Genetic Analysis of Innate Immunity in *Caenorhabditis elegans*

by

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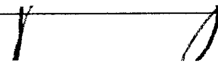
Signature of Author: 
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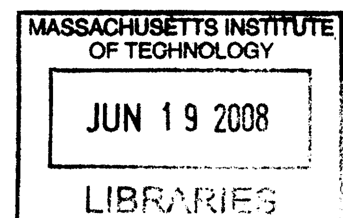

ARCHIVES

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Submitted to the Department of Biology on June 25, 2007 in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

ABSTRACT

Pathogen resistance in *Caenorhabditis elegans* is a model for studying innate immunity. Several conserved activators in the p38 PMK-1 pathway have been identified and are required for pathogen resistance to the bacterium *Pseudomonas aeroginosa*. Thus far, screens have been performed to identify mutants that are susceptible to pathogen infection. Here, I will discuss a *mek-1* suppressor screen that was performed to look for more components of the p38 PMK-1 pathway. Aided by the *agIs219* reporter, mutants with high levels of GFP expression were isolated and screened for suppression of the Esp phenotype of *mek-1*. Several independently isolated strong suppressor mutants were isolated and separated into possible complementation groups using pooled lysate SNP mapping. Interested to see if the mutants would also affect other stress response pathways in the worm, I tested the mutants for heavy metal sensitivity. Five out of the six strong Esp suppressor mutants did not suppress the heavy metal sensitivity of *mek-1*, suggesting that our mutants are more specifically affecting the pathogen resistance pathway and not the heavy metal stress response pathway. Future work will involve measuring PMK-1 phosphorylation levels using Western blots and fine mapping the mutants.

Thesis Supervisor: Dennis Kim
Title: Assistant Professor of Biology

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Table of Contents

Abstract 2

Acknowledgements 3

Table of Contents 4

CHAPTER 1: Introduction 5

References 10

CHAPTER 2: Materials and Methods 11

CHAPTER 3: The *mek-1* suppressor screen 13

CHAPTER 4: Figures 18

Figure 1: The relative phenotypes of Esp mutants 19
Figure 2: Heavy Metal Stress Response Assay 20
Figure 3: *vhp-1(km20)* suppresses the Esp phenotype of *mek-1(ks54)*. 21
Figure 4: *vhp-1(km20)* suppresses the low levels of GFP expression of *mek-1(ks54)* 22
Figure 5: *mek-1* Suppressor Screen Overview 23
Figure 6: GFP Fluorescence of strong *mek-1* suppressors 24
Figure 7: Esp phenotypes of *mek-1* suppressors 25
Figure 8: Relative fluorescence and Esp phenotype of *mek-1* suppressors 26
Figure 9: *vhp-1* complementation 27
Figure 10: SNP mapping strategy 28
Figure 11: SNP Mapping Results 29
Figure 12: *qd70* suppresses heavy metal sensitivity of *mek-1(ks54)* 30

CHAPTER 5: Future Work 31

CHAPTER 1

INTRODUCTION:

Innate Immunity, Signaling Pathways, and *C. elegans*

Innate Immunity and Model Organisms

For survival, it is essential that organisms are able to recognize microbial infection and defend against it. In vertebrates, there are two distinct immune responses, known as innate and adaptive, that defend against infection. In higher vertebrates, the adaptive immune response acts to target specific pathogens, using many specialized cells and antibodies. The innate immune response is thought to be made up of skin barriers, anti-microbial peptides, and macrophages that recognize invasion and work to slow infection using a general response. When the innate immune response is compromised as in patients with leukemia under chemotherapy, the result can be increased susceptibility to invasion by organisms which are quite common and normally do not harm humans. In studying the genetics of innate immunity, we hope to identify targets for therapies for patients with weak immune responses.

Model organisms are useful to study complex processes that would be difficult or unethical to study in humans. Invertebrates lack the adaptive immune response; their only defense against pathogen infection is their innate immune system. In recent years, scientists have begun to use invertebrate model systems to identify conserved components important for innate immunity. It was found that there is a great deal of conservation between the *Drosophila* and the mammalian innate immune systems (Hoffman, 2002, Jasper, 2002, Kimbrell, 2001). In *Drosophila* and mammals, Toll signaling and Toll-like receptors (TLRs) act to signal the innate immune response. (Akira, 2006, Kimbrell, 2001, Lemaitre, 1996)

Recently, scientists began to investigate pathogen resistance in *C. elegans* as a model for innate immunity. *Caenorhabditis elegans* is a hermaphroditic nematode that is normally found in the

soil. The advantages of using these organisms in genetic studies include a fast generation time and simple growth conditions. Under normal laboratory conditions, worms are kept on agar medium in Petri dishes and fed *Escherichia coli* OP50. An adult hermaphrodite can lay from 250 to 300 eggs and their development is completed in 2-3 days. The *C. elegans* lifespan is a little under 3 weeks when kept at 25 °C. The quick generation time enables scientists to perform large scale forward genetic screens in a short time. To study *C. elegans* microbial infection, a "slow-killing" assay was developed where live *Pseudomonas aeruginosa* is fed to the nematode and the numbers of worms alive and dead are recorded at different time points. Worms that are susceptible to infection die earlier than wildtype worms and are described as having an Esp phenotype (enhanced susceptibility to pathogen). (Tan, 1999)

Using this assay to find mutants susceptible to pathogen, it was discovered that *C. elegans* has a conserved p38 MAPK pathway that functions in response to infection. The MAP kinase kinase *sek-1* and its MAP kinase kinase kinase *nsy-1* mutants were identified in a forward genetic screen looking for mutants with an Esp phenotype. When inactivated, *pmk-1* also has an Esp phenotype. Other work has identified TIR-1 as a player in *C. elegans* innate immunity (Libertai, 2004). Unlike *Drosophila* and mammals, characterization of *tol-1*, the only known TLR in *C. elegans*, has shown that *tol-1* mutants do not have a Esp phenotype in the *Pseudomonas* slow killing assay. However, it has been suggested that *tol-1* does have a role in pathogen avoidance (Pujol, 2001). Further work is needed to identify other components of the *pmk-1* innate immune pathway.

