Genetic Studies on the Quick Avoidance Behavior to *Pseudomonas aeruginosa* in *Caenorhabditis elegans*

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Abstract

In most animals, avoiding pathogenic bacteria is crucial for better health and a long-life span. For this purpose, animals should be able to quickly sense the presence or uptake of pathogens. The intestine could be a candidate organ to induce escape behaviors, however, the intestinal signaling mechanism for acute regulation of neuronal activity leading to escape behaviors is not well understood. The intestinal microbiome is also known to regulate several neuronal functions for behavioral strategies and affect for neuronal disorders. Although many studies have revealed the involvement of the intestinal microbiome in chronic neuronal functions, the exact molecular and physiological mechanisms for acute neuronal regulation by intestinal bacteria are not clear yet.

To understand how intestinal bacteria affect animal behavioral changes, I used soil Nematode *Caenorhabditis elegans* as a model organism. Compared to other experimental animals such as mouse or fly, the bacterial species in the intestine of *C. elegans* can be easily controlled. Previous studies indicate that *C. elegans* shows avoidance behavior to several pathogenic bacteria such as *Pseudomonas aeruginosa* which is a common Gram-negative pathogenic bacterium and is known to affect health in many animals including the human. This avoidance should be important to select a better living environment. However, known avoidance to *P. aeruginosa* is not so quick; worms on the *P. aeruginosa* (PA14) lawn starts to avoid PA14 after 8 hours, or in some cases, after 16 hours they fed PA14. Avoidance behavior to PA14 is also known to be regulated by an associated learning with odors (secondary metabolites from the bacterium) and unidentified signals in the

body, probably stimuli in the intestine. In these backgrounds, I was curious about that how worms change their behavior avoidance to *P. aeruginosa*, and why it takes such long periods to induce this avoidance behavior. Alternatively, is it possible for worms to recognize PA14 as a pathogen in a shorter period and to induce an avoidance behavior before 8 hours' exposure? To answer these questions, I examined how quickly worms can respond to PA14 and what kind of molecules regulate this quick recognition and behavior.

To make clear the behavioral response to *P. aeruginosa* in the wild-type *C. elegans*, I first examined several experimental conditions in the *P. aeruginosa* avoidance assay and found that in one condition, adult worms showed an avoidance behavior to P. aeruginosa before 8 hours' exposure. In this condition, adult *C. elegans* responded to *P. aeruginosa* within 30 min of exposure, and avoidance of PA14 was gradually increased during exposure and reached a maximum at 6 hours of exposure. This behavior was quite fast compared to previously reported avoidance behaviors to *P. aeruginosa*. This behavior was named as "quick avoidance to *P. aeruginosa*". Exposure to *P. aeruginosa* induces upregulation of many genes. However, I found that the quick avoidance behavior is probably independent on both transcription and translation because of a transcriptional inhibitor Actinomycin D did not alter the behavior.

Secondary, I wondered whether quick avoidance behavior is regulated by genetic components or not. To examine what kind of molecules are involved in the quick avoidance response, I performed a genetic screen to isolate mutants with defective quick avoidance responses. Three candidate mutants were isolated, and I focused on the *ta218* mutant allele in this study. In the 30-min PA14 assay, 90 %

wild-type animals showed avoidance to PA14, but only 10 % animals showed quick avoidance behavior in the *ta218* mutant. The number of progeny in the *ta218* mutant animals was less than that of wild-type, but gross morphological and behavioral changes were not observed. The mutant animals showed normal locomotion on both *E. coli* OP50 and PA14, and their food preference and odor preference are also normal, suggesting the mutant worms can move normally to their preferable bacteria. However, the *ta218* mutant animals showed weak defect in learning behaviors, suggesting a role on neuronal functions. By SNP mapping and genome sequencing, I found a missense mutation on the C10C5.2 gene. Introducing wild-type genomic DNA covering the whole C10C5.2 region partially retained quick avoidance to PA14 in this mutant, suggest that the C10C5.2 is the responsible gene of the *ta218* allele. By cloning the gene, I found that the C10C5.2 encodes a novel protein with a F-box domain. So, I named the C10C5.2 gene *fbxc-58* (F-box C protein). The FBXC-58 protein consists of 332 amino acids, and one F-box domain is located at its N-terminus. No other known domains are found in this protein. Furthermore, no homologues are found in other organisms except for *Caenorhabditis* species. The F-box domain is known to mediate protein-protein interactions in various cellular signaling pathways such as ubiquitin protein degradation. The *fbxc-58* promoter activity was observed in glial cells and an identified neuronal cell in head region, body-wall muscles, intestinal cells, intestinal valve muscle cells, and vulval muscle cells. I generated a translational *fbxc-58*:: GFP fusion gene and expressed in the *ta218* mutant animals. This fusion protein could rescue slow avoidance to PA14 in the mutant, but GFP signal was not observed in any tissues. To know which tissues are required for quick

avoidance behavior to PA14, I expressed the FBXC-58 protein in tissue-specific manners and found that only the intestine-specific expression of FBXC-58 could rescue the avoidance defect in the mutant animals. The rescue ability by the intestinal FBXC-58 was partial, suggesting that simultaneous expression of FBXC-58 in several tissues may be required for normal avoidance response to *P. aeruginosa*.

Thirdly, I found that some but not all mutants in the p38-MAPK and insulin signaling pathways, which function in the immune response to pathogens in the intestine, were defective in the quick avoidance behavior to *P. aeruginosa*. This finding suggests that a novel signaling pathway constituted from identified proteins exists to regulate acute neuronal activity for a quick behavioral response.

In conclusion, I found that adult *C. elegans* show quite quick avoidance behavior when they meet with pathogenic bacteria *P. aeruginosa*. *C. elegans* which fed *P. aeruginosa* may detect some risk factors such as toxin or secondary metabolites from *P. aeruginosa* in the intestine, and the activity of nervous system may be altered based on the information from the intestine. Interestingly, though the p38-MAPK and insulin signaling pathways are well conserved between *C. elegans* and mammals, canonical signaling pathways may not regulate this behavior but a novel signaling mechanism by some components may be involved in quick avoidance. The findings in this study will contribute to elucidate an regulatory mechanism of the nervous system from the intestine in other organisms including our human.

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Chapter 1

General Introduction

1.1 The intestinal regulation of the nervous system.

The intestine plays important roles in the regulation of feeding behavior in animals. The intestine is known to secret several hormones in response to nutrients they get, and these intestinal signals are sent to the brain or other organs via bloodstream, vagal neurons and spinal sensory neurons (Figure 1) [1, 2]. For example, the linoleic acid infusion in the jejunum increases norepinephrine in the paraventricular hypothalamic nucleus, and this infusion also increases glucagon-like peptide 1 (GLP1), peptide YY and cholecystokinin in the rat's blood dose-dependently [3]. Moreover, sub-diaphragmatic vagotomy or midbrain transection prevents a change in food preference and total calorie intake by linoleic acid infusion [3].

In addition, a large number of recent studies have reported that the intestinal microbiome can affect neuronal development and maintenance (Figure 2) and is related to several neuronal disorders such as autism spectrum disorders [4], Parkinson's diseases [5] and Alzheimer's diseases [6]. As for neuronal development, for example, germ-free (GF) mice demonstrate several abnormalities such as an impaired neurogenesis of the hippocampus [7], a lower number of neuronal cell bodies per ganglion, and an increased number of nitrergic neurons [8]. Furthermore, these GF mice exhibit an increased locomotor activity

in the open-field activity box and a decreased anxiety behavior in the elevated plus maze test [9]. In addition to GF mice analyses, recent studies identified several short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate, which are produced from non-digestible carbohydrates in intestinal bacteria fermentation, as ligands on G-protein coupled receptors, free fatty-acid receptor 2 (FFAR2, previously called as GPR43) and FFAR3 (previously called as GPR41) (Figure 3). The both receptors are known to be expressed in the enteroendocrine cells by immunostaining [10, 11]. FFAR2 activates both the $G_{\alpha i}$ and $G_{\alpha q}$ pathways but FFAR3 activates only the $G_{\alpha i}$ pathway. When transfected in CHO-K1 cells these receptors, activate the mitogen-activated protein kinase ERK1/2 by propionate or acetate [12]. In *Ffar2* knockout mice, SCFA-dependent GLP1 secretion, which activates the insulin release in pancreatic β cells, is impaired [13]. Thus, the chronic mechanism of nervous system regulation has been studied intensively. However, acute regulatory mechanisms from the intestine to nervous system are not well understood yet. Because of complex and diverse intestinal environments, identifying specific ligand-receptor interactions which are involved in the signal transduction from the intestine to the nervous system is quite difficult even in widely-used experimental animals such as mouse and fly. However, *Caenorhabditis elegans* can be controlled its bacterial species in the intestine by arranging the rearing condition (Figure 4). In addition, the intestinal epithelium in *C. elegans* is comprised of just 20 fully differentiated cells, which is quite simple structure and easy to handle compared to that of mammals [14]. Therefore, I thought that I could understand the molecular and physiological mechanisms of nervous system regulation from the intestine by using *C. elegans.*

1.2 *C. elegans* as a model organism to study the nervous system regulation by the intestine.

C. elegans is known to show avoidance behaviors to several pathogenic bacteria such as *Pseudomonas aeruginosa, Serratia marcescens* and *Bacillus thuringiensis* (Figure 5) [15, 16]. In particular, avoidance behavior to *P. aeruginosa* has been studied actively. *P. aeruginosa* is a ubiquitous Gram-negative bacterium that is known to affect the health of many animals including *C. elegans* [17-19]. A clear avoidance behavior to *P. aeruginosa* is known as a result of an associated learning with odors (secondary metabolites from the bacterium) and unidentified signals in the body, probably stimuli in the intestine. (Figure 6) [20-23]. In these cases, *C. elegans* on a *P. aeruginosa* lawn started to avoid it after 8 hours [24], or in some cases, worms showed avoidance after 16 hours as they fed *P. aeruginosa* (Figure 7) [25]. I was curious about that how worms change their behavior avoidance to *P. aeruginosa*, and why it takes such long periods to induce this avoidance behavior. Alternatively, is it possible for worms to recognize *P. aeruginosa* as a pathogen in a shorter period than observed and to induce an avoidance behavior before 8 hours (Figure 6 and 7)? To answer these questions, I examined how quickly worms can respond to *P. aeruginosa* and what kind of molecules regulate this quick recognition and behavior.

1.3 Goals

This thesis represents the newly identified genetic components which are probably involved in the regulation of nervous system by the intestine of *C. elegans*.

The goal of this study is to:

1. Examine how quickly worms can avoid *P. aeruginosa* and build a novel assay method to observe quick avoidance.

2. Isolate mutants defective in avoidance behavior to *P. aeruginosa*.

3. Identify mutants defective in avoidance behavior to *P. aeruginosa* in existing worm mutant libraries.

For these goals, in Chapter 2, I tried to optimize the *P. aeruginosa* avoidance assay in order to investigate the period when significant avoidance can be observed. In Chapter 3, I explored the genes involved in quick avoidance behavior, which act in the detection of pathogenic bacteria in the intestine and/or signal transduction in the nervous system regulation by the intestine.

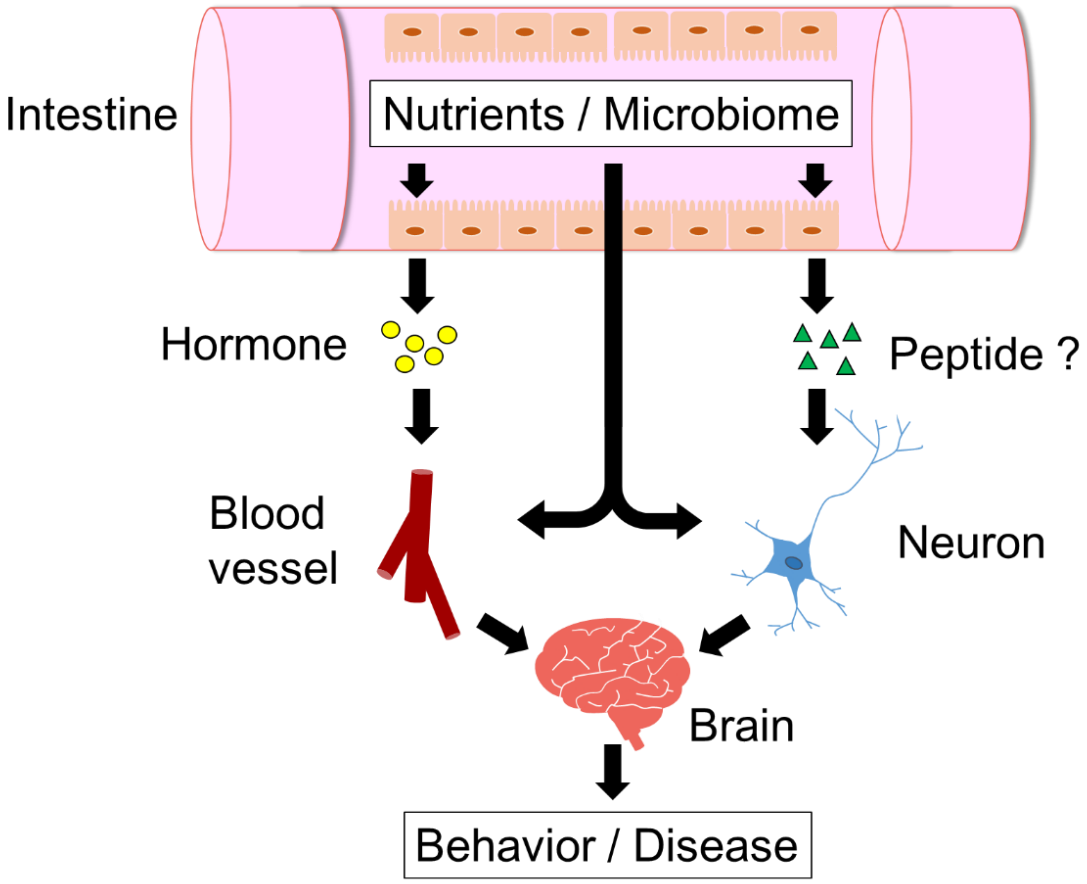

Figure 1: Signal transduction from the intestine to brain.

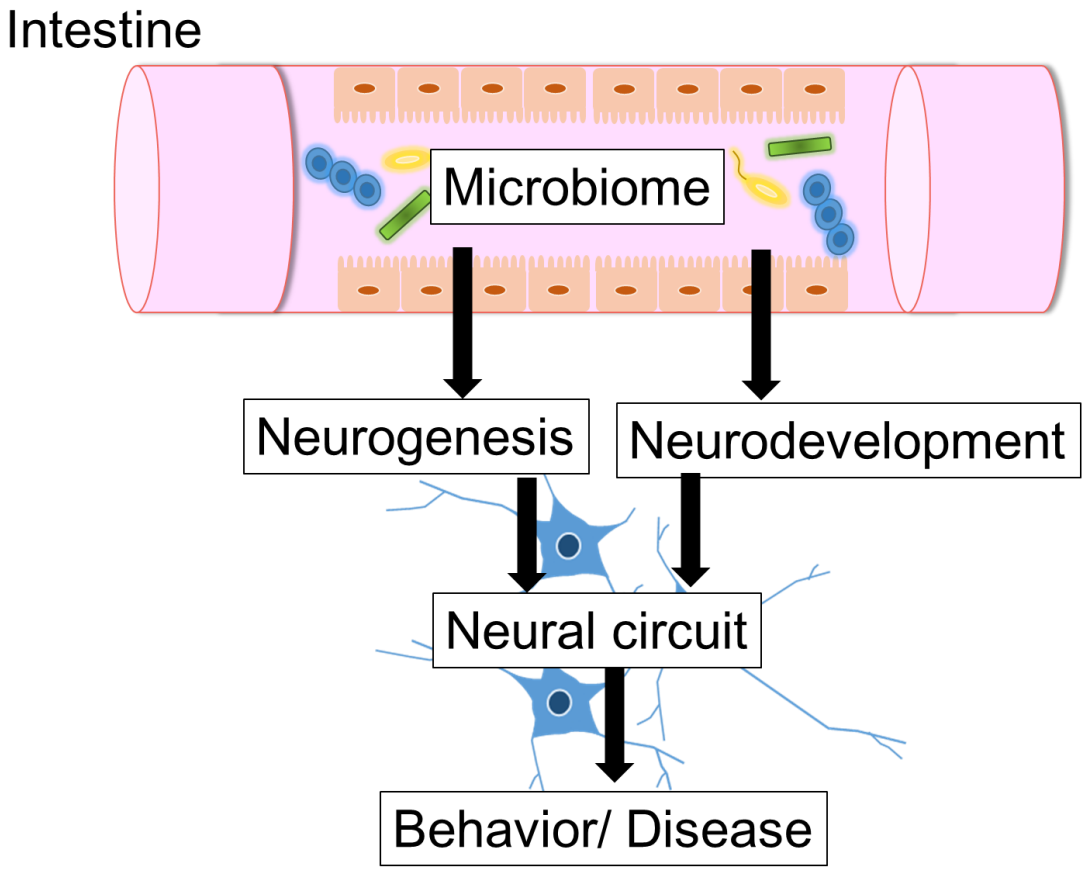

Figure 2: Effects on the neuronal events from intestinal microbiome.

