C. elegans Mutants with Decreased Pathogenic Response to Yeast

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Abstract

One of the largest problems in medicine today is fungal pathogenesis. The eukaryotic nature of fungi is similar to their hosts, which include humans, plants and animals. Due to these similarities it is difficult to develop novel drugs against their infection. Through the development of a host-pathogen system, host factors involved in resistance or sensitivity to infection may be identified. *Saccharomyces cerevisiae* causes a pathogenic response in wild type *Caenorhabditis elegans*, characterized as swelling of the anal region of the organisms. We developed an assay to test the immune response of mutant strains of *C. elegans*. Eight out of thirteen mutant strains displayed a deformed anal region (Dar) on the fourth day of the assay. One mutant strains, *bus-4*, had no visible pathogenic response to the *S. cerevisiae*.

Acknowledgements

We would like to thanks Professors Sam Politz and Reeta Prusty Rao for their help, guidance and insight on this project, as well as Professor Liz Ryder for her expertise on biostatistics. We would also like to thank Charu Jain for her help both in the lab, and on our poster and paper.

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Introduction

The nematode *Caenorhabditis elegans*, a widely used model organism, is one of the best known animals in terms of genetics. The easily observed organism can be maintained in lab settings, and is easily mutated to observe the effects of altered genes. Almost every aspect of biology has been studied using *C. elegans* mutations (Hodgkin, 2005).

The yeast *Saccharomyces cerevisiae* has been studied to better understand cellular and molecular processes. The genome sequence, which has been completely elucidated, contains many orthologs of genes involved in human diseases, and has similarities to many others. Many of the genes are involved in signal transduction processes. The organism's growth and division are efficiently controlled by environmental changes, and it is easily maintained in labs (Mager and Windericx, 2005).

Standard pathogenesis studies involve two organisms, a host and a pathogen. For this MQP we utilized *C. elegans* as a model host, against *S. cerevisiae* as a pathogen. Using mutant strains of *C. elegans* the effects of altered genes on the nematodes' response was observed.

Pathogenesis Assay

In a previous MQP, an assay to test the effects of S. *cerevisiae* as a primary food source for *C. elegans* was developed. In initial assays wild type *C. elegans* were observed to arrest growth at the L1 stage of development on plates containing only *S. cerevisiae* as a food source. It was concluded that the larval *C. elegans* could not ingest *S. cerevisiae* because the cells are substantially larger than *E. coli*, the food source commonly used to propagate *C. elegans* in the lab. A mixture containing ninety percent *S. cerevisiae* and ten percent *E. coli* was used to provide L1 stage larvae with a bacterial food source that they are able to consume. This combination allowed for exposure to the possible pathogen without an arrest in growth due to starvation (Gray and Lentz 2006).

Another problem that arose in the development of the assay was distinct differences in the developmental stages of *C. elegans* picked from stock plates to experimental plates. It was also observed that some *E. coli* was transferred with the organisms, providing the animals an unintended additional food source. To eliminate

these variables an egg preparation was developed. By treating a stock culture with a bleach solution, the larval and adult worms could be lysed leaving only the eggs. A controlled number of eggs could then be plated, and the resulting larvae developed synchronously. The bleach treatment also removed any *E. coli* contaminants from the stocks food source (Gray and Lentz, 2006).

Using this assay the team discovered that wild type *C. elegans* develop a Dar phenotype. Using the *S. cerevisiae* and *E. coli* mixture as the *C. elegans* primary food source, anal swelling or tail bumps would develop after 10 days. The Dar phenotype was observed in worms fed mutant strains of yeast as well as wild type. The team predicted that three mutant forms of worms would display an increased resistance to the pathogen and therefore resistance to an infection (Gray and Lentz, 2006).

Deformed Anal Region Phenotype

The Dar mutant phenotype was originally detected in 1986 because it displayed a deformed anal region (Hodgkins *et al*., 2000). After inspection of the affected *C. elegans* tails the phenotype was determined to be due to infection, not a genetic mutation as originally believed (Hodgkins *et al*., 2000). A patch of rod-shaped bacteria was visible around the tail swelling. By isolating the bacteria, a contaminant of the *E. coli* used to propagate the worms, it was found to be a member of the *microbacterium* genus, later named *Microbacterium nematophilum.* It was discovered during experiments to determine the type of genetic mutation causing the Dar phenotype, and it was found that wild type worms, when bred with Dar animals, would develop the Dar phenotype. Some progeny of Dar worms were also seen to have normal tails, with the phenotype reappearing in future generations (Gravato-Nobre *et al*., 2005).

The Dar phenotype is characterized by a swelling of the anal region, which can be seen under a dissecting microscope. The swelling is always located on the ventral side near the rectal opening. The worms also appear to be mildly constipated and grow at a slower rate. *C. elegans* strains that have mutations in the ERK MAP Kinase pathway have not been seen to exhibit the Dar phenotype. These mutants however appear severely constipated when in contact with *M. nematophilum,* as well as less viable and fertile (Gravato-Nobre *et al*., 2005).

Mutant C. Elegans

Alteration in the *C. elegans* genome may result in a change in expression of a Dar phenotype. To test this hypothesis we used thirteen different worm mutations. Two different categories of worm mutation were used, MAP kinase pathway mutations and surface-altered mutations. The surface mutants can be broken down into three types, *srf*, *bah*, and *bus*; these mutations have some overlapping phenotypes.

The surface coat of *C. elegans* is a negatively charged glycoprotein-rich layer overlying the epicuticle. This layer is believed to help with the worms' movement, prevent predator adhesion, and prevent desiccation. The expression of carbohydrate epitopes has been found to differ in the *srf* class mutants. Mutations in genes important for protein glycosylation have been shown to result in a weakened cuticle and altered adherence of some bacteria (Hoflich *et al*., 2004, Hodgkin, personal communication, York *et al*., 2007).

Bus (Bacterially UnSwollen) mutants were discovered with the bacterium *M. nematophilum* (Gravato-Nobre *et al*., 2005). Mutants in this class were observed to not develop a Dar phenotype when exposed to *M. nematophilum.* Many of these mutants appear to have little or no accumulation of *M. nematophilum* around their tail region, suggesting that something in their cuticle is altered. This alteration causes the bacteria to be unable to adhere to the *C. elegans*, and unable to initiate infection. One strain in particular, *bus-4*, is predicted to encode a galactosyltransferase (Gravato-Nobre and Hodgkin personal communication). It is also one of the *bus* mutants that shows no obvious alterations in surface coat, but reacts to pathogens as though a surface coat alteration is present (Gravato-Nobre and Hodgkin, 2005).

