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Isolation of a Dosage Dependent Suppressor Using *Saccharomyces Cerevisiae* as a Model System

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ISOLATION OF A DOSAGE DEPENDENT SUPPRESSOR USING
SACCHAROMYCES CEREVISIAE AS A MODEL SYSTEM

by

Rachel Chimner

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SACCHAROMYCES CEREVISIAE AS A MODEL SYSTEM

Rachel Chimner, M.S.

Western Michigan University, 2013

Human pathogenic *Yersinia* use a type three secretion system to deliver various effector proteins into host cells. Once these effector proteins are within the cell, they elicit a cascade of events that disrupt the normal immune response. One of these effectors, YopT, is known to disrupt actin distribution but it is currently unknown what YopT targets within the host cell. To investigate the cellular targets of the YopT effector, we use a yeast model system and a dosage-dependent suppression screen. The dosage-dependent suppression screen isolated three plasmids able to suppress YopT induced lethality within yeast. One of them, 2T9, was chosen for further analysis. Through the creation of several subclones, we determined that the genomic region within 2T9 was not necessary for suppression. From data collected through Western blotting and immunofluorescence, it was concluded that YopT levels were significantly reduced when the suppressor plasmids were present. Though we were unable to determine how or why suppression was occurring in the 2T9 plasmid, this research has provided proof that the genomic insert within 2T9 is not a YopT cellular target.

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Chapter I: Introduction

Yersinia Infections Today

Since the first recorded cases of *Y. pestis* during the 6th Century, this microbe has been responsible for an estimated 200 million deaths worldwide (Perry *et al.* 1997). *Y. pestis* is the causative agent of the bacterial infection commonly known as the plague, or Black Death. It has gained most of its recognition from historical events throughout the Dark Ages. During the 13th through 19th centuries, this disease killed approximately one third of the population in Europe (Perry *et al.* 1997). Though the majority of epidemics and pandemics occurred prior to the 19th century, this disease still remains a world health concern. Rural regions of Asia and Africa are experiencing a re-emergence of this infection (Stenseth *et al.* 2008). In 1994, western India experienced a *Y. pestis* outbreak that lasted two months and created widespread hysteria (Perry *et al.* 1997). More recently, in 2006 the Southwestern United States reported thirteen cases resulting in two deaths (Butler 2009).

The re-emergence of plague can be attributed to several factors. The first factor influencing the recent spread of plague is the poor living conditions found in rural areas of Africa. Most of the housing facilities in these areas are in close proximity to rodents. These rodents, which harbor the microbe, are often used as a food source. Commonly, these rural areas also lack adequate health care and the infected patient is unable to receive medical treatment in time. Second, countries undergoing political turmoil and social disorganization often have insufficient healthcare systems which can limit the amount of aid given to regions undergoing an

epidemic. The lack of protocols put in place to prevent the spread of infection leads to an increase in not only the number of cases, but the area to which the disease spreads as well. Another factor contributing to the re-emergence of plague is the change in geography. As we reshape the normal landscape, we allow for greater exposure to rodent reservoirs. Also, an increase in global travel allows for the spread of infection to new regions that have not previously been affected (Stenseth *et al.* 2008).

In the future, the risk associated with possible outbreaks increases. One reason for this is climate change. Warmer springs and wetter summers have been shown to increase the prevalence of *Yersinia* in its animal hosts (Stenseth *et al.* 2006). This type of climate change has been predicted for North America and Central Asia (Stenseth *et al.* 2008). Another factor contributing to the rising risk of plague outbreaks is the possibility of antimicrobial resistance. Currently, there is no vaccine for human pathogenic *Yersinia* and the main treatment consists of antibiotics. This creates the possibility of multidrug resistant *Yersinia* strains. An example of this occurred in Madagascar in 1995. The *Yersinia* strain isolated from this outbreak was shown to contain a self-transmissible plasmid that was resistant to eight different antibiotics. Another possibility is the acquiring of new ethological agents through homologous recombination with other pathogens (Welch *et al.* 2007).

Yersinia outbreaks elicit fear and panic among the public. Though the death toll from this infection has decreased in recent decades, the fear associated with it still remains. Most of this is due to the possibility of weaponizing the microbe. For centuries cultures have been weaponizing plague by throwing infected bodies over city

walls or dispersing infected fleas into populations. Another more recent tactic has been the creation of aerosol inhalants that spread the bacteria through respiratory droplets (Stenseth *et al.* 2008). The potential for weaponization of *Yersinia* leads to a greater need for research that aids in the understanding of how this microbe lives within its human host.

Though the possibility of a worldwide epidemic as devastating as the Black Death is unlikely, *Yersinia* still remains a threat to the world's population. Whether it's the change in climate, risk of increased antimicrobial resistance, or the fear of weaponization, *Yersinia* still has the potential to infect great numbers of people. Understanding the complete mechanism of pathogenesis and how *Yersinia* functions within its host is crucial to protect against future potentially devastating outbreaks.

Human Pathogenic *Yersinia* and the Yops

Yersiniae are gram negative coccobacilli belonging to the family *Enterobacteriaceae*. They are non-motile, non-spore forming facultative anaerobes. Of the eleven known *Yersinia* species, three have been identified as pathogenic to humans: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*. The most widely recognized of the three is *Y. pestis*, which causes the plague. The other two pathogens, *Y. enterocolitica* and *Y. pseudotuberculosis*, are less commonly known. Both species cause enteric infections, but *Y. pseudotuberculosis* can also manifest symptoms that resemble tuberculosis (Cover & Aber 1989). *Y. pestis* is spread to humans via an infected rodent, flea or another human. *Y. enterocolitica* and *Y. pseudotuberculosis* are spread only from human contact (Fallman *et al.* 2002). All

three of the pathogenic species grow in the lymph nodes and are closely related because they have similar mechanisms of pathogenesis in mammalian hosts (Straley *et al.* 1993).

Human pathogenic *Yersinia* species have several virulence factors that allow them to thrive in mammalian hosts. In a typical infection, once a bacterial cell has entered into the body, the immune response is initiated. The presence of different bacterial cell markers triggers professional phagocytes such as macrophages and leukocytes to ingest and degrade the foreign bacteria (Fallman *et al.* 2002). In order to evade this immune response, *Yersinia* cells produce effector proteins, called Yops (*Yersinia* outer proteins), which are secreted into the host cell via a type three secretion system (TTSS). This secretion system consists of approximately 27 proteins and uses a needle-like appendage, called an injectisome, to translocate effector proteins from the bacterial cell directly into the host cell cytoplasm (Broz *et al.* 2007). The formation of the injectisome and the translocation of its effectors, are triggered by the recognition of body temperature (Cornelis 2002). Once inside the cell, the Yops will work together to disrupt various cellular functions. Several of these effectors are directly involved in prevention of phagocytosis (Apefelbacher *et al.* 2007).

Along with the type three secretion machinery, there are six different Yop effectors that are encoded on a 70kb virulence plasmid called pYV (Apefelbacher *et al.* 2007). Of these six effectors, YopT, YopE, YopO and YopH all work together to prevent the formation of lamellipodia. Lamellipodia are arm-like projections on phagocytes that contain an actin cytoskeleton. These projections are used to surround

foreign material in order to bring it into the cell for degradation (Owen *et al.* 2007).

The Yops each use different molecular mechanisms in order to cause actin cytoskeleton breakdown and prevent formation of the lamellipodia.

YopE, YopO and YopT all target the Rho GTPase cycle, which regulates the actin cytoskeleton. YopE is a 23 kDa GTPase activating protein that down-regulates Rho GTP binding proteins (Apefelbacher *et al.* 2007). This down-regulation prevents the assembly of filamentous actin (Soon-Tuck & Manser 2011). YopO from *Y. enterocolitica* is an 82 kDa serine threonine kinase. The effector YdkA from *Y. pestis* and *Y. pseudotuberculosis* is a closely related homologue. It uses SycO as a chaperone, and auto-phosphorylates upon stimulation with actin (Apefelbacher *et al.* 2007). It interacts with Rac1 to inhibit the intrinsic guanine nucleotide exchange of GDP for GTP. This causes inactivation of Rac1 and prevents the formation of actin fibers. Another effector, YopT, is a cysteine protease that localizes to the cell membrane. It untethers G-proteins RhoA, Rac1 and CDC42 from the membrane through cleavage of an isoprene group (Trotsky *et al.* 2008). This cleavage leaves the GTPases inactive and no longer able to control actin cytoskeleton rearrangement. YopH is 51 kDa tyrosine phosphatase protein. It dephosphorylates focal adhesion kinase (Fak), paxillin, and Fyn-binding protein (Fib) (Apefelbacher *et al.* 2007). This dephosphorylation disrupts the interaction between the actin cytoskeleton and extracellular matrix-binding integrins, thereby preventing the formation of focal adhesions and disrupting phagocytosis.

The last two effectors, YopM and YopJ, are the least understood of the Yops. YopM is a 42 kDa protein that is thought to act as a scaffold for two kinases known as Rsk1 and Prk2. This scaffolding results in downstream activation of more kinases. It is the only Yop effector not to contain catalytic activity (Trosky *et al.* 2008). Lastly, YopJ from *Y. pestis* and *Y. pseudotuberculosis*, and YopP from *Y. enterocolitica*, are 31 kDa proteins that are not essential for *Yersinia* virulence (Straley *et al.* 1993). They disrupt the innate immune response and promote apoptosis of macrophages by targeting MAPK kinases and I κ B kinase- β (Trosky *et al.* 2008).

Though each Yop effector protein has its own function, they are thought to work together in order to prevent degradation of the bacterial cell after it has entered the body. With the exception of YopM, all of the effectors are essential for survival within its mammalian host. These effective virulence factors are what keep human pathogenic *Yersinia* thriving in different populations worldwide millennia after its first emergence. Even though there has been a recent outpouring of knowledge on the Yops, the complete mechanism to pathogenesis remains unknown. In order to gain a better understanding of how these Yops function, an effective and efficient model system must be used to study them.

Yeast History

For centuries budding yeast, also known as *Saccharomyces cerevisiae*, has been an important tool used in cultures around the world. Civilizations such as Babylonia and Sumer used yeast for brewing beer as early as 6000 B.C. (Hornsey 2003). In China, evidence of fermented beverages has been discovered as early as

7000 B.C. (Legras *et al.* 2007). *S.cerevisiae* was first observed under a microscope by Antony van Leeuwenhoek in 1680. Then, in the 1860's, Louis Pasteur discovered that yeast was a living organism and was the agent responsible for alcohol fermentation and dough leavening (Barnett 2010).