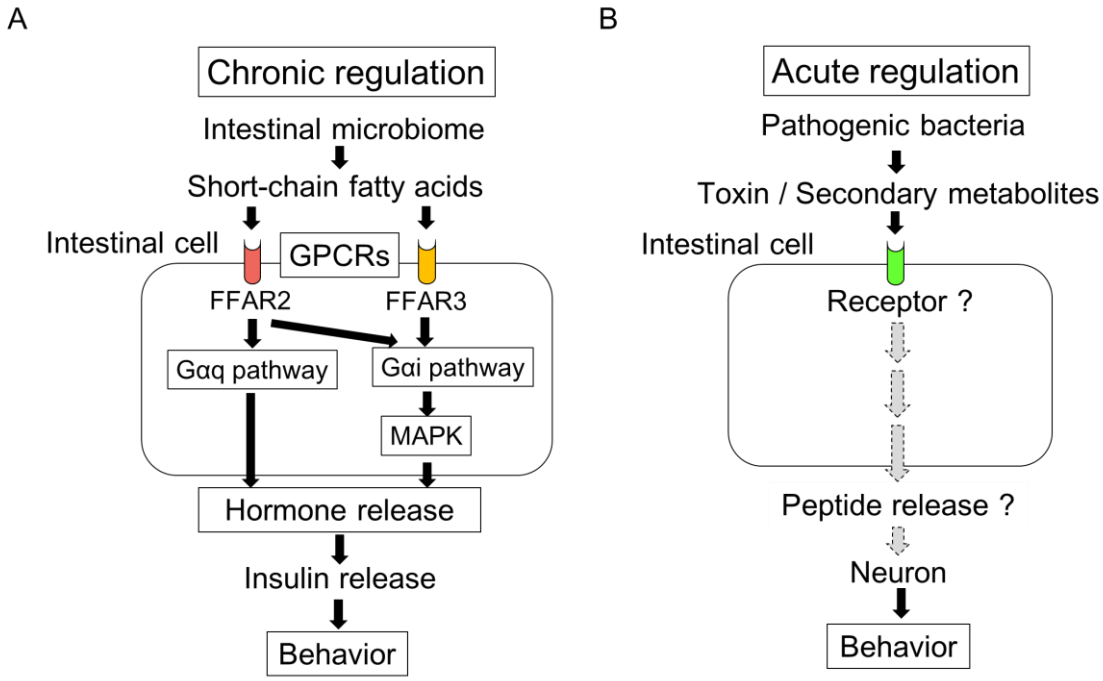

Figure 3: Molecular signaling in intestinal cells for second metabolites from microbiomes. Schematic illustration of chronic (A) and acute (B) regulatory mechanisms by intestinal microbiome.

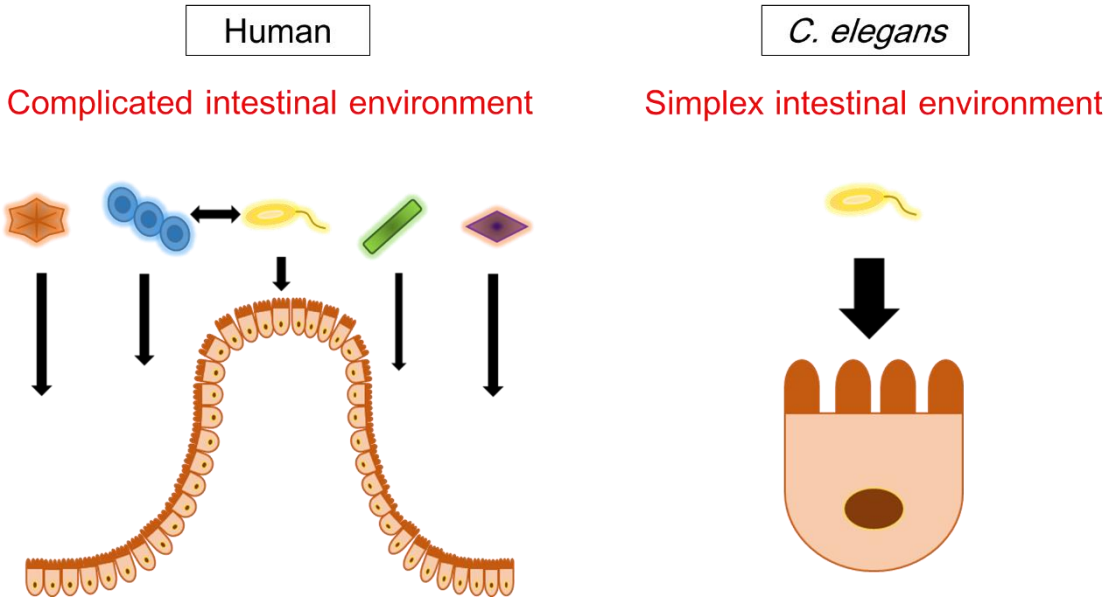

Figure 4: Comparison of intestinal environment between human and *C. elegans*.

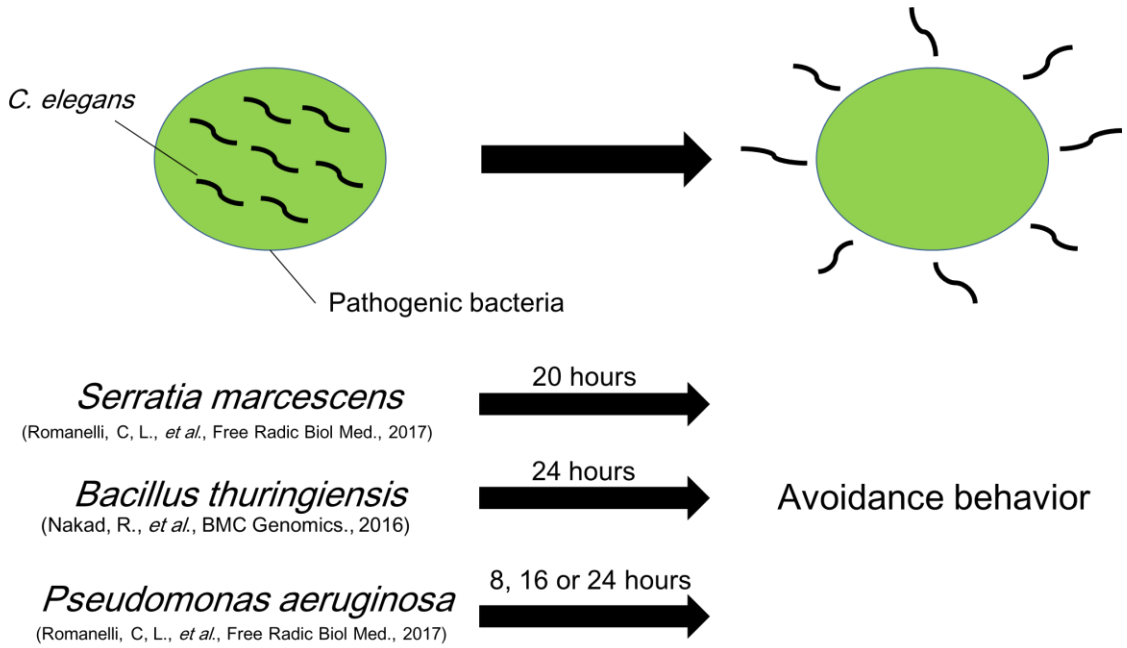

Figure 5: Avoidance behaviors to several pathogenic bacteria in *C. elegans*.

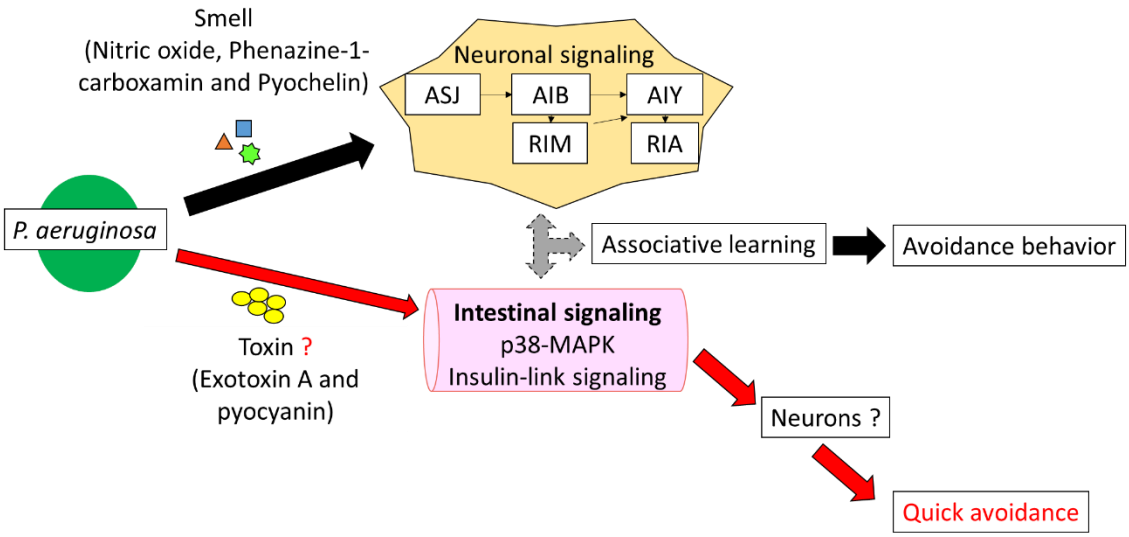

Figure 6: Predicted cellular mechanisms for associative learning and quick avoidance behaviors to *P. aeruginosa* in *C. elegans*.

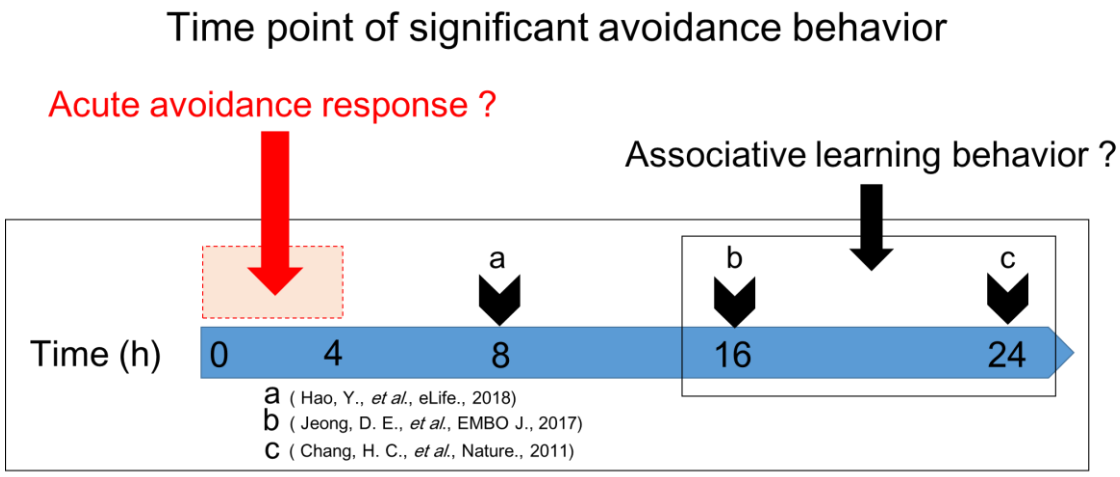

Figure 7: *C. elegans* timescale in response to *P. aeruginosa.*

Chapter 2

Construction of quick avoidance assay to *P. aeruginosa*

2.1 Introduction

To examine how animal behavioral changes are regulated by the intestinal bacteria, I used *Caenorhabditis elegans* as the host and *Pseudomonas aeruginosa* as a model pathogenic bacterium. *C. elegans*, a bacteria-feeding nematode, lives in the soil and is a convenient model organism because of its short life cycle and ease of genetic manipulation. *P. aeruginosa* is a ubiquitous Gram-negative bacterium that is also found in soil. *P. aeruginosa* has been reported to affect the health of many eukaryotic animals including *C. elegans* [17-19]. For example, *P. aeruginosa* kills worms over a period of 2–3 days on low salt medium plate, via an infection-like process that correlates with the accumulation of bacteria in the intestine [26, 27]. In contrast, on high salt and rich medium plates, *P. aeruginosa* can kill worms within 24 hours, in some cases within 4 hours of infection. This immediate effect of *P. aeruginosa* on worm health is caused by the production of a number of diffusible toxins [27, 28]. Thus, worms have to change their behavioral strategy to live longer when exposed to *P. aeruginosa*.

C. elegans is initially attracted to *P. aeruginosa* PA14, but, after a few hours, shows avoidance of *P. aeruginosa*. In some cases, worms on a PA14 lawn started

to avoid it after 8 hours [24], and in other cases, worms showed avoidance after 16 hours as they fed PA14 [25]. However, slow avoidance of *P. aeruginosa* does not increase worm fitness when the animals feed on PA14 on high salt rich medium plates. Prior to this study it was not known whether worms can show a behavioral response within 4 hours of exposure to *P. aeruginosa* in order to avoid the adverse effects of this pathogen.

2.2 Material and methods

2.2.1 *C. elegans* strain

C. elegans strain used in this chapter was N_2 Bristol. *C. elegans* was maintained at 15 or 20 ^{o}C on standard NGM agar plates seeded with *Escherichia coli* OP50.

2.2.2 *P. aeruginosa* PA14 avoidance assay

The *P. aeruginosa* avoidance assay in this study was modified from a previously reported method [29]. The bacterial strains used in this study were *E. coli* OP50 and *P. aeruginosa* PA14. Bacteria were grown overnight in three-ml of Luria-Bertani (LB) broth with shaking at 37^{o}C. Cultures were adjusted to an optical density (OD_{600}) of 1.0 with LB medium, and a 50-ul drop of medium was spotted onto a 60-mm NGM plate. Plates were incubated for 24 h at 25 ^{o}C. For the synchronization of assay animals, ten adult animals were transferred to fresh NGM plates and were removed after 6 h. Worms were grown to the young adult stage. Young adult animals were collected in M9 buffer and kept in M9 buffer for 30 min,

then washed three times with M9. Approximately 100 young adult animals were transferred to the center of the bacterial lawn. The numbers of animals present on the seeded and unseeded areas of the plates were counted at the indicated times. Percent avoidance was calculated using the following formula:

% avoidance = # animals in unseeded area/(# in seeded area + # in unseeded area) ×100.

2.2.3 Actinomycin D treatment

Young adult animals, which were grown on the OP50 culture plates, were collected and washed by M9. Washed animals were treated by Actinomycin D (0, 25, 50, 100, 200 μM) for 30 min. After treatment, animals were transferred to the center of *P. aeruginosa* PA14 avoidance assay plate. The numbers of animals on seeded and unseeded area of the plate were counted after 30 min.