Yersinia pestis and *Yersinia psuedotuberculosis* have also been shown to affect *C. elegans*. These bacteria secrete an extracellular matrix which adheres to the head of the worms. In order for the biofilm to adhere to *C. elegans* the worms must be in motion. As they move through lawns of this bacterium the matrix gathers on their head causing the worms to be unable to eat and slowing their growth (Darby *et al.* 2007). *Bah* (Biofilm Absent on Head) mutants exposed to the Yersinia bacteria did not suffer bacterial adhesion. These mutants have not been shown to develop the *srf* or *bus* phenotypes, except that *bah-3* has a *srf-6*-like phenotype. It has been speculated that these mutants

lack a surface component which allows the *Yersinia* to adhere to wild type worms (Darby *et al*., 2007). The *bus, bah,* and *srf* mutants are considered surface mutants, Table 1 shows a list of each strain utilized and the type of mutant they are considered.

MAP Kinase signaling pathways are believed to be involved in the innate immune responses of *C. elegans*. Three MAP kinase pathways are known in *C. elegans*: the JNK pathway, p38 pathway, and the ERK pathway. The *mek-1* and *jnk-1* mutants both alter steps in the worms JNK pathway. *Mek-1* is a possible activator of JNK-1 (Gravato-Nobre *et al*., 2005). The *mek-1* gene encodes a MAPKK. The *mek-1* larvae grow more slowly or are arrested in growth and are hypersensitive to starvation (Koga *et al*., 2000). *Jnk-1* encodes a homolog of the human stress activated protein kinases (SAPK) (Kawasaki *et al.*, 1999). Null mutations in *jnk-1* result in uncoordinated locomotion in *C. elegans*. The JNK-signaling components are also associated with the localization of synaptic vesicles. MEK-1 is found in the beginning of the pathway, while JNK-1 is found towards the end (Sakaguchi *et al*., 2004).

Nsy-1 encodes a MAPKKK, which belongs to the P38 pathway. NSY-1 is known to function in the asymmetry of neuronal cells, more specifically olfactory neuron fate. A loss of function in the NSY-1 protein will cause the STR-2 receptor to be expressed in both of the paired AWC neurons (Sakaguchi *et al*., 2004). In more recent studies with *Pseudomonas aeruginosa, C. elegans* that have an *nsy-1* mutation have an ESP (enhanced susceptibility to pathogen) phenotype and die faster than wild type C. elegans. *Pseudomonas aeruginosa* is pathogenic to *C. elegans* by proliferating in their intestines, causing death (Sakaguchi *et al.*, 2004). This suggests that *nsy-1* is also part of a MAP kinase cascade that is involved with innate immunity.

The third MAP kinase pathway that has been studies is the ERK MAP kinase pathway. Mutants in this pathway get sicker than the surface altered mutants when infected by *M. nematophilum*. *Mek-2* encodes an ERK MAPKK2 (Gravato-Nobre *et al.*, 2005). The MEK-2 protein is involved in the ERK pathway which is known to be required for the development of the Dar phenotype when *C. elegans* are infected with *M. nematophilum* (Nicholas and Hodgkin, 2004). The ERK MAP kinase pathway is directly involved in the development of the Dar phenotype with *M. nematophilum*, while the JNK and P38 pathways are not involved in the formation of Dar. The *jnk-1, mek-1, mek-2,*

and *nsy-1* mutants are considered MAP Kinase pathway mutants; Table 1 shows a list of each strain utilized and their grouping.

Table 1 Mutants Exposed to Pathogen

Strain	Mutant Type
bah-1 *(br1)*	Surface
bah-2 *(br7)*	Surface
bah-2 *(br8)*	Surface
bus-4 *(br4)*	Surface
bus-17 *(br11)*	Surface
srf-2 (br3)	Surface
srf-2 (br10)	Surface
srf-3 (br6)	Surface
srf-6 (yj13)	Surface
nsy-1 (ag3)	MAP Kinase
jnk-1 (gk17)	MAP Kinase
mek-1 (ks54)	MAP Kinase
mek-2 (n1989)	MAP Kinase

An analogous mutant screen on yeast is the thesis research of Charu Jain (graduate student), not part of our study.

Methodology

Stock Plate Maintenance

Stock plates of the thirteen mutant strains were kept at 16°C incubator. The plates were NGM agar (Sulston and Hodgkin 1988) with *E. coli* OP50 as a food source. Five to seven *C. elegans* were transferred to fresh plates every five to six days. Stock plates for egg preps were placed in the 20°C incubator four days before the egg prep was to be performed.

Strains

Thirteen different stains of mutant *C. elegans* were obtained from three different labs for this project. The MAP kinase mutants, *jnk-1 (gk17), nsy-1 (ag3), mek-1 (ks54)* and, *mek-2 (n1989),* came from the Caenorhabitis Genetics Center. The surface mutant *Srf-6 (yj13)* was obtained from the Politz lab. The other surface mutants, *bah-1 (br1), bah-2 (br7), bah-2 (br8), bus-4 (br4), bus-17 (br11), srf-2 (br3), srf-2 (br10)* and, *srf-3 (br6),* are from Creg Darby's lab.

Preparation of Experimental Plates

A single colony was picked from a streaked plate of *E. coli* OP50 and added to a test tube containing 5mL LB broth (Gray and Lentz, 2006). The tube was incubated at 37°C overnight. A single colony was picked from a *S. cerevisiae,* RPY101 strain, plate and added to a test tube containing 5mL YPD broth (Rose *et al.,* 1990). The tube was incubated overnight at 30°C.

Both tubes were removed from incubators, and the liquid was transferred to labeled sterile 15mL conical tubes and centrifuged for five minutes at 9,000xg. Two 1.5 mL eppendorf tubes were labeled and their weights recorded in mg. The supernatant was removed and each pellet was resuspended in 1mL of sterile deionized water (dH_2O) and transferred to its corresponding sterile eppendorf tube.

The eppendorf tubes were then centrifuged for five minutes in a microcentrifuge to harvest the *E. coli* and *S. cerevisiae*. Liquid was emptied from both tubes and a kim wipe was use to remove any excess water. The tubes and pellet were weighed to determine the weight of each pellet. The *E. coli* was resuspended in dH_2O to a working concentration of 200mg/mL of *E.coli* and the *S. cerevisiae* was resuspended in dH_2O to a

working concentration of 20mg/mL. The working solutions were then combined as follows. For the standard assay plates, per plate, 2.5µL of *E. coli* working suspension was combined with 7.5µL of *S. cerevisiae* working suspension. Per plate, 10µL of a 50mg/mL stock solution of streptomycin sulfate in dH_2O was added to the 10µL of *E. coli*: *S. cerevisiae* mix. For multiple plates, multiples of these volumes were prepared as needed. Twenty microliters of the mixture was pipetted onto each 60mm NGM agar (Sulston and Hodgkin 1988) plate and allowed to dry for an hour before addition of *C. elegans* eggs. In initial experiments to optimize the assay, relative volumes of *E. coli* and *S. cerevisiae* were varied, but the total volume of *E. coli* plus *S. cerevisiae* per plate was kept constant at 10µL.

Egg Preparation

Egg preps were performed in order to ensure that all *C. elegans* used in the experimental assay were at a similar developmental stage and did not introduce contaminants from stock plates to experimental plates. Stock plates of the different *C. elegans* mutant strains were prepared three to five days in advance, depending on the strain. The stock plates were examined prior to start of the egg prep to determine if there were a usable number of eggs on each plate. These plates were then washed with M9 solution (Brenner, 1974) to remove the eggs from the plate. The liquid was then transferred from each plate into a 15 mL conical tube. The plates were examined under a microscope to determine if the majority of the eggs had been removed from the plate.

