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A Liquid Assay for Screening Fungal Virulence Factors & kgb-1; fer-1 Double Mutant Construction

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A Liquid Assay for Screening Fungal Virulence Factors
&

$kgb-1; fer-1$ Double Mutant Construction

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By

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By

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Abstract

It is difficult to develop drugs against fungal infections due to the fact that both fungi and their hosts are eukaryotic. By using a host-pathogen system and an assay in high-throughput screens of genetic knockouts, fungal virulence factors as well as host immunity modulators can be identified and studied. We are developing a liquid culture assay for studying the effects of co-culturing Caenorhabditis elegans with Saccharomyces cerevisiae mutants. We tested several different conditions including different liquid media, E.coli to Yeast mass ratios, and C. elegans mutant strains. From the results of all the experiments, we concluded that there are other factors that can cause the death of the C. elegans in the assay, such as matricide, which are not S. cerevisiae-related. To exclude matricide in the experiment, a new C. elegans strain with kgb-1; fer-1 double mutant genotype was constructed by crossing.
Acknowledgements

We would like to thank Professors Sam Politz and Reeta Prusty Rao for their help, direction and insight on this project. We would also like to thank Charu Jain for her help in the lab, and Meijiang Yun for her help with Sigma Stat and information on her unpublished $kgb$-$I$ survival data.
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Introduction

In this project, we are developing a liquid culture assay for studying the effects of co-culturing *Caenorhabditis elegans* with *Saccharomyces cerevisiae* mutants. This host-pathogen model system allows us to do a high-throughput screen of the entire yeast genome. We expect to identify the fungal virulence factors involved in fungal infection, as well as host immunity modulators for innate immunity study.

Fungal Infections

Fungi are prevalent in the environment, many of which are pathogens that can infect plants and animals, including humans. For example, fungi are the most common cause of plant disease (Prescott *et al*., 2005). Fungi such as *Sclerotinia sclerotiorum* can cause infection of many crops (Huang *et al*., 2000). About fifty fungal species have been found to cause disease in humans (Prescott *et al*., 2005), among which *Candida albicans* is the most common human fungal pathogen (Heitman and Howlett, 2008). Statistics have also shown that more than half of AIDS patients have infections caused by pulmonary *Pneumocystis carinii* (Yang and Kerdel, 2006) and the occurrence of invasive fungal infections in organ transplant patients continues to be high with a high mortality rate (Gabardi *et al*., 2007). The rise of fungal diseases spurs many scientists on to the development of novel antifungal drugs. However, the task has not been easy due to the fact that fungal pathogens are also eukaryotic with cellular machinery very similar to that of their hosts’. This makes drug target identification difficult thereby slowing the process of drug development (Heitman, 2005).

*Saccharomyces cerevisiae*

*Saccharomyces cerevisiae* (*S. cerevisiae*) is a unicellular fungus, commonly known as baker’s yeast (Daum, 2000). As a widely used eukaryotic genetic model, it was chosen as the pathogen in this project. *S. cerevisiae* was used instead of a human pathogen, such as *Candida albicans*, because *S. cerevisiae* is the best studied eukaryotic cell (Prescott *et al*., 2005), with its genome fully sequenced and its biology well
understood (Forsburg, 1999). Compared with *S. cerevisiae*, the genomic sequence determination of human fungal pathogen genomes is proceeding very slowly, one reason being that the genome of *S. cerevisiae* is very similar to that of human fungal pathogens, so it is unnecessary to find the genome of the human fungal pathogens (Goffeau et al., 1996). *S. cerevisiae* has 16 chromosomes and a small genome size of about 12 Mbp. About 6000 genes are predicted to be in the genome, and the function of 80% of the predicted genes has been characterized (Miller-Fleming, 2008). Major pathways such as cell cycle, regulation and signal transduction first identified in *S. cerevisiae* are also conserved in higher eukaryotes (Cooper and Hausman, 2007). This has made yeast a very good genetic model for studying higher eukaryotes with more complex genomes. Besides all these advantages, yeast is a safe, fast, and facile organism to culture in the laboratory that makes it an attractive model system. Its fast reproduction cycle accelerates experiment completion (Angier, 1986). Another advantage of using *S. cerevisiae* is that it provides us with an available deletion library of more than 5000 genes that can be used in the screening process of the project.