Since becoming the first eukaryotic genome to be sequenced in 1996, researchers have utilized yeast as an important tool in scientific research (Dujon 1996). Scientists began using *S. cerevisiae* as a model organism in the mid 1930's, and it has become one of the most popular model organisms used in research today (Roman 1981). For example, yeast has played a vital role in the understanding of mitochondrial genetics as well as vacuolar function (Borkovich and Ebbole 2012). The large success of yeast as a model organism can be attributed to the many unique features of yeast that make it advantageous.

Yeast as a Model System

Many properties of *S. cerevisiae* make it an ideal model organism for molecular research. First, *S. cerevisiae* is a simple unicellular fungus, which provides the advantage of working with an organism that needs few nutrients to survive, but still contains a nucleus. Also, many molecular processes such as cellular repair and replication are conserved throughout eukaryotes (Guthrie and Fink 1991). This homology between eukaryotes allows researchers to make comparisons between yeast and higher order organisms such as mammals (Feldmann 2010).

There are various advantages of *S. cerevisiae* that make it more favorable than other organisms to work with in the laboratory. First, yeast is nonpathogenic to

humans. Therefore, it can be handled with limited precautions and without special equipment (Sherman 1991). Other model microorganisms, such as *Escherichia coli*, require biosafety precautions that do not apply when working with yeast. Another attractive feature of yeast is the ability to grow on defined media. This enables the researcher to have complete control over environmental parameters. Also, it allows for colony selection through the use of nutritional auxotrophs.

Another benefit of *S. cerevisiae* is its quick generation time. Yeast reproduces by generating a complement daughter cell through budding. This method of reproduction is advantageous because it allows for a fast doubling time of ~90 minutes at 30°C (Watson *et. al* 1987). This fast generation time permits yeast to be cultured quickly, in mass amounts, and at a low economic cost. Also, mutants can be created and selected quickly since many generations of progeny can be created in a short time.

Another unique feature of yeast is its life cycle. *S. cerevisiae* is viable in both haploid and diploid states, depending on nutrient availability. When the cell is in an adequate nutrient environment, two haploid cells of differing mating types (α and a) can mate to form a diploid cell. When the cell is in a nutrient deprived environment, it undergoes meiosis to form four haploid spores that are encapsulated in a thick walled sac called an ascus (Watson *et. al* 1987). The ability to exist in the haploid state allows for recovery of recessive mutations while the diploid state can be used for complementation tests and homologous recombination. Since yeast can exist in both states, there is a large capacity to carry out multiple methods of experimentation.

Yeast is known to contain 16 chromosomes with over 6,000 open reading frames that are predicted to produce protein products (Sherman 1991). The genome is 12.8 Mb, which makes it larger than *E. coli* (4.6 Mb), but significantly smaller than the human genome (3.2 Gb) (Blattner *et al.* 1997, Guthrie and Fink 1991). Also, yeast contain extra-chromosomal elements such as the 2 μ plasmid sequence which is a 6.3 kb circular DNA sequence that is found at about 50-100 copies per cell (Strathern 1981). This plasmid sequence provides the ability to easily overexpress genes within the cell through plasmid selection.

Lastly, yeast is advantageous over other model organisms because of the large amount of information currently available to researchers. Since the completion of the yeast genome project, scientists have created several online yeast databases containing a wealth of knowledge (Dujon 1996). The accessibility of information from these online databases has led to a greater availability of perfected genetic protocols. One example is the high efficiency yeast transformation which allows for easy addition or deletion of genes through homologous recombination (Orr-Weaver *et al.* 1981). This allows for specific location integration of plasmids containing foreign sequences (Sherman 1991). Another example is the two-hybrid screen that is used to determine protein-protein interactions.

All of these factors combined make yeast a very effective model organism. The low economic cost, ability to control environmental parameters, and the ease of working with a nonpathogenic organism all have made yeast an important tool in biological research. In the last century, *S. cerevisiae* has become one of the most

efficient model organisms in molecular cloning experiments due to its small genome size and the availability of perfected genetic protocols. In the future, yeast will continue to be a valued model system due to the large availability of information that is accessible through online databases.

Conclusion

The recent re-emergence of plague has led to an immediate need for research on how this pathogen infects and survives within its human hosts. In the last decade, a slew of literature has been published regarding the pathogenesis of *Yersinia* and its Yop effectors. However, with this new information, many new questions arise. What do these Yop effectors target within the host cell? How do they work together? What other genes are necessary for proper functioning? Do these effectors need buffers or chaperones in order to reach the intended cellular targets? All of these questions are important for understanding the establishment of *Yersinia* infections. To begin to gain answers to these questions, researchers are developing new ways to study gene interactions without the limitations of single mutant phenotypic studies. One example, the Synthetic Genetic Array (SGA), can be used to determine genetic interactions through the use of a systematic screen. This new experimental approach can be used to investigate the cellular targets of the Yops using yeast as a model system, and thus give insight into how this prevalent pathogen survives.

Chapter II: Study Design and Results

Introduction to Systematic Genetic Screening

Synthetic Genetic Array

With the recent wealth of knowledge gained from genome sequencing projects, scientists are beginning to investigate the functions of thousands of highly conserved genes. Also, they are examining new ways to determine how these genes work together within complex biochemical pathways and cellular processes. The use of single mutant phenotypic studies as an effective way to determine gene function is limited by the realization that most genes act as buffers for other genes within the same pathway. For example, *Saccharomyces cerevisiae* haploid cells can still survive when 5,000 of the 6,000 predicted genes are deleted (Baetz *et al.* 2006). Therefore, some cellular processes may be directed by several genes that work in unison.

By the end of the 20th century, there was a strong need for an efficient approach to investigate genetic interactions. The emergence of a Synthetic Genetic Array (SGA) technique provided researchers with a systematic way to screen for genetic interactions. SGA can be divided into two types of approaches: synthetic lethal and synthetic dosage lethal arrays. In synthetic lethal arrays, a gene of interest is mutated so that it no longer functions properly. Systematically mutating all other non-essential genes, and creation of a double mutant that has a phenotype that is distinct from that of either single mutant, indicates the presence of a genetic interaction. For example, when a single mutation causes cells to be less viable than wild type, and the

addition of a second mutation that is not essential for viability in a different gene causes lethality, the two genes may interact with other (Baetz *et al.* 2006).

Another type of assay, synthetic dosage lethal, uses the same principle as synthetic lethal, but instead of a loss-of-function mutation, the genes are over-expressed. The over-expression of the genes themselves have no known effect on wild type, but a different phenotype can be observed if a mutation lies in another gene within the same biochemical pathway. For example, the expression of a gene of interest causes lethality, while the over-expression of a different gene within the same pathway will suppress the lethality. Since the increased amount of the second gene was able to compensate for loss from the lethality of the first gene, then the two genes may interact with one another. The process of systematically screening all over-expression phenotypes is referred to as dosage-dependent suppression screening and can be used to identify cellular targets of proteins (Baetz *et al.* 2006).

Dosage-Dependent Suppression Screening

The dosage-dependent suppression screen has been shown to be effective in the model system *Saccharomyces cerevisiae* (DeChamps *et al.* 2005, Burger *et al.* 2000). It can also have implications in other organisms as well. For example, it is currently known that 30 percent of the genes that are identified to play a role in human disease have yeast orthologues. This is mostly due to the fact that the components of DNA repair machinery and cell division are highly conserved (Foury 1997). Because of this homology, researchers can use bioinformatical analysis to look for orthologues in other organisms.

The dosage-dependent suppression screen in yeast uses a multicopy plasmid library. Each clone within the library contains a fragment, or insert, of the yeast genome and a 2 μ sequence that allows for over-expression of the inserted genes. Each library clone is transformed into yeast, and the over-expression phenotype is examined. Thus, the phenotype of only a few genes at a time can be viewed, as opposed to the entire genome. This suppression screening technique has many applications in the field of biotechnology and its methodology has been proven to be effective.

Dosage-dependent suppression screening has useful implications for cancer treatment. One example is a study conducted on cisplatin resistance by Burger and colleagues in 2000. Cisplatin based chemotherapy is frequently used to fight against cancer but, over time, cellular resistance can occur. In order to isolate resistance genes, a plasmid library was created by cloning 5-20 kb fragments of the yeast genome into multicopy vectors. The plasmid library was transformed into cisplatin-sensitive mutant yeast cells and plated onto selective media containing cisplatin. Colonies that could grow in the presence of cisplatin were considered to contain the genes responsible for the resistance phenotype and were selected for further analysis. Sequencing and bioinformatical analysis revealed the presence of the *PDE2* gene in resistant strains. This gene is important because it is known to induce cisplatin resistance in mammalian cells as well (Liu *et al.* 1998). This provides proof that the experimental approach used for this study was appropriate to isolate cisplatin resistance genes using a multicopy genomic library.

In 2005 Dechamps *et al.* used suppression screening to investigate vesicular trafficking genes in yeast. The *Saccharomyces cerevisiae* genes *msb3* and *msb4* have been previously shown to act as GTPase-activating proteins by facilitating exocytosis and actin cytoskeleton rearrangement in vesicular trafficking (Gao *et al.* 2003 & Albert and Gallwitz 2000). To investigate other possible interacting genes/proteins in the process of *msb3* and *msb4*, a suppression screen was conducted on cells containing an *msb3 msb4* double mutation. The yeast genome was isolated into 2-5 kb fragments and the multicopy library was then transformed into yeast harboring the double mutation and grown on media containing DMSO or caffeine. Colonies that could overcome growth inhibition in the presence of caffeine were considered to contain suppressor plasmids. From this screen, six suppressor genes were identified, classified, and the phenotypes were obtained. This method of multicopy suppression screening has proven to be useful in determining cellular components that are involved in poorly understood biochemical pathways.

Conclusion

Over the last decade, advancements have been made to aid in the understanding of complex biochemical pathways. The emergence of synthetic lethal and synthetic dosage lethal assays has allowed researchers the ability to screen for specific genetic interactions within these pathways. This technique does not encompass the limitations that can be found in single mutant phenotypic studies and it can be performed on essential genes. The use of a dosage-dependent suppression

screening technique allows researchers to begin to identify the cellular targets of genes that work together to carry out various cellular processes.