After all strains were transferred into corresponding labeled tubes, they were centrifuged for two minutes at 900xg. The supernatant was then removed from the tube using a Pasteur pipette, without disturbing the eggs. Ten milliliters of bleach solution, containing 0.25M NaOH dissolved in a 1:4 dilution of commercial bleach in dH_2O, was added to all of the tubes to digest adult worms; the eggs, which are slightly more resistant to the bleach then adult worms and hatched larvae were not digested. The egg prep also sterilized the eggs for plating. The tubes were rocked for two to five minutes depending on the strain that was being used prepared. They were then centrifuged for another two minutes at 900xg and the supernatant was quickly removed without disturbing the eggs. The eggs were washed by adding M9 solution (Brenner, 1974) and centrifuged for two minutes at 900xg and removing supernatant.

After the supernatant was removed from the second wash, the pellet was suspended in 3mLof M9 buffer (Brenner, 1974). Two 10µL drops were added to an unused NGM agar (Sulston and Hodgkin 1988) plate and eggs were counted to find the amount of eggs per micro-liter. An aliquot containing between twenty and twenty-five eggs was then transferred onto each experimental and control plate. The plates were placed in a 20°C incubator and worms were allowed to mature.

Assay Procedure

Experimental and control plates were incubated at 20°C for four days, and observed once per day for development of *C. elegans*. On the fourth day the total number of *C. elegans* was recorded, as well as the number of organisms displaying the Dar phenotype. From these numbers the percent of individuals with tail bumps was determined. The plates were incubated for two more days and observed for any additional changes in phenotype. Data was not collected from plates contaminated by mold.

Data Analysis using SPSS

Accessing the SPSS Program

A computer program, SPSS, was used to statistically analyze the data. SPSS is a statistical computer program that can perform a variety of statistical tests very quickly. It also has the ability to perform post hoc tests. This software can either be downloaded from the WPI site for use off campus or can be remotely accessed for use on campus.

For this project the program was accessed on campus using remote access. Remote desktop was selected from the accessories section or in communication in the accessories section according to the access path below.

Start Menu -> Programs -> Accessories -> Communication

To connect to the remote desktop windows.wpi.edu was entered into the command line before clicking “connect”. Some computers asked for user log in with school username and password immediately. Other computers connected to the remote computer then ask for a log in. SPSS was found in the WPI academic software section of the start menu.

Creating an SPSS File

Once open the SPSS program was used to statistically analyze data in a new file. There are two different windows in SPSS, the data view and the variable view. In variable view all of the variables and groups that were used were specified. The Names of the Variables and groups were entered under the Name heading. The group name used was Mutants, and the variable name used was Percent Tail Bumps. Under the Type heading Numeric was chosen because the data to be entered are in numeric form. Under the Value heading mutant strains were assigned a number to represent each strain for analysis. Clicking on a cell under Value, opens a new window, where the groups were entered and given an integer value (N2 = 1, bah-1 (br1) = 2). By assigning numbers to the different variable groups the program decreases necessary data entry and simplifies analysis of data. Label can be an abbreviation or explanation of groups; this can be helpful when interpreting the data but is not necessary. The Missing, Columns, Align, and measure fields can be left in their default setting.

In the data view the number of variables entered was listed after filling in the variable view. For this project the variables listed were Percent Bumps and Mutants. In this sheet the data can be entered for analysis in the proper column. After all data points are entered analyze, then general linear model, were selected by mouse click Under general linear model univariate analysis of variance was chosen. The variable factor (percent bumps) was selected to be in the dependent variable box and group factor (mutant) to be in the fixed factors. Under options a significance level was entered before clicking continue. Post Hoc was chosen in univariate and group was pushed to Post Hoc test by selecting it and clicking on the arrow. Under equal variance assumed, LSD, Bonferroni, S-N-K, and Dunnet were selected. Control was in the first in groups and that position was selected for the Dunnet test. Under test, 2-sided was selected before clicking continue, and OK in main univariate. The final screen displays test results and enters group labels in place of numbers.

Results

Assay Development

In order to determine the correct ratio of *E. coli* to *S. cerevisiae* that would result in a robust Dar phenotype in wild type *C. elegans,* about twenty wild type *C. elegans* were transferred from stock plates to experimental plates. In order to determine an optimal *E. coli* to *S. cerevisiae* combination five types of plates with different mass ratios of yeast to *E. coli* were tested as seen in Table 2 below.

Table 2 *E. coli* to *S. cerevisiae* Ratios Tested

Plate	Volume of 20mg/mL yeast per plate	Volume of 200mg/mL *E. coli* per plate
1	10µL	0µL
2	7.5µL	2.5µL
3	5µL	5µL
4	2.5µL	7.5µL
5	0µL	10µL

Worms on all plates were able to grow, however few to no Dar phenotypes were observed on plate 3 and 4. The optimal ratio for the development of the Dar phenotype was observed on plate 2. It was surmised that *E. coli* may have been transferred with the worms, allowing growth even when the plate initially contained 100% yeast. For future experiments egg preps were used so *C. elegans* were at similar developmental stages and stock plate contaminants were removed.

During the experiments it was found that the *E. coli* would still grow in the yeast plates. This was a concern that the worms would still have *E. coli* to eat after passing the early developmental stages and that the *E. coli:* yeast ratio would increase during the experiment. The previous MQP had been using gentamicin and ampicillin in their experiments but discovered in the end that it was hindering worm growth. Streptomycin was chosen for our experiment because it would stop the *E. coli* from growing but still allow the worms to develop.

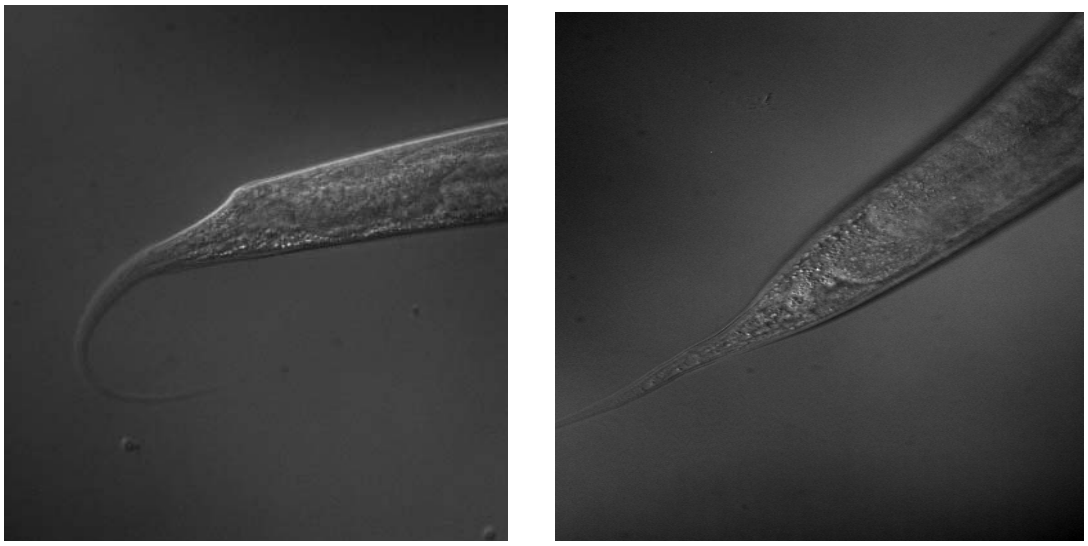
The above experiment was repeated using an egg prep, plating between 15 and 25 eggs on the different percentages of *S. cerevisiae* and *E. coli*. The eggs plated on 100 percent yeast hatched, and arrested at the L1 stage of development, similar to the results