Figure 1: *S. cerevisiae* Life Cycle

*S. cerevisiae* has a budding mode of growth. It is normally a diploid with a long G1 phase and overlapped S and M phases. When the cells have enough nutrients, they
will proceed with the cell cycle. When there is a lack of nutrients, the cells will either go into a quiescent state or begin sporulation to become haploid. Haploid cells can go through conjugation to become diploid again (Pringle, 1997).

**Caenorhabditis elegans**

*C. elegans* are free-living in soil and are about 1mm in length. In the lab, they can be maintained on agar plates or liquid cultures. They eat bacteria in the soil and are fed with *E. coli* in the laboratory (http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm).

There are two sexes, hermaphroditic and male. The hermaphroditic is the most abundant sex and males compose only about 0.1% of the time (http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm).

**Figure 2: Anatomy of a C. elegans adult hermaphrodite**

The main shape is an unsegmented, cylindrical body that is tapered at the ends (shown in Figure 1). In the body wall, there is a cuticle which surrounds the worm. Within the wall there are four different systems; epithelial, nervous, muscle and excretory. The internal organs are incorporated into two systems; alimentary and reproductive. The alimentary includes the pharynx, intestine, rectum and anus. The Reproductive System consists of the somatic gonad, the germ line and the egg-laying apparatus (http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm).
Figure 3: Life cycle of *C. elegans*

Figure 3 shows the life cycle of a *C. elegans* hermaphrodite. This entire cycle takes only three days at 20 degrees C. The stages of the life cycle are the embryonic stage, larvae (L1-L4) stages, and adult (http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm).

The embryonic stage can be separated into two parts; proliferation and organogenesis/morphogenesis. During the four postembryonic larval stages, the development of the nervous system and the reproductive system occurs. If the egg hatches and there is no food available, the larva stays at the L1 stage. During L4 stage, cells for the vulva start to generate, followed by tissue morphogenesis. A transparent region can be seen in L4 larva’s body where the vulva is to be formed. The adult is the fully developed reproductive form (http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm).

The dauer larva is formed under conditions of food limitation, starvation, or a high temperature. This stage is non-aging if the environment stays unfavorable for growth. When the dauer larva is exposed to food or a more favorable environment, then
the worm exits the dauer stage and develops to the L4 and then into an adult (http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm).

Figure 4: C. elegans male

Figure 4 shows anatomical structure of male C.elegans. Male C.elegans differ from hermaphrodites in a way that only one X chromosome is present in male, while two are present in hermaphrodite. The posterior half of a male C.elegans body is characterized by arrow-shaped tail, a structure used during mating with hermaphrodite (http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm).

Model Host

C. elegans has been used to provide important insights into how animals perceived threats in the environment and defend themselves against infection. This nematode has been very useful in many different aspects in biology such as developmental biology, neurobiology, and ageing (Gravato-Nobre and Hodgkin, 2005).

C. elegans is an ideal model host for many reasons. From the practical standpoint the advantages are low cost, easy maintenance, minimal lab space, and suitability for automated animal sorting (Gravato-Nobre and Hodgkin, 2005).

They also have a small genome size. This has helped scientists to determine the complete DNA sequence of the genome. Self-fertilization is an important aspect of C. elegans for genetic analysis. Another aspect that is important for studying development is that their body is transparent allowing observation of all cells and cell divisions from egg to adult. This also makes it easier to observe the infection process (Gravato-Nobre and Hodgkin, 2005).