Previous Research

Expression of Effector Proteins in Yeast

Constructs containing one of three Yop effector proteins, YopO, YopE and YopT, have been created for expression in *Saccharomyces cerevisiae*. When expressed, these effectors cause lethality, or cell death (Nejedlik *et al.* 2004 and Nejedlik, L. unpublished data). The similarity in phenotype between the three Yops has led researchers to propose that the three Yops have the same or similar cellular targets. The study of YopO expression in yeast concluded that growth inhibition is not due to the arresting of the cell during a specific phase within the cell cycle, but rather that YopO kills the cell regardless of its place within the cell cycle.

Furthermore, YopO localizes to the cell periphery where it disrupts normal actin distribution (Nejedlik *et al.* 2004). It is currently unknown whether YopE or YopT also locate to the periphery to cause the same or a similar effect (Lesser *et al.* 2001).

Though three of the six Yop effector proteins are lethal in yeast, not all type three secretion system effectors inhibit growth of *Saccharomyces cerevisiae*. One example of this is found in the plant pathogen *Pseudomonas syringae*. *Pseudomonas syringae* use a type three secretion apparatus to secrete over 30 different Hop effectors, or Hrp Outer Proteins, across host cell membranes (Buell *et al.* 2003, Grant 2006). The Hops deactivate the normal plant immune system and establish infection of the bacterium. In a study published by Munkvold *et al.* in 2008, 27 Hop effectors

were tested for lethality in yeast. Of these 27, only five were shown to inhibit growth while two elicited cell death. The remaining effectors neither inhibited cell growth nor elicited cell death (Munkvold *et al.* 2008). In order to further investigate these results, 3 Hop effectors (HopAO1, HopAF1 and HopM1) were tested for lethality in yeast. Of these three, HopAO1 and HopAFI were found to inhibit growth while HopM1 was not lethal in yeast (Revindrin, V. unpublished data). Therefore, it is not simply the expression of the effectors themselves that causes lethality in yeast.

Suppression of YopT

The *Yersinia* effector, YopT was chosen for the dosage-dependent suppression screen to identify cellular targets. For this screen, a Yeast Genomic Tiling Collection of plasmids was used. This plasmid library consists of over 1, 500 unique plasmids that make up an overlapping collection of the yeast genome. The plasmids contain a yeast-*E. coli* shuttle vector (pGP564) that is comprised of a *LEU2* selectable marker along with a 2 μ sequence for over-expression. The average insert size is 10Kb and contains approximately 4-5 genes. This plasmid library was cloned into *Saccharomyces cerevisiae* along with an expression plasmid harboring the YopT gene under control of the *GALI* inducible promoter.

To conduct the dosage-dependent suppression screen, the growth phenotypes of all the library plasmids were observed. In colonies that displayed growth inhibition, a cellular target was not likely to be contained within that plasmid. This is due to the fact that YopT was able to interact with its cellular target normally to elicit cell death. Conversely, in cultures where growth was not inhibited, that library plasmid is a

suppressor plasmid and was thought to contain a possible cellular target. This is due to the fact that while YopT interacts with its cellular target, the over-expression of the suppressor plasmid create extra copies of the target within the yeast cell that can compensate for the YopT induced lethality. Therefore, if a plasmid is able to suppress lethality, it is thought to contain a cellular target. The phenotypes of all the library plasmids were observed and three plasmids were found to suppress YopT lethality: 2T9, 10T14, and 10T15 (Geiser, J.R. unpublished data). From these three, 2T9 was selected for further analysis.

The plasmid 2T9 contains 3 yeast genes in its insert: *SUL1*, *VBA2* and *PCAI*. The first gene, *SUL1*, is a high affinity sulfate permease. It transports sulfate across the plasma membrane so that it can be assimilated into S-amino acids. Also, it is a member of the SulP anion transporter family (Smith *et al.* 1997). The next gene *VBA2*, is a permease that mediates transport of amino acids into the vacuolar membrane. This gene will transport basic amino acids such as histidine, arganine and lysine. Also, *VBA2* can be used to transport tyrosine. It is considered a member of the basic amino acid transporter family which is a subset of the larger major facilitator superfamily, or MFS (Shimazu *et al.* 2005). The last gene, *PCAI*, is a cadmium transporter P-type ATPase. *PCAI* functions as an efflux pump to remove toxic metals such as cadmium and copper from the cell. It is a member of the P_{1B}-type ATPase family of heavy metal transporters (Adel *et al.* 2007). Interestingly, all three of the yeast genes isolated on the 2T9 plasmid function as membrane transporters. Though

each gene has a separate and unique function, they all seem to act as the pore, or pump, through which molecules are translocated.

Specific Aims

- 1. To identify the gene of the cellular target of YopT that resides in the genomic insert of the 2T9 plasmid.** The unique 2T9 library plasmid is able to suppress YopT induced lethality; therefore, the genomic insert contained within this plasmid is thought to contain a possible cellular target gene. There are three yeast genes located within this plasmid: *SUL1*, *VBA2* and *PCAI1*. The region required for suppression may consist of a single gene, a fraction of a gene, or multiple genes working together. The goal of this research is to isolate the region within 2T9 needed for suppression. This will be accomplished by the creation of several subclone constructs through standard molecular cloning procedures (Sambrook *et al.* 1989). These subclones will then be tested for suppression of YopT lethality by assessing growth phenotypes through serial dilution replica plating. Once the fragment of 2T9 that is necessary for suppression has been isolated, we can begin to understand how this suppression is occurring.
- 2. Identify the sub-cellular location of the YopT protein.** To give insight into the mechanism of suppression of YopT induced lethality, it is important to localize YopT within yeast cells. Then, we can examine if the putative suppressor has an effect on YopT localization. The goal of this research is to determine if the location of YopT and the identified suppressor coincide.

Cellular localization will be accomplished by immunofluorescence as previously described (Nejedlik *et al.* 2004). The putative suppressor may be deactivating YopT by delocalization, or degradation of the protein. It is also possible that the suppressor is able to repress the *GALI* promoter, thus preventing expression of YopT. Once it has been determined where YopT is located in the presence of the suppressor, we can begin to theorize how the suppressor is preventing lethality.

Methods and Materials

Media

All yeast media (YPD, SD-leu, SD-ura, SD-ura-leu, Sgal-ura, Sgal-leu, Sgal-ura-leu) were prepared as previously described (Guthrie and Fink, 1991). All LB media (LB + ampicillin, LB + Kanamycin) were prepared as previously described (Sambrook and Russell 2001).

Yeast Strains

Table 1: Yeast strains used in this study.

Strain	Chromosomal
JGY4	<i>MATα lys2-801 his3-Δ200 leu2-3,112 ura3-52</i>
JGY3	<i>MATα ade2-801 his3-Δ200 leu2-3,112 ura3-52</i>
JGY709	<i>MATα/MATα ade2-101/ADE2 LYS2/lys2-801 his3--200/his3-200 leu2-3,112/leu2-3,112 ura3-52/ura3-52</i>

Yeast Transformation

Transformations of yeast strains were performed essentially as described (Gietz and Schiestl, 1991).

Bacterial Transformation

Plasmid DNA was transformed into chemically or electrically competent DH5 α or XL-1 blue cells. Colonies were grown overnight on LB+ampicillin or LB+kanamycin media and an alkaline lysis miniprep was performed to isolate plasmids DNA (Sambrook and Russell, 2001).

Serial Dilution Replica Plating

Transformants were grown overnight in S raffinose-ura-leu minimal media to a final raffinose concentration of 2%. Cultures were serially diluted by 40-fold and induced by plating into Sgal-ura-leu media. They were incubated for 3 days at 30°C.

Culture Preparation

Yeast strains were grown overnight with shaking at 30°C in S raffinose-ura-leu minimal media (2% final concentration of raffinose). Cultures were then diluted to 30 klett in fresh S raffinose-ura-leu media. These diluted cultures were then grown at 30°C for ~5 hours until mid-log phase was reached (~80 Klett). Samples were collected for immunofluorescence and western immunoblotting at time point zero, and the cultures were then induced with galactose to 2% final concentration. Cultures were allowed to continually grow for ~4 additional hours and samples were collected at two, three and four hour time points.

Immunofluorescence

Immunofluorescence was performed essentially as described (Nejedlik *et al.* 2004). Slides were viewed using a Leica DM5500 microscope using a Q-Imaging Retiga Exi 1394 Fast camera and Image Pro 6.0 software.

Sample Preparation

Samples were collected in 15ml aliquots and Phenylmethanesulphonylfluoride (PMSF) was added to a final concentration of 1 mM, to inhibit proteases. Samples were washed with 1 ml of water, and spun in a microcentrifuge. The supernatant was removed and the resulting pellet was resuspended in 500 μ l of GFO with protease cocktail. GFO is made up of 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM MgCl₂ and 0.2% Tween 20. The protease cocktail consists of 1 mM PMSF, 1 μ g/ml pepstatin and 1 μ g/ml leupeptin. Approximately one half of the total volume of acid-washed glass beads were added to each sample. The samples were then bead beaten by vortex at 30 second intervals for 10 minutes at 4°C in order to break down the cell wall. The samples were placed on ice for 30 seconds in between intervals. The samples were then centrifuged for 10 minutes in a microcentrifuge at 14000 rpm at 4°C. The supernatant was removed and placed in a fresh eppendorff tube.

Western Blotting

Western immunoblotting of proteins was performed as previously described (Nejedlik *et al.* 2004).

Yop Expression Plasmids

Table 2: Yop effector expression plasmids used in this study.

Expression Plasmid	Yeast Markers	Bacteria Markers
pJG495	CEN6 ARSH4 URA3 PGAL1-YopT-V5-6H-CYC1term	bla f1 attR1 attR2
pJG494	CEN6 ARSH4 LEU2 PGAL1-YopT-V5-6H-CYC1term	bla f1 attR1 attR2
pLN5	CEN6 ARSH4 URA3 PGAL1-YopO-V5-6H-CYC1term	bla f1 attR1 attR2
pJG491	CEN6 ARSH4 URA3 PGAL1-YopE-V5-6xHis-CYC1 _{term}	bla f1 attR1 attR2
pJG485	CEN6 ARSH4 URA3 PGAL1-V5-6H-CYC1term	bla f1 attR1 CmR ccdB attR2
pJG484	CEN6 ARSH4 LEU2 PGAL1-V5-6H-CYC1term	bla f1 attR1 CmR ccdB attR2

Creation of 2T9 Sub-clones

All digestions were performed per New England Biolabs enzyme standard reaction protocols. All reactions were run on a 1% agarose gel.