Another MAPK kinase kinase, *mek-1,* is necessary for complete activation of PMK-1 in *C. elegans* immunity. MEK-1 was initially identified to be involved in the heavy metal stress response when the mutant *mek-1(ks54)* was identified as unable to survive on heavy metal enriched media (Koga, 2000). The *mek-1(ks54)* mutant was found to have a moderate Esp phenotype and affects levels of PMK-1 phosphorylation *in-vivo*, suggesting MEK-1 is important for full activation of PMK-1 (Figure 1) (Kim, 2004).

Regulation of the p38 MAPK pathway

Regulation of the p38 MAPK pathway is of interest in our quest to better understand pathogen resistance. Many serine threonine phosphatases, tyrosine phosphatases, and dual-specificity MAPK phosphatases (MKPs) have been considered to be involved in the negative regulation of MAPK signaling pathways. However, there have been few studies examining the negative regulation of p38 and JNK MAPK *in vivo*. One study in *Drosophila* identified a MKP, *puckered*, that has a role in regulating JNK activity in dorsal closure (Martin-Blanco, 1998). So far, only one phosphatase, VHP-1, was identified as a negative regulator of p38 in *C. elegans.* Dennis Kim took a candidate approach and, using RNAi, examined all the known dual-specificity phosphatases in the *C. elegans* genome. The Esp phenotype of *mek-1* can be suppressed by feeding RNAi of *vhp-1*, a phosphatase that negatively regulates PMK-1 activity as well as KGB-1 activity in the heavy metal response pathway. The other members of the MKP phosphatase family were tested with feeding RNAi and found not to suppress *mek-1 (*Kim, 2004).

My project's goal was to identify novel p38 pathway components by performing a forward genetic screen for mutants that suppress the Esp phenotype of *mek-1*. Here, I describe my results so far and suggest future experiments to continue this study.

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CHAPTER 2

MATERIALS AND METHODS

Worm Cultures:

Worms are kept at 20°C on nematode growth agar seeded with *E. coli* OP50 bacteria.

Heavy Metal Assay:

In the heavy metal stress response assay, three to four young adult worms were placed on each of the 100 micromolar $CuSO_4$ plates seeded with OP50. Eggs were laid overnight and the adult worms were removed. The eggs were counted and after 3 days, the number of adults was counted. The percentage of eggs that grew up was calculated and presented in Figure 2.

Slow Killing Assay:

Pseudomonas aeroginosa PA14 was grown in LB media overnight and six microliters were dropped onto small agar plates. The plates were placed at 30 degrees overnight and then placed at room temperature for a day. Thirty L4 *C. elegans* were placed on the plates and the number alive and dead were recorded at different intervals over the next few days. Worms which left the plates are not counted in the total.

agIs219 Reporter:

The GFP reporter used in this study is the promoter of T24B8.5 fused to GFP. T24B8.5 is a gene differentially regulated by PMK-1 and was identified from microarray data (Troemel, 2006). It is thought to be a short secreted cysteine-rich peptide; it may be produced by the worm in its defense against pathogen. The *agIs219* strain contains an integrated construct containing this promoter fused to GFP; the integration event took place on chromosome III.

CHAPTER 3

RESULTS:

Isolation and Preliminary Characterization of *mek-1* Suppressor Mutants

A *mek-1* Suppressor Screen

Initial Steps and Rationale

Up until now, screens done in our lab have focused on finding mutants that are more susceptible to pathogen. To look for more components of the PMK-1 pathway that would be difficult to identify by looking for susceptible mutants, we chose to do a suppressor screen. Past attempts at a *sek-1* suppressor screen were unsuccessful and turned up no candidates, suggesting that *sek-1* may be necessary for any PMK-1 activation (Dennis Kim, communication). Because *mek-1* has an intermediate Esp phenotype, we chose to use it as an optimized background to do a suppressor screen. The only known *mek-1* suppressor was VHP-1 and all previous data was obtained using VHP-1 RNAi. Kirthi Reddy and I obtained the *mek-1; vhp-1* mutant from the Matsumoto lab and tested its phenotype to check for confirmation of the RNAi phenotype (Figure 3). We also show that the fluorescence of the *agIs219* reporter correlates with suppression of the Esp phenotype (Figure 4). This difference in fluorescence was apparent by eye and could be, with moderate to high success, sorted using the Worm Sorter. This correlation suggested that we might be able to find other components of the p38 pathway by initially identifying suppressor mutants with higher *agIs219* GFP expression and later testing them to identify those mutants that live longer than *mek-1* in our slow killing assay. Therefore, we chose to use a *mek-1(ks54); agIs219* background for our screen. We looked for mutants that are "bright", fertile, and are resistant to pathogen infection

Screen

We performed a forward genetic screen of about 20,000 genomes. We separated the P0's into 20 pools, egglayed for 4 hours, and eggprepped the F1's to obtain synchronized F2's. We selected

411 bright F2 worms, both by eye under the fluorescence scope and also by using the Horvitz lab's worm sorter (Figure 5). Out of those 411 F2 worms, 288 were fertile. Out of the 288 fertile bright F2 worms, we obtained 24 bright F3 populations, where all of the progeny were bright. These 24 bright isolates came from 12 different pools, suggesting that we have at least 12 independent isolates. Out of these 24 bright strains, 22 of them showed moderate to strong suppression of the Esp phenotype of *mek-1.* (Figures 6, 7) The other fertile populations out of the 288 that did not give bright progeny may have been mistakenly picked by the worm sorter, or may be generally sick.

We ranked the mutants in relation to their fluorescence and their suppression phenotypes and there is some correlation. (Figure 8) The strong mutants tended to have higher agIs219 GFP expression. We decided to focus on further characterizing our mutants, focusing on the strongest suppressors.

Phosphatase RNAi Screen

It was possible that there were other phosphatases that would suppress *mek-1.* Alongside our forward genetics screen, we investigated whether there were any other phosphatases in *C. elegans* that suppress the GFP expression and Esp phenotypes of *mek-1.* We screened all 166 phosphatases available in the Ahringer library with the help of Jessica Chiang and Claire Richardson. We found that 15 were brighter than *mek-1;* however, when these were tested for an Esp phenotype, only *vhp-1* RNAi suppressed Esp phenotype of *mek-1.* This suggests that it is most likely that there are few, if any, phosphatases in *C. elegans* that act on the p38 pathway as VHP-1 does. The 14 other phosphatases which caused higher GFP expression may cause the

worms to be sick, which does cause the worms to be "brighter", or they may cause changes in general transcription of transgenes.