2.2.4 Heat-shock experiment

The taEx224 [*Phsp-16.2*::GFP, *Podr-1*::DsRed and *lin-15*(+)] transgenic animals were mounted with M9 solution containing NaN3 (10mM) onto slide-glasses with a 2 % agarose pad. These slide-glasses were incubated at 30 ºC for 0, 0.5 and 5 hours.

2.2.5 Plasmid construction

To generate the plasmid pDK750 (*Phsp16.2*::GFP), the *hsp16.2* promoter region in the pPD49.78 plasmid DNA (a kind gift from Andy Fire) was inserted between BamHI and HindIII site of the pPD95.77 plasmid DNA.

2.2.6 Transgenic animals

Transgenic worms were generated using standard microinjection methods for *C. elegans*. The pbLH98 [*lin-15*(+)] plasmid and pDK300 (*Podr-1*::DsRed) were used as a co-injection marker at 45 ng/µl. The pDK750 plasmid was injected at 10 ng/µl for heat-shock experiment.

2.2.7 Microscopy

Animals in M9 solution containing NaN_3 (10mM) were mounted onto slides with a 2 % agarose pad. The slides were observed with an epifluorescence microscope (OLYMPUS BX53) with 20×/0.75 objective lens, equipped with a CCD camera in a 16-bit format.

2.2.8 Statistical analysis

Statistical analyses were performed using the GraphPad Prism7 software. The statistical significance was analyzed by *t*-test or Mann-Whitney's *U* test, and statistically significant differences were defined as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.3 Results

Exposure of *C. elegans* to pathogenic bacteria is known to cause severe health problems and death after a certain period of infection. To understand when and how worms quickly respond to the pathogenic bacterium *P. aeruginosa*, I examined the avoidance behavior of *C. elegans* to *P. aeruginosa* PA14 (Figure 8).

I investigated a cultivation condition of PA14 on assay plates (Figure 9 and 10). On 50 µl PA14 lawn, worms showed the strongest avoidance behavior to PA14 compared to 100 or 200 µl PA14 lawn in 3 hours (Figure 9). In addition, on the 25 ^{o}C cultured assay plates in which the 1.0 OD PA14 was spread, worms show the clearest avoidance behavior compared to other cultivation condition in 30 min (Figure 10). Here I found the best condition of the assay plate which enable us to observe a quick avoidance behavior to PA14 compared to previously reports, and these data suggests that avoidance behavior is affected by lawn size and cultivation condition of PA14. Furthermore, I carefully observed worm's behavior on PA14 lawns, and I found that adult *C. elegans* tend to show a strong avoidance behavior to PA14 within 30 min of exposure (Figure 7B and 11). Worms do not show any avoidance of *E. coli* OP50 at 30 min (only 0.14 % worms were present in the unseeded area of OP50 lawns). However, a significant number of worms (39.5 %) showed an avoidance behavior in response to PA14 (Figure 11). Previous reports using L4 larval animals showed that significant avoidance of PA14 was observed a few hours after PA14 exposure. My data suggest that adult animals can quickly avoid PA14. Avoidance of PA14 was gradually increased during exposure and reached a maximum approximately 6 hours after exposure (Figure 11). These results suggest that *C. elegans* can respond to PA14 and start to avoid it in less than

30 min. I call this behavior quick avoidance of *P. aeruginosa*.

I wondered whether quick avoidance behavior is regulated by the transcription and translation of certain genes. Therefore, I performed avoidance assay at 30 min using a transcriptional inhibitor Actinomycin D (Figure 12). In the treated worms, quick avoidance behavior was not significantly different compared to the non-treated worms (Figure 12). Furthermore, using transgenic animals expressing a *Phsp-16.2*:: GFP reporter, I examined whether the translation is activated in 30 min. The *hsp-16.2* promoter is known to be activated by temperature rises. In our experiment, GFP fluorescence was significantly increased after 5 hours at 37 ^{o}C (Figure 13A). However, by the heat-shock for 30 min, transgenic animals did not show the significant increase of GFP fluorescence (Figure 13). There data suggest that the regulation of quick avoidance behavior may not depend on both transcription and translation of certain genes.

2.4 Discussion

In this study, I found that *C. elegans* starts to avoid the pathogenic bacterium *P. aeruginosa* within 30 min of exposure. This avoidance seems to be a quite quick response compared to previous reports concerning on avoidance to PA14, which those were clearly observed from 8 to 16 hours after exposure [24, 25]. This data suggests that worms have two avoidance behavioral strategies to live longer, and may select a suitable strategy dependent on their to circumstances.

I first wondered whether this quick avoidance behavior is regulated by transcription and translation. My data show that quick avoidance behavior was not

affected by Actinomycin D treatment, and GFP fluorescence was not increased for 30 min incubation at 37 ºC. In addition, genes expression levels are known to change after 4 hours of exposure to *P. aeruginosa* [30]. As for time periods in protein modifications such as treatment with tetanus toxin, phosphorylation levels of Trk, Akt and ERK 1/2 are increased more than 20 fold at 5 min in rat brain [31]. My data and previously reports suggest that quick avoidance behavior may be regulated through short-term processes such as protein phosphorylation and degradation, but not regulated through long-term processes such as transcription and translation.

The most intriguing result in this study was the rapid (< 30-min) response to PA14 by adult worms, in contrast to the > 8-hour response time of L4 worms. The avoidance assay protocol was not largely altered from that in previous reports, suggesting that the difference is probably due to the life stage of the worms. Adult worms tend to show a high chemotaxis index to the food-associated odor diacetyl compared to larval worms, and this high chemotaxis index in adults depends on germline growth [32]. In addition, larval worms show an olfactory response to diacetyl only in the AWA sensory neuron, whereas adult worms show the response in other AWB, ASK and AWC sensory neurons in addition to AWA [33]. Thus, my results suggest that the sensitivity to pathogenic bacteria is also higher in adults than larva, although it is not clear whether this altered sensitivity is caused by neuronal cells or intestinal cells. This age-dependent change of sensitivity may be required to select a better environment that is suitable for species conservation.

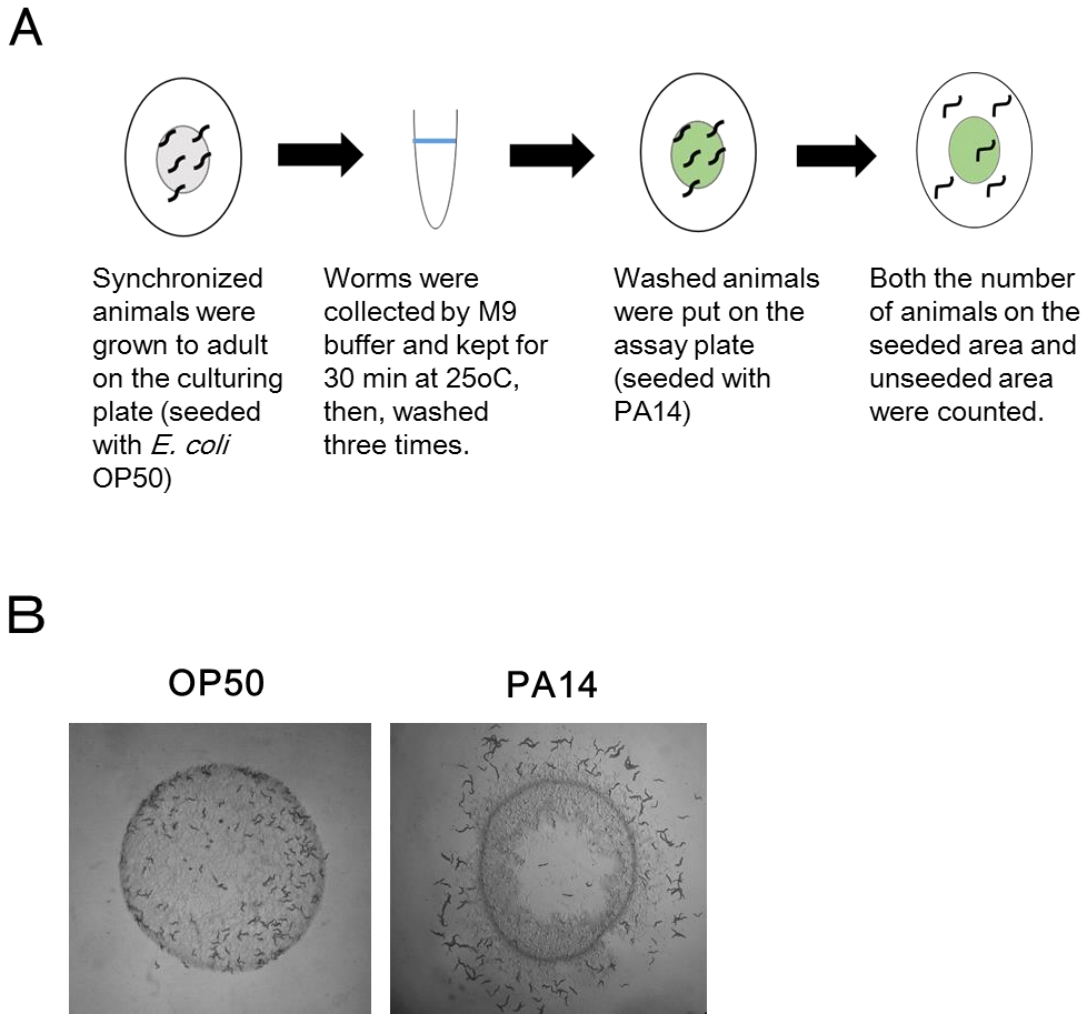

Figure 8: Avoidance behavior in response to *P. aeruginosa*. (A) Schematic illustration of our avoidance response assay. The percentage of avoidance was calculated as the number of animals on unseeded area dividing by the total number of animals on the plate. (B) Responses of wild-type animals after 30-min exposure to *E. coli* OP50 or *P. aeruginosa* PA14.

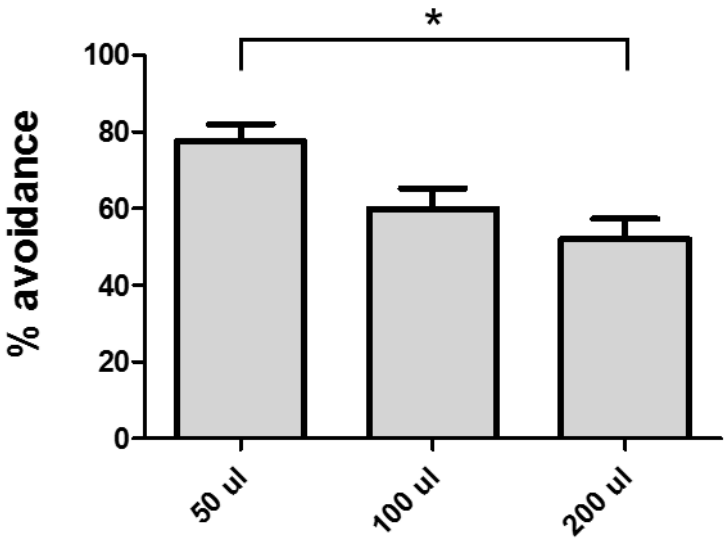

Figure 9: Drop volume of *P. aeruginosa* on assay plate affects avoidance response. A 50, 100 or 200 µl drop of *P. aeruginosa* were cultured on the NGM plate and used as assay plates. The percent avoidance of PA14 lawn in 3 hours are shown. *, $p < 0.05$ determined by Mann-Whitney's *U* test. Error bars represent SEM, N= 3 replicates.

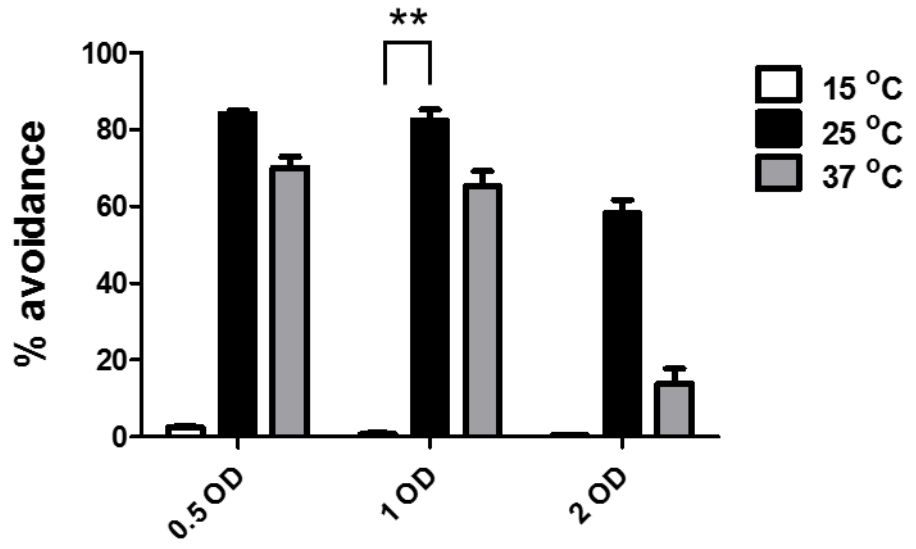

Figure 10: The cultivation condition of *P. aeruginosa* alters avoidance behavior. The percentage of avoidance in 30 min are shown. A drop of 0.5, 1.0 or 2.0 OD culture media on the NGM plate were incubated at 15, 25 or 37 ºC for 24 hours. **, $p < 0.01$ determined by Mann-Whitney's *U* test. Error bars represent SEM, N= 5-6 replicates.

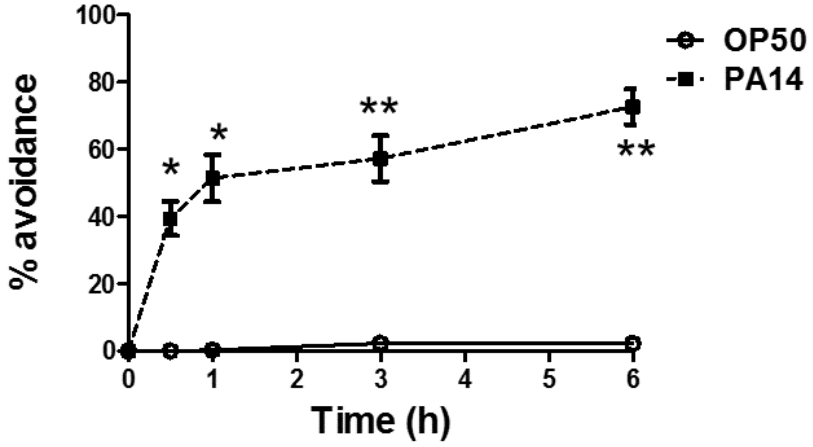

Figure 11: Quick avoidance behavior in response to *P. aeruginosa*. The percentage of avoidance to OP50 or PA14 are shown. **, $p < 0.01$; *; $p < 0.05$ determined by t-test. Error bars represent SEM, N=3 replicates for OP50 and N= 9 replicates for PA14.

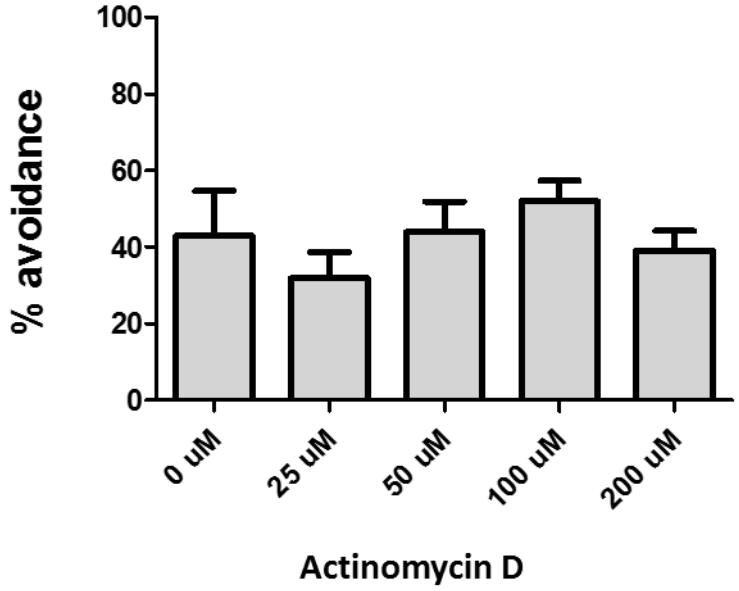

Figure 12: Treatment of transcriptional inhibitor Actinomycin D does not affect on a quick avoidance behavior to *P. aeruginosa*. The avoidance percentage of PA14 lawn in 30 min are shown. Worms were treated by Actinomycin D for 30 min. Error bars represent SEM. N= 3 replicates.