To create pRC1 and pRC8, pJG551 (2T9) was digested with HindIII. The 9.2 kb band was isolated and reclosed through ligation. To create pRC2, pRS426 was digested with HindIII and SpeI and the 5.7 kb band was isolated. Next, pJG551 (2T9) was digested with HindIII and XbaI and the 1.5 kb band was isolated. The 5.7kb and 1.5 kb bands were then ligated together. To create pRC3, first pRS426 was digested with NotI and EcoRI and the 5.7 kb band was isolated. Then, pRC1 was digested with NotI and EcoRI and the 1.7 kb band was isolated. The 5.7 kb and 1.7 kb band were then ligated together. For creation of pRC4, pGP564 was digested with XhoI and SacI and the 7.1 kb band was isolated. Next, pRC1 was digested with XhoI and

SacI and the 1.7 kb band was isolated. Then, the 7.1 kb and 1.7 kb bands were ligated together.

To create pRC5, pRC1 was digested with HindIII. After 1 hour, 1 μ L of T₄ DNA polymerase and 1 μ L of nucleotides were added and allowed to sit at 37°C for 1 additional hour. From this reaction the 7.1 kb band was isolated. Next, pRC3 was digested with EcoRI for 1 hour, then with addition of 1 μ L of T₄ DNA polymerase and 1 μ L of nucleotides for 1 hour. The reaction was then heat shocked for 15 minutes at 65°C. Lastly, NotI was added and the reaction continued for 1 additional hour. From this reaction the 1.7 kb band was isolated. The 7.1 kb and 1.7 kb fragments were ligated together. For creation of pRC6, pRC1 was digested with HindIII and NotI for 1 hour. Then, 1 μ L of T₄ DNA polymerase and 1 μ L of nucleotides were added and the reaction continued for an additional hour. The 7.2 kb band was isolated and ligated. In order to create pRC7, pGP564 was digested with NotI and the 6.6 kb band was isolated and ligated. Lastly, for creation of pRC9, pGP564 was digested with NotI and the 6.6 kb band was isolated and ligated. All created plasmid constructs were confirmed by sequencing to assure that no mutations had been incorporated during experimentation. Table 3 summarizes the creation of all subclones.

Table 3: Summary of creation of the 2T9 subclones.

2T9 subclones	Template(s)	Markers	Enzyme(s)	Plasmid Size
pRC1	pJG551 (2T9)	<i>KanR LEU2</i> <i>2micron ARS228</i>	HindIII	9.2 kb
pRC2	pRS426	<i>AmpR URA3</i> <i>2micron ARS228</i>	HindIII, SpeI	7.2 kb
	pJG551 (2T9)	<i>KanR LEU2</i> <i>2micron ARS228</i>	HindIII, XbaI	
pRC3	pRS426	<i>AmpR URA3</i> <i>2micron ARS228</i>	NotI, EcoRI	7.2 kb
	pRC1	<i>KanR LEU2</i> <i>2micron</i>	NotI, EcoRI	
pRC4	pGP564	<i>KanR LEU2</i> <i>2micron</i>	XhoI, SacI	8.8 kb
	pRC1	<i>KanR LEU2</i> <i>2micron</i>	XhoI, SacI	
pRC5	pRC1	<i>KanR LEU2</i> <i>2micron</i>	HindIII, NotI, T4 DNA Polymerase	8.8 kb
	pRC3	<i>AmpR URA3</i> <i>2micron ARS228</i>	EcoRI, NotI, T4 DNA Polymerase	
pRC6	pRC1	<i>KanR LEU2</i> <i>2micron</i>	HindII, NotI, T4 DNA Polymerase	7.2 kb
pRC7	pGP564	<i>KanR LEU2</i> <i>2micron</i>	NotI	6.6 kb
pRC8	pJG551 (2T9)	<i>KanR LEU2</i> <i>2micron ARS228</i>	HindIII	9.2 kb
pRC9	pJG551 (2T9)	<i>KanR LEU2</i> <i>2micron</i>	NotI	6.6 kb

Results

YopE and YopO Lethality Suppression

Previous research has discovered that YopE, YopO and YopT effector proteins cause growth inhibition when expressed in yeast (Nedjedlik 2004 and Geiser, J.R. unpublished data). These results have led us to propose that these three Yops have the same or similar cellular targets. To determine if the suppression of yeast

have the same or similar cellular targets. To determine if the suppression of yeast lethality seen by the 2T9 library plasmid was specific to YopT, lethality by the other two Yops was examined as well. The suppressor subclone pRC6 was expressed in yeast cells containing a YopT, YopO or YopE expression plasmid. The growth phenotypes were then examined by serial dilution replica plating on SD-ura-leu and Sgal-ura-leu medium. Serial dilution replica plating is a growth assay that assesses cell viability. This assay is able to distinguish between complete growth inhibition versus partial growth inhibition of colonies. Since the YopT gene is under control of the *GALI* promoter, plating on media that contains either dextrose or galactose allows for growth phenotypes to be examined in un-induced and induced cultures. As shown in Figure 1, the pRC6 suppressor was able to suppress lethality in all three of the Yop effectors. Therefore, the suppression of Yop effector induced lethality is not unique to YopT. The consistency of suppression between the Yops by the pRC6 plasmid suggests that suppression of YopO, YopE and YopT may occur via a similar mechanism.

Suppression of YopT Lethality by 2T9 Sub-clones

To investigate the cellular targets of the Yops, it is important to understand what region of the 2T9 library plasmid is responsible for suppression of YopT induced lethality. Each library plasmid clone is unique and only three plasmids were able to suppress lethality, therefore, the genomic region located within 2T9 is thought to harbor a Yop target gene. There are three yeast genes located within the 2T9 plasmid:

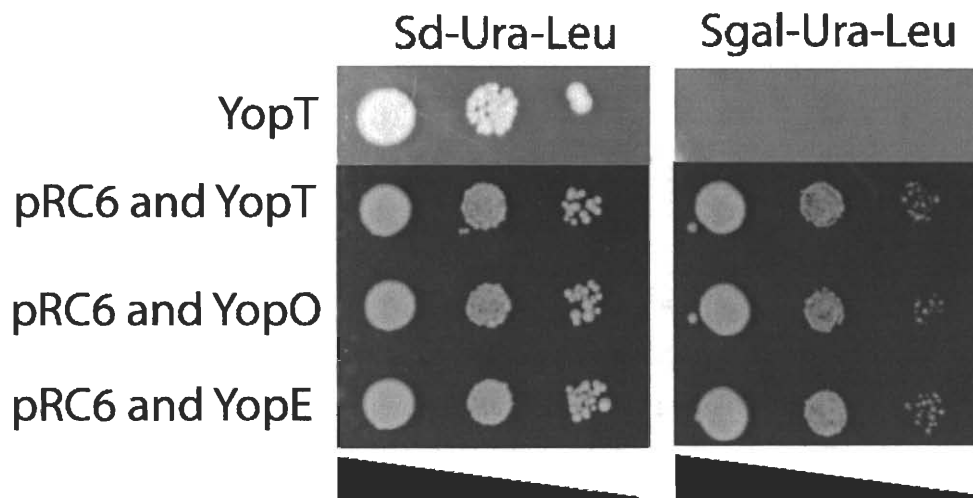


Figure 1: Suppression of lethality in *Yersinia* effectors. Yeast strain JGY4 containing either YopT (pJG495) with a control plasmid (pJG485), YopT (pJG495) with the suppressor plasmid pRC6, YopE (pJG491) with pRC6 or YopO (pLN5) with pRC6 were replica plated onto SD (SD-ura-leu) or Sgal (Sgal-ura-leu) medium. Cultures were incubated at room temperature for 3 days. The auxotrophic markers Ura and Leu were used to select for cells that contained both the expression plasmid as well as the control or suppression plasmids. The black triangle indicates that each spot of cells is a 40-fold dilution of the cells in the previous spot.

In order to isolate the specific region of 2T9 needed for suppression, several subclone constructs were created. Subcloning uses restriction enzyme digestion and molecular cloning techniques to isolate specific DNA fragments within the plasmid. Then, fractions of the 2T9 plasmid can individually be tested for suppression. Once the region within 2T9 that is necessary for suppression has been isolated, we can begin to investigate how this suppression is occurring.

Confirmation of YopT induced lethality was conducted to establish growth parameters (Figure 2). To achieve this, each plasmid was transformed into yeast via lithium acetate transformation as previously described (Gietz and Schiestl 1991). Serial dilution replica plating was performed on Sd-Ura-Leu and Sgal-Ura-Leu media.

As shown in Figure 2, when two empty vectors were present within yeast cells, wild-type growth was present. When YopT was expressed in cells, growth is inhibited. When 2T9 was expressed, cellular growth was present as expected. When YopT and 2T9 are both present within the cell, lethality of YopT is suppressed. Lastly, when the cloning vector was expressed in the presence of YopT, growth was inhibited. Therefore, cells with empty vectors grow normally, while YopT expression is lethal. Also, the 2T9 plasmid is capable of suppressing YopT lethality and the library cloning vector alone was not able to suppress lethality. These results are consistent with data previously conducted on YopT (Gesier, J.R. unpublished data).

Several subclones of 2T9 were created to examine ability to suppress YopT. Each of these subclones was transformed into yeast via lithium acetate transformation as previously described (Gietz and Schiestl 1991). Most of the subclones were transformed into haploid yeast strain JGY4; however, some of the subclones could not be transformed into haploid yeast. For subclones pRC2 and pRC3, haploid JGY4 containing the expression plasmid was mated with haploid JGY3 containing the subclone. This mating produced diploid yeast cells. For pRC4, haploid mating was not successful so subclones were transformed into diploid yeast strain JGY709. Table 4 summarizes the yeast strain used for the transformation of each subclone.