VHP-1 Complementation

To further characterize and identify the mutants, we complemented a few of them to *mek-1; vhp-1*. We showed that *qd68, qd69, qd71, and qd73* complement *vhp-1*. (Figure 9) Male mating within our strains had a very low success rate and was quite difficult to perform, so we decided the best use of time would be to work on mapping our mutants and characterizing their metal sensitivity.

SNP Mapping

To map the mutants, we constructed a modified strain of CB4856 that has *mek-1(ks54); agIs219* in the background and began to perform SNP mapping of the suppressor mutants. We used the strategy outlined in Figure 10. We found that *qd69, qd73,* and mutant *K17.1* (allele number unassigned as of 6/8/07) all map to the same region on the left arm of chromosome I. To identify the gene in that region affected, we screened the Ahringer and Vidal Unique feeding RNAi libraries in this region and no RNAi caused a suppression of *mek-1*'s fluorescence and *esp* phenotype. Our mutant *qd70* maps to the same region on chromosome II as *vhp-1*, suggesting that *qd70* may be an allele of *vhp-1*. (Figure 11)

We are continuing to finely map *qd69, qd73,* and K17.1 using SNP mapping to find the gene on chromosome I. We will sequence *vhp-1* in *qd70; mek-1(ks54); agIs219.*

Heavy Metal Stress Assay

It was possible that the mutants we isolated would affect other stress response pathways, as well as the pathogen resistance pathway. MEK-1 and VHP-1 are involved in the pathogen resistance as well as the heavy metal stress response pathway. To further characterize the mutants, we performed heavy metal stress assays on all of the mutants as outlined in Figure 2. We found that one allele, *qd70*, suppressed *mek-1*'s sensitivity to heavy metal stress in similar ways as *mek-1; vhp-1*. All other mutants showed very little or no suppression of the heavy metal stress sensitivity, suggesting that all of the mutants, except possibly *qd70,* are most likely not alleles of *vhp-1* and they may regulate the p38 innate immune response pathway and not the heavy metal stress response pathway (Figure 12).

CHAPTER 4

FIGURES

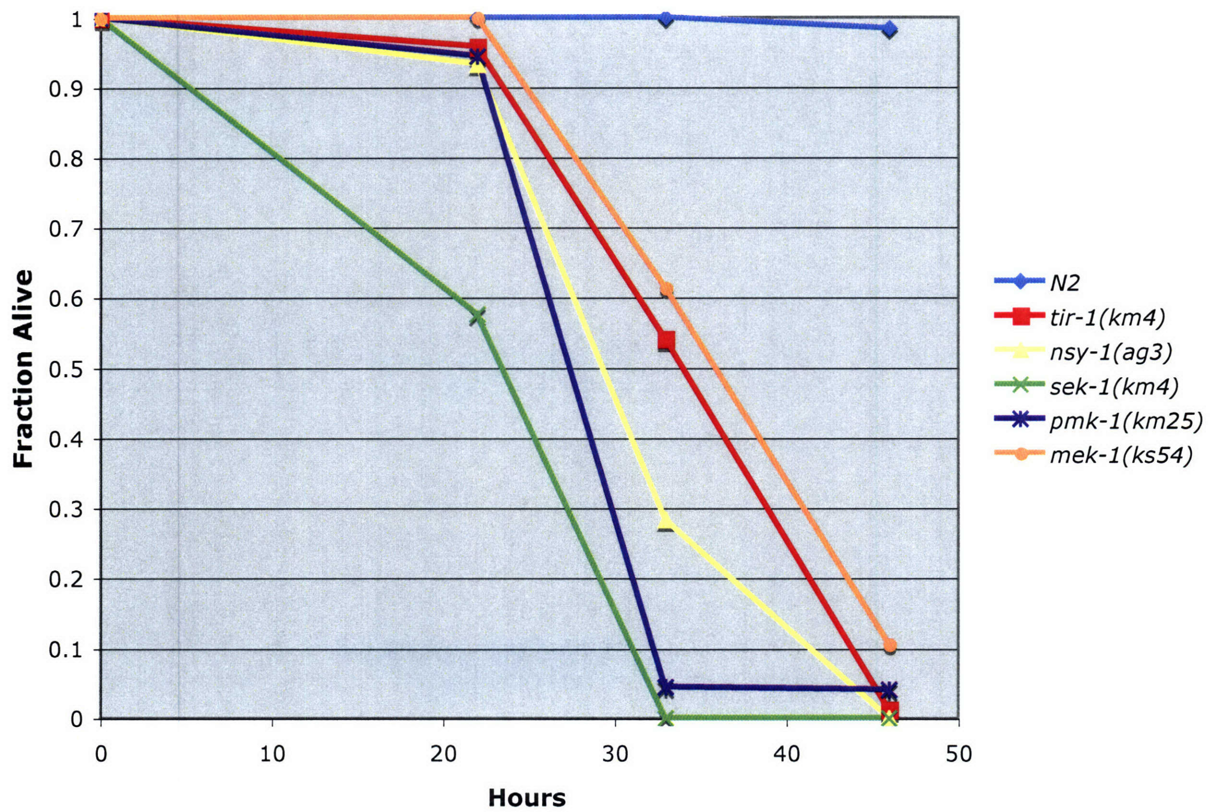


Figure 1: The relative phenotypes of mutants known to affect PMK-1 activity in pathogen resistance