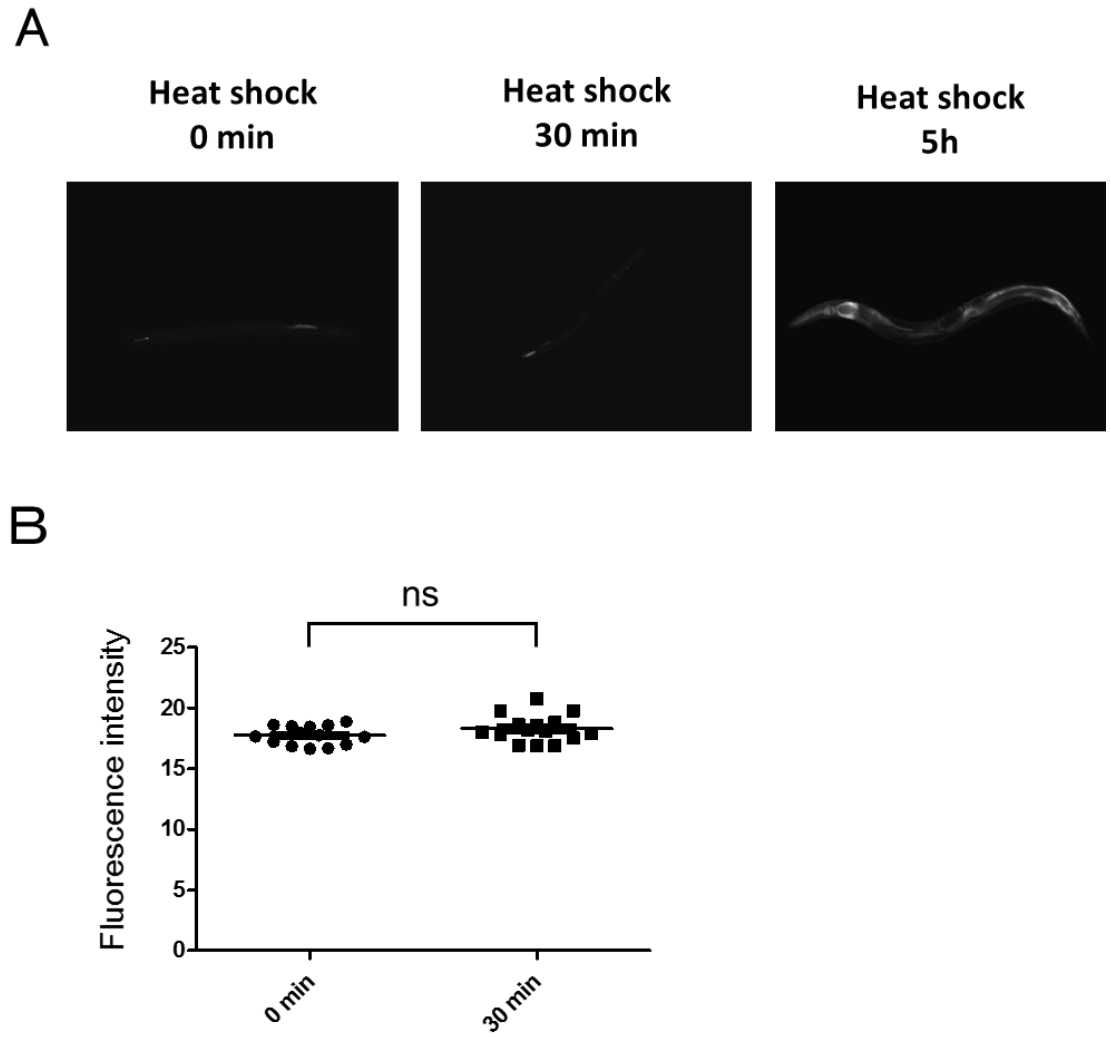

Figure 13: The activity of heat-shock promoter was not observed in 30 min at 37 ºC. (A) Expression patterns of transgenic animals expressing *Phsp-16.2*::GFP. (B) Fluorescence intensity after heat-shock treatment for 0 and 30 min. ns, not significant determined by t-test. Error bars represent SEM, N= 14 replicates for 0 min and N= 15 replicates for 30 min.

Chapter 3

Genetic and molecular analyses of genes involved in the *C. elegans* quick avoidance behavior.

3.1 Introduction

In chapter 2, to understand whether worm can quickly respond to a pathogenic bacterium *P. aeruginosa*, I examined their behavioral responses at early time points after they start to feed PA14 and found that worms show a quick avoidance behavior of *P. aeruginosa*.

As for slow avoidance behavior to PA14, this behavior is known to be regulated by an associative learning between odors (secondary metabolites from the bacteria), and several uncomfortable stimuli. The sensory neurons which sense secondary metabolites and their innervating interneurons, molecules acting those neurons, were identified [20-23]. For example, the INS-6/INS-7 pathway in the ASI neurons is required for olfactory learning behavior to *P. aeruginosa*, and the URX neurons modulates learning ability by regulating the RIA neuronal activity [20]. Furthermore, the insulin-like neuropeptide INS-11 is demonstrated that it negatively controls the insulin-like signaling pathways and also serotonin synthesis in sensory neurons for associative learning behavior. The transcriptional expression of *ins-11* is controlled by both the transcription factor *hlh-30* and p38 mitogen-activated protein kinase (MAPK) pathway in the intestine [23]. Thus,

regulatory mechanisms in slow avoidance behavior to PA14 have been actively revealed. However, as for quick avoidance behavior to PA14, what kind of molecular mechanism is involved? Therefore, I examined molecules which are involved for the regulation of the quick avoidance response in *C. elegans*. Furthermore, I tried to understand how these molecules control behavioral changes when worms meet PA14.

3.2 Material and methods

3.2.1 *C. elegans* strain

C. elegans strains used in this study were N_2 Bristol, CB4856 Hawaii, *daf-2*(*e1370*), *ins-1*(*tm1888*), *daf-28*(*sa191*), *age-1*(*hx546*), *akt-1*(*ok525*), *akt-2*(*ok393*), *sgk-1*(*ok538*), *pdk-1*(*mg142*), *daf-16*(*mu86*), *tir-1*(*ok1052*), *nsy-1*(*ok593*), *sek-1*(*km4*), *pmk-1*(*km25*), *atf-7*(*gk715*), *skn-1*(*zj15*). *C. elegans* was maintained at 15 or 20 ºC on standard NGM agar plates seeded with *E. coli* OP50.

3.2.2 *P. aeruginosa* PA14 avoidance assay

Young adult animals were collected in M9 buffer and kept in M9 for 30 min, then washed three times with M9. Approximately 100 young adult animals were transferred to the center of bacterial lawn in the assay plate. See the chapter 2.2 for details.

3.2.3 Isolation and identification of the *ta218* allele

Mutagenesis was performed using ethylmethanesulfonate (EMS). L4 animals were treated with 50 mM EMS for 4 h and allowed to recover for 1 h on NGM plates with OP50. Ten L4 animals were transferred to a 60-mm NGM plate seeded with OP50 and allowed to lay eggs for 1 day. After one day, the P0 animals were removed, and F1 animals were grown to adulthood. To isolate mutant alleles that caused defective avoidance behavior in response to PA14, F2 animals were placed on the PA14 plate and animals that did not show avoidance behavior were collected as mutant candidates. Their F3 progenies were also examined to confirm their avoidance phenotype. After backcrossing to N_2 five times, SNP mapping was performed using the Hawaiian CB4856 strain. From the obtained candidates, the *ta218* mutant allele was mapped to the middle region of the right arm on chromosome IV, and I found a missense mutation in the coding region of the C10C5.2 gene. I named the C10C5.2 gene *fbxc-58* (F-box C protein). The full-length cDNA fragment was reverse-transcribed from N_2 RNA and sequenced.

3.2.4 Food choice assay

Ten µl aliquots of PA14 or OP50 (OD_{600}=1.0) were spotted at opposite sides of 60-mm NGM plates, and the plates were incubated for 24 h at 25 ºC. Young adult animals that had been grown on OP50 culture plates were collected, and approximately 100 animals were transferred to the center of the assay plate. The number of animals on the PA14 or OP50 lawns were counted after 30 min. The choice index was calculated by using the following formula:

Choice index = (# animals in PA14 - # in OP50)/(# animals in PA14 + # in OP50).

3.2.5 Olfactory preference assay

The Olfactory preference assay was performed using 60-mm assay plate (5 mM KPO_4, pH 6.0, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 2% agar). Each odorant and 0.5 M NaN_3 were dropped on the edge in the plate, and control diluent (ethanol) and 0.5 M NaN_3 were dropped on the opposite side of edge in the plate. Young adult animals, which grown on the OP50 culture plates, were collected and washed four times with S-Basal buffer. In addition, worms were washed with water and were transferred to the center of the assay plate and incubation for 30 min. The preference index was calculated by the following formula:

Preference index = (A- B)/(A + B).

A was the number of animals in 1 cm circle of high salt gradient, B was the number of animals in 1 cm circle of high salt gradient.

3.2.6 Salt Chemotaxis learning assay

The Salt Chemotaxis assay was performed using 90-mm assay plate (5 mM KPO_4, pH 6.0, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 2 % agar), and a salt gradient had been formed by placing an agar containing 50 mM NaCl (6 mm diameter) on the edge of the plate at overnight. 0.5 M NaN_3 was also dropped on the edge in the plate. Young adult animals, which grown on the OP50 culture plates, were collected and

washed four times in conditioning buffer (5 mM KPO_4, pH 6.0, 1 mM $CaCl_2$, 1 mM $MgSO_4$). Washed animals were incubated in a conditioning buffer with 20 mM NaCl (conditioning) or without NaCl (mock) for 1 hour. After the incubation, animals were washed three times by a conditioning buffer and transferred to the center of the assay plate and incubation for 1 hour. The chemotaxis index was calculated by the following formula:

Chemotaxis index = (A- B)/(A + B).

A was the number of animals in 1.5 cm circle of high salt gradient, B was the number of animals in 1.5 cm circle of low salt gradient.

3.2.7 Plasmid construction

To generate the rescue plasmid pDK856, which fully covers the *fbxc-58* coding region, a DNA fragment containing a 5.0 kb promoter region, 1.6 kb coding region and 1.5 kb 3' UTR region was amplified from N_2 genomic DNA using the following primers (5'- GAGAGACCTGCAGGCTGAAATTACTGAACAATCG -3' and 5'- CTCTCTGCTAGCGCGTAGTTTCATGGAACAACATAGATC -3'). This fragment was inserted between the NheI and MscI sites of pPD49.26 (a kind gift from Andy Fire). To generate the transcriptional reporter plasmid pDK811 (*Pfbxc-58*::GFP), the same *fbxc-58* promoter fragment with pDK856 (-5,000 to +11 from the ATG start codon) was amplified from N_2 genomic DNA using the following primers (5'- GAGAGACCTGCAGGCTGAAATTACTGAACAATCG -3' and 5'- ACCGGTGGCCACGACATTCTAAAACTCGAC -3'), and the amplified fragment was inserted between the SbfI and AgeI sites of the pPD95.77.

To generate the translational reporter plasmid pDK839 (*Pfbxc-58*::*fbxc-58*::GFP), the 5.0 kb promoter region and full-length *fbxc-58* coding region was amplified from N_2 genomic DNAs using following primers (5'- GAGAGACCTGCAGGCT-GAAATTACTGAACAATCG -3' and 5'- CTACC-GGTCCATAACTATAATAA-AATGG -3'), and the fragment was inserted between the SacI and AgeI sites of pPD95.77. For tissue-specific rescue constructs, the *elt-2* promoter for the intestine, *myo-3* promoter for the body-wall muscles, and *rimb-1* promoter for neurons, were PCR amplified and inserted into the pPD vector. Both the full-length *fbxc-58* cDNA fragment amplified from the N_2 cDNA library and the GFP fragment were inserted in frame downstream of each promoter sequence.

3.2.8 Transgenic animals

Transgenic worms were generated using standard microinjection methods for *C. elegans*. The pbLH98 [*lin-15*(+)] or *Pmyo-2*:: mCherry plasmid was used as a co-injection marker at 30 ng/µl for the pbLH98 and 1 ng/µl for *Pmyo-2*:: mCherry. Each fusion plasmid was injected at 5 ng/µl for rescue experiments, and 50 ng/µl for transcriptional reporter analyses. At least three independent stable transgenic lines were used in each experiment.

3.2.9 Microscopy

Animals in M9 solution containing NaN_3 (10mM) were mounted onto slides with a 2 % agarose pad. The slides were viewed with an epifluorescence microscope (OLYMPUS BX53) with 20×/0.75 or 60×/1.40 (oil) objective lens,

equipped with a CCD camera in a 16-bit format.

3.2.10 Statistical analysis

Statistical analyses were performed using the GraphPad Prism7. The statistical significance was analyzed by *t*-test or Mann-Whitney's *U* test, and statistically significant differences were defined as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.3 Results

Since this quick avoidance behavior in response to PA14 has not been reported previously, the cellular and molecular mechanisms that regulate quick avoidance behavior in *C. elegans* are also unknown. Therefore, to identify genes involved in this quick avoidance behavior, I performed a genetic screen to isolate candidate mutants defective in quick avoidance response to PA14 (see Methods and Figure 14). From this screening, I isolated 5 candidate mutants. After backcrossing to wild-type five times, I examined avoidance behaviors in response to OP50 or PA14 lawn in these 5 candidate mutants. There 5 candidate mutant animals showed normal responses to OP50, same with wild-type animals (Figure 15). However, *ta218* mutant animals did not show quick avoidance behavior in response to PA14. About half of wild-type animals showed quick avoidance behavior in 30 min (Figure 16), whereas only 13 % of *ta218* mutant animals showed quick avoidance behavior to PA14 in the same period (Figure 16). In addition, in 2.0 OD PA14 which was cultured at 15 ºC, *ta221* mutant animals showed significantly lower

avoidance behavior in 6 hours (Figure 17). Furthermore, *ta219* mutant animals showed a strong avoidance behavior in 3 hours in 2.0 OD PA14 which was cultured at 25 ^{o}C (Figure 18).