of the previous MQP (Gray and Lentz, 2006). The *C. elegans* were observed for any developmental differences, or phenotypic changes, when compared to the plate containing 100% *E. coli.* Table 3 below shows the resulting changes in phenotype and development, for each type of experimental plate.

Table 3 Growth of wild type *C. elegans* from eggs in different *E. coli* to *S. cerevisiae* ratios

Days After Plating	Plate	Number live worms	Notes
2	E0	19	Worms appear to have all hatched on all plates. The E0 plate worms are slightly smaller than on other plates.
	E25	15	
	E50	22	
	E75	22	
	E100	23	
3	E0	13	E0 plate worms are still in early stages of growth and appear unhealthy and thin. Worms on plates with some *E. Coli* appear to be growing and healthy. E25 is appearing slightly skinnier then other plates.
	E25	14	
	E50	23	
	E75	23	
	E100	23	
4	E0	7	E0 plate worms never reached adulthood; more may have been hidden beneath yeast, so count may be inaccurate. The E25 worms may also been an inaccurate count because of the yeast growth. Worms on this plate have developed tail bumps. Other plates appear healthy and active.
	E25	15	
	E50	21	
	E75	25	
	E100	24	

In the development of subsequent assays, plates were examined four days after eggs were plated. The Dar phenotype was seen when *C. elegans* were exposed to *S. cerevisiae* in our assays. The Dar phenotype was observed mostly on the 3:1 mass ratio of *E. coli* to yeast plates. The Dar phenotype has been cited as a possible example of an innate immune response to infection in *C. elegans* such that worms which develop this phenotype, do not suffer as severely from the infection (Gravato-Nobre *et al.,* 2005). Figure 1 shows an example of the Dar phenotype, resulting from exposure to *S. cerevisiae*, next to a control wild type worm. The Dar phenotype was only observed in the adult stage of *C. elegans*. On previous days, Dar was not observed in larval stages.



Left panel: worm grown on *E. coli*: yeast mixture. Right Panel: worm grown on *E. coli* alone.

Figure 1 Dar in Wild Type *C. elegans* after four days of growth.

General Results

Each of the surface altered mutants was plated on a 3:1 mass ratio of *E. coli* to *S. cerevisiae.* They were observed for four days for developmental changes and evidence of the Dar phenotype. Each strain of worm was also plated on a control plate of 100% *E. coli* so that infected worms could be compared to healthy *C. elegans* of the same strain. The Dar phenotype was observed in five separate assays, and the data from the separate plates was compiled. The raw data and observations of the experimental plates can be found in appendix 1.

Bus Mutants

Two *bus* strains, *bus-4* and *bus17*, were tested for the Dar phenotype. The bar graph in Figure 2 shows the percentage of worms that developed the Dar phenotype in each *bus* strain.

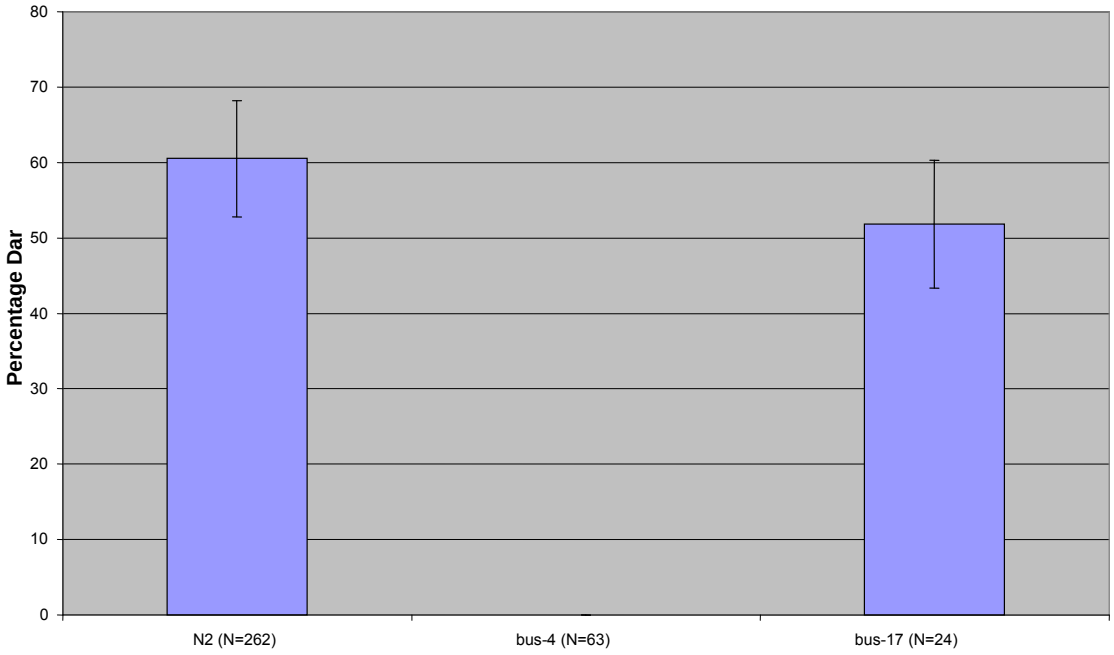


Figure 2 Percentage of Dar Phenotype in *bus* Mutants

Bars represent the percentage of each strain which developed the Dar phenotype. *C. elegans* were observed and counted on the fourth day after eggs plated on Yeast: *E.coli* experimental plates. Error bars represent standard deviations.

In the *bus-4* strain, the Dar phenotype was not observed on any worms. This strain appeared smaller and had a much slower growth rate then other strains on the experimental plates. While *bus-17,* on experimental plates, also appeared to have a slower growth rate, than on control plates, *bus-4* appeared to be developing even slower. As with both *srf* and *bah* strains the worms appeared sluggish and didn't move around as much, or as quickly as the worms on the control plates.

Srf Mutants

Four *srf* mutants were observed for the Dar phenotype; *srf-2 (br3), srf-2 (br10), srf-3(br3)* and *srf-6 (yj13).* The bar graph in Figure 3 shows the percentage of worms that developed the Dar phenotype in each *srf* strain.