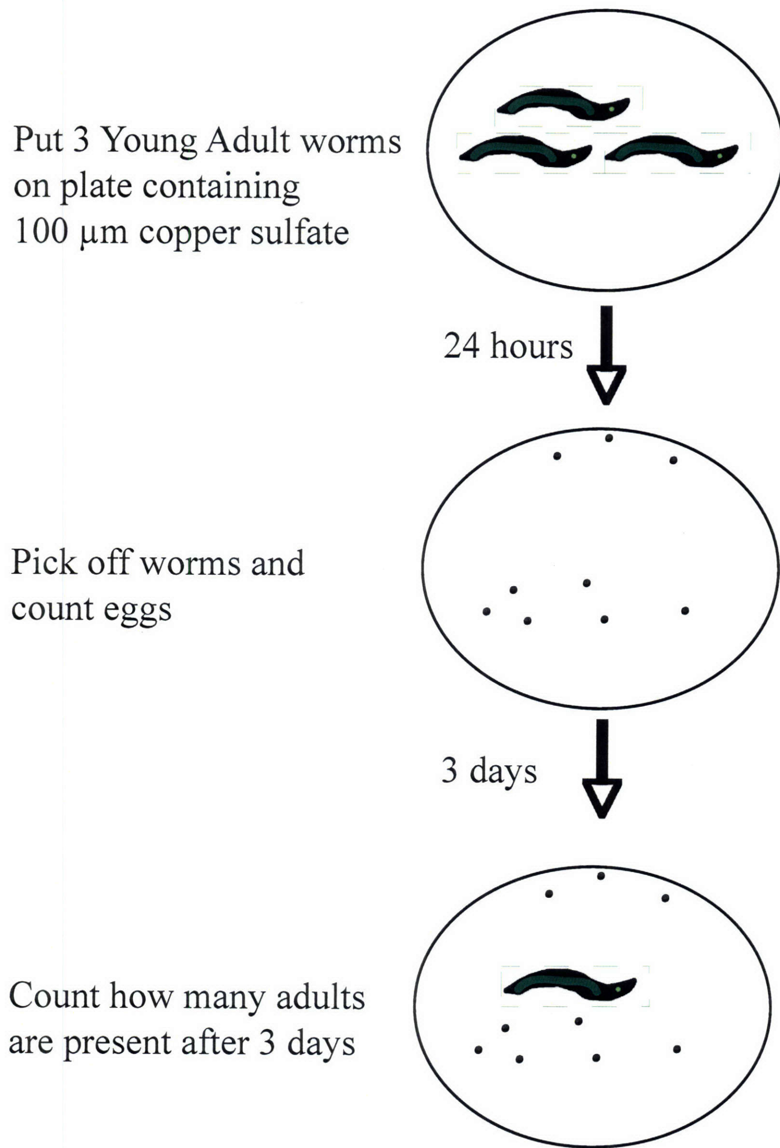


Figure 2: Heavy Metal Stress Response Assay

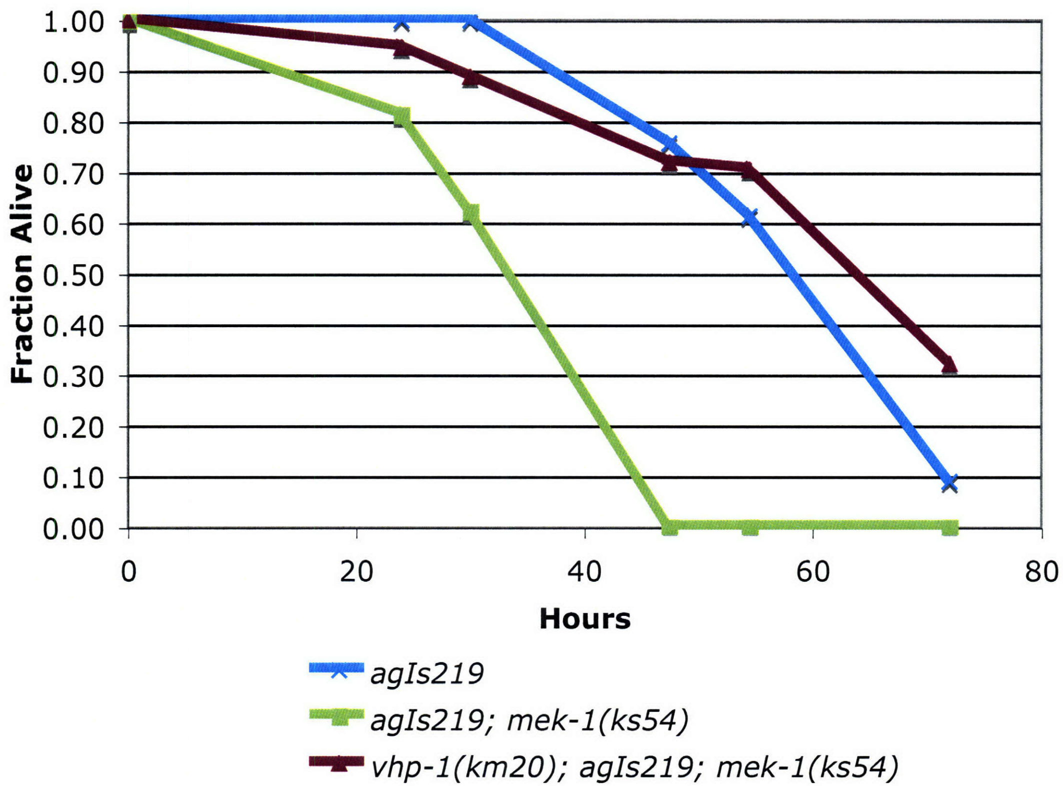


Figure 3: *vhp-1(km20)* suppresses the Esp phenotype of *mek-1(ks54)*.