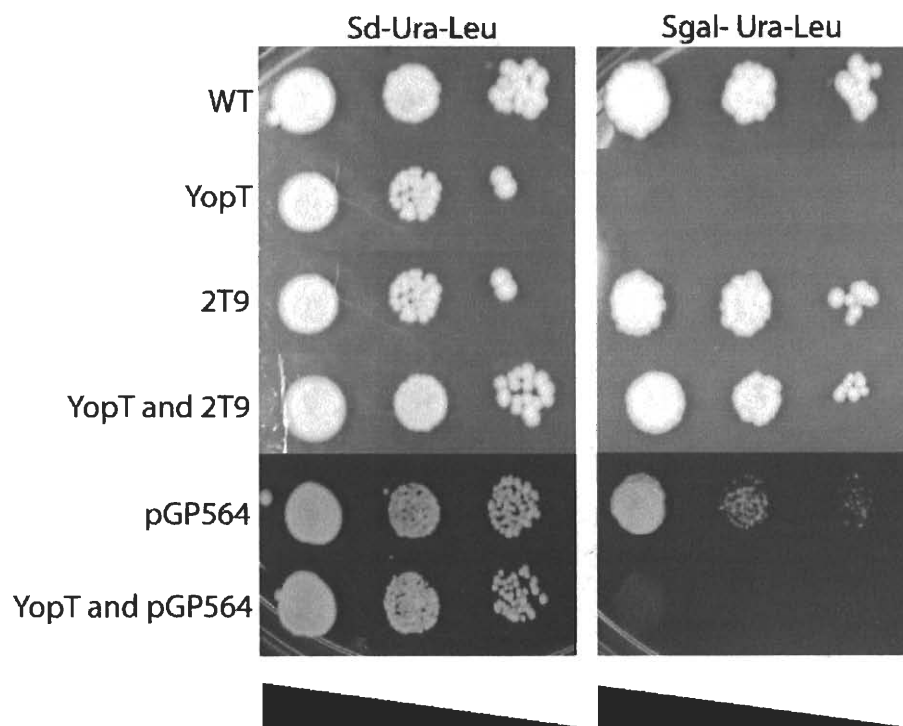


Figure 2: YopT suppression controls. Yeast strain JGY4 containing either two empty vectors (pJG485 and pJG484), YopT (pJG495) and an empty vector (pJG484), 2T9 (pJG551) and an empty vector (pJG485), 2T9 (pJG551) and YopT (pJG495), the library cloning vector (pGP564) and an empty vector (pJG485) or the library cloning vector (pGP564) and YopT (pJG495) were replica plated onto SD (SD-ura-leu) or Sgal (Sgal-ura-leu) medium. Cultures were incubated at room temperature for 3 days. The auxotrophic markers Ura and Leu were used to select for cells that contained both the expression plasmid as well as the control or suppression plasmids. The black triangle indicates that each spot of cells is a 40-fold dilution of the cells in the previous spot.

Table 4: Haploid and diploid strains used for yeast transformations of 2T9 subclones.

JGY4	Mated JGY4 and JGY3	JGY709
pRC1, pRC5, pRC6, pRC7, pRC8, pRC9	pRC2, pRC3	pRC4

The growth phenotype of all 2T9 subclones was assessed by serial dilution replica plating on Sd-Ura-Leu and Sgal-Ura-Leu medium (Figure 3). Cells containing

each sub clone and either a YopT expression plasmid or an empty vector were analyzed. The empty vector was used to determine that the subclone plasmid alone had no effect on cellular growth. The first subclone, pRC1, was created by removing the *SUL1* and *VBA2* genes from the 2T9 plasmid. Construct pRC8 was also created using the same procedures as pRC1 and was used to confirm the results seen with pRC1. As shown in Figure 3A, when pRC1 and pRC8 were expressed in conjunction with YopT, the cells were able to overcome growth inhibition. Therefore, cells were able to suppress YopT induced lethality. From these results we can conclude that *SUL1* and *VBA2* genes are not necessary for suppression. Through the process of elimination, it was then thought that the *PCAI* gene was needed for suppression. To test this, pRC2 was created by isolating the *PCAI* gene from 2T9 and placing it into a new cloning vector (pRS426). As shown in Figure 3B, subclone pRC2 was not able to suppress lethality. In another attempt to remove the *PCAI* gene, the *PCAI* gene was isolated from pRC1 and placed into a new cloning vector (pRS426) to create pRC3. As shown in Figure 3B, pRC3 was unable to suppress lethality. Again, in another attempt to isolate the *PCAI* gene, *PCAI* from pRC1 was isolated and placed into the pGP564 library cloning vector to create pRC4. As shown in Figure 3B, pRC4 was unable to suppress lethality. Therefore, when *PCAI* is isolated and placed into a new vector, no suppression occurs. Thus, the *PCAI* gene is not required for suppression. To further confirm these results, pRC6 was created by removing the *PCAI* gene from pRC1. As shown in Figure 3A, pRC6 is able to suppress lethality. These results again suggest that the *PCAI* gene is not needed for suppression. Also,

the *PCAI* gene was isolated from pRC3 and placed back into pRC1 in order to create pRC5. As seen in Figure 3A, pRC5 is able to suppress lethality. Table 5 summarizes the results of the suppression screen used for these subclones.

Table 5: Summary of suppression of YopT lethality by various 2T9 subclones.

Sub-clone	Suppression of YopT Lethality
pGP564	No
2T9	Yes
pRC1, pRC8	Yes
pRC2	No
pRC3	No
pRC4	No
pRC5	Yes
pRC6	Yes
pRC7	Yes
pRC9	No

An interesting finding was the suppression of the subclone pRC7. In an attempt to remove the multiple cloning site from the library cloning vector, pGP564 was cut with NotI and the 2.1 kb band was supposed to be removed. However, for the creation of pRC7, that band was accidentally ligated back into the cloning vector and therefore, was never removed. As shown in Figure 3A, pRC7 is able to suppress lethality. Then, pRC9 was made by removing the entire multiple cloning site from the library cloning vector. As shown in Figure 3B, pRC9 is not able to suppress lethality.

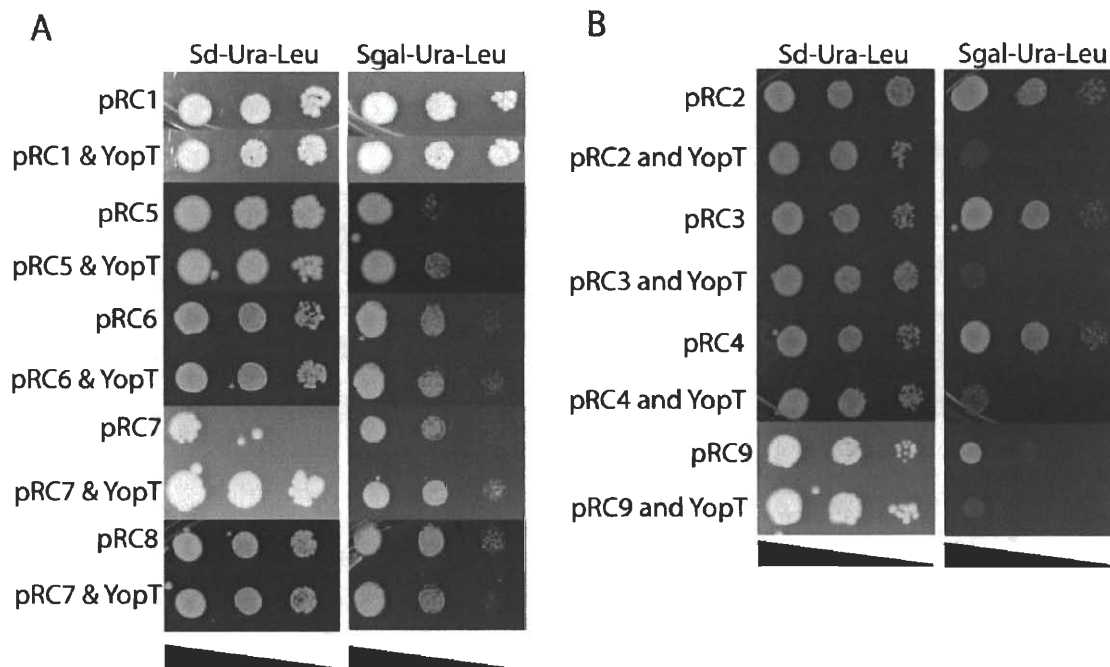


Figure 3: YopT suppression by 2T9 subclones. Yeast strain JGY4 containing each sub-clone in the presence of an empty vector (pJG484 or pJG485) or the presence of YopT (pJG494 or pJG495) were replica plated onto SD (SD-ura-leu) or Sgal (Sgal-ura-leu) medium. **A.** The 2T9 subclones that were able to suppress YopT lethality. **B.** The 2T9 subclones that were not able to suppress YopT lethality. Cultures were incubated at room temperature for 3 days. The auxotrophic markers Ura and Leu were used to select for cells that contained both the expression plasmid as well as the control or suppression plasmids. The black triangle indicates that each spot of cells is a 40-fold dilution of the cells in the previous spot.

To assess for a mutation, the OpenBio2 promoter of the cloning vector and pRC7 were sequenced in the forward and reverse directions by Genewiz, Inc (Figure 4). As seen in Figure 4, sequencing results indicate that both the cloning vector (pGP564) and pRC7 share complete sequence homology through the multiple cloning site. Therefore, this mutation might lie on another region of the subclone plasmid that has not been sequenced. Also, since pRC9 was not able to suppress lethality, these

results indicate that suppression is not occurring simply through the process of cutting the cloning vectors with restriction enzymes.

Since all three of the yeast genes located within the 2T9 plasmid have been removed and suppression still occurs, these genes are not needed for suppression. This suggests that another piece of the 2T9 plasmid is necessary for suppression. To investigate the contents of the multiple cloning site within 2T9 and the subclones, the sequences were evaluated. As shown in Figure 4, along with pGP564 and pRC7, 2T9 and pRC6 share complete sequence homology through the multiple cloning site. The only exception is the deletion of approximately 50 bp in pRC6. The only other two noticeable differences occur outside of the multiple cloning site. In the suppressing constructs, there is a deletion of one adenine base. Also, there is an alteration from a guanine to an adenine base in 2T9 and pRC6. These results purpose that the multiple cloning site within 2T9 is not causing suppression.

