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Interkingdom Communication: The Role of Cyclic Di-nucleotides, Specifically 3',5'-Cyclic Diguanylate, in Attraction and Immunomodulation of *Caenorhabditis elegans*

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INTERKINGDOM COMMUNICATION: THE ROLE OF CYCLIC DI-NUCLEOTIDES, SPECIFICALLY 3',5'-CYCLIC DIGUANYLATE, IN ATTRACTION AND IMMUNOMODULATION OF *CAENORHABDITIS ELEGANS*

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ABSTRACT

Cyclic di-nucleotides are important secondary signaling molecules in bacteria that regulate a wide range of processes. Recently, the role of these molecules has expanded to the eukaryotic domain where they act in modulating the innate immune response. In this study, we have shown that *Caenorhabditis elegans* are able to detect and are attracted towards numerous signaling molecules produced by *Vibrio cholerae*, even though this bacterium kills the host at a high rate. Of these molecules, it seems that CDNs are playing an important role, specifically the 3',5'-cyclic diguanylate (c-di-GMP), and the recently described hybrid molecule produced by *V. cholerae*, c-GMP-AMP (c-GAMP). The chemoattraction of *C. elegans* towards these molecules occur in a concentration dependent manner. However, c-di-GMP was the only CDN present in *V. cholerae* cell lysate or supernatant, revealing its importance in this novel communication pathway. C-di-GMP is sensed through *C. elegans* olfactory AWC neurons which then evokes a series of signal transduction pathways that lead to reduced activity of two key stress response transcription factors, SKN-1 and HSF-1, and a weakened innate immunity. Taken together, our study elucidates the role of c-di-GMP in interkingdom communication, *i.e.* bacteria produce c-di-GMP to attract a host and impair its immune response, which in turn promotes bacterial invasion and survival.

DEDICATION

I would like to dedicate this work to my family and friends who have helped me through my academic journey. My parents, Carol and Gary, who have always been there to support me in pursuing my goals and have pushed me to do my best. They have taught me valuable life lessons and continue to be the foundation to my success in the future. They will always be my source of inspiration. To my friends and loved ones, there are too many to name, but each one of you has played a major part during my pursuit of obtaining a PhD. Your faith and confidence in me sometimes outweighed my own, but has provided me with the resiliency needed to complete this process. Thank you all.

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Introduction

1.1 Quorum sensing in bacteria

Bacteria communicate with one another through hormone-like signals (1-5). More specifically, bacterial cells produce chemicals called autoinducers for cell-cell communication, and this allows bacteria to behave in a specific manner depending on the signal (1, 3, 6, 7). This cell-cell communication in bacteria is known as quorum sensing (QS). Quorum sensing was discovered in the 1990's, and relies on cell-density dependent signaling to aid in the survival and proliferation of bacteria (8). Autoinducers accompanied with acyl-homoserine lactones have been shown to regulate QS, and this was first observed in the mechanism of light production in *Vibrio fischeri* (9).

Since the discovery of quorum sensing, the LuxI/LuxR signaling system (and its homologs) has been well studied in Gram-negative bacteria for its roles in cell-density dependent functions (10). LuxI is the autoinducer synthase that synthesizes acylated homoserine lactones (AHLs), and LuxR is the transcriptional activator that binds the AHLs and induces gene expression. AIs can differ depending on the bacteria species that produce them, and different

chemical structures of AIs have been discovered with the increase in quorum sensing research (11).

Gram-positive bacteria do not utilize AHLs in their signaling pathways, nor do they use the LuxI/LuxR signaling system. Instead they operate through the autoinducing polypeptide system (AIP). These signals are produced by a dedicated ATP-binding cassette exporter protein, and the subsequent peptides are recognized by two-component sensor kinase proteins (12). Specific quorum sensing genes can then be transcribed after AIPs reach a certain concentration causing induction of the system.

While initial research found that specific autoinducers produced by bacteria allowed for intraspecies communication, there is also signaling that allows for communication across different species. Both Gram-positive and Gram-negative bacteria can produce the autoinducer AI-2 through the LuxS synthase (13). AI-2 can exist in many forms in the environment that allows it to act as a signaling molecule with different species and these are usually interconvertible cyclic furanone compounds (14). Of note, there is another autoinducer, AI-3, that was initially found in *E. coli* (EHEC) O157:H7, but I will discuss its role later on in this chapter.

When studying these quorum-sensing pathways it was found that these signaling molecules are not only implicated in communication amongst bacteria, but can also be sensed by eukaryotes (host).

1. 2 *Vibrio cholerae*

Vibrio cholerae is a Gram-negative, comma-shaped bacterium that is known to cause cholera in humans and other mammals. It possesses a single polar flagellum, is highly motile, and can tolerate alkaline environments while being sensitive to acid. It is usually found in brackish waters and can easily attach to chitin on shells, making it a potential food-borne pathogen. It is transmitted through the fecal-oral route. There are two serogroups of *V. cholerae,* O1 and O139, and two biotypes of O1, classical and El Tor. Strain C6706 is a bacterium of *V. cholerae* El Tor biotype in the seventh pandemic that has strong hemolytic activity. Cholera is endemic or occurs in epidemic in areas with poor sanitation and is a common problem in developing nations (15).