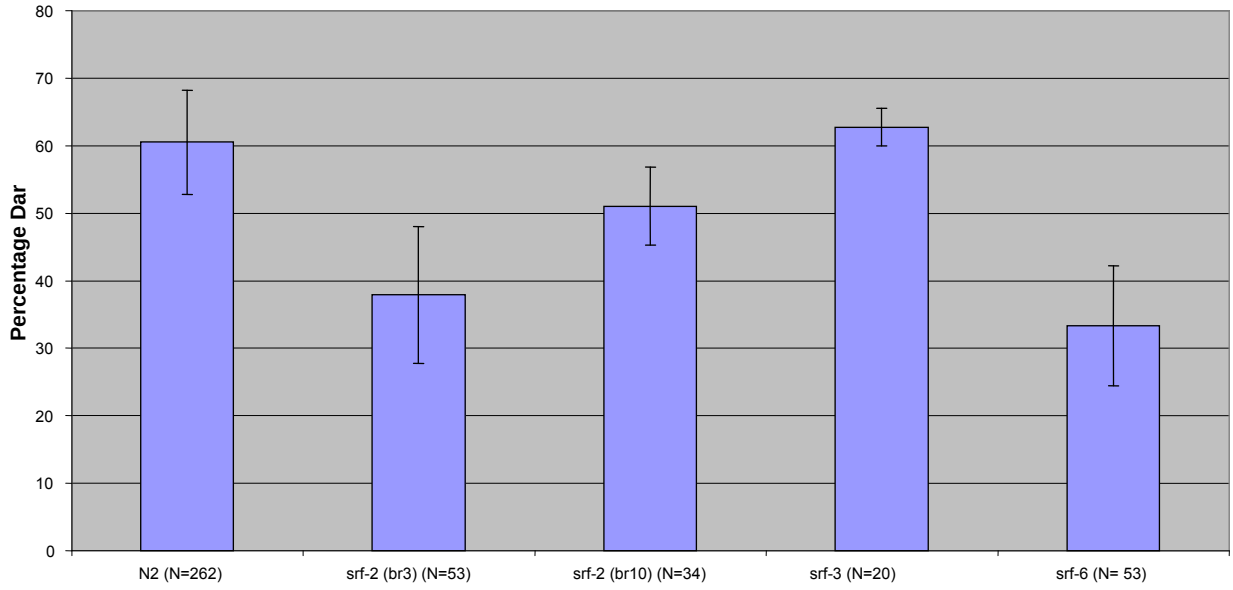


Figure 3 Percentage of Dar Phenotype in *srf* Mutants

Bars represent the percentage of each strain which developed the Dar phenotype. *C. elegans* were observed and counted on the fourth day after eggs plated on Yeast: *E.coli* experimental plates. Error bars represent standard deviations.

In the *srf-6* strain, a rare phenotype, swelling in the vulva region, was observed. This phenotype had also been noticed in some N2 *C. elegans* but only when the Dar phenotype was present. In the *srf-6* mutants this phenotype was observed in some worms that did not display the Dar phenotype. Vulva swelling was observed in approximately 40-50% of *srf*-6 adults tested. It was unclear whether this phenotype was due to infection. In all of the strains the *C. elegans* on experimental plates appeared to have a slower growth rate than those on control plates. Many of the worms appeared sluggish and didn't move around as much, or as quickly as the *C. elegans* on the control plates.

Bah Mutants

Three *bah* mutants, *bah-1, bah-2 (br7)* and *bah-2 (br8)* mutants were observed for the Dar phenotype. The bar graph in Figure 4 shows the percentage of worms that developed the Dar phenotype in each *bah* strain.

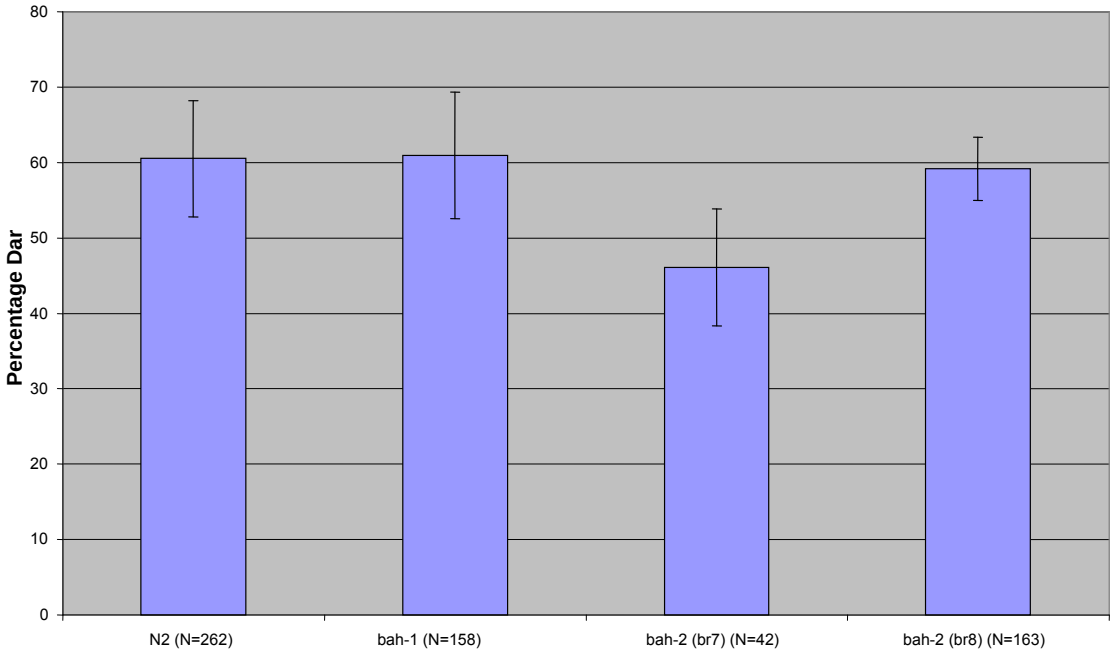


Figure 4 Percentage of Dar Phenotype in *bah* Mutants

Bars represent the percentage of each strain which developed the Dar phenotype. *C. elegans* were observed and counted on the fourth day after eggs plated on Yeast: *E.coli* experimental plates. Error bars represent standard deviations.

Similar to the *srf* mutants all of the *bah* strains on experimental plates appeared to have a slower growth rate than those on control plates. Many of the worms appeared sluggish and didn't move around as much, or as quickly as the *C. elegans* on the control plates. Other than arrested growth and sluggishness, no unique phenotypes were observed in the *bah* mutants. Two of the *bah* strains, *bah-1* and *bah-2(br8)*, had Dar percentages very close to that of N2.