Either in the soil or in the laboratory, C. elegans eats bacteria, which constitutes a convenient route for infection by microbes, as well as the application of RNA interface-based gene silencing (Gravato-Nobre and Hodgkin, 2005).
C. elegans is a very important model in studying innate immunity. Several pathways are involved in the nematode’s innate immunity, such as DBL-1 and MAP kinase pathways (http://www.wormbook.org/chapters/www_signalingimmuneresponse/signalingimmuneresponse.html) with a common theme in which protein cascades transduce signals in response to various extracellular signals (Mizuno et al., 2004). Three MAP kinase pathways, p38 pathway, JNK pathway, and ERK pathway, are shown to be important for growth control and resistance to cellular stresses. Each cascade contains the following three classes of protein kinase: extracellular signals activate MAPKKK to phosphorylate the MAPKK, which can activate MAPK in turn (Koga et al., 2000). JNK-1 (MAPK) and JKK-1 (MAPKK) have been identified in the JNK pathway, a pathway involved in several biological functions in mammalian cells (Mizuno et al., 2004). All three of these MAP kinase pathways have been shown to be required for resistance of C. elegans to bacterial infections (http://www.wormbook.org/chapters/www_signalingimmuneresponse/signalingimmuneresponse.html; Kim et al., 2004).

Previous Projects in the Politz and Prusty Rao laboratories

Previous studies have identified S. cervisiae mutants that are virulent as well as worm mutants that show altered susceptibility to yeast infection. It was found that S. cerevisiae could infect C. elegans and induce the Dar phenotype. C. elegans produces reactive oxygen species (ROS) which are produced by the product of the bli-3 gene, a NADPH-dependent oxidase required for normal cuticle formation. Preliminary evidence suggests that production of ROS by BLI-3 may protect C. elegans against yeast pathogenesis (Charu Jain and R. Prusty Rao, unpublished). S. cerevisiae may then use gene products of YAP 1 and SOD 1 to neutralize the effect of ROS. YAP 1 is a transcription factor in S. cerevisiae that is critical for oxidative-stress response (Maeta et al., 2004). SOD1 encodes the protein superoxide dismutase, which can neutralize ROS by converting superoxide radicals to hydrogen peroxide and molecular oxygen (Cox et al., 2002).
These results suggest that a high-throughput assay would allow us to conduct an unbiased whole genome screen for novel fungal virulence factors as well as host immunity modulators. However, experiments on agar plates are labor intensive and time consuming. In order to screen the deletion libraries of *S. cerevisiae*, a more efficient assay is needed. We decided to try to develop a liquid culture assay in microtiter plates.
Methodology

Stock Plate Maintenance

The stocks of *C. elegans* strains were kept on 60 mm agar plates of NGM medium with an *E. coli* OP50 lawn at 16°C. Three hermaphrodites were transferred every 5 to 7 days to fresh plates. The plates were transferred to 20°C four days before egg preparations were done (Kelly and O’Brien, 2007).

Mutant *C. elegans*

*Fer-1*(b232) I mutant *C. elegans* is temperature-sensitive fertilization defective. At 20 °C, *fer-1*(b232) hermaphrodites are infertile. The *fer-1* gene product is required for Ca\(^{2+}\)-mediated membrane fusion during *C. elegans* spermiogenesis. *Fer-1* mutation alters the sensitivity to Ca\(^{2+}\), which affects the membranous organelle fusion and results in abnormal sperm (Washington and Ward, 2006).

*Kgb-1* encodes a JNK-like MAPK in a JNK-like MAPK pathway. This JNK-like MAPK pathway is involved in the stress responses to heavy metals (Mizuno et al. 2004). The *kgb-1*(km21) IV mutant is hypersensitive to copper (Cu\(^{2+}\)) and cadmium (Cd\(^{2+}\)) heavy metal ions. Most of the *kgb-1* mutants fail to grow to adult stage on plates containing 75 μM Cu\(^{2+}\) or more in four days, while the wild type *C. elegans* grow very well under these conditions (Mizuno et al., 2004).

*C. elegans* Egg Preparation

Egg preparations were used to start developmentally synchronous liquid cultures in microtiter wells. Use of egg preparations also helped avoid contamination due to the use of bleach treatment to lyse worms, which sterilized the egg preparation. To make sure the worms were exposed to yeast are at the same stage of development, eggs were harvested as follows.