Lastly, since a few of the subclones could not be transformed into haploid cells, two suppressor plasmids were transformed into haploid and diploid cells to determine if ploidy had an effect on suppression. Library plasmid 2T9 and subclone pRC1 were transformed into diploid cells and serial dilution replica plated on SD-Ura-Leu and Sgal-Ura-Leu media (Figure 5). As shown in Figure 5, both pRC1 and 2T9 were able to suppress lethality in diploid cells as well. Therefore, the ploidy of yeast cells has no effect on suppression.

pGP564	AGAAATCACAGCCG	AAGCCATTAAGGTTCTTAAAGCTATTTCTGATGTTGACTGGTTGCACCA
pRC7	AGAAATCACAGCCG	-AAGCCATTAAGGTTCTTAAAGCTATTTCTGATGTTGACTGGTTGCACCA
pRC6	AGAAATCACAGCCG	-AAGCCATTAAGGTTCTTAAAGCTATTTCTGATGTTGACTGGTTGCACCA
2T9	AGAAATCACAGCCG	-AAGCCATTAAGGTTCTTAAAGCTATTTCTGATGTTGACTGGTTGCACCA

pGP564	GTNGACCGTTTTGCTGCTANAAGAATCCCCGCTGTAATCTTACCATATAGGAACTTTATATATATATATAACG
pRC7	GTNGACCGTTTTGCTGCTANAAGAATCCCCGCTGTAATCTTACCATATAGGAACTTTATATATATATATAACG
pRC6	GTNGACCGTTTTGCTGCTANAAGAATCCCCGCTGTAATCTTACCATATAGGAACTTTATATATATATATAACG
2T9	GTNGACCGTTTTGCTGCTANAAGAATCCCCGCTGTAATCTTACCATATAGGAACTTTATATATATATATAACG

pGP564	AGACTTTACATTTTCCATTCTTTTCAATCTTTCATTCTGCTAACGATTGGTGGATAACCTTTTTTGTATCCAGG
pRC7	AGACTTTACATTTTCCATTCTTTTCAATCTTTCATTCTGCTAACGATTGGTGGATAACCTTTTTTGTATCCAGG
pRC6	AGACTTTACATTTTCCATTCTTTTCAATCTTTCATTCTGCTAACGATTGGTGGATAACCTTTTTTGTATCCAGG
2T9	AGACTTTACATTTTCCATTCTTTTCAATCTTTCATTCTGCTAACGATTGGTGGATAACCTTTTTTGTATCCAGG

pGP564	AATTTATTATAACAGTTGAAGTTCATAACTACGTTGCTAAATCAGTACTTGCGAAGAGAT ATACTTTTCGG
pRC7	AATTTATTATAACAGTTGAAGTTCATAACTACGTTGCTAAATCAGTACTTGCGAAGAGAT ATACTTTTCGG
pRC6	AATTTATTATAACAGTTGAAGTTCATAACTACGTTGCTAAATCAGTACTTGCGAAGAGAT ATACTTTTCGG
2T9	AATTTATTATAACAGTTGAAGTTCATAACTACGTTGCTAAATCAGTACTTGCGAAGAGAT ATACTTTTCGG

pGP564	CCAAGGCCGCGAGAGTGGAAAGGAAAAAGAGGGTAAAAAGTCAACTTTTTCCATATAC GCAGTCGCTGG
pRC7	CCAAGGCCGCGAGAGTGGAAAGGAAAAAGAGGGTAAAAAGTCAACTTTTTCCATATAC GCAGTCGCTGG
pRC6	CCAAGGCCGCGAGAGTGGAAAGGAAAAAGAGGGTAAAAAGTCAACTTTTTCCATATAC GCAGTCGCTGG
2T9	CCAAGGCCGCGAGAGTGGAAAGGAAAAAGAGGGTAAAAAGTCAACTTTTTCCATATAC GCAGTCGCTGG

pGP564	AGATCCTAGTCACCTACCGTTTATTACTAAAATAAACTGACTATCACTGGACAAGCAACGTTGTTGTTAACT
pRC7	AGATCCTAGTCACCTACCGTTTATTACTAAAATAAACTGACTATCACTGGACAAGCAACGTTGTTGTTAACT
pRC6	AGATCCTAGTCACCTACCGTTTATTACTAAAATAAACTGACTATCACTGGACAAGCAACGTTGTTGTTAACT
2T9	AGATCCTAGTCACCTACCGTTTATTACTAAAATAAACTGACTATCACTGGACAAGCAACGTTGTTGTTAACT

pGP564	ATTCGTTACGAAGAATATTACGGTTGAAATATGTTCTTTTGCACCCAGCCGCGCGGGTGGGAAGCGGGTTATG
pRC7	ATTCGTTACGAAGAATATTACGGTTGAAATATGTTCTTTTGCACCCAGCCGCGCGGGTGGGAAGCGGGTTATG
pRC6	ATTCGTTACGAAGAATATTACGGTTGAAATATGTTCTTTTGCACCCAGCCGCGCGGGTGGGAAGCGGGTTATG
2T9	ATTCGTTACGAAGAATATTACGGTTGAAATATGTTCTTTTGCACCCAGCCGCGCGGGTGGGAAGCGGGTTATG

pGP564	CGTTTGCGGAGGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGGGCTGACCTT
pRC7	CGTTTGCGGAGGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGGGCTGACCTT
pRC6	CGTTTGCGGAGGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGGGCTGACCTT
2T9	CGTTTGCGGAGGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGGGCTGACCTT

pGP564	TCGCCC TCACTCGGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATAC
pRC7	TCGCCC TCACTCGGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATAC
pRC6	TCGCCC TCACTCGGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATAC
2T9	TCGCCC TCACTCGGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATAC

BssHII

PvuII

OpenBio2 Promoter



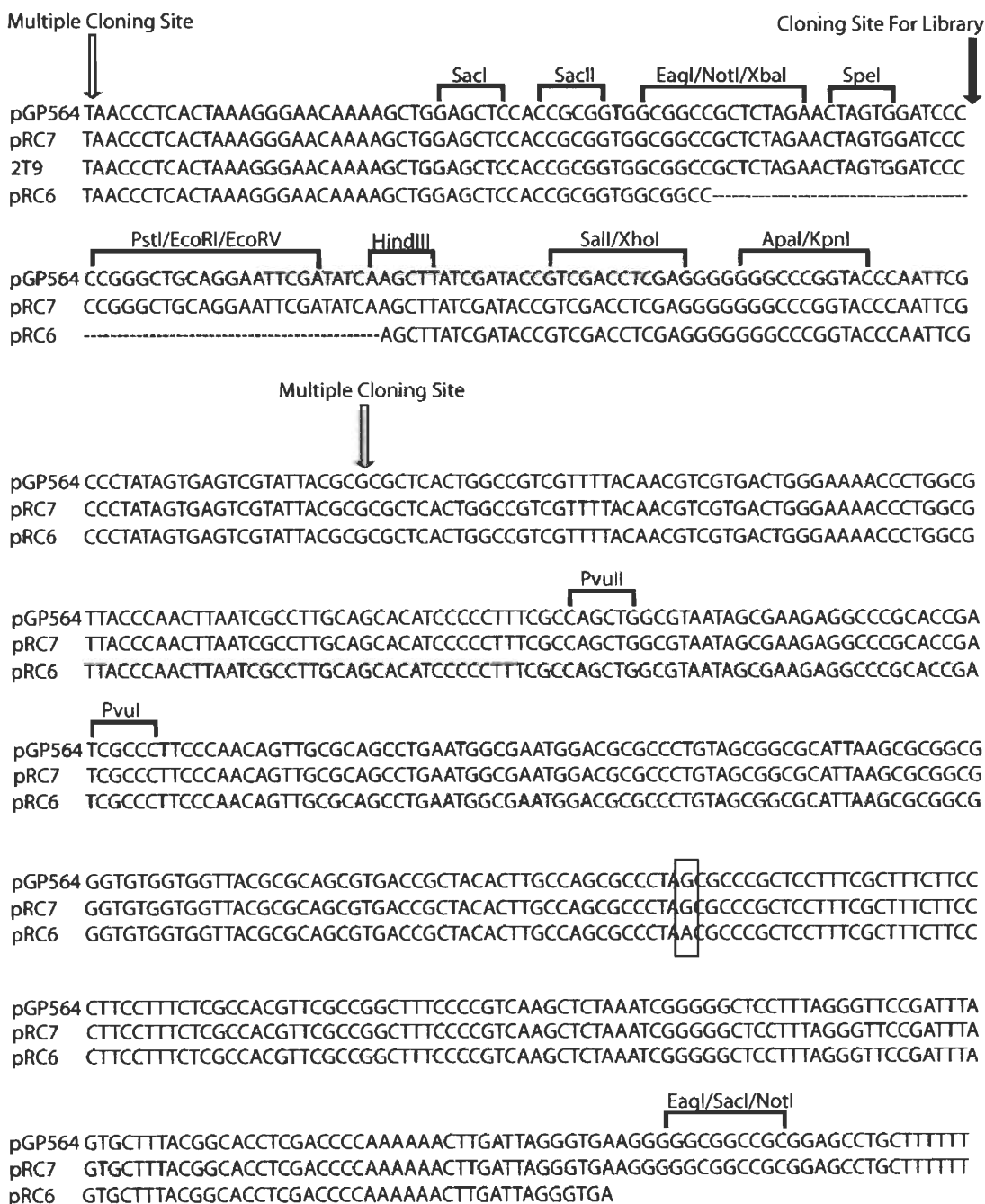


Figure 4: YopT suppressor sequences. The cloning vector (pGP564), 2T9, pRC7 and pRC6 were all sequenced in the forward and reverse directions of the OpenBio2 promoter. The OpenBio2 promoter and the library cloning site are indicated by black arrows while white arrows denote the ends of the multiple cloning site. The brackets designate specific restriction enzyme sites and dashes represent bases that are not present. The two areas of variation between sequences are outlined with a rectangle.

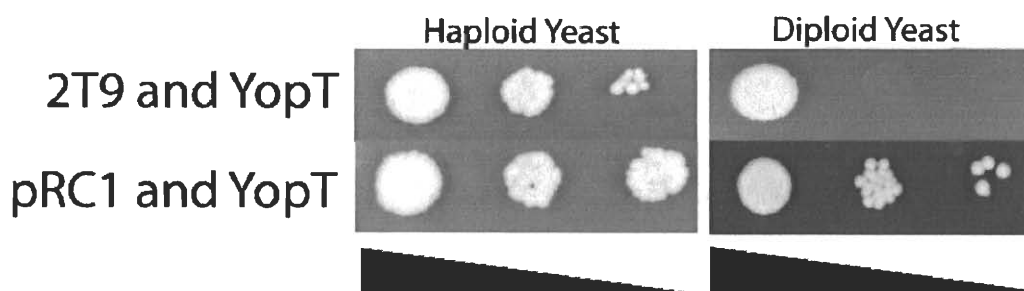


Figure 5: Effects of ploidy on suppression. Yeast strains JGY709 (diploid) and JGY4 (haploid) containing either 2T9 (pJG551) and YopT (pJG495) or pRC6 and YopT (pJG495) were replica plated onto SD (SD-ura-leu) or Sgal (Sgal-ura-leu) medium. Cultures were incubated at room temperature for 3 days. The auxotrophic markers Ura and Leu were used to select for cells that contained both the expression plasmid as well as the control or suppression plasmids. The black triangle indicates that each spot of cells is a 40-fold dilution of the cells in the previous spot.