Statistical Results

The SPSS program was used to analyze the percentage of each strain with respect to the Dar phenotype. Each mutant strain was compared to wild type *C. elegans* to determine if they were significantly different. The results of the Least Significant Difference (LSD) test of significance suggested three mutant strains were significantly different then the N2 strain. The LSD test is a liberal test but is useful in seeing differences among multiple groups. Even without the test the significance of the *bus-4* strain was evident. The *srf-6* mutants were found to have a significance of 0.018 when compared to the wild type *C. elegans*. The appearance of anal swelling in these mutants

was 33% in our assays. The *srf-2 (br3)* mutant was also observed to have a higher rate of Dar phenotype, 38%, and had a significance value of 0.030 compared to wild type *C. elegans*. Since the *srf-6*, *srf-2 (br3)* and *bah-2 (br7)* strains had a lower N value then many of the other strains, their significance may change with an increased N.

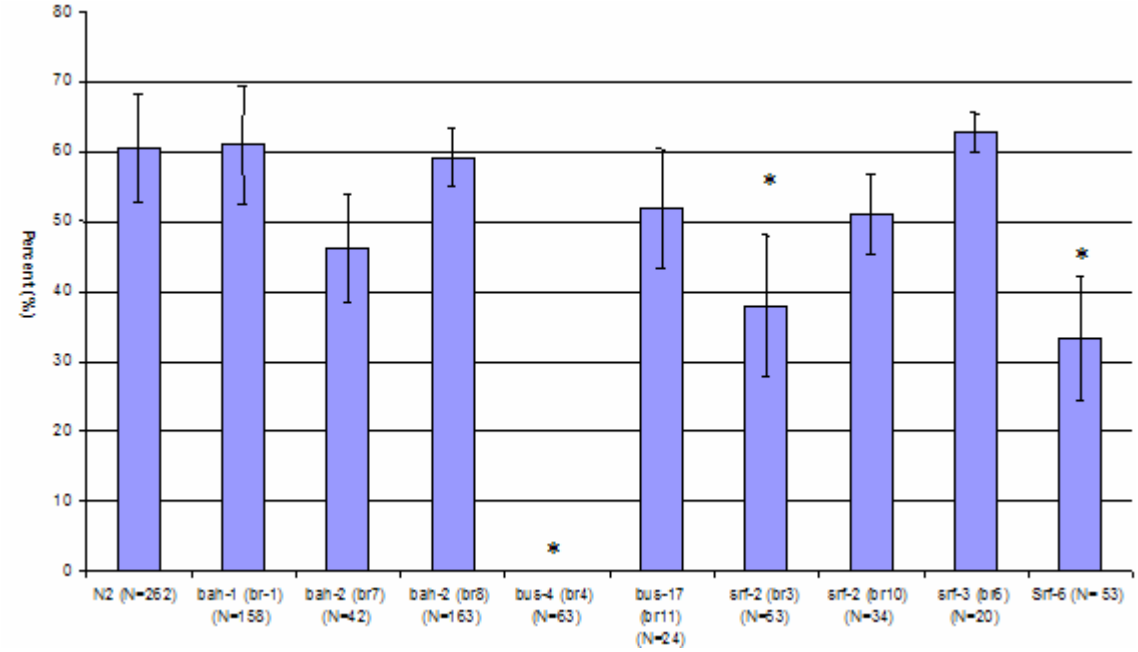


Figure 5 Percent Dar in mutants compared to wild type.

Strains whose Dar percentages were significantly different according to the LSD test from that of wild type are indicated by an asterisk.

The Dunnett, SNK and the Bonferroni tests were also used. The Dunnett test showed that both *srf-6* and *srf-2 (br3)* were approaching significance but had *p* values that were higher than 0.050. The SNK test was useful in comparing the mutants' statistical ranks but not individual significance. The Bonferroni test, which was the most conservative of the tests used, suggested that *bus-4* was the only significant strain.

Table 4 shows the number of each mutant plated in total. It also shows the total number of tail bumps that were observed as well as the average percentage of tail bumps found on each strain of *C. elegans*.

Table 4 Surface Mutant Total Tail Bumps

Surface Altered Mutants Plated On Yeast		
C. Elegans Strain	Total Number	Number of Tail Bumps
N2	262	184
bah-1	158	97
bah-2 (br7)	42	20
bah-2 (br8)	163	103
bus-4	63	0
bus-17	24	14
srf-2 (br3)	53	25
srf-2 (br10)	34	19
srf-3	20	12
srf-6	53	20

Discussion

Exposure to *S. cerevisiae* results in the Dar phenotype in wild type *C. elegans*. Using a four to five day assay developed with wild type *C. elegans,* mutant strains of *C. elegans* can be screened for a reaction to *S. cerevisiae*. By observing the reaction of different mutant strains to *S. cerevisiae* as a food source, predictions can be made as to which genes maybe involved in the development of the Dar phenotype in *C. elegans.*

Surface Protein Alteration Mutants

Three of the nine surface mutants tested were significantly different in percentage of Dar phenotype when compared to wild type *C. elegans*: *bus-4*, *srf-6* and *srf-2*. The complete absence of the Dar phenotype was striking in *bus-4.* The *bus-4* mutant strain did not show any tail bumps but did appear more sluggish and delayed in development compared to the *bus-4* mutants on control plates. It has been found that *bus-4* encodes a galactosyltransferase (J. Hodgkin, personal communication) suggesting that this protein is required for the pathogenic reaction to *S. cerevisiae*. The strains which showed a significant reduction all have a Bus phenotype when exposed to *M. nematophilum* (Darby *et al.*, 2007) which suggests that surface alterations in these mutants may be important for both *S. cerevisiae* and *M. nematophilum* infection. The remaining *srf* and *bus* mutants, however, displayed the Dar phenotype on yeast, suggesting that *S. cerevisiae* and *M. nematophilum* may have different requirements for host recognition.

Most of the surface mutants tested developed the Dar phenotype, even though their mutations inhibit the adherence of other microbes. These results suggest that *S. cerevisiae* may adhere to *C. elegans* differently then *M. nematophilum*, or that its adherence is not necessary for a pathogenic response, as it is with *M. nematophilum* or *Yersinia*. In order to determine if the adherence to the *C. elegans* is necessary, GFP tagged yeast could be used to determine the location of the *S. cerevisiae* on the infected worms. It may also be useful to determine if the swelling decreases if *C. elegans* are no longer in contact with the pathogen.

Future Experiments

The results produced by observing *S. cerevisiae*'s effect on mutant strains of *C. elegans* has raised even more questions about the infection pathways of *S. cerevisiae*.

Several of the surface mutants should be retested to increase the N, and checked for any changes in significance. The experiments that were done showed statistical significance with the LSD test, which is one of the more liberal statistical tests available. In order to see something with a more conservative statistics test, like Bonferroni, a greater N value needs to be obtained. The mutants that should be tested are the ones that are significant now or are close to significance.

All of the MAP kinase pathway mutants should be retested to allow enough examples for statistical analysis, as well as reproducibility. The MAP Kinase pathway mutants also showed the most interesting results because of their previously demonstrated involvement in innate immunity. Their results were seen on the fifth day as opposed to the fourth day observations in the surface mutant experiments.