The egg preparation stock plates were prepared three to five days earlier to obtain the maximum number of eggs on plates. Plates were not used if they did not contain enough eggs or if the plates had starved. These plates were washed with M9 buffer to
remove eggs and worms. Then the liquid was transferred to a 15mL conical tube. It was centrifuged into a pellet for two minutes at 900xg. The supernatant was removed using a Pasteur pipette. Twelve milliliters of bleach solution, which consisted of 0.25M NaOH dissolved in a 1:4 dilution of commercial bleach in dH₂O, was added to the conical tube to lyse the worms. The eggs were more resistant to the bleach solution, so they stayed intact. The tube was rocked for three minutes and centrifuged for another two minutes at 900xg. The supernatant was removed quickly, and then sterile M9 was added to wash the eggs free of traces of bleach. Between washes, samples were centrifuged for two minutes at 900xg and the supernatants were removed.

After the second wash, the pellet was resuspended in 5 mL of M9 buffer. Three 10µL drops were added to an unused agar plate to determine the average egg count for a given volume. A volume containing about fifteen to twenty eggs was then transferred into the microtiter wells and incubated at 20°C to mature.

**Preparation of Liquid Assay**

A single colony from a streaked plate of *E. coli* OP50 was added to 5 mL of LB and incubated in 37°C for three days. A single colony from a streaked plate of *S. cerevisiae*, such as RPY101 strain, was added to 5 mL of YPD and incubated at 30°C for three days.

After the three days, the tubes were removed from the incubators and the absorbance at 600 nm was taken. For *S. cerevisiae*, YPD was used as a blank and for *E. coli*, LB was used as a blank. The dilution for the absorbance was 1:10. It is important to take into account that *S. cerevisiae* yeast cells are very large compared to *E. coli* cells. To determine the number of cells per milliliter for *S. cerevisiae* and *E. coli*, the absorbance that was determined was converted to cells/mL using the factor 8 x 10^8 cells/OD unit for *E. coli* and 2 x 10^7 cells/OD unit for *S. cerevisiae*, also taking into account the dilution factor, which was 10.

The mass ratio was determined by divided the calculated value of *S. cerevisiae’s* cells/mL by the calculated value of *E. coli’s* cells/mL, then multiply the result by 10 since one *S. cerevisiae* cell weigh 10 times more than an *E.coli* cell.
The total volume of liquid in each well was 750 μL. The volume of S. media added equals to the total volume minus the volume of S. cerevisiae and E. coli together. To each well there was an addition of 1 μL streptomycin 50 mg/mL to stop the growth of E. coli and bacterial contaminants. Components were added to each sample in the amounts indicated in Table 1.

<table>
<thead>
<tr>
<th>Component</th>
<th>Added Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>50 μL</td>
</tr>
<tr>
<td>Yeast</td>
<td>15 μL - 150 μL (depending on absorbance and concentration)</td>
</tr>
<tr>
<td>S. medium</td>
<td>750 μL – (yeast + E. coli)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1 μL of 50 mg/mL</td>
</tr>
<tr>
<td>Eggs</td>
<td>5-10 μL (depending on the egg count)</td>
</tr>
<tr>
<td>Total Volume</td>
<td>750 μL + egg volume + Strep volume</td>
</tr>
</tbody>
</table>

*Table 1: Assay Set-Up*

**Assay Procedure**

The microtiter plates were incubated at 20°C for ten to twelve days, during which observations and data were recorded daily. The total number of live and dead worms was recorded. Worms, that were not moving or that had larvae hatched inside of bodies, were considered to be dead.

**Data Analysis using Sigma Stat**

A computer program called Sigma Stat was used to plot survival percentage as a function of time. The statistics that were used is called LogRank. This compares the survival distributions of two samples. It constructed by comparing the observed and expected number of events in one of the groups at each observed event time and then adding these to obtain an overall summary across all time points where there is an event.