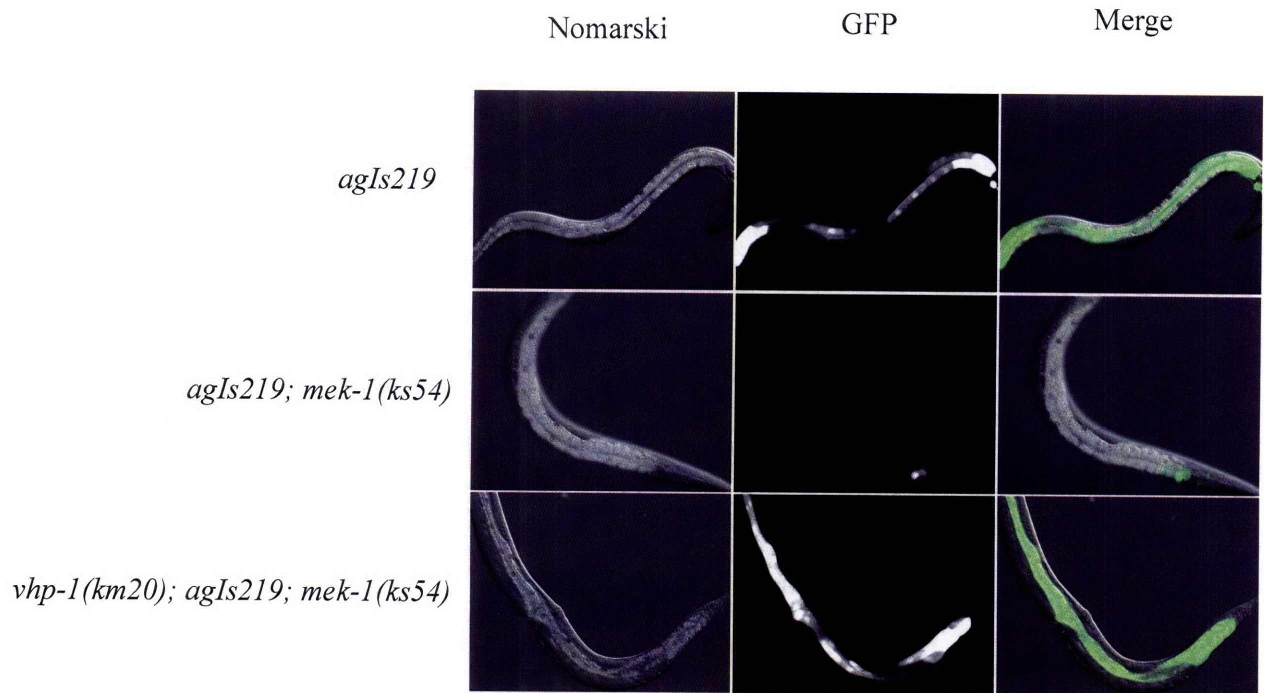


Figure 4: *vhp-1(km20)* suppresses the low levels of GFP expression of *mek-1(ks54)* .