The four MAP kinase mutants are mutants of pathways believed to be involved in the innate immune response of *C. elegans* to bacterial infections. Two of these mutants are from the JNK pathway. M*ek-1* is upstream in the JNK pathway from *jnk-1*. *Jnk-1* was observed to have some tail bumps after the fifth day of incubation with *S. cerevisiae*. *Mek-1,* however, did not display the Dar phenotype and many of the *mek-1* mutants died by the fifth day of observation. It is unclear what caused death. However, if the increase in death is due to infection it can be hypothesized that *mek-1* activates other pathways that assist in the immune response and overall resistance of *C. elegans* to *S. cerevisiae.*

The other MAP kinase pathways also showed a disruption of the Dar phenotype in the mutants. The mutations in the P38 and the ERK MAP kinase pathways disrupt the development of the Dar phenotype in bacterial infections as well. This suggests that these pathways are utilized by the *C. elegans* response to both *S. cerevisiae* and bacteria.

Several other experiments could be done to help determine details about the *S. cerevisiae* - *C. elegans* relationship. One possible experiment is a survivorship study, to see how long the worms survive when exposed to *S. cerevisiae*. This would also demonstrate whether an interaction with yeast changes the worm's lifespan. This would involve having yeast plates ready every for several consecutive days. After the initial yeast to *E. coli* mixture plate the rest of the plates could be spotted just with yeast, since later in the experiment the *C. elegans* will be large enough to eat the yeast. Then it can be determined how long *C. elegans* survive with *S. cerevisiae* as their only food source.

Another experiment that could be done is a food source preference experiment. *C. elegans* that grew up on yeast could then be transferred to a plate that had separate yeast and *E. coli* spots, to observe for preferences in a food source. In similar experiments using pathogenic bacteria the worms preferred the non-pathogenic food source. (Zhang *et al.*, 2005) A qRT PCR of the infected *C. elegans* is also another possible experiment to do. This can help to study changes in gene expression when *C. elegans* are exposed to yeast.

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Appendix 1: Raw Data

Date	Plate	Number live worms	Carcasses on edges	Notes
9/29/2006	E0	10	0	The E0 plate worms are slightly smaller than other plates. Very little food appears to be left on high percentage E. coli plates
	E25	12	0	
	E50	7	0	
	E75	7	0	
	E100	26	0	
09/31/2006	E0	10	0	E0 plate worms are still in early stages of growth and appear unhealthy and thin. E100 plate appears starved. E100 also has some mold contamination.
	E25	12	0	
	E50	7	0	
	E75	7	0	
	E100	57	0	

Date	Plate	Number live worms	Notes
10/9/2006	E0	19	Worms appear to have all hatched on all plates. The E0 plate worms are slightly smaller then other plates.
	E25	15	
	E50	22	
	E75	22	
	E100	23	
10/10/2006	E0	13	E0 plate worms are still in early stages of growth and appear unhealthy and thin. Worms on plates with some E. Coli appear to be growing and healthy. E25 is appearing slightly skinnier then other plates.
	E25	14	
	E50	23	
	E75	23	
	E100	23	
10/11/2006	E0	7	E0 plate worms never reached adulthood; more may have been hidden beneath yeast, so count may be inaccurate. The E25 worms may also been an inaccurate count because of the yeast growth. Worms on this plate have developed tail bumps. Other plates appear healthy and active.
	E25	15	
	E50	21	
	E75	25	
	E100	24	

Date	Plate	Number live worms	Number of eggs	Carcasses on edges	Notes
	C1	36	4	0	
	C2	42	1	0	
	C3	36	4	0	
	HK1	34	5	0	
	HK2	42	6	0	
	HK3	60	0	0	Heat killed yeast and control yeast appear similar, some eggs have not yet hatched from egg prep. Yeast plate appears to not be growing as quickly as control and heat killed.
	Y1	37	8	0	
	Y2	26	5	0	
11/2/2006	Y3	34	4	0	
	C1	43	0	0	
	C2	42	0	0	
	C3	40	0	0	
	HK1	40	0	0	
	HK2	42	0	0	
	HK3	58	0	0	
	Y1	54	0	0	Heat killed yeast and control yeast still appear similar.Yeast plate appears to beginning opf tail bumps developing on most worms.
	Y2	30	0	0	
11/3/2006	Y3	41	0	0	
	C1	43	110	0	
	C2	42	132	0	
	C3	40	96	0	
	HK1	40	107	0	
	HK2	42	84	0	
	HK3	58	97	0	Heat killed yeast and control yeast still appear similar.Yeast plate has evident tail bumps on worms. There may be more eggs hidden in yeast and egg counts may not be accurate.
	Y1	54	6	0	
	Y2	30	8	0	
11/4/2006	Y3	41	8	0	

Date	Plate	Number live worms	Tail Bumps	Notes
11/13/2006	N2 C	38	0	majority of the eggs appear to have hatched. There was a large variation in the number of eggs plated. Egg preps may need a higher dilution then was used.
	N2 Y	80	0	
	DC4 C	24	0	
	DC4 Y	28	0	
	Srf 6 C	27	0	
	Srf 6 Y	25	0	
	DC6 C	24	0	
	DC6 Y	13	0	
11/14/2006	N2 C	53	0	The N2 control plate appears to be running low on food. All the mutants appear to be growing more slowly then the wild type plates, especially on the yeast. There is some mold contamination on the plates.
	N2 Y	80	0	
	DC4 C	40	0	
	DC4 Y	25	0	
	Srf 6 C	25	0	
	Srf 6 Y	30	0	
	DC6 C	33	0	
	DC6 Y	17	0	
11/15/2006	N2 C	53	0	Mutant yeast plates are at similar stages of growth on each plate. Control plates have varying stages and appear to be out of food. The yeast on the DC4 plate seems thinner then other experimental plates. Mutants appear less active on both control and yeast plates as compared to the wild type.
	N2 Y	72	0	
	DC4 C	30	0	
	DC4 Y	25	0	
	Srf 6 C	25	0	
	Srf 6 Y	30	0	
	DC6 C	28	0	
	DC6 Y	17	0	
11/16/2006	N2 C	38	0	Worms on 3 of the 4 yeast plates developed tail bumps. A higher percentage of wild type worms developed tail bumps then the mutants. The DC4 worms show no visible signs of infection.
	N2 Y	65	52	
	DC4 C	30	0	
	DC4 Y	24	0	
	Srf 6 C	22	0	
	Srf 6 Y	25	13	
	DC6 C	30	0	
	DC6 Y	17	10	