Data were arranged in three columns; group, time and status. The group was each condition to be compared. Time was the number of days since plating of eggs. Status was alive or dead. For each day, each death was recorded in a separate line in the spreadsheet. Each worm had to be entered in as an individual. For every worm that had died that day there would be a one for the status and the number of rows for that day would coincide with the number of worms that died that day. For each row there would
be the group name, the day, and a one for the status. If no worms died within a 24 hour period then there would be a group name, the day, and for the status it would be left blank.

To plot the graph and calculate significance between groups, the log-rank test was used. Groups to be compared pairwise were chosen, and because worms could not escape the microtiter wells, no data were censored (the set-up is also shown in Appendix 2).

**Cu$^{2+}$ plate preparation**

Agar plates of NGM medium with 75 μM Cu$^{2+}$ were prepared by adding cupric sulfate (CuSO$_4$) into standard NGM medium before pore the plates. The amount of cupric sulfate added was calculated using the formula below:

Weight of cupric sulfate =

$$75 \mu M \times \text{Volume of the solution} \times \text{molecular weight of cupric sulfate}$$

The calculated amount of cupric sulfate was dissolved in dH$_2$O first. The volume of dH$_2$O used was then deducted from the NGM medium recipe. For example, if 75 mL dH$_2$O was used to dissolve cupric sulfate, 75 mL less of dH$_2$O was added when mixing NGM medium. The cupric sulfate solution was added into NGM medium after being autoclaved.

**N2 male C. elegans**

N2 males were obtained by leaving L4 stage larvae at 27.5°C overnight. The elevated temperature could increase the occurrence of males in the population. Ten L4 were placed on a new plate. The plate was moved back to 20°C on the second day. The progeny of these ten worms were observed and selected for males. A male stock plate were started with putting ten N2 hermaphrodites (L4 stage or younger) and five N2 males, matching the age of hermaphrodites, at 16°C. This procedure was repeated daily until a good stock of N2 males was obtained.

**Fer-1; kgb-1 double mutant construction**

Since kgb-1 males did not mate well with any hermaphrodites in our preliminary tests, ten kgb-1 hermaphrodites (L4 stage or younger) and five N2 males were put on one
plate for crossing first. Five of the resulted male progeny, heterozygous for $kgb$-$1$, were then crossed with ten $fer$-$1$(b232) *hermaphrodites* (L4 stage or younger) at $20^\circ$C on another plate. In this way, the offspring on this plate could only be from crossing, because the $fer$-$1$ hermaphrodites are infertile at $20^\circ$C. Ten of the F1 hermaphrodite offspring were picked onto plates, one hermaphrodite per plate. The F1 hermaphrodites were picked unbiased for $kgb$-$1$. The plates were kept at $20^\circ$C to ensure that they were not offspring from self-fertilized $fer$-$1$ hermaphrodites, and were moved to $16^\circ$C as soon as fertilized eggs (F2) were found on plates. F2 worms were allowed to grow to L4 stage and were then singled onto new plates. Approximately sixty F2 hermaphrodites were picked, six from each F1 plate. The F3 eggs of each F2 were used in Cu$^{2+}$ and temperature sensitivity tests. F2 plates were marked with numbers for future reference and maintained at $16^\circ$C.

In the Cu$^{2+}$ sensitivity test, for each F2, about twenty eggs were picked onto a plate containing 75 μM Cu$^{2+}$, labeled with the number on the F2 plate. The plates were left at $16^\circ$C for growth and observed after a week. If all worms on a single plate failed to reach the adult stage, the F2 that laid these eggs was determined to be homozygous $kgb$-$1$.

After $kgb$-$1$ homozygous F2 were found, eggs of F3 left on the same F2 plate were picked again for the temperature sensitivity test. Again, for each $kgb$-$1$ homozygous F2, about twenty eggs were picked onto a standard NGM plate, labeled with the number on the F2 plate. The plates were left at $20^\circ$C for growth and observed after about five days. If all the eggs laid by F3s were infertile, then the F2 was determined to be *fer*-$1$ homozygous. Plates with both fertile and infertile eggs were determined to be heterozygous for *fer*-$1$. *The* temperature sensitivity test could be carried out again to find additional homozygous *fer*-$1$, by singling offspring from the F2 plates accordingly and testing the eggs of these plates. After performing Cu$^{2+}$ and temperature sensitivity tests, the $kgb$-$1; fer$-$1$ double mutant could be identified.