In this study, I focused on the *ta218* mutant allele which showed the slowest avoidance to PA14. The locomotion behavior of the *ta218* mutant on both bacterial lawns seemed to be normal (Figure 19), suggesting that quick avoidance to PA14 does not result from locomotion defects. I also wondered if weak avoidance to PA14 could result from an altered food preference. However, the *ta218* mutant animals showed normal food preferences similar to those of wild-type animals, meaning that mutant worms prefer OP50 to PA14 (Figure 20). As for odor preference, moreover, the *ta218* mutant animals showed normal preferences like wild-type (Figure 21). These data indicate that the *ta218* mutant animals do not show a quick avoidance response to PA14, but that their locomotion behavior, food preferences and odor preferences are intact. However, the *ta218* mutant animals which fed PA14 for 12 hours showed abnormal food preference compared to wild-type (Figure 22). In addition, the *ta218* mutant animals showed abnormal leaning behavior in salt chemotaxis leaning assay (Figure 23). These data suggest that the *ta218* mutation may affect some neuronal functions for several behaviors. By SNP mapping and next-generation sequence analysis, I found a point mutation (G493A) in the C10C5.2 gene of the *ta218* genome which causes Glu (165) to Lys amino acid change (Figure 24A). To confirm relationship between the C10C5.2 mutation and the mutant phenotypes, I performed a rescue experiment using the wild-type C10C5.2 gene (Figure 24A). Transgenic animals harboring the wild-type C10C5.2 gene showed significantly higher avoidance than that in the *ta218* mutant and

animals not harboring the transgene, although the rescue was partial (Figure 24B). These results suggest that the C10C5.2 gene is required for the quick avoidance behavior to PA14 in *C. elegans*. The C10C5.2 protein consists of 332 amino acids and has one F-box domain at its N-terminus (Figure 25). Five key amino asides in the F-box domain of C10C5.2 are well conserved in other proteins from different species (Figure 26) [34]. The F-box domain is known to mediate protein-protein interactions in various cellular signaling pathways such as ubiquitin protein degradation. Other known domains are not identified in this protein. Therefore, I named this C10C5.2 gene as *fbxc-58* (F-box C protein). The *C. elegans* genome encodes several similar F-box proteins and most *Caenorhabditis* species have *fbxc-58* orthologs (Table 1). However, *fbxc-58* homologs are not found in mammalian genomes including human. Thus the cellular functions of FBXC-58 have not yet been examined well in any animals. To determine in which tissues the *fbxc-58* gene is expressed, I observed expression and patterns of a GFP-fusion transcriptional reporter (Figure 27A). Strong GFP fluorescence was observed in several tissues from the embryonic to adult stages (Figure 27B). In the larvae stage, the GFP fluorescence was observed in glial cells, a neuronal cell in the head, body-wall muscle cells, intestinal cells, and intestinal valve muscle cells. These expressions and expression in vulval muscle cells were also observed in adults (Figure 27B and 28). These findings suggest that *fbxc-58* may be expressed in these cells. I also tried to observe GFP fluorescence using a translational reporter construct (Figure 29A). Using this reporter, however, no GFP fluorescence was observed in any tissues such as intestinal cells and body-wall muscle cells where the promoter activity was observed. Surprisingly, this fusion protein could rescue

the *fbxc-58* mutant phenotype in *P. aeruginosa* avoidance assays (Figure 29B), suggesting that the FBXC-58-GFP fusion protein is functional in the quick avoidance behavioral response to PA14, even in small amount of expression in animals. To determine in which tissue of FBXC-58 regulates the quick avoidance behavior, I performed tissue-specific rescue experiments. The abnormal avoidance behavior to PA14 by the *fbxc-58* mutant was rescued best when *fbxc-58* was expressed in the intestine (Figure 29B). This suggests that the intestine is the main site of action for FBXC-58 to regulate quick avoidance behavior.

In *C. elegans*, both the p38 mitogen-activated protein kinase (MAPK) pathway and insulin-like signaling pathway are known to function in anti-pathogenic bacteria responses in the intestine and are required for higher viability on pathogenic bacteria [35-38]. Therefore, I wondered whether these immune-response pathways are also involved in the quick avoidance behavior to PA14. I investigated the quick avoidance behavior in mutant animals in the p38-MAPK and insulin-like signaling pathways (Figure 30 and 32). The mutant alleles used to test the response were null or strong loss-of-function mutations except for *daf-28*, *daf-2*, and *pdk-1*, which confer partial loss-of-function. In the p38-MAPK pathway, the signal from TIR-1 to PMK-1 activates transcription factors (TFs) ATF-7 and SKN-1, and these TFs upregulate the expression of immunity genes (Figure 30A). In my avoidance assay to PA14, however, only *nsy-1*, *sek-1*, and *atf-7* mutant animals did not show quick avoidance behavior to PA14 within 30 min. Other *tir-1*, *pmk-1*, and *skn-1* mutant animals showed normal quick avoidance behavior similar to that in the wild-type (Figure 30B). Interestingly, all mutants showed normal avoidance behavior to PA14 in 6 hours (Figure 30B). These data suggest

that *nsy-1*, *sek-1*, and *atf-7* are required for the quick avoidance behavior to PA14, but are not necessary for the avoidance behavior after long exposure to PA14. Although *pmk-1* mutant animals showed normal quick avoidance behavior, another MAP kinase gene *pmk-2* is found in the *C. elegans* genome. *pmk-2* is highly homologous to *pmk-1*, both proteins share 62 % amino acids [39]. Furthermore, in a previous report, both the *pmk-1* and *pmk-2* single mutant animals show the normal survival response to *P. aeruginosa*, but the *pmk-1; pmk-2* double mutant animals show an altered survival response to *P. aeruginosa* [40], suggesting genetic complementation between two genes. To examine whether the *pmk-1; pmk-2* double mutant animals show quick avoidance behavior to *P. aeruginosa* or not, I examined the response in the double mutant animals and compared it with each single mutant animals. As a result, the *pmk-1; pmk-2* double mutant animals showed normal quick avoidance behavior same as wild-type (Figure 31). However, the *pmk-1* mutant animals which cultured at 25 ºC did not show quick avoidance (Figure 31B). These data suggest that both *pmk-1* and *pmk-2* are not involved in quick avoidance behavior to *P. aeruginosa*, and that the *km25 pmk-1* mutant allele may cause a temperature-sensitive phenotype in quick avoidance behavior to *P. aeruginosa*. The insulin-like signaling pathway is activates by the DAF-28 binding to its receptor DAF-2, and this binding activates the downstream transcription factor DAF-16 for the expression of immunity genes (Figure 32A). The INS-1 peptide has an antagonistic role in this DAF-2 signaling. In mutant animals of this pathway, *ins-1*, *daf-28*, *age-1*, and *daf-16* mutant animals did not show quick avoidance behavior to PA14 within 30 min, but the other mutants showed normal quick avoidance behavior (Figure 32B). Furthermore, even after 6 hours, *ins-1*,

daf-28, and *age-1* mutants showed significantly lower avoidance to PA14 (Figure 32B). These data suggest that only *ins-1*, *daf-28*, *age-1*, and *daf-16* may be involved in the quick avoidance behavior to PA14, and *ins-1*, *daf-28*, and *age-1* are also involved in the avoidance behavior after long exposure to bacteria.

3.4 Discussion

I isolated a mutant defective in this quick avoidance behavior to PA14. In addition, some but not all mutants both in the p38/MAPK and insulin-like signaling pathways also showed defective avoidance of PA14. These data suggest that quick avoidance behavior is genetically controlled.

From tissue-specific rescue experiments, I found that FBXC-58 in the intestine functions in the quick avoidance behavior to PA14. In addition, several but not all genes involved in the p38-MAPK and insulin-like signaling pathways are also required for quick avoidance behavior to PA14. These immune-response pathways are activated in the intestine by the infection of pathogenic bacteria. These data suggest that in intestinal cells the above molecules probably act to regulate neuronal activities for an avoidance behavior in *C. elegans*. An unidentified signal released from the intestine may control both circuits to accelerate moving out from the PA lawn and to suppress reentering onto the lawn. However, rescue by both the introduction of the wild-type gene and a tissue-specific rescue construct of FBXC-58 was partial. For full rescue, simultaneous expression in both the intestine and other tissues such as neurons may be required.

The specific roles of FBXC-58 and/or the several proteins in the p38-

MAPK/insulin-like pathways for the quick avoidance behavior are not yet clear. However, the expression of immunity genes under the control of the p38-MAPK pathway is increased around 12 hours after exposure to PA14 [41], and the expression of *fbxc-58* is also upregulated more than two-fold in response to PA14 at 12 hours [30]. These gene expression data suggest that both FBXC-58 and several proteins in the p38-MAPK pathway may not act for transcription and translation in the quick avoidance to *P. aeruginosa*, and several proteins in the p38-MAPK/insulin pathways may be involved in novel signaling pathway which is different from the conserved immune-response pathway, (Figure 33).

How does the FBXC-58 protein regulate quick avoidance behavior in *C. elegans*? FBXC-58 contains a F-box domain, which is known to mediate protein-protein interaction in several cellular events such as ubiquitination. Ubiquitination is involved in proteasomal degradation, gene expression, DNA repair, nuclear export, and endocytosis [42]. In the sensory neuron OLL of *C. elegans*, the E3 ubiquitin ligase HECW-1 regulates avoidance behavior to *P. aeruginosa* [43]. In mammalian cells, exotoxin T, which is produced by *P. aeruginosa*, was degraded by interaction with the E3 ubiquitin ligase Cbl-b [44]. A similar mechanism may function in intestinal cells: FBXC-58 may mediate interaction with an E3 ubiquitin ligase and exotoxin T for ubiquitination and resulting degradation of the toxin (Figure 30). Finally, intestinal cells may release decomposed exotoxin T as a kind of modulator for neurons to elicit quick avoidance behavior (Figure 34).

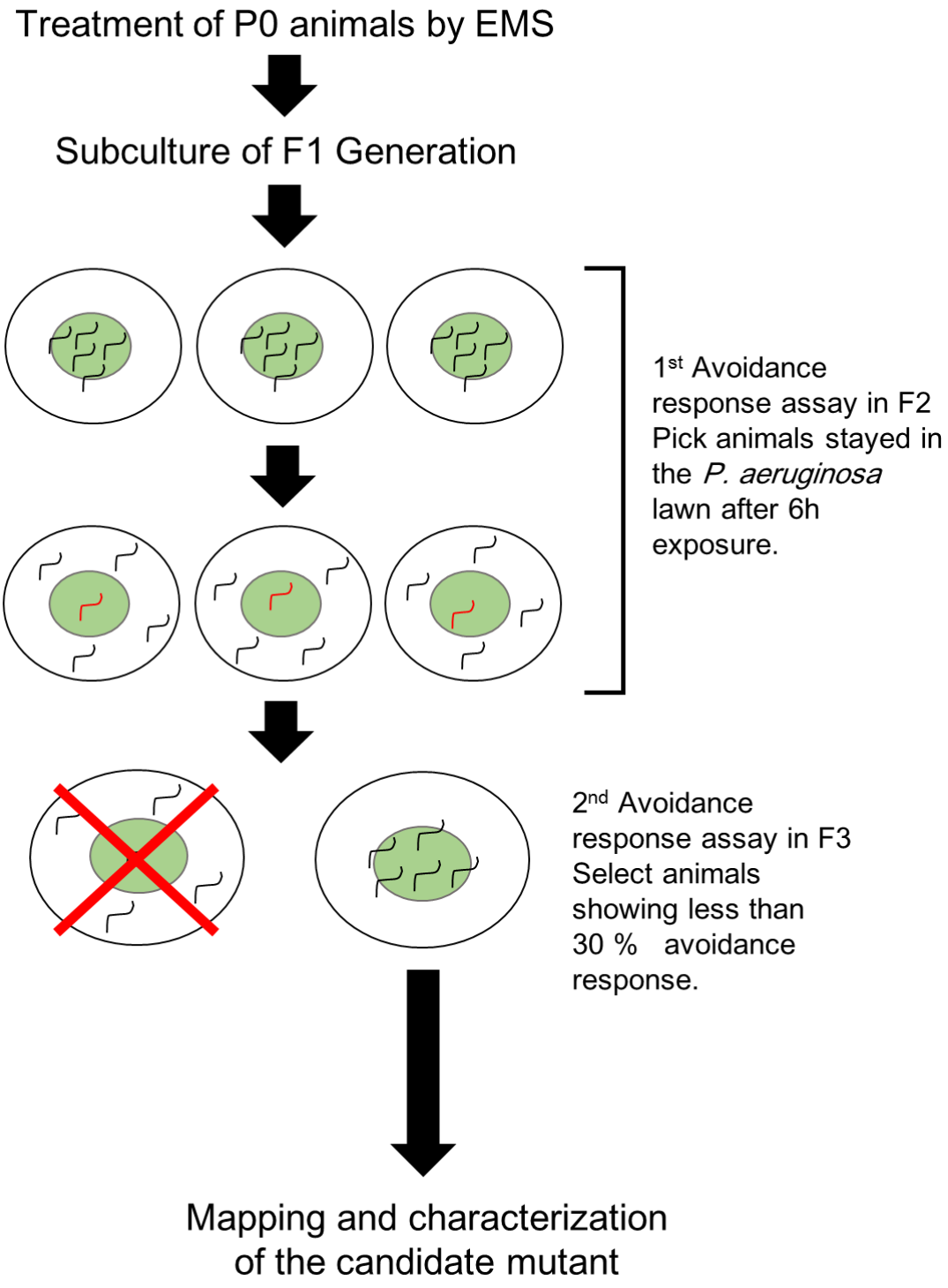

Figure 14: Isolation of mutants defective in the avoidance to *P. aeruginosa*. Shown is the screening scheme to isolate mutants defective in quick avoidance behavior.

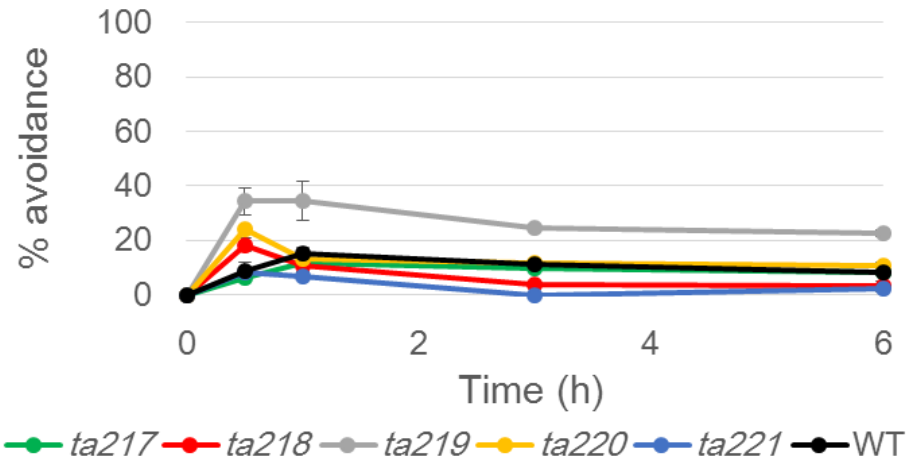

Figure 15: The response to *E. coli* is normal in the candidate mutants. Avoidance responses to OP50 in the candidate mutant animals are shown at indicated time points. N= 3-5 replicates.

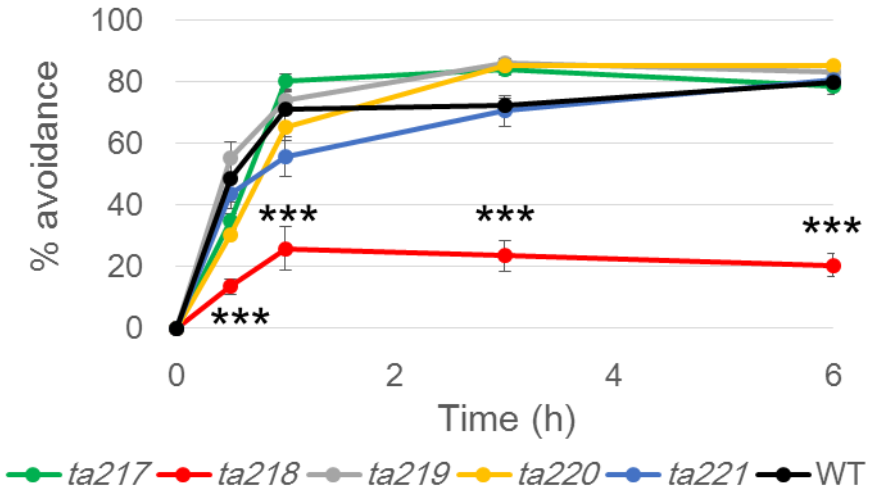

Figure 16: Avoidance response to *P. aeruginosa* in the candidate mutants at 20 ºC. Avoidance responses in candidate mutant animals were examined at each time point. Worms were kept at 20 degree and PA14 growth was optimized at OD 1.0. ***, $p < 0.001$ determined by Mann-Whitney's *U*. Error bars represent SEM, N= 3-5 replicates.