Being sensitive to acid, most die in the stomach, but the remaining that survive are able to establish and colonize the small intestine through the toxin co-regulated pilus (TCP). Upon establishment, the bacterium secretes the cholera enterotoxin (CT) (16). Downstream effects of the toxin in intestinal epithelial cells cause them to produce cAMP which leads to massive secretion of anions with inhibition of sodium chloride. This causes a great loss of fluids in infected patients through watery diarrhea usually accompanied by vomiting. Several liters of fluid can be lost, leading to hypovolemic shock. The infection usually runs its course in 2 to 7 days with adequate water and electrolyte repletion. In severe cases, antibiotics are used to reduce duration of infection and fluid requirements.

Most persons infected with *V. cholerae* have mild diarrhea or no symptoms at all. Only about 5-10% of persons infected with *Vibrio cholerae* O1 may have illness requiring treatment at a health center. Death can occur however from hypovolemic shock, uremia, and metabolic acidosis. If left untreated, 25%-50% of cases can be fatal. In 2016 the FDA approved a single-dose live oral cholera vaccine that can be administered to adults planning to travel to areas of active cholera transmission (15, 17).

Quorum sensing is vital for the success of *V. cholerae* and the utilization of these signaling molecules allow for robust biofilm formation, establishment in the small intestine, and increased pathogenicity. It also seems that the production of the molecules used in quorum sensing also has an effect on the host in regards to its immune response. Two autoinducers produced by *V. cholerae* have been characterized so far. The first is CAI-1, which is an intra-species molecule, and the second is AI-2, which is inter-species (8). CqsA and LuxS produce these autoinducers, respectively. This, along with other second messengers, allows *V. cholerae* to control biofilm formation, virulence, and other traits (18-21). A depiction of the pathways involved in quorum sensing by *V. cholerae* was described by Higgins et al. in 2007 (22). This is provided in Figure 1.1. Briefly, LuxP and LuxQ function together to detect AI-2, and CqsS detects CAI-1. The signals are transduced through LuxO, and this protein controls the levels of HapR, the master transcription factor in *V. cholerae*. Contrary to some bacteria, at low cell densities HapR is being repressed, which allows for

transcription of virulence factors and biofilm formation genes. This allows the bacterium to better survive in the environment and also effectively colonize a host. The opposite happens at high cell density when AIs are being produced, and it is proposed that this happens so *V. cholerae* can disperse and exit a host into the environment where it can begin another infection cycle (22, 23). The fact that AIs are produced after successful colonization and function in the dispersal of to the pathogen, this makes AIs potential therapeutic targets.

