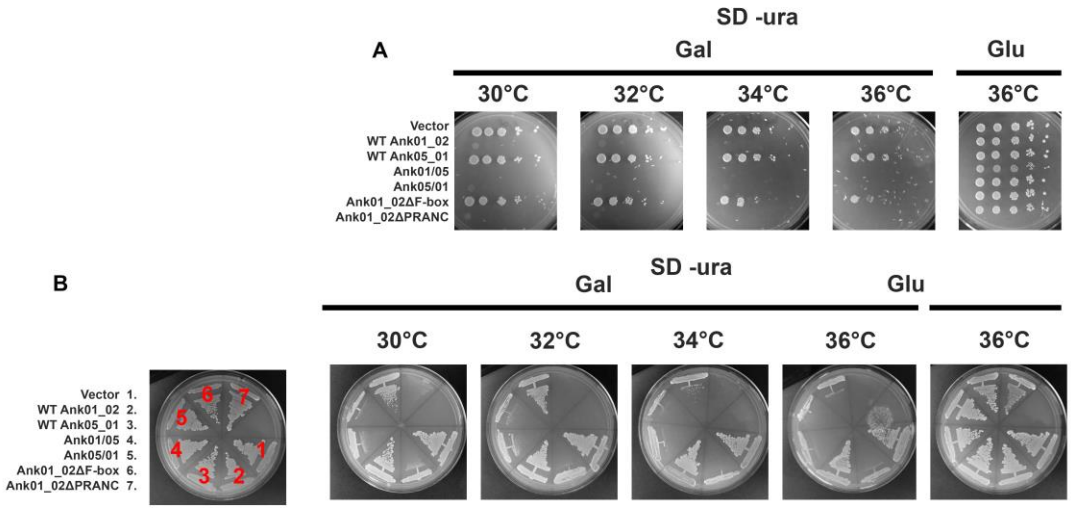

Figure 2.6: Streak plate analysis comparing the N-terminal Flag tag to C-terminal tags. The N-terminal Flag-tag decreased the toxicity of Ank01_02, while the C-terminal Flag-tag and Ha-tag had a similar toxicity as with the wild type Ank01_02.