YopT Effector Protein Expression

The previously stated results demonstrate that some of the 2T9 subclone creations were able to suppress YopT induced lethality, while others could not. The suppressor subclones may be deactivating YopT by delocalization, or degradation of the protein. It is also possible that the suppressor is able to repress the *GALI* promoter, thus preventing expression of YopT. We investigated how the suppressors were able to overcome YopT induced lethality using Western immunoblotting as previously described (Sambrook *et al.* 1989). Western immunoblotting is an assay that detects relative size and concentration of proteins through the use of antibody staining. Since the YopT expression plasmids contain a V5 epitope, the anti-V5 antibody and a horseradish peroxidase reagent fluorescent tag were used to detect the YopT protein.

First, normal YopT expression levels within yeast cells were examined (Figure 6). To detect YopT, yeast cells containing a YopT expression plasmid and an empty

vector were grown to mid-log phase in minimal selective media. Protein expression was induced with galactose and extracts were prepared from each aliquot as previously described (Kahana et al. 1998). As shown in Figure 6, YopT migrates in the gel at approximately 35 kDa. This is consistent with data previously published (Iriarte and Cornelis 2002).

Next, it was important to investigate if the suppressing subclones had an effect on YopT expression (Figure 7). It was hypothesized that the possible mechanism for suppression was the degradation of YopT by the subclones. As shown in Figure 6, when a suppressor is present within the cell, there are no detectible levels of YopT. For consistency, YopT protein expression in all of the subclones was observed. As seen in Figure 7, YopT in all non-suppression subclones (pGP564, pRC2, pRC3, pRC4, pRC9) migrates to approximately 35kDa within the gel. Conversely, no YopT is detected in the suppressor subclones: 2T9, pRC1 and pRC5. This is consistent with the theory that YopT is being degraded by the suppressor, but it was also possible that YopT was not being produced within the cells. Therefore, further investigation was needed to determine exactly how YopT was deactivated. Another interesting finding is that pRC7 was able to suppress lethality, but YopT is detected within the gel at 35kDa when pRC7 was present.

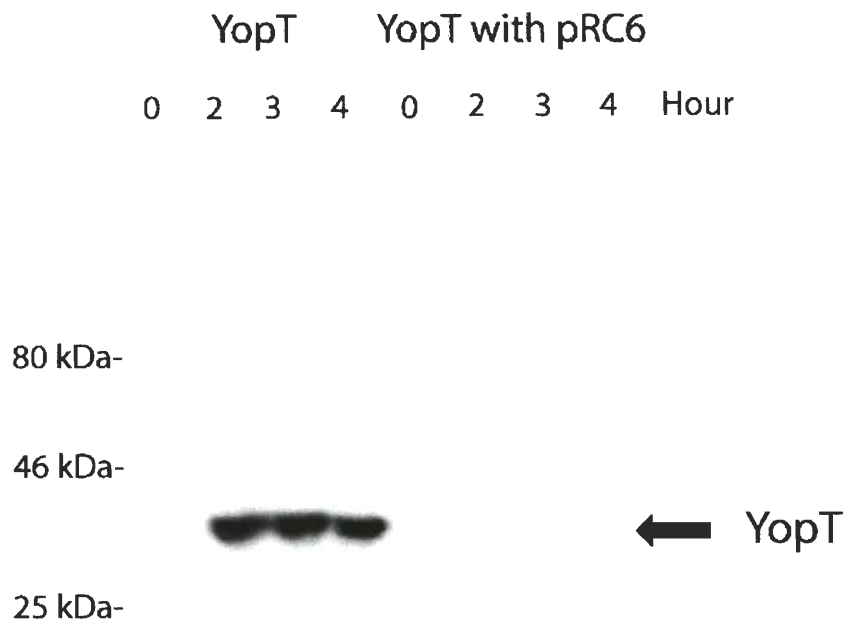


Figure 6: YopT expression in yeast cells. Yeast strain JGY4 containing YopT (pJG495) in conjunction with an empty vector (pGJ484) or suppressor (pRC6) were grown in S raffinose-Ura-Leu media. Cultures were induced with 2% galactose for 4 hours. Aliquots were taken at respective time points and protein extracts were prepared as previously described by Nejedlik *et al.* 2004. The same concentration of YopT protein was added to each well and YopT was detected with a V5 epitope by immunoblotting. Molecular weight marker is shown. Arrow indicates the expected molecular weight of YopT protein.

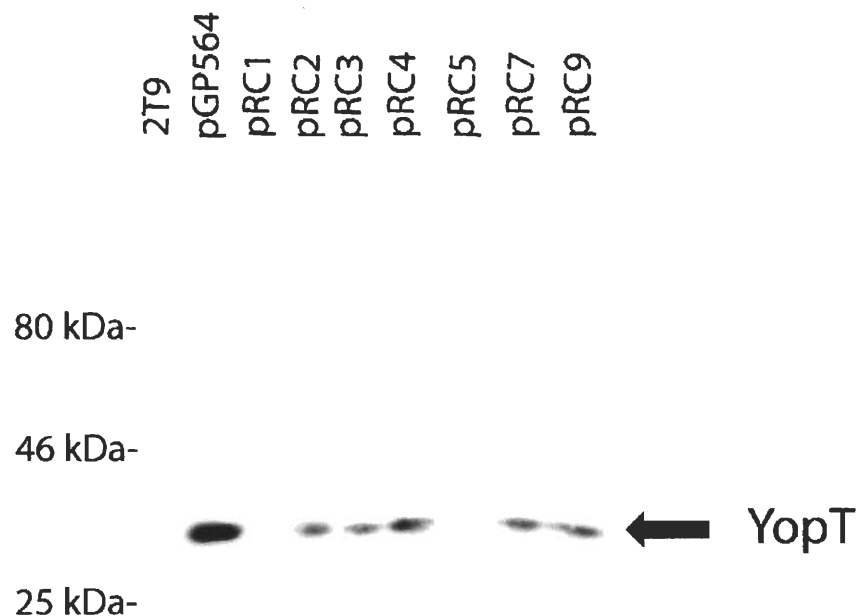


Figure 7: YopT expression in subclone constructs. Yeast strain JGY4 containing YopT (pJG495 and pJG494) in conjunction with either 2T9 (pJG551), the library cloning vector (pGP564), pRC1, pRC2, pRC3, pRC4, pRC5, pRC7 or pRC9. Cultures were induced with 2% galactose for 3 hours. Aliquots were taken at hour 3 and protein extracts were prepared as previously described by Nejedlik *et al.* 2004. The same concentration of YopT protein was added to each well and YopT was detected with a V5 epitope by immunoblotting. Molecular weight marker is shown. Arrow indicates the expected molecular weight of YopT protein.

Localization of YopT Effector

To further investigate the mechanism of YopT lethality suppression, the cellular localization of YopT was examined (Figures 8 and 9). It is currently unknown what compartment of the cell YopT localizes to within yeast cells. It may be found in the periphery, in certain organelles, or diffused all over the cytoplasm. A clear picture of where it is located can give insight into how it functions within the cell. Cellular localization was examined by immunofluorescence as previously described (Nejedlik *et*

al. 2004). Indirect Immunofluorescence allows for visualization of the effector protein within the cell through the use of antibody staining. Similar to western blotting, anti-V5 antibody was used in conjunction with a fluorescent tag. Yeast cells containing a YopT expression vector along with an empty vector were induced with galactose and samples were collected at various time points. The samples were then prepared for indirect immunofluorescence as previously described (Nejedlik *et al.* 2004). As shown in Figures 8 and 9, YopT can be found diffused and localized all over the cell.

Numerical counts of cells expressing YopT were performed at hours 3 and 4. As shown in Table 6, at hour 3, 87% of cells contained detectible levels of YopT and at hour 4, 81% contained YopT. Therefore YopT is abundantly localized all over the cell when expressed in yeast. Also, shown in Figure 9, DNA is localized the nucleus of cells and mitochondrial DNA can found on the periphery of cells.

Table 6: Cellular counts of YopT effector expression.

	Time Point	% of cells containing YopT	% of cells not containing YopT
YopT with Empty Vector	Hour 3	87	13
	Hour 4	81	19
YopT and pRC6	Hour 3	7	93
	Hour 4	6	94

The cellular localization of YopT was also examined in the presence of a suppressor to determine if there was a detectible change in localization. Yeast cells containing a YopT expression plasmid and a suppressor plasmid (pRC6) were induced with galactose and samples were collected at various time points. The samples were

then prepared for indirect immunofluorescence as previously described (Nejedlik *et al.* 2004). As shown in Figures 8 and 9, very low amounts of YopT were present at all time points. To confirm these results, numerical counts of cells expressing YopT were conducted at hours 3 and 4. As shown in Table 6, only 7% of cells at hour 3 and 8 % at hour 4 had visible YopT expression. Therefore, when a suppressor plasmid is present, YopT expression is significantly reduced. These data is consistent with the protein expression levels seen in the previous Western blots. Since detectable levels of YopT are still present in a few cells, it is unlikely that the suppressor is preventing YopT expression by repressing the *GALI* promoter. Also, we can conclude that suppression is not due to a malfunction of the cloning vector or subclone constructs.

Lastly, immunofluorescence was conducted on diploid and haploid cells to determine if ploidy had an effect on YopT localization (Figure 10). As shown in Figure 10, in both haploid and diploid cells, YopT is found diffused all over the cell. These results indicate that there is no difference in YopT expression in haploid versus diploid cells. Therefore, the ploidy of yeast cells has no effect on the localization of YopT within yeast cells.