A simplified diagram of construction procedure is shown below.
**Freezing kgb-1; fer-1 double mutant**

Freezing of *kgb-1; fer-1* double mutant was performed as follows. Larvae of *kgb-1; fer-1* double mutant were washed off from a recently starved plate, with a majority of larvae being L1 or L2. 2.2 ml M9 buffer (Brenner, 1974) was used and the suspension was placed in a sterile conical tube with cap. Equal volumes of sterile 2X S medium (http://www.med.yale.edu/mbb/koelle/protocols/protocol_liquid_culture.html) and sterile 30% glycerol (w:v) were mixed to obtain 1X S medium containing 15% glycerol (w:v). 1.6 ml of larval suspension and 1.6 of the S. medium-glycerol solution were drawn into a new conical tube for mixing. This mixture was then separated equally into six freezer vials, which later were placed at -80°C overnight. One vial was taken out and thawed the next day to determine viability of the frozen stock. After a stock plate was successfully started from this vial, the rest of the vials were moved into liquid nitrogen for storage.

![Figure 5: Double mutant construction strategy](image-url)
Results
All the Figures in this section are survival curves plotted using Sigma Stat.

_C.elegans arrested at L1 stage when S. cerevisiae served as the only food_

In wells that _C.elegans_ were fed with 100% _S. cerevisiae_, the eggs hatched but were all arrested at L1 stage in development (data not shown). This is consistent with results from previous projects on agar plates.
**S. cerevisiae Mutants**

Wild type *S. cerevisiae* (Y101), *S. cerevisiae SOD1* mutant and *Sigma* strain (Y196) were used in this experiment. *Sod1Δ* is a superoxide dismutase deletion mutant. *Sigma* (Y196) is a wild strain that is more virulent than *Y101*. The mass ratio of all *S. cerevisiae* to *E.coli* was 1:30. NGM was used in all the wells.

![Figure 6: Survival curve of C. elegans with S. cerevisiae mutants and E.coli in NGM](image)

The Y axis is the percentage of live *C.elegans* each day. The X axis is the time of experiment in days. The *C.elegans* were counted every day for 8 days. The mass ratio of *S. cerevisiae: E.coli* was 1:30.

Results of this experiment are shown in Figure 6. *C. elegans* grown on *Y101* and *SOD1* were significantly different (*P*<0.001). *Sigma* and *Sod 1Δ* were significantly different from each other (*P*<0.001). *Sigma* was not significantly different from the *Y101* control (*P*<0.141). However, *C elegans* grown on *Sigma*, which were expected to die faster than *Sod 1Δ*, actually had a higher survival percentage by the end of the experiment. This was probably due to difficulty encountered in counting. *S. cerevisiae* became very dense in the center of wells during experiments, which made the field under the microscope unclear for counting for all the conditions.
**Varying Mass Ratios**

The mass ratios used for the first experiment were 1:30, 1:60, 1:120, and 1:24 *S. cerevisiae: E. coli*. After this first experiment the media was changed because the *S.cerevisiae* was very thick due to the use of rich medium NGM. S. medium was used subsequently because it contains no carbon or nitrogen source for *S. cerevisiae* to grow and the counting would be more accurate.

![Survival curve](image)

*Figure 7: Survival curve of C. elegans with different mass ratio of S. cerevisiae to E. coli in S. medium*

Y1E30 is a 1:30 mass ratio of *S. cerevisiae* to *E.coli*. The same notation was used for the other ratios of 1:60, 1:120, and 1:240. The Y axis is the percentage of live *C.elegans* each day. The X axis is the time of experiment in days. The *C. elegans* were counted every day for 8 days.