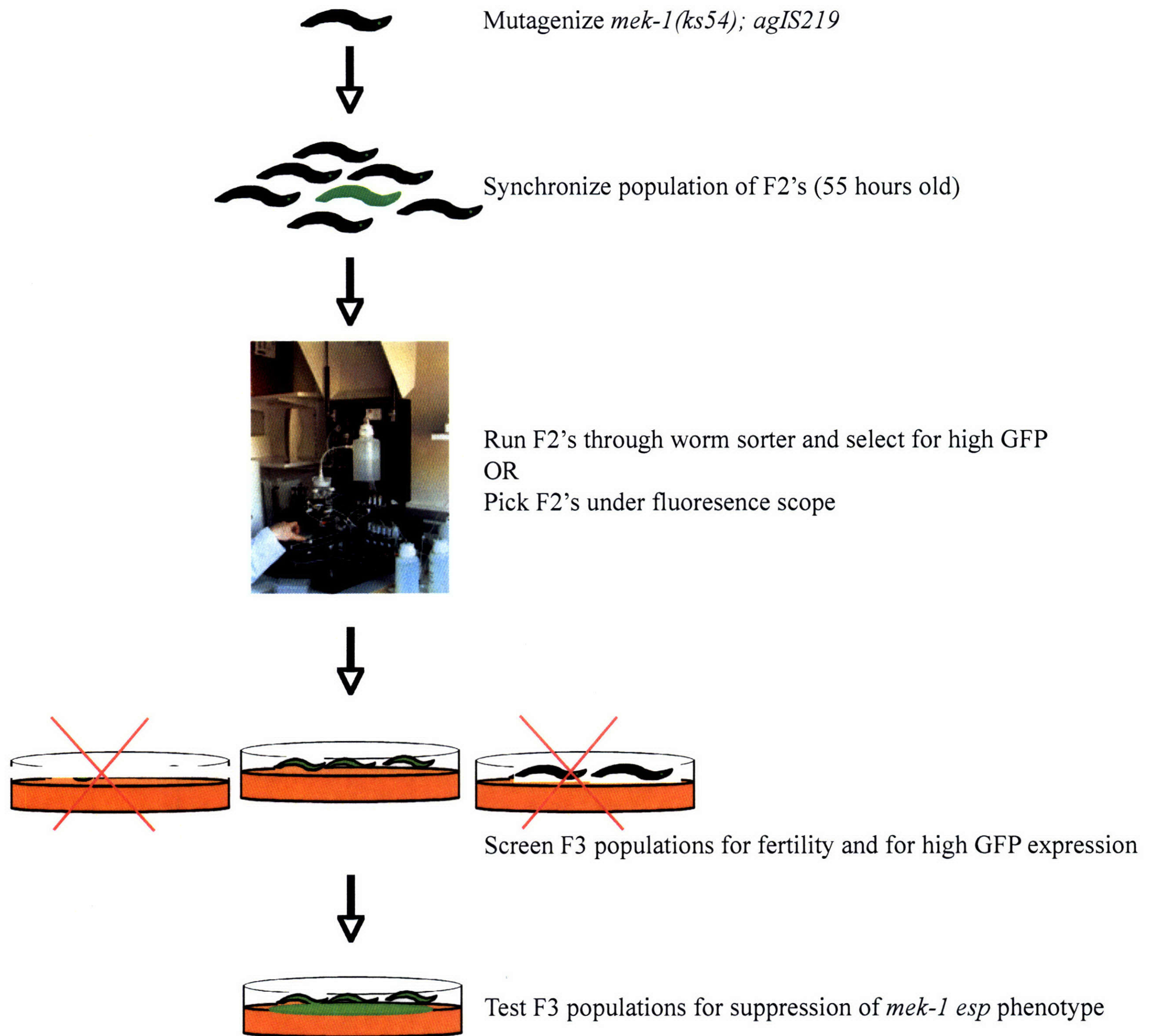


Figure 5: *mek-1* Suppressor Screen Overview

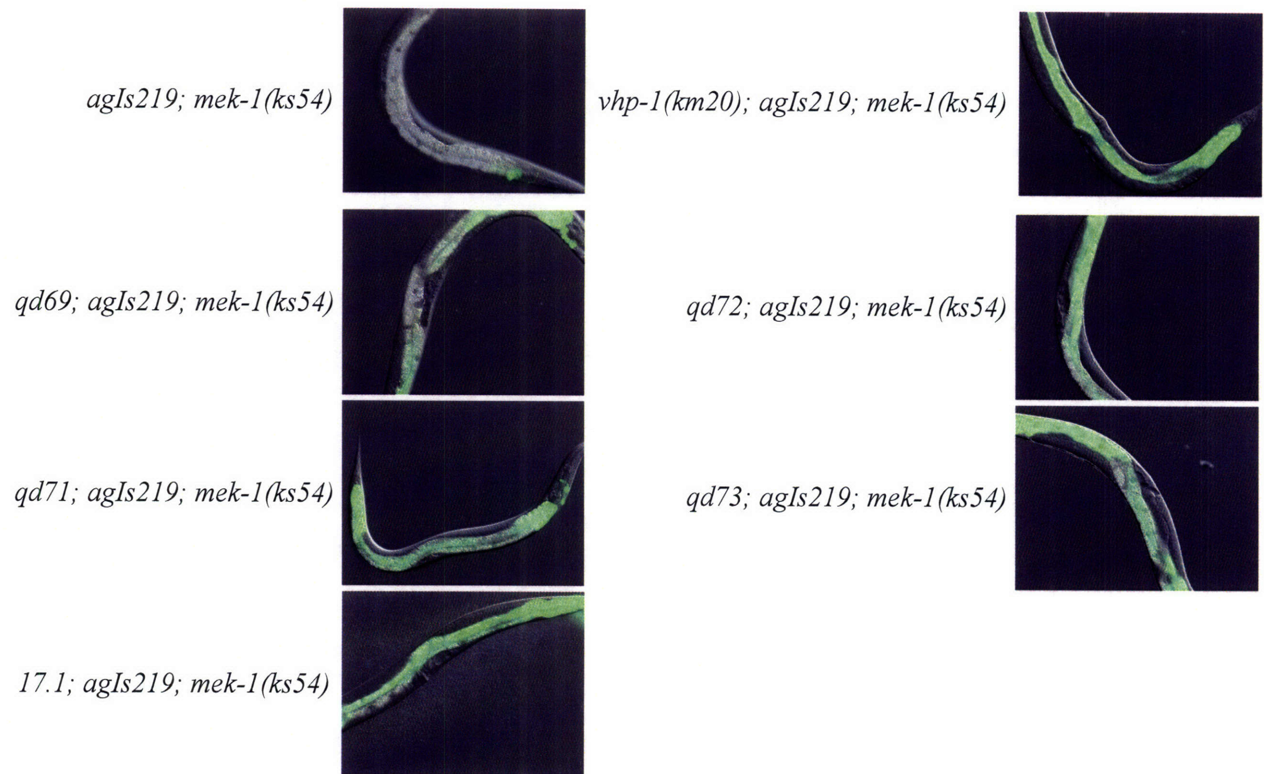
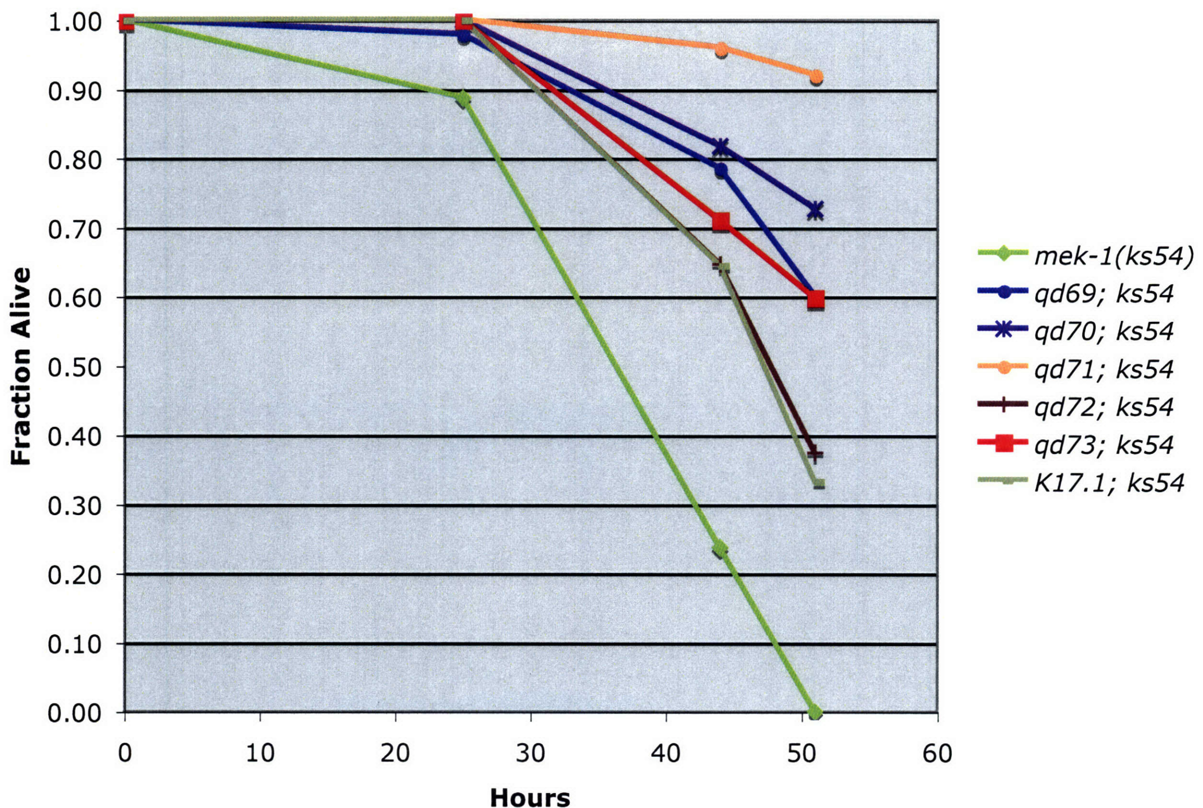