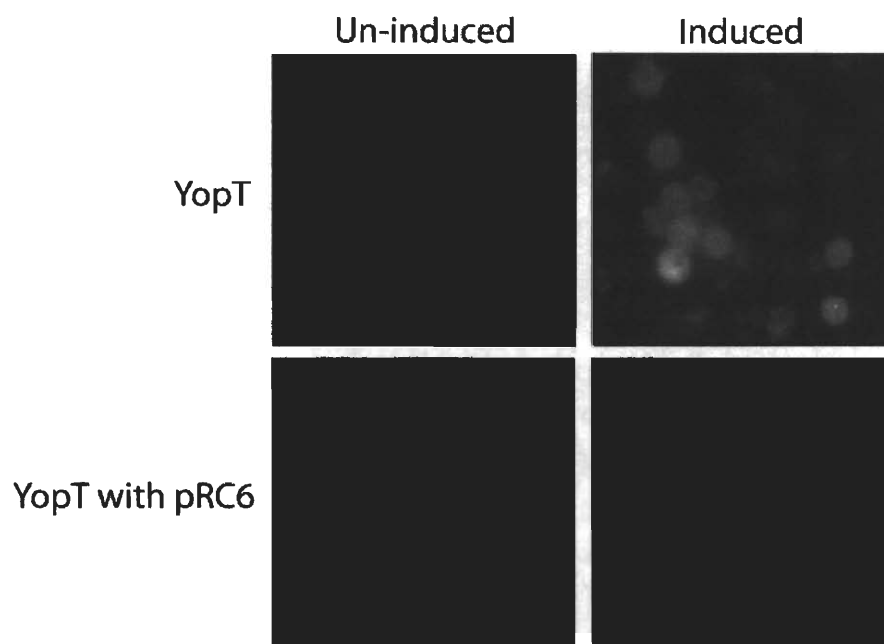


Figure 8: Localization of YopT. Yeast strain JGY4 containing YopT (pJG495) and either a control plasmid (pJG484) or a suppressor plasmid (pRC6) was grown in selective media containing 2% raffinose. YopT was induced with 2% galactose after time point zero. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 18 hours at 4°C. Immunofluorescence was used to visualize the V5 epitope as previously described by Nejedlik *et al.* 2004.

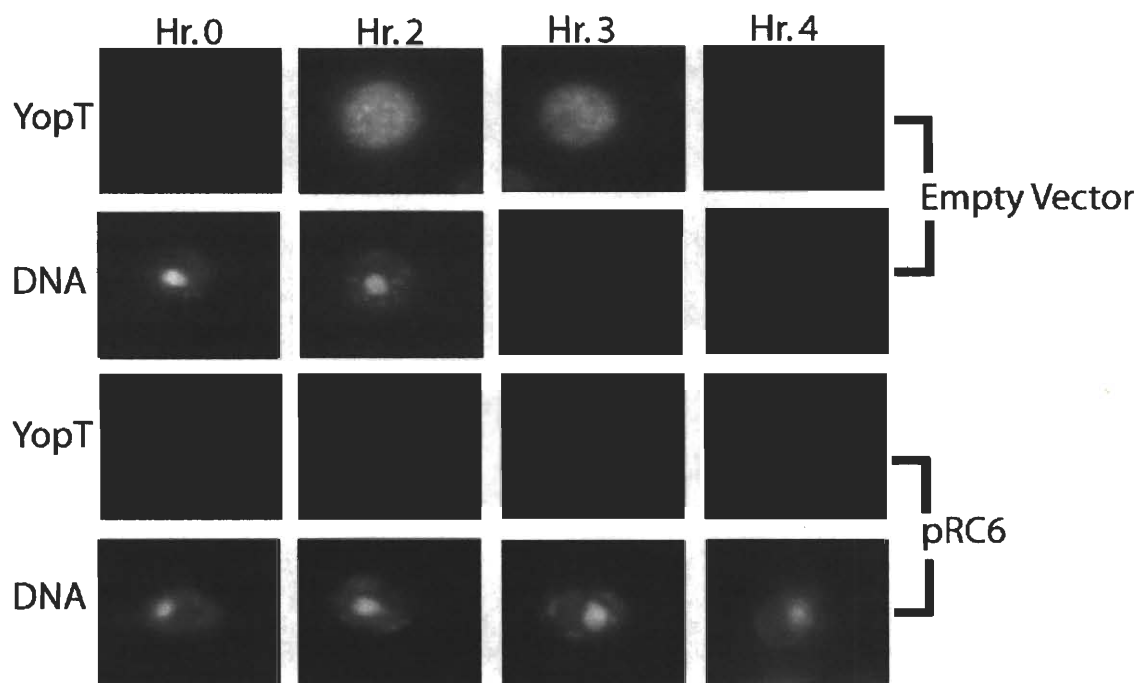


Figure 9: Individual cell view of localization of YopT. Yeast strain JGY4 containing YopT (pJG495) along with either an empty vector (pJG484) or a suppressor plasmid (pRC6) were grown in selective media containing 2% raffinose. YopT was induced with 2% galactose after time point zero. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 18 hours at 4°C. Immunofluorescence was used to visualize the V5 epitope and DAPI was used to visualize DNA as previously described by Nejedlik *et al.* 2004.

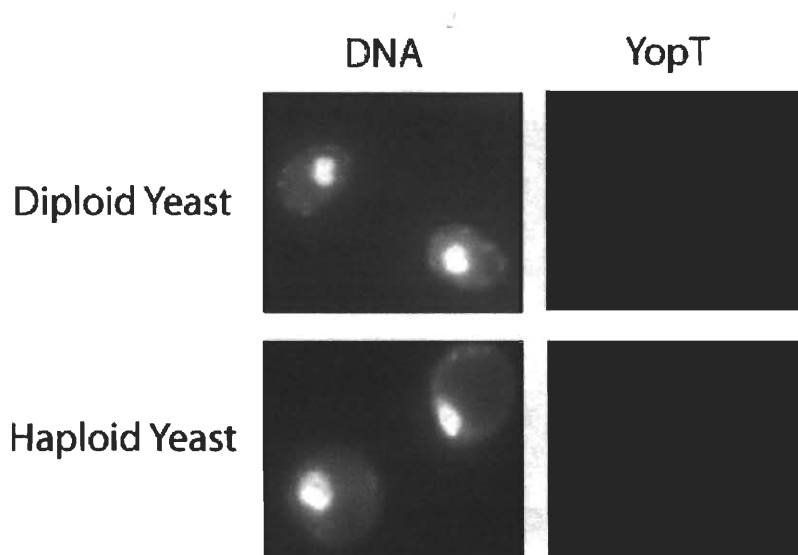


Figure 10: Effects of ploidy on YopT localization. Yeast strains JGY4 and JGY709 containing YopT (pJG495) and control plasmid (pJG484) were grown in selective media containing 2% raffinose. YopT was induced with 2% galactose after time point zero. Aliquots were taken at each time point and fixed with 3.7% formaldehyde for 18 hours at 4°C. Immunofluorescence was used to visualize the V5 epitope and DAPI was used to visualize DNA as previously described by Nejedlik *et al.* 2004.

Chapter III: Discussion

Through the use of a yeast model system, the cellular targets of the *Yersinia* outer proteins, or Yops, were investigated by the use of a dosage-dependent suppression selection. From this selection, 2T9 was identified as a plasmid that could suppress YopT lethality. There are three yeast genes located within 2T9; *SUL1*, *VBA2* and *PCAI*. Our preliminary hypothesis was that a single gene, multiple genes, or part of a gene, were necessary for suppression of YopT lethality. However, upon creation of several 2T9 subclones, it was discovered that when all three yeast genes were removed, suppression still occurred. Therefore, suppression was not caused by one of the yeast genes. We then analyzed sequencing data to determine if there was an aspect within the multiple cloning site, other than the yeast genes, that may be causing suppression. As presented in the sequencing data, there is no difference in the DNA sequence within the multiple cloning site, between the library cloning vector and the suppressing constructs. Therefore, the unique genomic insert within the 2T9 plasmid is not responsible for suppression.

After the creation of pRC9, we made many attempts to remove various fragments from the 2T9 plasmid and several of the subclone constructs. Unfortunately, we were unable to cut DNA from the multiple cloning site near the OpenBio1 promoter. The restriction enzymes were not functioning within this region despite the sequencing data proving that these sites existed. Hence, we were unable to narrow down the region within the 2T9 plasmid needed for suppression. It is possible

that this region may be found outside of the multiple cloning site, however, it is more likely that suppression is occurring by means not currently apparent to us.

The conclusion that suppression is not caused by the genomic DNA within the 2T9 plasmid is reinforced by the data collected on subclone pRC7. It was noted that pRC7 was able to suppress lethality though we could not detect a difference in the DNA sequence between this subclone and the library cloning vector it was made from. Thus, a non-suppressing construct became a suppressing construct with no known cause. Another interesting finding was the results from the Western blot assay conducted on YopT in the presence of pRC7. When YopT was expressed in the presence of all other suppressor subclones, expression levels were decreased. However, YopT was still present in cells that contained the pRC7 suppressor. Therefore, it is possible that pRC7 was not suppressing YopT through the same mechanism as the other suppressor constructs.

When YopT was visualized within yeast cells, it diffused all over the cell. It was not localized to any one compartment within the cell. Also, when YopT was visualized in the presence of a suppressor plasmid, protein expression was greatly reduced. Therefore, YopT was still produced, but it was possibly degraded by the suppressor.

Lastly, our results proved that suppression by 2T9 was not specific to YopT. Rather, 2T9 could suppress YopO and YopE induced lethality as well. This indicates that the suppressor effects are not unique to YopT. These results suggest that the mechanism for suppression is not occurring through the involvement of the specific

interactions of YopT within in the cell, such as the untethering of RhoA, Rac1 and CDC42. Rather, suppression may be occurring through the interaction with an intermediate that all three Yops interact with within the Rho GTPase cycle.

From the information gathered through this research, we were unable to determine why the unique 2T9 plasmid was able to suppress YopT induced lethality. Hence, we were unable to assess the possible mechanisms of lethality suppression. From this, we have concluded that our dosage-dependent suppression selection in yeast may identify false positives that need to be controlled for. In the future, it is important to isolate the region of the 2T9 plasmid needed for suppression. To do this, the entire 2T9 plasmid, along with the pGP564 cloning vector, need to be sequenced to assess for differences. Any difference in the sequence data may indicate a possible region necessary for suppression.

Another future direction for this research is to investigate how suppression of YopT lethality is occurring. Suppression may be occurring through the destabilization of the YopT expression plasmid or degradation of the YopT protein. Another possibility is the prevention of YopT protein expression through repression of the *GALI* promoter. However, since 6-7% percent of cells in cultures containing both YopT and the suppressor plasmid show YopT staining, it is unlikely that YopT is not being expressed within the cells. Also, through plasmid selection using auxotrophic markers, it is unlikely that the yeast cultures are not maintaining the YopT expression plasmid. Finally, there may be an additional factor causing suppression that is not currently apparent to us. Further research to isolate the region within 2T9 needed for

suppression, and examination of how that region is causing suppression, can give good insight into what YopT targets within yeast cells to cause lethality.

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