Results of an experiment to test the effects of different mass ratios of *S.cerevisiae* and *E.coli* are shown in Figure 7. With a better view of the plate, 1:60, 1:120 and1:240 mass ratios of *S. cerevisiae* to *E.coli* were significantly different from1:30 (*P = <0.001*), using pair wise comparisons. However, the majority of dead *C.elegans* had larva hatched
within their bodies. Whether the *C. elegans* died from *S. cerevisiae* infection or from the hatching larva was unclear.
For the next experiment (Figure 8), *C.elegans* were fed on the third day of the experiment with the same amount of *S. cerevisiae* used to start the experiment. Control wells with *E.coli* only were fed with same amount *E.coli*.

Figure 8: Survival curves of N2 *C.elegans* at different mass ratios of *S. cerevisiae* to *E. coli* with re-feeding

Y1E30 is the mass ratio of *S. cerevisiae* to *E.coli*, 1:30. The same notation is used for 1:60 mass ratios. The Y axis is the percentage of live *C.elegans* on each day. The X axis is the time of experiment in days.
C. elegans in re-feeding wells did live longer than the non-feeding ones in the E. coli only condition (P = 0.038). But in 1:30 and 1:60 mass ratios, there was no significant differences between re-feeding and non-feeding conditions (for 1:30, P = 0.176; for 1:60, P=0.134). However, dead C. elegans with larva hatched within bodies were still observed during the experiment.

**C. elegans Mutants**

The C. elegans mutants that were used were mek-1(ks54) and fer-1(b232). Mek-1 encodes a MAP kinase that is required for resistance to killing by S. cerevisiae in agar plate cultures (Meijiang Yun, unpublished results).
Figure 9: Survival curves of N2 and mek-1 C.elegans at different mass ratios of S. cerevisiae to E.coli with re-feeding

The Y axis is the percentage of live C.elegans on each day. The X axis is the time of experiment. The number of worms was counted each day for 11 days.

The experiment was done with three different conditions: an amount of equivalent to that mixed with yeast in other samples, a mass ratio of S. cerevisiae to E. coli of 1:30, and a mass ratio of S. cerevisiae to E. coli of 1:60. Results are shown in Figure 8. Mek-1 died significantly faster than N2 (P = <0.001) in both E.coli control and 1:60 mass ratio conditions. The P value of 1:30 mass ratio was 0.958, from which we concluded that the
1:30 survival curve is not great enough to exclude the possibility that mek-1 lived longer than the N2 strand is due to random sampling variability. Both mek-1 and N2 had many dead C.elegans with larva hatched inside the bodies.

*Fer-1* is a *C. elegans* mutant that is temperature-sensitive fertilization defective. At 20 °C, *fer-1(b232)* hermaphrodites are infertile.
**Figure 10: Survival curves of N2 and fer-1 C. elegans at different mass ratios of S. cerevisiae to E. coli at 20°C with re-feeding**

The Y axis is the percentage of live *C. elegans* on each day. The X axis is the time of experiment in days. The *C. elegans* were counted everyday for 12 days.

The experiment was done with three different conditions: same amount of *E. coli*, a mass ratio of *S. cerevisiae* to *E. coli* of 1:30, and a mass ratio of *S. cerevisiae* to *E. coli* of 1:60. Y1E30 notation means a *S. cerevisiae* to *E. coli* mass ratio of 1:30. The same notation is used for mass ratio of 1:60. *Fer-1* lived significantly longer than *N2* in the
experiment (All curves have a P value of <0.001). About 80% of the fer-1 C.elegans lived till the 13th day of the experiment.

**Kgb-1; fer-1 C. elegans double mutant was constructed successfully**

After performing the construction strategy described in Methodology, three clones of kgb-1; fer-1 double mutant were found. As expected for a kgb-1 mutant, eggs of the putative double mutant strain failed to reach adult stage on plates containing 75 μM Cu^{2+}. As expected for a fer-1 mutant, when grown at 20°C, their offspring did not lay fertilized eggs. These results verified that the three clones are true kgb-1; fer-1 double mutants.
Discussion

Using a liquid culture assay would be ideal for identifying fungal virulent factors because it would make the high-throughput screening of the deletion library more efficient. However, the assay has not been fully developed.