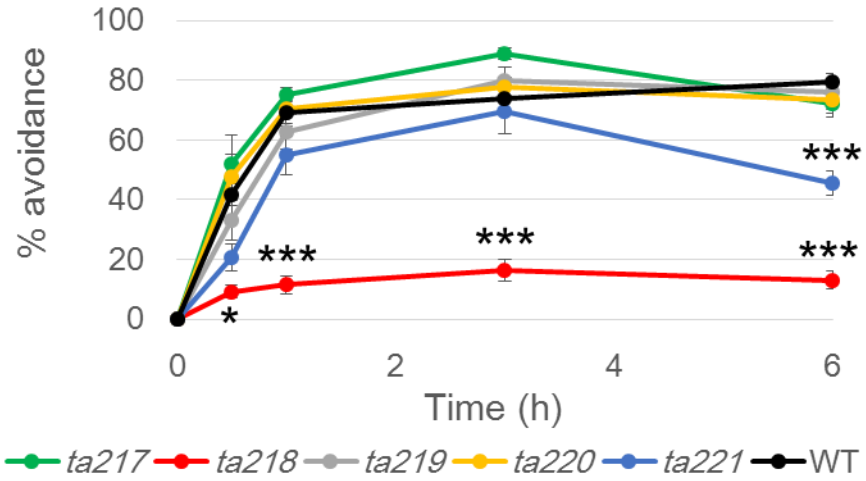

Figure 17: Avoidance response to *P. aeruginosa* cultured at 15 ºC and optimized at 2.0 OD. *P. aeruginosa* was cultured at 15 ºC and the 2.0 OD culture media was spread on assay plates. Avoidance responses in candidate mutant animals are shown at each time point. ***, $p < 0.001$ determined by t-test. Error bars represent SEM, N= 3-5 replicates.

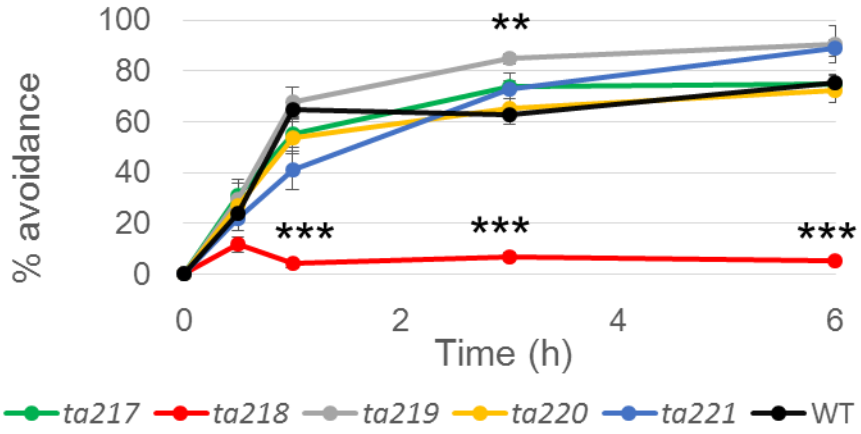

Figure 18: Avoidance response to *P. aeruginosa* cultured at 25 ºC and optimized at 2.0 OD. *P. aeruginosa* was cultured at 25 ºC and the 2.0 OD culture media was spread on assay plates. Avoidance responses in candidate mutant animals are shown at each time point. ***, $p < 0.001$; **, $p < 0.01$ determined by t-test. Error bars represent SEM, N= 3-5 replicates.

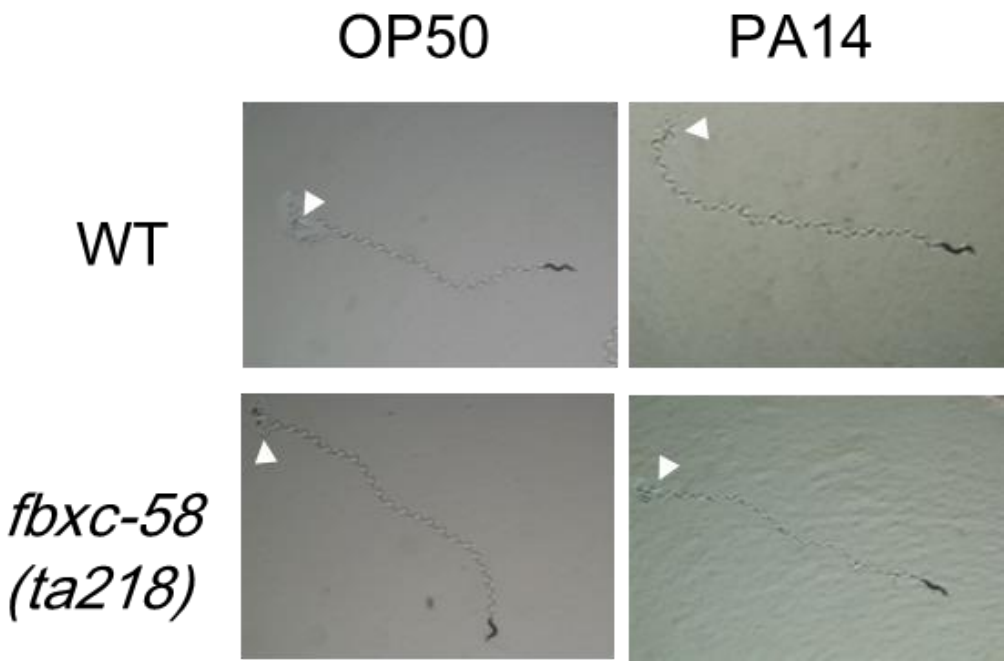

Figure 19: The locomotion behavior in the wild-type or *fbxc-58* mutant animals on the *E. coli* and *P. aeruginosa* lawn. Worms were placed on the OP50 or PA14 lawn, and their locomotion trajectories were imaged after 1 min. The *fbxc-58* mutant animals showed normal locomotion on the OP50 and PA14 lawn. Arrowheads indicate the start point.

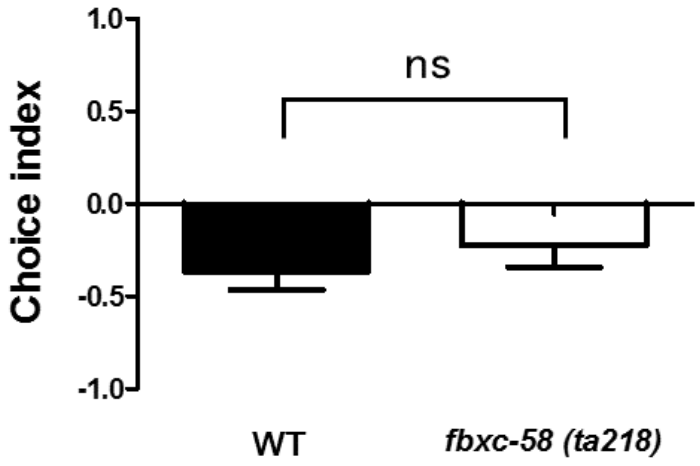

Figure 20: Normal preference to food in the *fbxc-58* mutant animals. Food choice response to OP50 or PA14 in *fbxc-58* mutant animals were examined. ns, not significant, determined by t-test. Error bars represent SEM, N = 5 replicates.

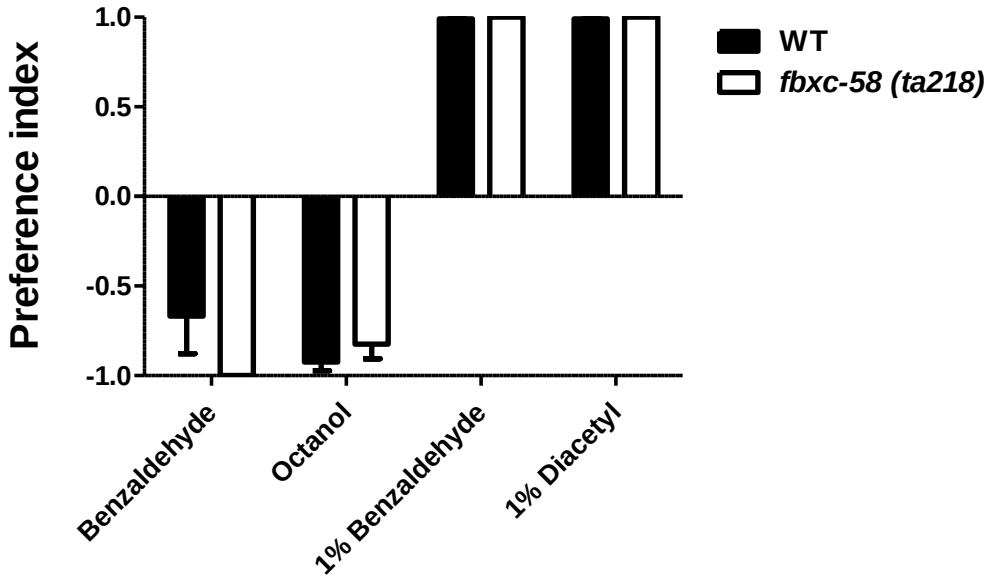

Figure 21: Normal preference to odors in the *fbxc-58* mutant animals. The preference index to Benzaldehyde, Octanol, 1 % Benzaldehyde and 1 % Diacetyl were examined in the *fbxc-58* mutant animals. Error bars represent SEM. N= 5-6 replicates.

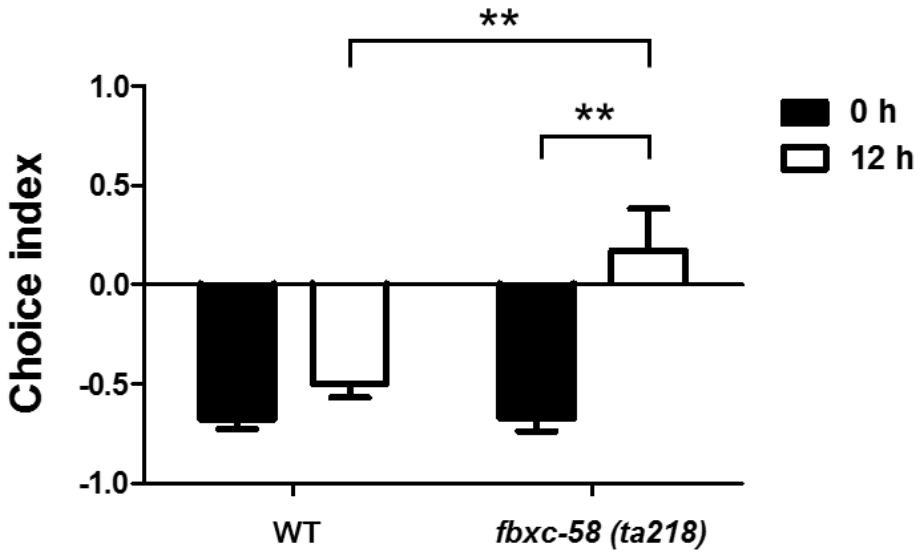

Figure 22: Altered food choice behavior in the *fbxc-58* mutant animals after the *P. aeruginosa* treatment. Food choice preference indices in the *fbxc-58* mutant animals are shown. **, $p < 0.01$ determined by Mann-Whitney's *U* test. Error bars represent SEM, N= 3 replicates.

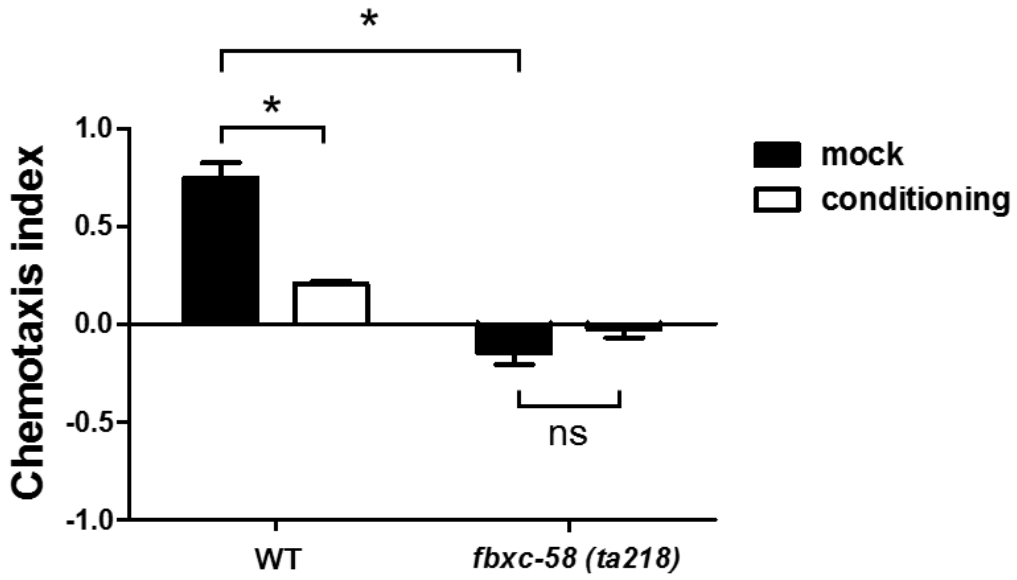

Figure 23: Defective salt-learning behavior in the *fbxc-58* mutant animals. Worms were incubated in a conditioning buffer with 20 mM NaCl (conditioning) or without NaCl (mock) for 1 hour. The chemotaxis index in *fbxc-58* mutant animals were examined. *, $p < 0.01$; ns, not significant, determined by t-test. Error bars represent SEM, N= 5 replicates.

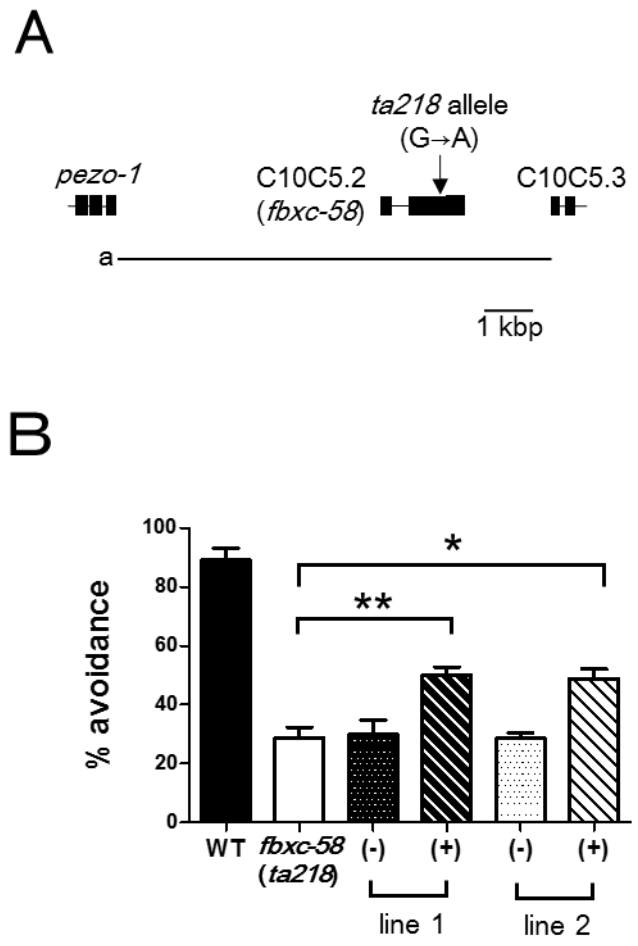

Figure 24: Slow avoidance response in the *ta218* mutant animals was rescued by the expression of *fbxc-58*. (A) Physical map of the *fbxc-58* (C10C5.2) genomic region on the right arm of chromosome IV. The mutation of the *ta218* allele is indicated. Lower bars indicate DNA regions used for the rescue experiment. (B) The rescue ability in transgenic animals harboring the full-length *fbxc-58* genomic region shown in (A). Two independent transgenic lines were examined. (-), non-transgenic animals; (+), transgenic animals. **, $p < 0.01$; *, $p < 0.05$ determined by *t*-test. Error bars represent SEM, N = 3-5 replicates.