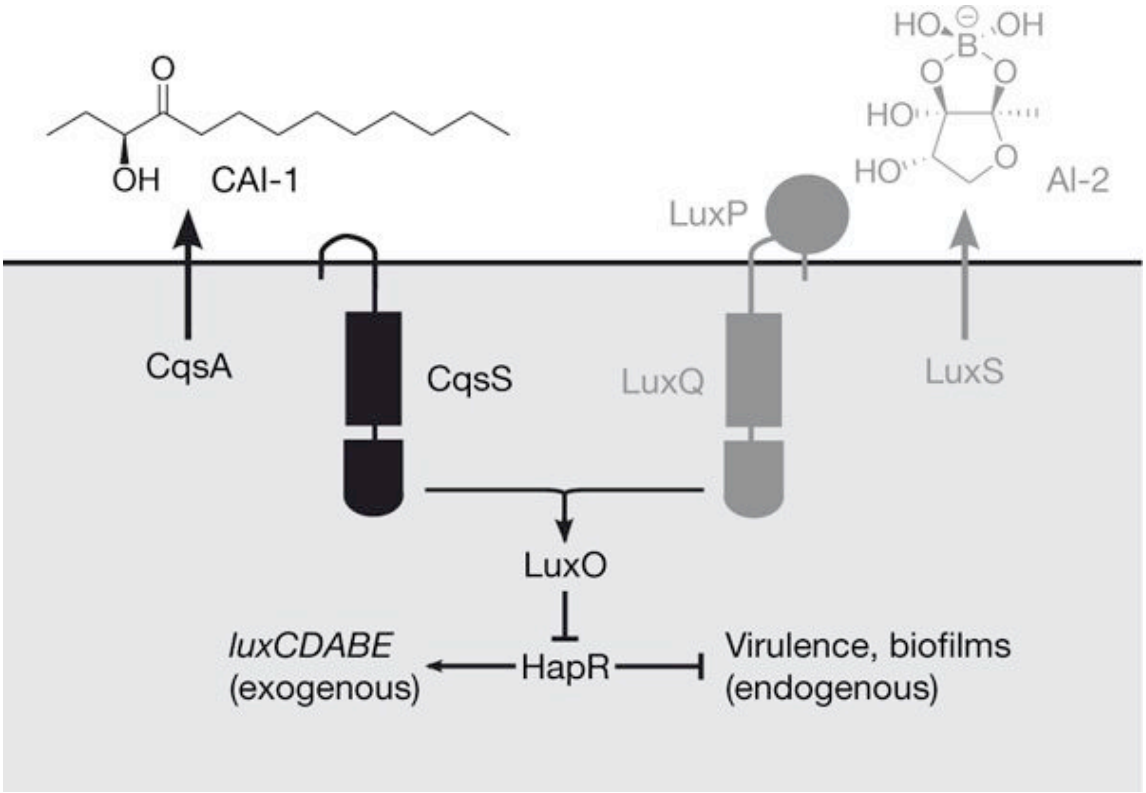


Figure 1.1. Representation of quorum sensing signaling network of *V. cholerae* described by Higgins et al. (22).

1.3 Interkingdom Communication

Recently, there has been a lot interest in trying to understand the phenomenon that has been termed interkingdom communication. This is defined as the process in which bacteria and eukaryotes interact via small signaling molecules (2, 8). Prokaryotes and eukaryotes have coexisted for millions of years, and numerous bacterial cells are present within humans, making up their endogenous bacterial flora. Humans have a symbiotic relationship with their respective flora, but through signaling and other factors this relationship can either become detrimental (pathogens) or beneficial (probiotics). It has been shown that eukaryotes can detect these autoinducers, and this is vital for the success of the organism to locate food, avoid predators, etc. (19, 24-33). This process is known as chemotaxis, and it has been well studied in the nematode, *Caenorhabditis elegans.* Previous studies have looked at how *C. elegans* are able to sense and respond to specific autoinducers that are produced by different bacteria (18, 34). A study done by Zhang et al. (2012) showed that through the TGF-β pathways, DBL-1 is essential in *C. elegans* to actually learn and avoid the smell of certain pathogenic bacteria (35). Hasshoff et al. (2007) also showed that behavioral responses of *C. elegans* to avoid pathogenic strains of the Gram-positive *Bacillus thuringiensis* is dependent on the insulin-like signaling pathway, which is conserved in higher organisms including humans (36). Studying these interactions is thus important in understating how eukaryotes are able to detect and interpret environmental cues. I will go into more detail on the intricacies

involved in interkingdom communication as well as how it has been studied using our model organism later in this chapter.

1.4 *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans is a soil dwelling nematode that grow to about 1 mm long, have a transparent body, and are capable of both sexual and asexual reproduction. Today, *C. elegans* is used in a variety of laboratory experiments to study cell signaling, gene regulation, aging, etc. (37). With its transparent body, it makes it easy to observe the internal organs of *C. elegans* using a simple dissection microscope. This organism is of interest to researchers because they are easy to handle, have a low-cost compared to other model organisms, and have a relatively short lifespan (~14 days). They go through four larval stages and reach adulthood within 42 hours when grown at 25^{o}C (optimal incubation temperatures are closer to room temperature).

C. elegans is a bacterivore, which means they obtain their energy and nutrients from the consumption of bacteria. In laboratory settings, they typically feed on *E. coli* OP50, an uracil auxotroph, that is plated on Nematode Growth Media (NGM). *C. elegans* is grown and maintained on NGM, which contains minimal bactopeptone, NaCl, and agar that allows the nematodes to move freely and easily observed under a microscope. Since *E. coli* OP50 is an uracil auxotroph, its growth is limited on NGM and this allows *C. elegans* to move more freely on the plate to observe behavior (38, 39).

C. elegans possess highly conserved molecular and cellular pathways, and its genome is surprisingly similar to that of humans (>30% homology) (37). Being a hermaphrodite, this makes the organism more stable genetically across

generations (40). *C. elegans* lacks an adaptive immune system, but many of the innate immune pathways, such as p38 MAPK, insulin-like signaling, TGF-β, etc., are also conserved in humans (3, 37). Many *C. elegans* mutants are available for biological research, and this allows for extensive genetic studies to be conducted. Their genome is completely sequenced, and its neuronal network has been studied extensively. For these reasons, *C. elegans* is a great organism to use for this project.

1.5 Effects of Quorum Sensing signals on Mammalian Hosts

Mentioned earlier, I described briefly the phenomenon of interkingdom communication. In this chapter I will highlight some of the known effects these quorum sensing molecules have on mammalian hosts. This will shed some light on how these signals produced by bacteria are able to influence immunity.