Figure 6: GFP Fluorescence of strong *mek-1* suppressors



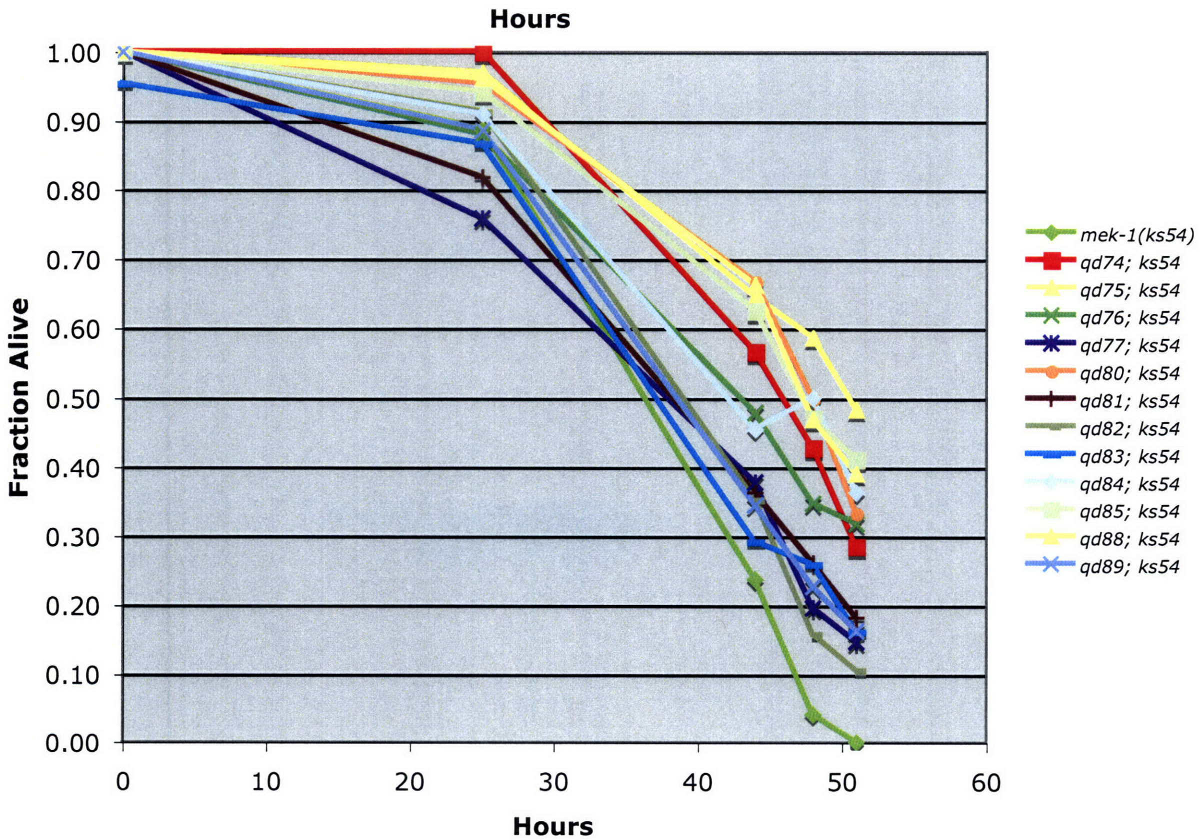


Figure 7: Strong (top) and moderate (bottom) suppressors of the Esp phenotype of *mek-1(ks54)*

Strong suppression	Moderate suppression	Little suppression
qd69 ++	*qd68* ++	*qd86* ++
qd70 ++	*qd74* ++	*qd87* ++
qd71 +++	*qd75* +	
qd72 ++	*qd76* +	
qd73 ++	*qd77* +	
17.1 ++	*qd78* +++	
	qd79 +	
*all 6 are from independent pools	*qd81* ++	
	qd82 +	
	qd83 +++	
	qd84 +	
	qd85 ++	
	qd88 +	
	qd89 +	
	2.6 +	
	21.8 +	

Figure 8: Relative fluorescence and Esp phenotype of mek-1 suppressors.