A

FBXC-58 (332 amino acid)

```
  1  M S W P T L T V R L Q Q K V I R Y L D Y E S R C N L R I C S  30
 31  K D D K D S V D S V K F N P K T L M L Y E I T S D M S E E K  60
 61  T I V R M Q I D T F K M W F I G K N N I T K V D K S W N G E  90
 91  L V D E L S E L K Q E N R Y D V I R R Y L T R M S L D G I I  120
121  Q A E S I K L D D L S F S P P E T L K F K C D N L E A S Y I  150
151  G N N H D A L W M K Q F I L E F K N L T I I K Y H P H E D I  180
181  G F T Y S E L K V S N S L N L N C D I G I T D K D L L E I K  210
211  A T S L N I S S Q L I T V E G A K K A L E R F L K R G K K T  240
241  D T F V L E I R R P E N F N A Q E H I P K S L V I R K I I R  270
271  R G E T E D E Y Y G K I F E R G F E N E N G V Q D V R D F N  300
301  S S L M G D K L I S C S V Y E K S E T P C Q L Y P F Y Y S Y  330
331  G *
```

B

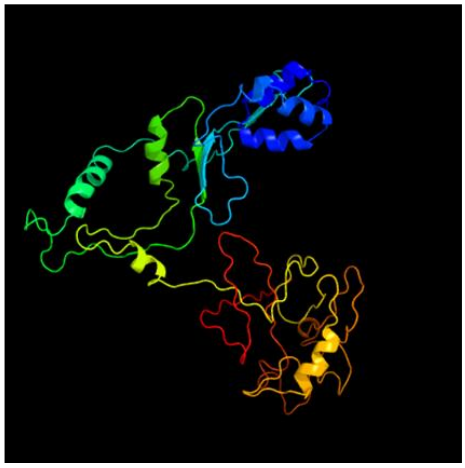

Figure 25: Sequence alignment and three-dimensional structure of FBXC-58. (A) The full-length amino acid sequence of FBXC-58 protein is shown. Marked region represents the F-box domain. (B) Predicted three-dimensional structure of FBXC-58 protein by website Phyre[2].

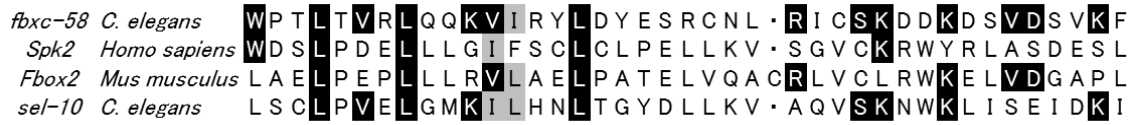

Figure 26: Sequence alignments of the F-box motif. Conserved and similar amino acids in the F-box domain of FBXC-58, compared to *Homo sapiens Skp-1*, *Mus musculus Fbox-2* and *C. elegans sel-10*, are shown in black and gray boxes respectively.

Ortholog	Organism
fbxc-58	***Caenorhabditis elegans***
CBN29082	*Caenorhabditis brenneri*
CBN15036	*Caenorhabditis brenneri*
CBN18189	*Caenorhabditis brenneri*
CBG22138	*Caenorhabditis briggsae*
CBG22119	*Caenorhabditis briggsae*
CRE03731	*Caenorhabditis remanei*
CRE03732	*Caenorhabditis remanei*

Table 1: *Caenorhabditis* species have orthologs of FBXC-58. Table shows the orthologous genes found in other *Caenorhabditis* species to *fbxc-58*.

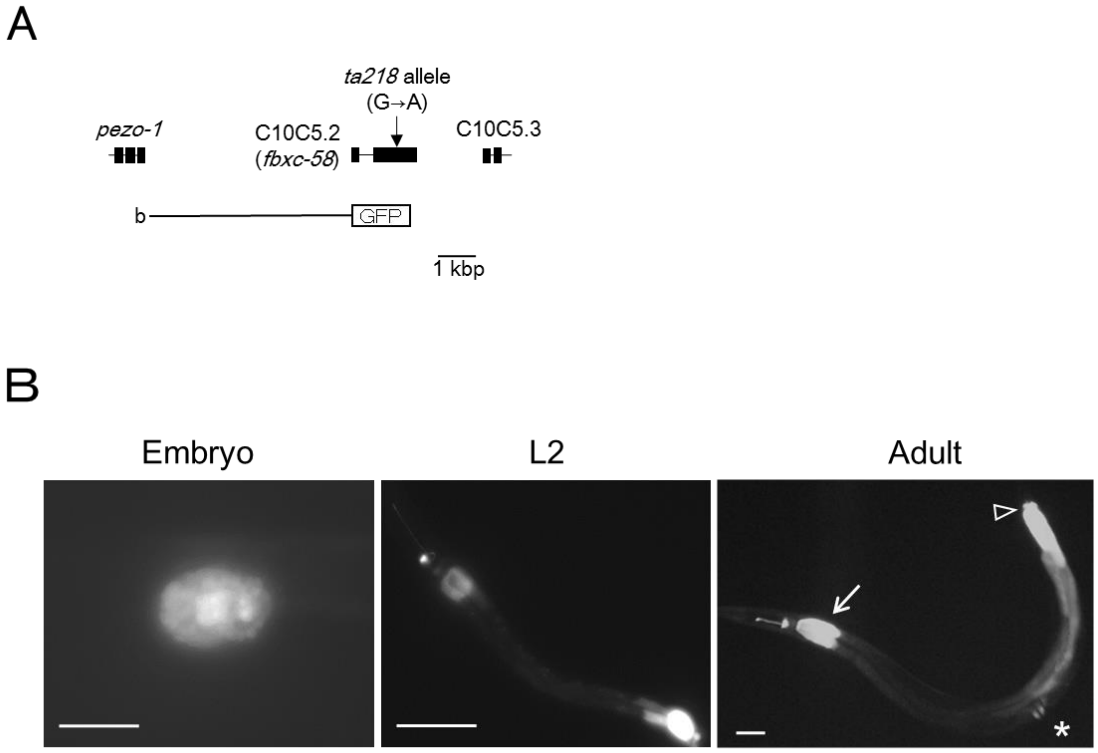

Figure 27: The *fbxc-58* promoter activity is observed from embryonic to adult stage. (A) Physical map of the *fbxc-58* (C10C5.2) genomic region. The mutation of the *ta218* allele is indicated. Lower bars indicate DNA region of the transcriptional reporter. (B) Expression patterns of the transgenic animals expressing transcriptional reporter gene in (A). Arrow, star and arrowhead indicate intestinal cells, vulval muscle cells and intestinal valve muscle cells, respectively, Scale bar indicates 50 μm.

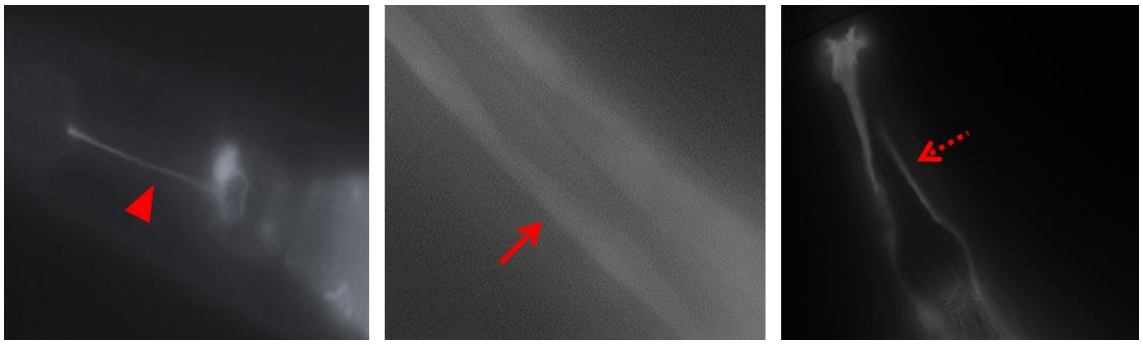

Figure 28: The *fbxc-58* promoter activity is observed in a head neuronal cell, body muscle cells and glial cells. Expression patterns of transgenic animals expressing transcriptional reporter gene. Arrowhead, arrow and broken arrow indicates head neuronal, body muscle cells and glial cells, respectively.

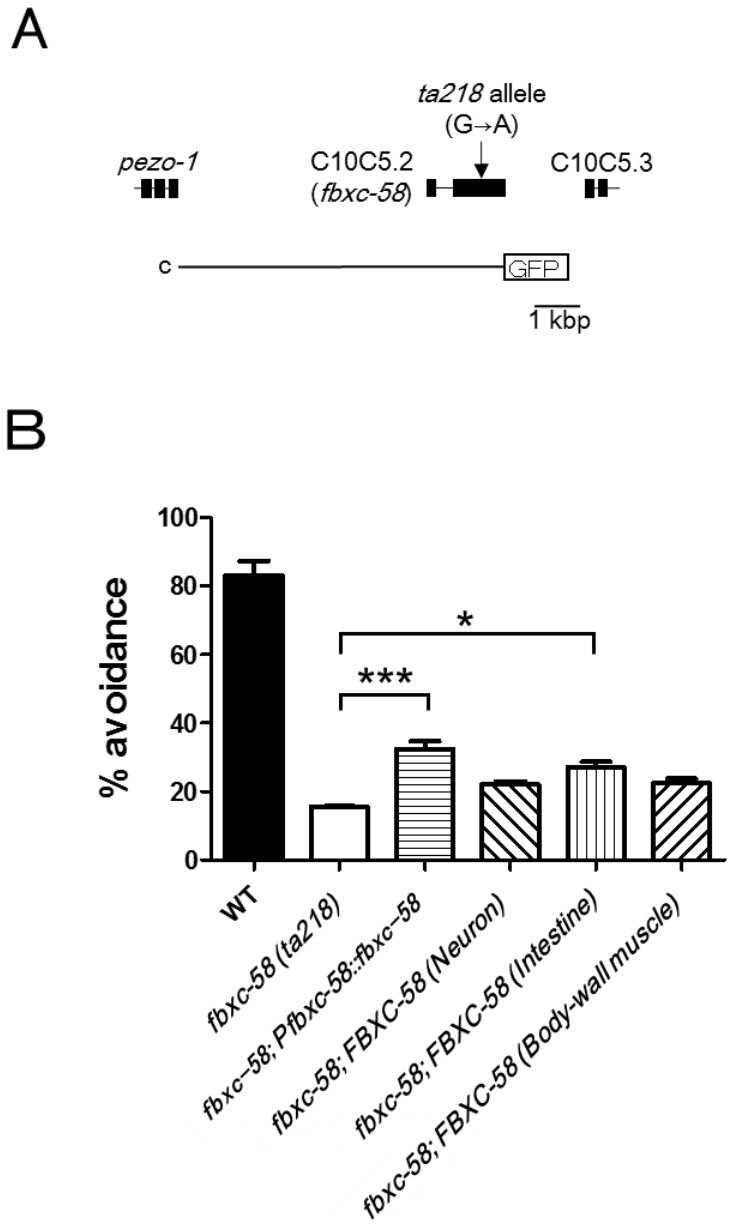

Figure 29: The intestinal expression of *fbxc-58* retains normal quick avoidance behavior to *P. aeruginosa.* (A) Physical map of the *fbxc-58* (C10C5.2) genomic region on the right arm of chromosome IV. The mutation of the *ta218* allele is indicated. Lower bars indicate DNA region of the translational reporter. (B) Rescue of quick avoidance behavior by transgenic animals expressing a translational fusion construct or tissue-specific expression constructs. ***, $p < 0.001$; *, $p < 0.05$ determined by Mann-Whitney's *U* test. Error bars represent SEM, N = 4-17 replicates.

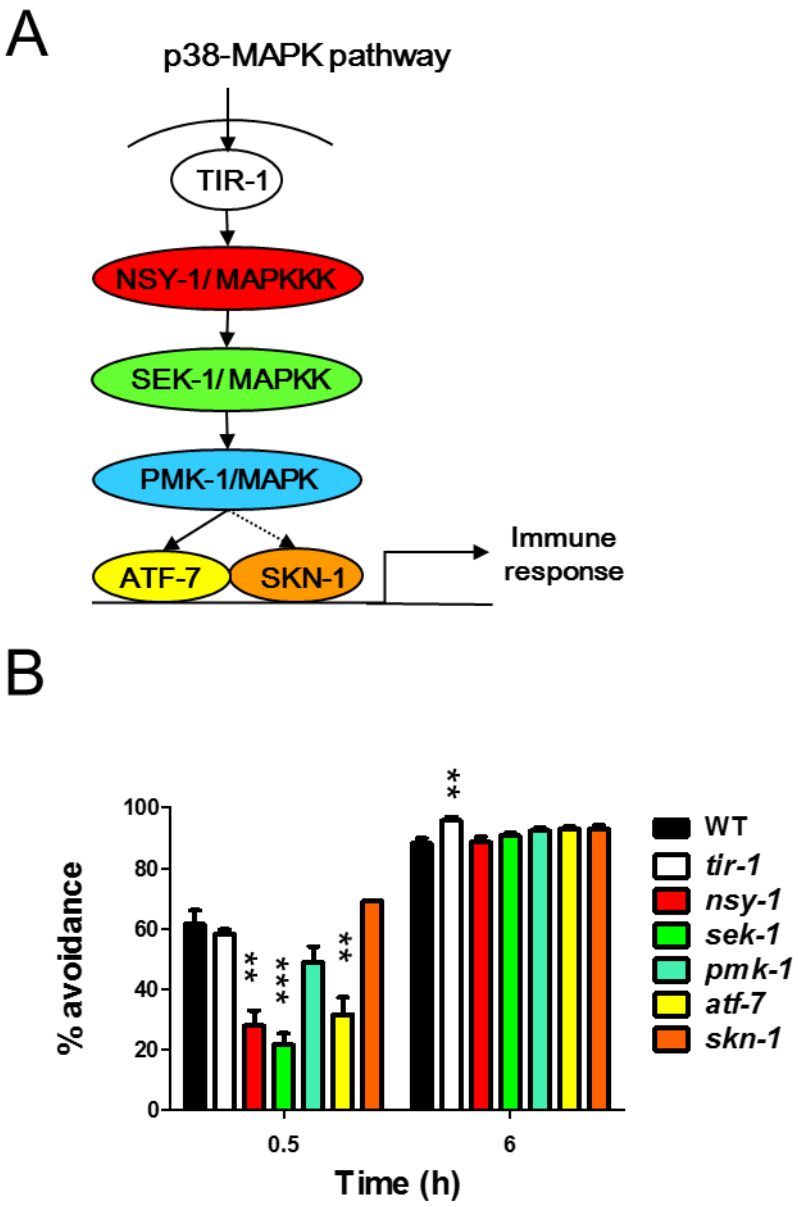

Figure 30: Avoidance response to *P. aeruginosa* in the p38-MAPK mutants. (A) The canonical p38-MAPK signaling pathway identified for immune-response. (B) Percent avoidance responses to PA14 in the p38-MAPK signaling mutants. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ determined by Mann-Whitney's *U* test. Error bars represent SEM, N = 7-10 replicates.