1.5.1 PQS signaling in *P. aeruginosa*

The quorum sensing system of *P. aeruginosa* is not only seen to have effects on *C. elegans*, but has also been implicated in interaction with a mammalian host. *P. aeruginosa* is an opportunistic human pathogen that is able to colonize in the lungs of patients with cystic fibrosis. Along with production of its two AHL quorum-sensing molecules, it is also able to use alkyl quinolones for signaling (41). Production of the *Pseudomonas* quinolone signal (PQS) is able to induce gene expression that allows for production of pyocyanin. The production of pyocyanin then induces neutrophil apoptosis and epithelial cell damage to allow it to occupy the lungs and increase pathogenesis (42). In a different manner, it was seen in mice that the opioid dynorphin that is released in times of stress is able to synergize with PQS and increase expression of virulence genes (43). In the same study, dynorphin also caused increased virulence of *P. aeruginosa* against *C. elegans.*

1.5.2 AI-3, epinephrine, and norepinephrine signaling

Briefly mentioned earlier, AI-3 is produced by *E. coli* (EHEC) O157:H7, and other commensal bacteria (44). It was noticed that this pathogen is capable of hijacking epinephrine and norepinephrine produced by the host so that it can colonize effectively and induce virulence genes (45). QseBC is the two component QS system that is able to detect AI-3, epinephrine, and norepinephrine through the histidine sensor kinase QseC (46). This leads to phosphorylation of the response regulators QseB, QseF, and KdpE that regulate gene expression for flagella biosynthesis, formation of attaching and effacing lesions, SOS stress response, and other genes involved in virulence (47).

Interstingly, QseC is a functional analog of an adrenergic receptor and can subsequently be blocked by an antagonist (46). It still remains unclear if AI-3 produced by bacteria is able to bind to mammalian receptors, but this could be another way in which bacteria can manipulate the environment within a host for its own benefit. The conservation of QseC in a number of bacterial species highlights its importance in signaling and colonization of a host.

1.5.3 Indole as a bacterial signaling molecule and its role in Interkingdom communication

Indole is a by-product of metabolism of tryptophan that can be carried out by free-living bacteria, and bacteria within animals. Because tryptophan is an essential amino acid for all animals, the production of indole is a valuable cue for

different organisms (48). Cells are able to detect indole and other tryptophan metabolites that can lead to changes in gene expression levels. This molecule is able to bind and activate the aryl hydrocarbon receptor (AhR) in animals that can stimulate expression of genes that function in immune response (i.e. cytochrome P450) (49). Indole also closely resembles human and plant hormones that are used in signaling.

Indole is produced by at least 27 different bacterial genera, and is considered a compound for cell-to-cell signaling because it is produced extracellularly and recognized by a specific receptor (50). It also generates a concerted response in delaying cell division and can control biofilms (51). This shows an interspecies role of indole, because bacteria that cannot synthesize it are able to detect the molecule. An example is that it is able to repress the multidrug efflux pump in *P. aeruginosa* and genes involved in pyocyanin synthesis in guinea pigs (52).

In communication with a host, indole has also been seen to be beneficial. It is one of the first compounds made by commensal bacteria in the mammalian GI tract and it can tighten gut epithelial cell junctions (53). This prevents the invasion of certain pathogens and it benefits both the host and the commensal bacteria in the gut.

1.6 Immune Response in *Caenorhabditis elegans*

When it comes to studying innate immunity, *C. elegans* serves as a great model organism. As mentioned earlier, these nematodes possess highly conserved pathways (>30% homology with humans), and many innate immune pathways (p38 MAPK, insulin-like signaling, TGF-β, etc.) are conserved in humans (37). In this section I will discuss what we know about the immune response in *C. elegans* thus far by highlighting the major pathways involved, and the interplay that exists between them. It is worth noting that *C. elegans* possesses defense mechanisms such as behavioral responses, physical barriers, and physiological mechanisms to defend against certain pathogens. I will try to focus my review on the physiological defense mechanisms of *C. elegans*. Based on previous knowledge, it is known that bacterial products and signals are playing a role in immunity, and if we want to study this interaction with more detail we need to understanding the intricacies of these pathways. *C. elegans* is a great organism to utilize to study these potential interactions.

1.6.1 Insulin/IGF-1-like (IIS) Signaling Pathway

The insulin-signaling pathway is an interesting one in that it is able to regulate pathogen resistance as well as longevity in *C. elegans*. This pathway was initially characterized in the dauer formation of *C. elegans*. Under unfavorable conditions the worms become highly resistant and long-lived (54). DAF-2 was first described as the main regulator in this pathway for dauer

formation, but also regulates the innate immune response. For innate immunity, DAF-2 is an insulin-like receptor that when activated by insulin-like peptides leads to subsequent activation of AGE-1 (phosphoinositide kinase PI3K) (55). AGE-1 activity leads to the conversion of PIP_2 to PIP_3 that will activate the serine/threonine-directed kinases PDK-1 and AKT-1/AKT-2. Serum- and glucocorticoid-inducible kinase, SGK-1, is also activated by this activity. These are all phosphorylation events that will ultimately prevent translocation of DAF-16 (FOXO homolog) into the nucleus, which is essential for stress response. It is also of note that *C. elegans* DAF-2 mutants are more resistant to pathogen infection than compared to the wild-type (55).

The transcription factor SKN-1 is vital in early development, and is also required for healthy lifespan and stress response (56). When IIS is repressed, SKN-1 is able to accumulate in intestinal nuclei where it can control stress response independently of *daf-16* (57). Phosphorylation of SKN-1 by AKT-1,-2 and SGK-1 does not allow it to be present in the nucleus. When SKN-1 is not localized in the nucleus, stress resistance is significantly reduced and pro-longevity mechanisms are not initiated (57).