Date	Plate	Number live worms	Tail Bumps	Notes
11/20/2006	N2C1	11	0	Eggs on DC11 plates appear un-hatched, may not have survived the egg prep as well as other. Hatched worms on all plates appear to be developing normally
	N2C2	8	0	
	N2Y1	8	0	
	N2Y2	7	0	
	DC3C1	12	0	
	DC3C2	11	0	
	DC3Y1	6	0	
	DC3Y2	3	0	
	DC7C1	19	0	
	DC7C2	10	0	
	DC7Y1	8	0	
	DC7Y2	11	0	
	DC11C1	6	0	
	DC11C2	4	0	
	DC11Y1	3	0	
	DC11Y2	0	0	
11/21/2006	N2C1	11	0	All plates excluding DC11 appear to be developing well. DC11 worms appear smaller and unhealthy even on control plates.
	N2C2	8	0	
	N2Y1	9	0	
	N2Y2	7	0	
	DC3C1	12	0	
	DC3C2	11	0	
	DC3Y1	6	0	
	DC3Y2	3	0	
	DC7C1	16	0	
	DC7C2	10	0	
	DC7Y1	9	0	
	DC7Y2	10	0	
	DC11C1	7	0	
	DC11C2	4	0	
	DC11Y1	3	0	
	DC11Y2	0	0	
11/22/2006	N2C1	8	0	All plates are reaching L4 stage. DC11 still appears unhealthy. Some worms appear to have beginnings of tail bumps on experimental plates.
	N2C2	7	0	
	N2Y1	11	0	
	N2Y2	5	0	
	DC3C1	10	0	
	DC3C2	13	0	
	DC3Y1	5	0	
	DC3Y2	4	0	
	DC7C1	16	0	
	DC7C2	11	0	
	DC7Y1	9	0	
	DC7Y2	8	0	
	DC11C1	6	0	

	DC11C2	4	0	
	DC11Y1	3	0	
	DC11Y2	0	0	
11/23/2006	N2C1	8	0	Plates have all reached adulthood, some still in L4 stage. Experimental plates appear smaller than control plates. Wild type and DC7 experimental worms appear very sluggish
	N2C2	7	0	
	N2Y1	11	7	
	N2Y2	5	2	
	DC3C1	10	0	
	DC3C2	13	0	
	DC3Y1	5	1	
	DC3Y2	4	1	
	DC7C1	11	0	
	DC7C2	11	0	
	DC7Y1	9	4	
	DC7Y2	8	2	
	DC11C1	6	0	
	DC11C2	4	0	
	DC11Y1	3	1	
	DC11Y2	0	0	

Date	Plate	Number live worms	Tail Bumps	Notes
12/3/2006	DC1C	48	0	Number of eggs plated was underestimated. All eggs appear to have hatched. Some eggs may have been hidden in carcasses. DC10 plates were discarded due to mold contamination.
	DC1Y1	64	0	
	DC1Y2	70	0	
	DC8C	40	0	
	DC8Y1	93	0	
	DC8Y2	69	0	
	N2C	50	0	
	N2Y	120	0	
12/4/2006	DC1C	50	0	Worms appear to be developing normally. Control plates are already starved
	DC1Y1	66	0	
	DC1Y2	75	0	
	DC8C	40	0	
	DC8Y1	92	0	
	DC8Y2	69	0	
	N2C	86	0	
	N2Y	121	0	
12/5/2006	DC1C	55	0	Control plates are arrested due to starvation. Yeast plates appear to be developing normally
	DC1Y1	66	0	
	DC1Y2	77	0	
	DC8C	40	0	
	DC8Y1	93	0	
	DC8Y2	69	0	
	N2C	86	0	
	N2Y	120	0	
12/6/2006	DC1C	54	0	Control plates are completely starved and sluggish. Yeast plates, even with tail bumps appear healthier. Controls may need a higher percentage of E. coli
	DC1Y1	66	38	
	DC1Y2	77	51	
	DC8C	40	0	
	DC8Y1	92	56	
	DC8Y2	69	46	
	N2C	86	0	
	N2Y	120	89	

Date	Plate	Number live worms	Tail Bumps	Notes
	N2 C	20	0	
	N2 Y	18	0	
	N2 Y	32	0	
	DC6 C	5	0	
	DC6 Y	1	0	
	DC3 C	15	0	
	DC3 Y	20	0	
	DC3 Y	18	0	
	DC10 C	6	0	The count was done early in the day, not all eggs from the egg prep appear to have hatched. Those that have are growing normally. DC6 may have had problems during egg prep.
	DC10 Y	20	0	
	DC10 Y	9	0	
	Srf 6 C	16	0	
	Srf 6 Y	10	0	
1/21/2007	Srf 6 Y	8	0	
	N2 C	26	0	
	N2 Y	25	0	
	N2 Y	40	0	
	DC6 C	10	0	
	DC6 Y	2	0	
	DC3 C	15	0	
	DC3 Y	22	0	
	DC3 Y	20	0	
	DC10 C	9	0	
	DC10 Y	25	0	
	DC10 Y	9	0	
	Srf 6 C	16	0	Worms appear to be growing normally between the L2 and L4 stages.
	Srf 6 Y	11	0	
1/22/2007	Srf 6 Y	11	0	
	N2 C	47	0	
	N2 Y	25	0	
	N2 Y	46	0	
	DC6 C	10	0	
	DC6 Y	3	0	
	DC3 C	15	0	
	DC3 Y	22	0	Worms seem to be growing normally most are in L4 or adult stage but have not developed tail or vulva bumps. On the N2, Srf 6, and DC10 experimental plates the worms appear thinner then worms on control plates
	DC3 Y	20	0	
	DC10 C	9	0	
	DC10 Y	25	0	
	DC10 Y	9	0	
	Srf 6 C	21	0	
	Srf 6 Y	11	0	
1/23/2007	Srf 6 Y	16	0	
1/24/2007	N2 C	47	0	Worms on yeast plates appear thinner in all strains. Three DC 10 worms with tail bumps also appeared to
	N2 Y	25	mold	
	N2 Y	46	27	
	DC6 C	10	0	

	DC6 Y	3	2	have vulva bumps. Nine Srf 6 worms had Vulva bumps but only five appeared to have both vulva and tail bumps. One of the N2 experimental plates developed mold by the last day and was thrown out.
	DC3 C	15	0	
	DC3 Y	24	10	
	DC3 Y	20	13	
	DC10 C	12	0	
	DC10 Y	27	16	
	DC10 Y	7	3	
	Srf 6 C	21	0	
	Srf 6 Y	12	2	
	Srf 6 Y	16	5	

Date	Plate	Number live worms	Tail Bumps	Notes
	N2 Y	15	7	DC4 *bus-4* appeared more sluggish then the wild type. They also appeared to be developing slower then other strains. All experimental plates appeared sluggish when compared to their controls.
	N2 C	47	0	
	DC4 C	30	0	
	DC4 Y	19	0	
	DC4 Y2	21	0	
	DC7 C	Mold		
	DC7 Y	5	3	
	DC7 Y	20	11	
	DC11C	Mold		
	DC11Y	9	5	
	DC11Y	12	8	
	DC1C	54	0	
	DC1 Y1	10	4	
	DC1 Y2	5	4	
	DC8C	40	0	
2/6/2007	DC8 Y	2	1	

MAP Kinase Mutants Plated on Yeasr			
C. Elegan Strain	Total Worms	Number of Tail Bumps	Number of Dead Worms
Mek-1	48	0	19
Mek-2	40	0	2
Nsy-1	11	0	0
Jnk-1	88	8	0