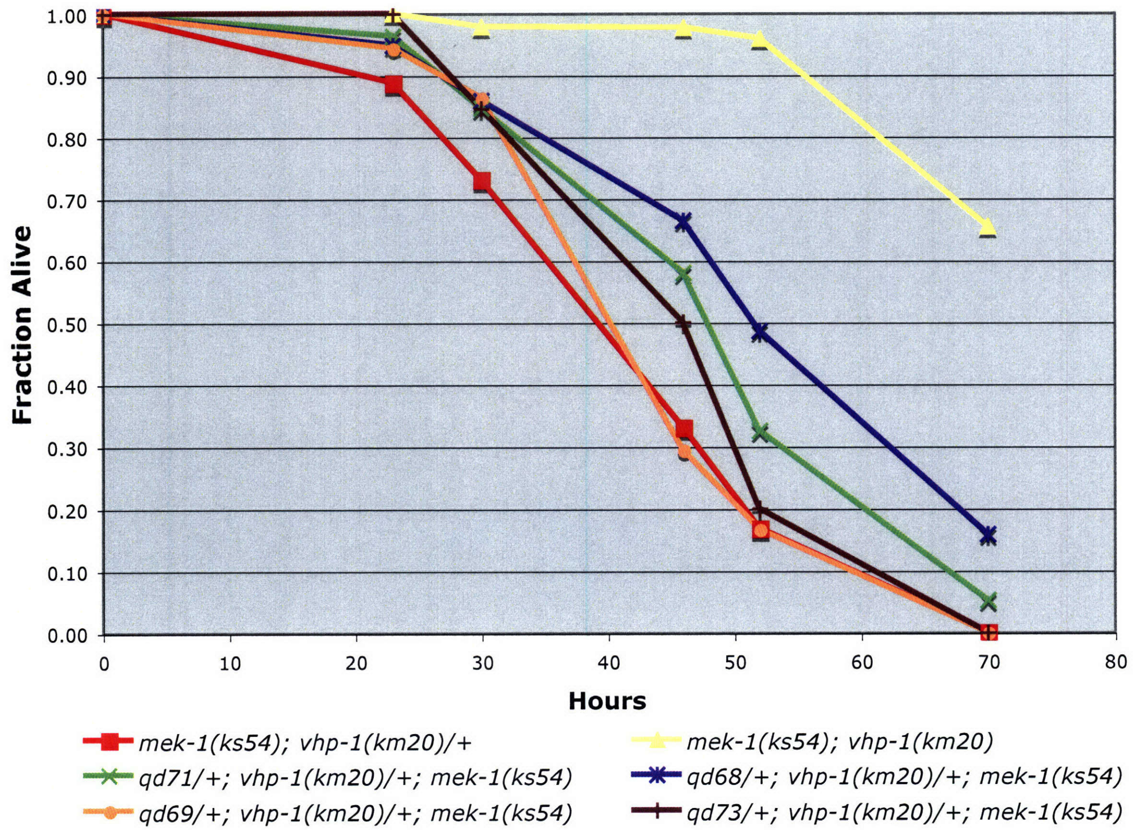


Figure 9: *vhp-1* complementation with selected strong suppressors

qd70; agIs219; mek-1(ks54) x *CB4856; agIs219; mek-1(ks54)*

F1 *qd70* / + *agIs219* / *agIs219* *mek-1(ks54)* / *mek-1(ks54)*

single F2 worms

qd70 / *qd70* *agIs219* / *agIs219* *mek-1(ks54)* / *mek-1(ks54)*

\+ / + *agIs219* / *agIs219* *mek-1(ks54)* / *mek-1(ks54)*

Pick "bright"
F3 populations

Pick "dark"
F3 populations

SNP PCR on pooled lysate

SNP PCR on pooled lysate

Figure 10: SNP mapping strategy

Suppressor	Maps to
qd70; mek-1(ks54)	Near -2 on II
qd69; mek-1(ks54)	Near -19 on I
qd73; mek-1(ks54)	Near -19 on I
17.1; mek-1(ks54)	Near -19 on I
qd72; mek-1(ks54)	NA

Figure 11: SNP Mapping Results

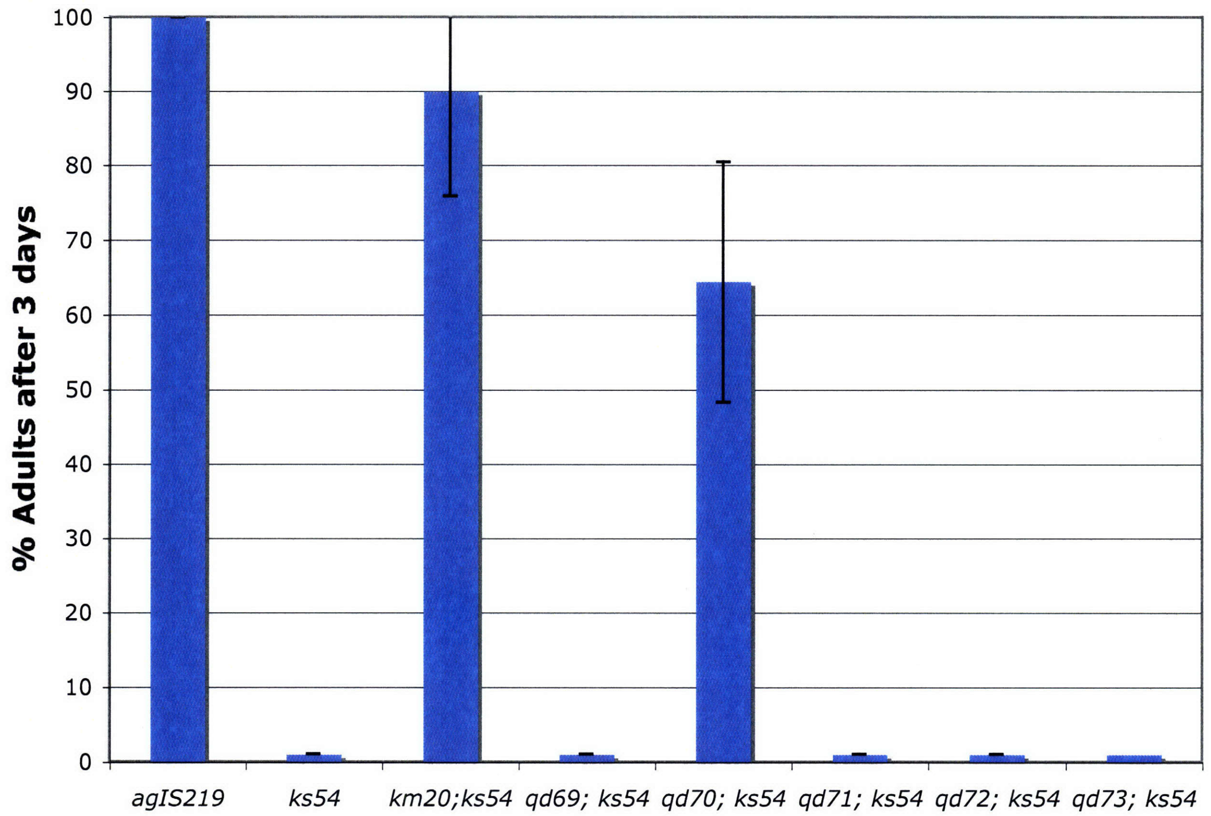


Figure 12: *qd70* suppresses heavy metal sensitivity of *mek-1(ks54)* like *vhp-1(km20)*

CHAPTER 5

FUTURE WORK

Our results so far suggest that there are important regulators of the PMK-1 pathway that can be identified using the suppressor screen approach. As many of the genes so far identified in this pathway are highly conserved between *C. elegans* and mammals, identifying which genes are negative regulators of the pathogen resistance pathway might help scientists to identify potential drug targets to improve innate immune function in animals and in humans.

This project will be continued by other members of the Kim lab. Cloning new signaling components that negatively regulate innate immunity is important in our understanding of what components of an organism's environment affects its survival. Organisms we study in the lab are living in ideal conditions. Learning about their responses to stress and resistance to infection will teach scientists new ways to design experiments that can take more variables into account. As new components are discovered that exist in both *C. elegans* and in mammals, scientists will better understand functional conservation of genes across species.