When using different mass ratios of *S. cerevisiae* to *E. coli*, *C. elegans* did not die significantly faster at a lower concentration of *S. cerevisiae* compared to a higher concentration of *S. cerevisiae*. Death could be accounted for fertilized eggs hatching inside the body. While viewing this, there was uncertainty of whether the *C. elegans* died because of *S. cerevisiae* or because of starvation. When the environment is not ideal, *C. elegans* does not lay eggs (Schafer, 2005).

When observing the results obtained with *N2* and *mek-1, mek-1* died faster compared to *N2*. There were other factors that influenced the dying of the *C. elegans* such as larva hatching inside of the body. This mutation in the MAP kinase cascade in the immune system may still be useful for high throughput assays because it is weaker and more susceptible to infection.

To eliminate death due to fertilized eggs hatching within the worm’s body, we used *fer-1*, a temperature-sensitive fertilization-defective mutant. At the restrictive, 20°C, *fer-1(b232)* sperm are inactivated and eggs consequently are not fertilized. This would prevent the effects of *S. cerevisiae* infection from being obscured and allow the real effects of yeast to be observed. The *fer-1* *C. elegans* stayed alive throughout the experiment; only about 20% of the *fer-1* *C. elegans* died within 12 days while more than 70% of the *N2* died. The only reason that 100% of the *N2* did not die is that the experiment was terminated at 12 days. This experiment allowed us to conclude that wild type *C.elegans* was not dying due to *S. cerevisiae* but due to the fertilized eggs hatching within the body.

From the results of experiments using *fer-1 C. elegans* mutants, a *kgb-1; fer-1* double mutant of *C. elegans* would be a good strain in which to study fungal infections in worms. *Fer-1(b232)* mutant *C. elegans* do not lay any fertilized eggs at 20°C, the temperature used for previous experiments, which helps excluding the death-causing factor of fertilized eggs hatching inside *C. elegans* bodies from experimental results. *Kgb-1* mutant *C. elegans* was shown by Meijiang Yun (unpublished results) to show
enhanced susceptibility to fungal infection; a kgb-1 mutant was previously shown to be important for innate immune responses to bacterial infections (Kim et al., 2004). Therefore if kgb-1; fer-1 also shows enhanced susceptibility to yeast infection, it is likely to be because of a defect in innate immunity.
Future Experiments

With the newly available \textit{kgb-1; fer-1 C. elegans} double mutant, observational analysis on intestinal lipofuscin auto-fluorescence intensity, using BY4742 yeast strain expressing RFP, can be done to visualize the infection caused by \textit{S. cerevisiae}. In the absence of matricide from internal hatching, more significant features of yeast pathogenesis might be revealed. Survival experiments on agar plates can also be done to see if this double mutant is as susceptible as a \textit{kgb-1} single mutant. By comparing the results from these experiments, a better view of intestinal infection in \textit{C. elegans} can be obtained, and the real death-causing factor, either matricide or fungal infection, can be determined. Therefore, the effect of \textit{S. cerevisiae} on \textit{C. elegans} is expected be represented more accurately in future experiments.
## Appendix 1: Raw Data

### Various Mass Ratios Without Feeding

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<th></th>
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### Various Mass Ratios With Feeding

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### Different Yeast Mutants and Stains

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*mek-1 and N2 With Feeding*

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*fer-1 and N2*

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Appendix 2: SigmaStat Set-Up

Step 1: Enter Data in Columns
Step 2: Select LogRank Test
Step 3: Select Data Format
Step 4: Select Group, Time, and Status Columns
Step 5: Select Groups for Comparison
Step 6: Select Event (nothing is censored) and Click Finish
References

Angier, N. (1986) A Stupid Cell with All the Answers. Discover 7: 70-83


Introduction to C. elegans Anatomy. (2006)
http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm


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