Active DAF-16, when DAF-2 is not induced, will enter the nucleus and regulate a number of detoxifying genes (*mtl-1; sod-3*) and antimicrobial genes (*lys-7/8; spp-1*) (58). Heat shock factor (*hsf-1*) is also an important transcription factor that lies downstream of the ILS and is known to play a role during *V. cholerae* infection (59). This gene regulates the activity of many stress response

genes in the nucleus. Other downstream target genes of DAF-16 are all genes that are named based on their regulation by DAF-16: (Downstream of DAF-16) *dod-3, -17, -19,* and *-22* and (DAF-16/FOXO Controlled, germline Tumor affecting) *dct-7, -16, -17,* and *-18* (60).

1.6.2 p38 Mitogen-activated Protein Kinase (MAPK)

The p38 MAPK signaling pathway is known as a major player in anti-microbial defense in *C. elegans*. This pathway is also orthologous to the similar mammalian pathway that is involved in initiating innate immune responses (59). In the nematode, the pathway consists of a cascade of neuronal symmetry family member 1 (*nsy-1)*, a SAPK/ERK kinase 1 (*sek-1*), and a MAPK (*pmk-1)* (61). In response to a pathogen, this pathway undergoes a phosphorylation cascade in which NSY-1 phosphorylates SEK-1, and SEK-1 phosphorylates PMK-1. Although a specific receptor has not been identified in activation of this pathway, TIR-1 (Toll/IL-1 resistance domain protein) has been shown to act upstream of PMK-1 and is a prime candidate for the most upstream member of this pathway (62).

Through this innate immune pathway, different downstream responses can be initiated. Some of these downstream targets include *skn-1* (transcription factor skinhead-1), *mef-2* (myocyte specific enhancing factor 2), *ced-9* (cell death abnormality-9), as well as C-type lectins, CUB-like proteins, and other anti-microbial peptides (56, 63). SKN-1, as mentioned previously, is a transcription

factor that has the ability to control detoxification in response to different pathogens. CED-9 is a negative regulator of programmed cell death in *C. elegans*, and it lies downstream of PMK-1 (64).

1.6.3 ERK MAPK Pathway

While PMK-1 of the p38 MAPK pathway seems to be a major player in the immune response of *C. elegans*, there is also another MAPK, MPK-1 (ERK1/2 MAPK homolog), that can be activated in response to certain pathogens (65). The ERK pathway is evolutionarily conserved in eukaryotes, and has been found to be important in transducing signals from cell surface to the nucleus. The receptor(s) for identifying signals and activating this pathway in *C. elegans* has not been discovered. However, the signal transduction occurs through a series of phosphorylation events, starting with LIN-45 (RAF1 homolog), MEK-2 (MEK1/2 homolog), and ending with MPK-1 (66). A downstream target of MPK-1 upon infection is the invertebrate lysozyme ILYS-3 that can be activated in distal tissues from pharyngeal activation of MPK-1 (67).

While little has been discovered in regards to MPK-1 and immunity in *C. elegans*, the ERK pathway in eukaryotes is known to play a role in cell differentiation, proliferation, and can also extend the replicative life cycle of human diploid cells (68). In *C. elegans* it has been observed that MPK-1 phosphorylates SKN-1 that allow it to accumulate in the nucleus and regulate DAF-2/DAF-16 signaling. This, in turn, causes repression of insulin-like peptide expression that causes repression of IIS pathway and correlates to increased

longevity(66). Although the involvement of ERK pathway in *C. elegans* is not completely understood, it has been shown to aid in the response to certain pathogens and in the overall health of *C. elegans*.

1.6.4 cJun Kinase (JNK) Pathway

The JNK signaling pathway is another subgroup of the MAPK family, and is known undergo signal transduction and regulate different genes under a variety of different stresses (69). JNK-1 is one of the known JNK MAPK homologs in *C. elegans*, and just like other MAPKs it undergoes phosphorylation for activation and downstream effects. When JNK-1 is active, it is able to interact and phosphorylate DAF-16 that allows its translocation into the nucleus(70). From here it can activate a variety of stress response genes. This is different than what is seen in the IIS pathway in that DAF-16 in its phosphorylated state will not enter the nucleus, however JNK-1 phosphorylates DAF-16 at a different site.

Another JNK pathway in *C. elegans* is the KGB-1 (JNK-like MAPK homolog) pathway that offers protection against heavy metals, protein-folding stress, and regulates the response when in the presence of bacterial pore-forming toxins (71, 72). Upstream of KGB-1 lies the MAPKKK homolog and the MAPKK homolog, MLK-1 and MEK-1 respectively. KGB-1 is under negative regulation by the MKP, VHP-1, which in turn can also negatively regulate PMK-1 of the p38 MAPK pathway (73). MEK-1 in the JNK pathway has also been found to play a role in the PMK-1 pathway, and is needed for full physiological

activation of that MAPK (73). This can allow the organism to further utilize the MAPK pathways to aid in its survival by producing a stronger immune response.