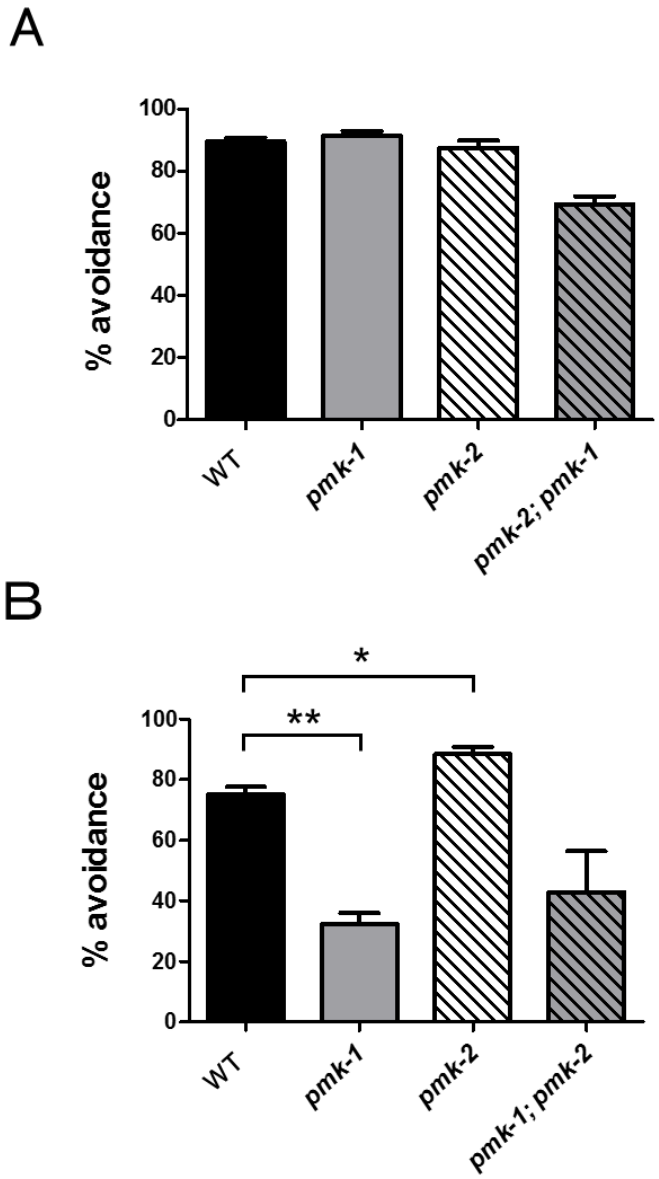

Figure 31: Genetic complementation between *pmk-1* and *pmk-2* in avoidance behavior to *P. aeruginosa*. (A and B) Avoidance responses to *P. aeruginosa* in *pmk-1*, *pmk-2* and *pmk-1; pmk-2* mutant animals. Each mutant animals were cultured at 15 ^{o}C (A) or 25 ^{o}C (B). **, $p < 0.01$; *, $p < 0.05$ determined by *t*-test. Error bars represent SEM, N= 5 replicates for (A) and N= 3-5 replicates for (B).

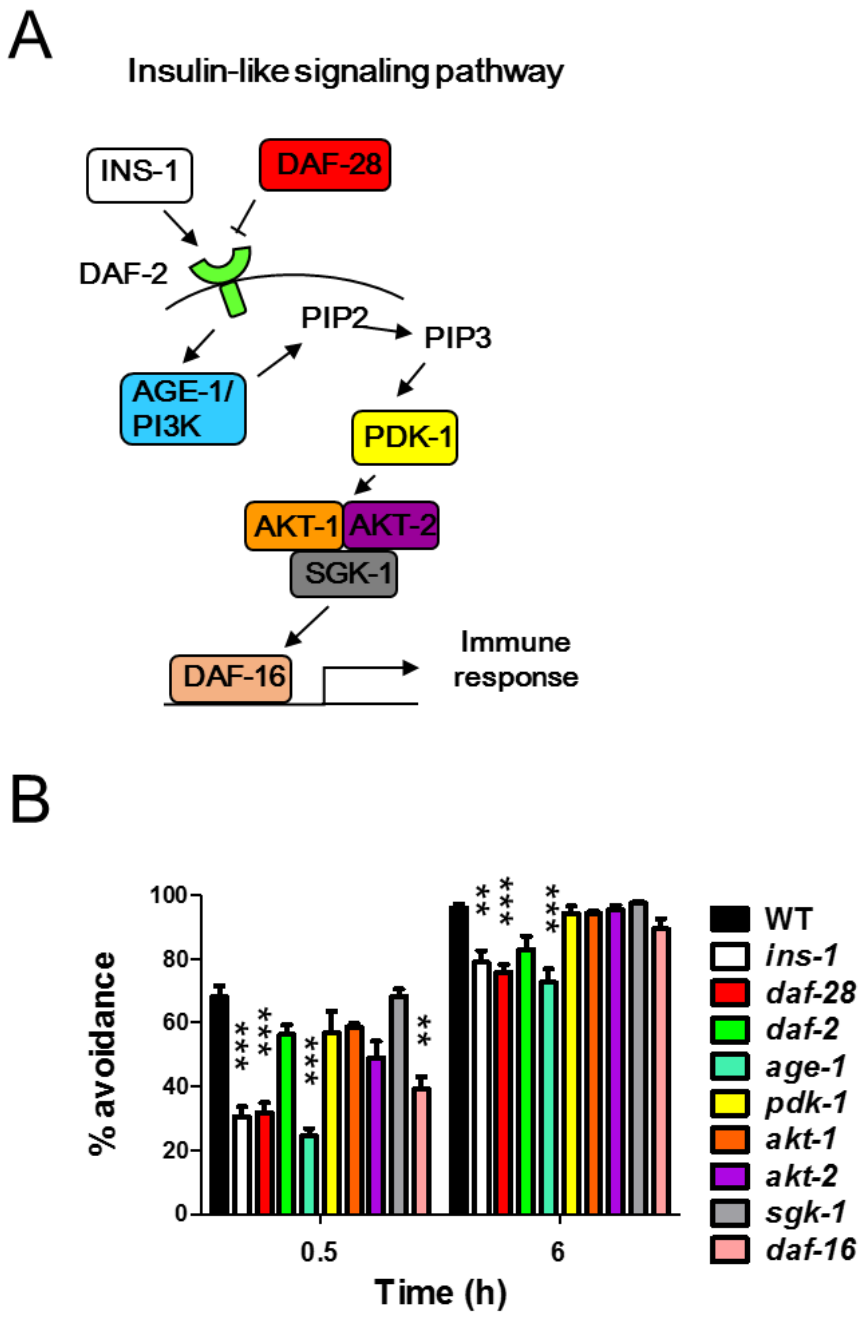

Figure 32: Avoidance response to *P. aeruginosa* in the mutants defective in insulin-like signaling pathway. (A) The insulin-like signaling pathway for immune response. (B) Percent avoidance responses to PA14 in the insulin-like signaling pathway. ***, $p < 0.001$; **, $p < 0.01$ determined by Mann-Whitney's *U* test. Error bars represent SEM, N = 8-15 replicates.

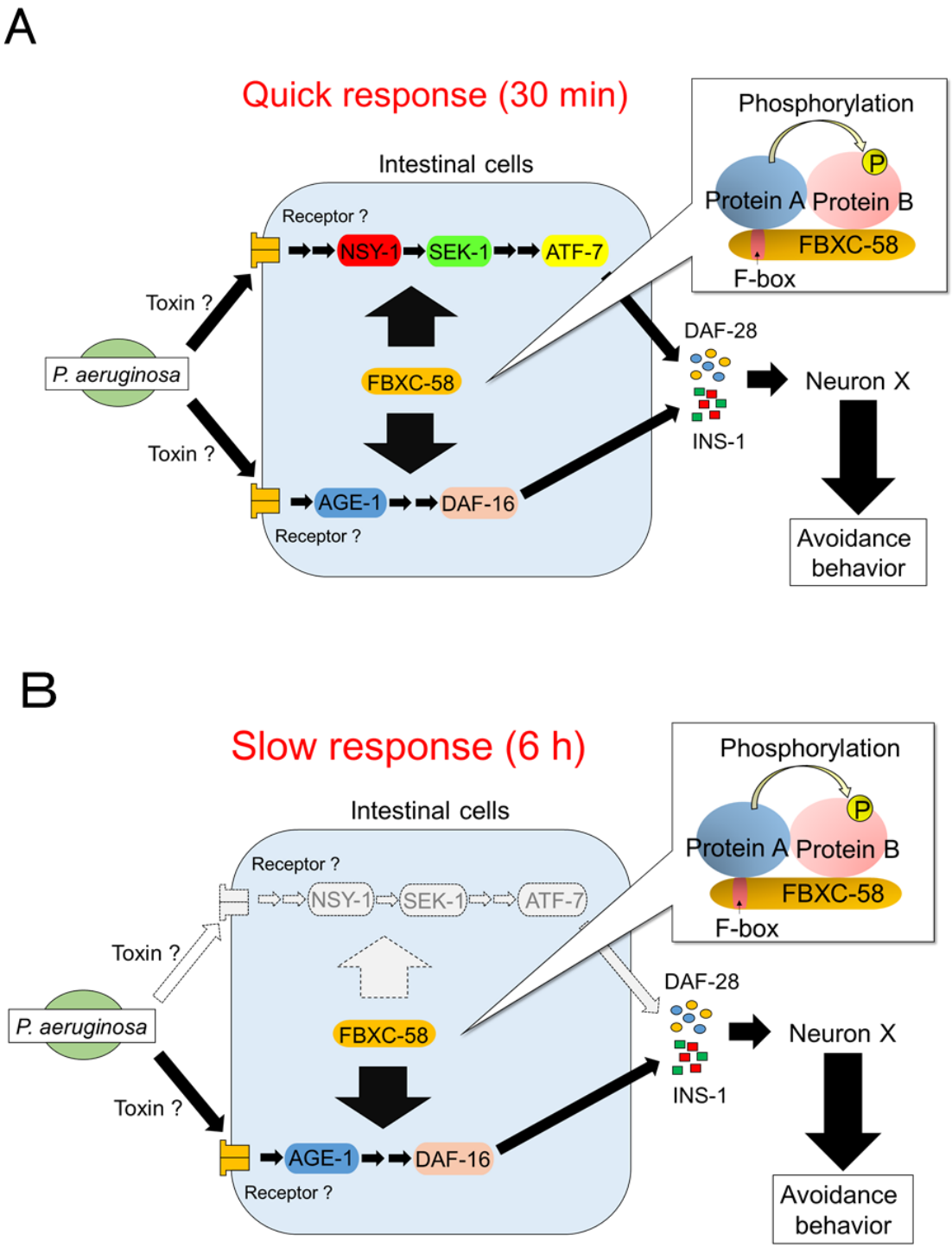

Figure 33: Predicted novel signaling pathway for quick avoidance behavior to *P. aeruginosa*, by identified genes. (A) The quick avoidance response to *P. aeruginosa* within 30 min. (B) The slow avoidance response after long exposure to bacteria.

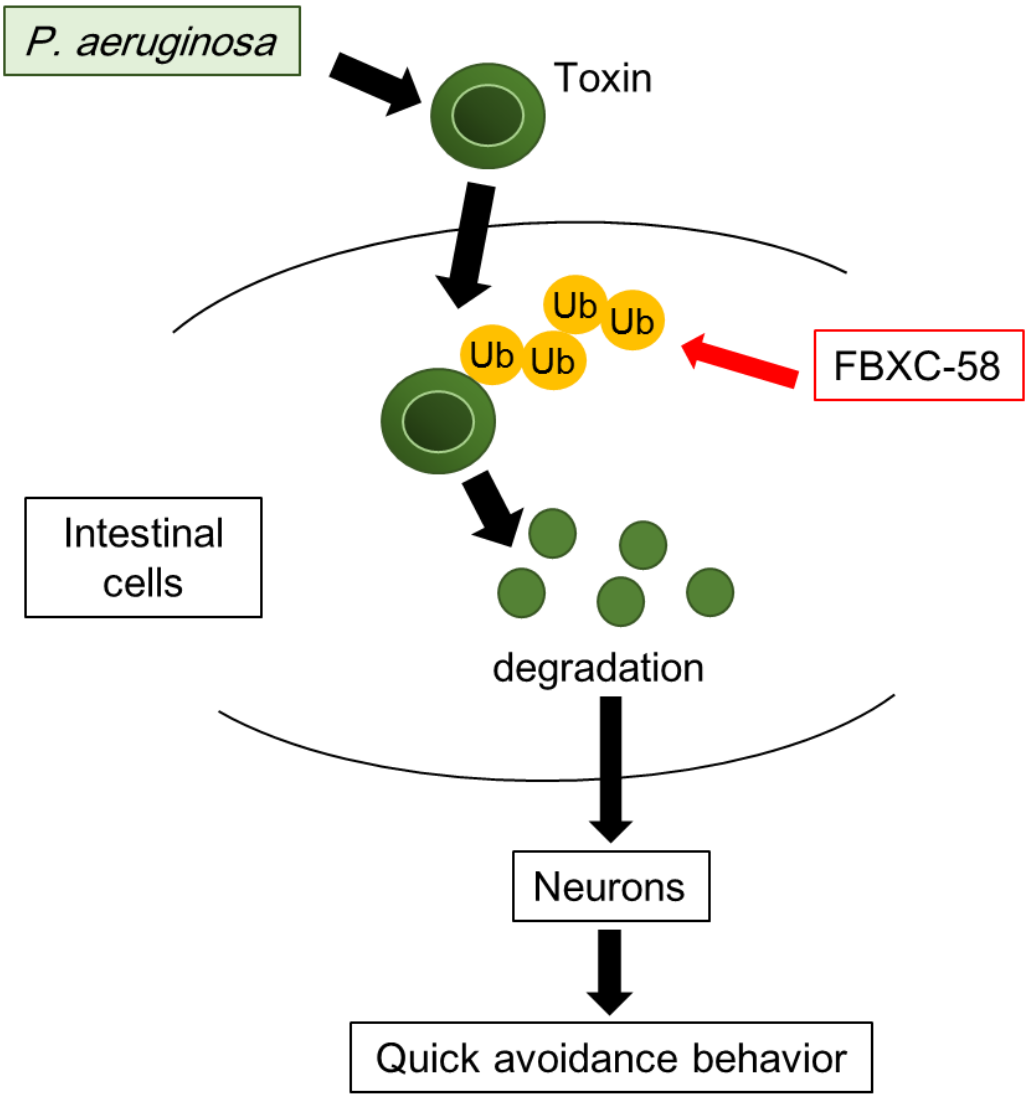

Figure 34: Predicted FBXC-58 function for in quick avoidance to *P. aeruginosa.*

Chapter 4

General discussion and conclusion

Regulation of the nervous system by the intestine is important for not only the recognition of risk factors such as pathogenic bacteria, but also proper neuronal functions including neurogenesis, neurodevelopment, and even for behaviors. In this thesis, I aimed to elucidate what kinds of intestinal signals are expressed and released, and how those signals functions for nervous system development and activity. I tried to identify genes affecting nervous system activity from the intestine of *C. elegans*, using originally-designed *P. aeruginosa* avoidance assay.

In chapter 2, to examine how quickly worms can respond to *P. aeruginosa*, I modified several culturing conditions for both worms and bacteria from the existed *P. aeruginosa* avoidance assay [29]. I found the existence of quick avoidance behavior to *P. aeruginosa* in *C. elegans*. In chapter 3, I succeeded to isolate 3 candidate mutants (*ta218*, *ta219*, *ta221*) defective in avoidance behavior to *P. aeruginosa,* compared to the wild-type animals. In this study, I focused on the *ta218* mutant allele and identified *fbxc-58* as the responsible gene for *ta218* mutant phenotypes. In tissue-specific rescue experiments, abnormal avoidance behavior to PA14 in the *fbxc-58* mutant was rescued by the expression of FBXC-58 protein in the intestine. Based on these results, I hypothesized that *C. elegans* which fed *P. aeruginosa* may detect some risk factors such as toxin or secondary metabolites secreted from *P. aeruginosa* in the intestine. On the basis of information from intestine, neurons may evaluate a degree of risk. After that, *C. elegans* may decide

its behavioral response, quick or slow avoidance behavior.

The brain is thought to sense intestinal stimuli via the passive release of hormones [1, 2]. In *C. elegans*, the connection between intestinal cells and neuronal cells has not observed up to now. However, I believe that regulatory mechanisms for neural function by the intestine are probably conserved between worms and mammal. As shown in chapter 3, I found that several genes in the p38-MAPK pathway and insulin-like signaling pathway are required for quick avoidance behavior to *P. aeruginosa*. These genes are well-known to be conserved in mammals. Furthermore, previous report shows that the p38-MAPK pathway is activated by bacterial metabolites in mammals [45]. I believe that knowledges obtained from this study will contribute to elucidate a signaling mechanism for the microbiota-intestine-brain interaction.

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