1.6.5 TGF-β / DBL-1 Pathway

There are 3 known TGF-β-like pathways in *C. elegans.* One of the pathways containing DBL-1 (decapentalegic/bone morphologenic protein-like-1) has been shown to aid in resistance and the initiation of an immune response to different pathogens (74, 75). During signal processing of this pathway DBL-1 (TGF-β homolog) binds to SMA-6/DAF-4 (serine/threonine protein kinase receptor), and triggers phosphorylation of SMA-3, -2, and -4 (known as the SMAD complex). Receptors and SMAD proteins associated with the immune response of this pathway are more strongly expressed in the *C. elegans* primary sites for immune response, such as the intestine and pharynx. Downstream targets for this pathway include several families of genes involved in the immune response like lysozymes, C-type lectins, and lipase (60, 76).

1.6.6 Toll-like Receptor

Unlike its mammalian counterparts, the Toll-like receptor (TLR) in *C. elegans,* TOL-1, does not seem to play an important role in innate immunity. Loss of function in *tol-1* does not affect *C. elegans* defense against a number of different pathogens (except *S. enterica*) (77, 78). But, as mentioned before, the Toll/IL-1 resistance domain protein, TIR-1, has been shown to function upstream

of NSY-1 in the p38 MAPK pathway, and loss of function in this gene results in susceptibility to numerous pathogens (62).

While not directly involved in the immune response in *C. elegans*, TOL-1 has been shown to be required for some pathogen avoidance behavior. Nematodes defective for *tol-1* were unable to respond and move away from *S. marcescens*, and even laid eggs in the pathogen lawn (78). Thus, it is worth noting that although downstream immune pathways are not controlled by TOL-1, it could be responsible in detecting certain bacterial components that allow *C. elegans* to avoid harmful bacteria.

1.6.6 Other Interplay between pathways

Based on the information provided above, *C. elegans* possesses distinct innate immune pathways that are able to respond to a variety of stresses, and there seems to be some crosstalk that exists between these pathways depending on the signal(s) received. During exposure to infection, these pathways are utilized for antimicrobial responses to control expression of innate immune effectors like C-type lectins, CUB domain containing proteins, antimicrobial peptides, etc. Different pathogens are able to induce expression of different antimicrobial genes. It has been found that when *C. elegans* are introduced to Gram-negative pathogens that some similar antimicrobial genes are induced, but a completely different set of genes are induced when encountering a Gram-

positive pathogen. Through studying the PMK-1, IIS, and DBL-1 pathways, it was noted that *clec-85* (C-type lectin) and *lys-8* (lysozyme) were regulated by all three pathways. Certain members of the *duf-141* (domain of unknown function 141) have also been shown to be induced by certain pathogens and lie downstream of both the IIS and PMK-1 pathways (*dod-22* and K08D8.5) (79).

Through utilization of multiple pathways that regulate distinct responses to different bacteria, it is clear that the innate immune pathway of *C. elegans* is complex and more specific than previously thought. Much work still needs to be done to elucidate the interplay that exists between these pathways and how they regulate certain antimicrobial genes. It is also critical to identify the pathogenic compounds detected by *C. elegans* and the unique receptors for these compounds so we are able to better understand differential response mechanisms. Figure 1.2 displays a rough schematic of the immune pathways in *C. elegans* described above.

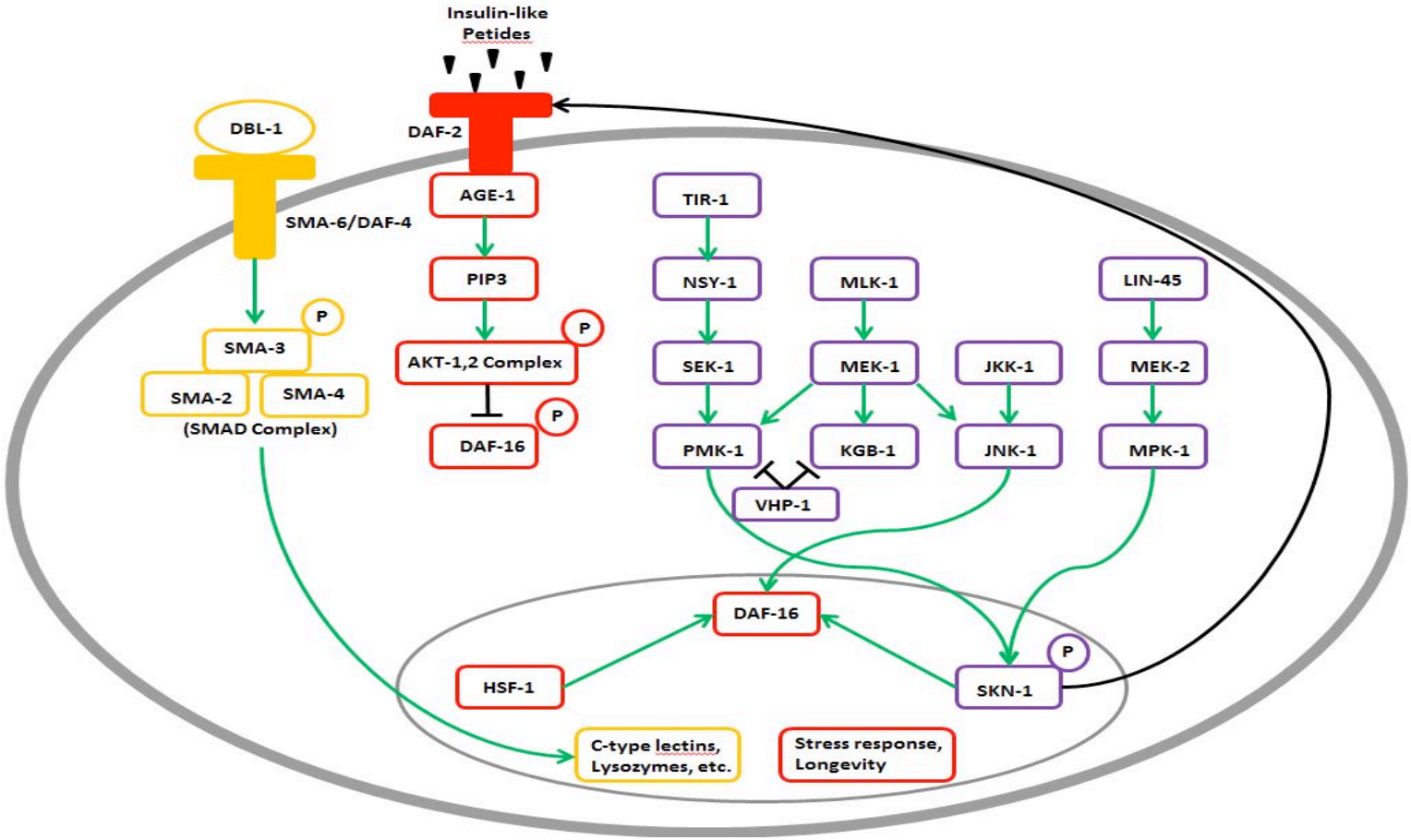


Figure 1.2. Rough overview of immune response pathways. IIS pathway is outlined in red. MAPK pathways are outlined in purple. DBL-1 pathway is outlined in gold. Green arrows represent activation/regulation, while black arrows show negative regulation.

1.7 Initial Insights: Utilizing *C. elegans* as a model to study interkingdom communication

Early studies were conducted on *C. elegans* to determine which cues in the environment allow it to locate food (bacteria). Since there can be numerous cues in the environment at any given time, the neuronal network in *C. elegans* is able to distinguish between the types of cues and this information is used to elicit a specific response. An early study by Ward showed that *C. elegans* are attracted towards cyclic nucleotides (cAMP and cGMP), anions, cations, and alkaline pH environments (80). Through ion channels and cNMP-gated channels, microbes are able to respond to their environment in such a way that allows for their survival, and *C. elegans* is able to detect these products and is attracted towards areas that will provide them food and likely survival. Several possible attractants were screened and those that yielded the strongest attraction of *C. elegans* were cAMP, cGMP, Cl^-, Na^+, and basic pH. The attractive behavior noticed towards these stimuli all happen in a concentration-dependent manner. The attraction towards cyclic nucleotides is not surprising because bacteria are able to synthesize and produce cAMP and, more recently discovered, cGMP (81). In initial tests, it was also noticed that *C. elegans* was attracted to biotin and lysine (82).

Through laser ablation studies of different neurons in *C. elegans* it was determined how these different attractants could be sensed. The ASE neurons seem to be most responsible in attraction towards cAMP, biotin, Na^+, and Cl^-

(82). ASG, ASI, ADF, and ASK also have an effect on the sensation of these different water-soluble attractants, just to a lesser extent than ASE, showing the overlapping function of these neurons.

Since *C. elegans* is typically found occupying the soil, it can be exposed to both water-soluble signals and also volatile chemicals. In a soil environment, bacteria are likely to be decomposing material constantly and during this process the microbes can produce alcohols, esters, aromatic compounds, etc. Many of these chemicals were tested to see if *C. elegans* was able to sense them, and a number of them were found to be attractant and others repellent (83). *C. elegans* displays a high sensitivity to these volatile odors (in the nanomolar range) when compared to water-soluble attractants (micro- to millimolar range) (80). The AWA and AWC neurons were found to be responsible in the attraction of *C. elegans* towards certain volatile compounds, whereas the AWB neuron was responsible for repulsion (84).

The changing decomposition conditions of bacteria in the soil can give rise to fluctuations in gasses in the environment like oxygen. Since *C. elegans* is an aerobic animal, it requires oxygen to live and has been shown to seek out and respond to environments that have ideal levels of O_2 (85). The sensation of varying oxygen levels happens through the UXR, AQR, and PQR neurons that use a specific guanylate cyclase (GCY-35) for sensory transduction of cGMP-gated channels (86).

Nitric oxide (NO) is an important, small signaling molecule in living organisms. In animals, it can modulate the activity of different proteins, and in bacteria it can function to protect against stress and antibiotics (87, 88). When NO is present it can also activate guanalyl cyclase enzymes that in turn produce cGMP, which can modulate specific events within a host (87). *C. elegans* does not possess the ability to synthesize NO, so instead relies on the NO produced by bacteria for its overall wellbeing.

NO produced by bacteria is able to diffuse through the tissues of *C. elegans* and through a transcriptional response can extend its lifespan (89). This response is dependent on the activity of DAF-16 and HSF-1. The activation of these transcription factors regulates the expression of many genes that in turn improve overall health of the organism.

These are just some of the ways in which *C. elegans* was initially shown to respond to environmental cues. The changing environment in which *C. elegans* inhabits makes it vital for this animal to locate areas in which bacteria are available and offers best chance for survival. The sensation of numerous bacterial products through specific pathways creates a great model of host-microbe interactions. By briefly discussing the environmental cues and bacterial products/byproducts sensed by *C. elegans* I hoped to provide sufficient background knowledge on the complexity and specificity of host-microbe interactions.

1.8 *C. elegans* can sense bacterial autoinducers

It was first described that *C. elegans* can sense autoinducers produced by bacteria through studies with *Pseudomonas aeruginosa*. As mentioned before, *C. elegans* is a good model organism to study chemotaxis behavior because it uses its chemosensory neurons to identify food sources and it can respond to a wide range of chemicals. It was found that, when given a choice, the nematodes migrated greatly towards *P. aeruginosa* strain that was able to produce AHLs over a strain that had deletions in autoinducer synthase genes (90). Nematodes were also able to migrate towards pure, synthetic AHLs produced by the bacterium. While *C. elegans* is initially attracted towards this pathogen through detection of AHLs, they can learn to avoid the lawn after initial exposure.

Another study looked at a similar phenomenon in *Vibrio cholerae* (18). *V. cholerae* can produce two AIs. One is an intraspecies molecule, CAI-1, and the other is the interspecies molecule, AI-2. CA-1 is the dominant quorum-sensing signal in *V. cholerae*, but both of these AIs are important in controlling virulence, biofilm formation, and regulation of other genes (10). Through this study it was found that *C. elegans* is readily attracted towards *V. cholerae* most notably through detection of CAI-1, and to a lesser extent AI-2. Studying the attractive behavior in different *C. elegans* mutants revealed that the sensory neuron, AWC^{ON}, is needed for detection and response to CAI-1 production. The structure of CAI-1 was important for recognition, and the length of carbon chain in the molecule is also a prominent factor (shorter carbon chains do not elicit a strong

attractive response). Interestingly, when AI production is removed from *V. cholerae* there is still an attraction exhibited towards the lawn by *C. elegans*, suggesting other signaling molecules other than AIs are detected by the host (18).

The relationship that exists between this model organism and the signaling molecules produced by bacteria can allow us to better understand host-microbe interactions at the molecular level. This can provide insight on mechanisms conserved in higher organisms and how these communication pathways affect both the host and the bacterium.

1.9 The role of secondary signaling molecules in bacterial signaling and implications in Interkingdom communication

The secondary signaling molecules, cyclic AMP and cyclic GMP, have been known and studied for over 50 years. Their role in bacterial signaling has been well defined over the years, but the more recent discovery of the secondary messenger cyclic di-GMP (c-di-GMP), has been of particular interest to researchers. c-di-GMP was first described as an activator of cellulose synthase in *Gluconacetobacter xylinus* (53). Since then, c-di-GMP has been shown to be a ubiquitous Gram-negative bacterial signal that has roles ranging from cell growth to virulence and persistence (91). Other CDNs have been discovered in the past 10 years, such as cyclic di-AMP (c-di-AMP) and the hybrid molecule cyclic GMP-AMP (c-GAMP). These secondary signals are synthesized by specific cyclases and degraded by phosphodiesterases with specific domains. These may be small signaling molecules, but the roles that they play in bacteria are profound and widespread.

c-di-GMP has been well studied for its role in biofilm formation, especially pertaining to *Vibrio cholerae*. The specific mechanisms are still unknown, but high levels of c-di-GMP produced by *V. cholerae* repress the function of a polar flagellum which results in a decrease in motility (92). The same type of mechanism has been shown to be present in other pathogens such as *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (93-97). Recent work in our lab has also found that

treatment with water-soluble cranberry extract down-regulates genes involved in c-di-GMP synthesis and this resulted in decreased biofilm formation (98, 99). Biofilm formation aids in the survival of bacteria in a limited nutrient environment, but in certain pathogens it also helps in virulence. A good example of this is the chronic infection of *P. aeruginosa*, which has elevated levels of c-di-GMP production and thus helps biofilm formation and virulence (100). Although not directly involved in virulence, during late stage infection of *V. cholerae* c-di-GMP is produced to prepare for transition through the host and survive/proliferate in the environment (101).

c-di-AMP, which is widely utilized by Gram-positive bacteria, has recently become an attractive drug target because it is essential for growth in a number of Gram-positive pathogens (102). In *Listeria monocytogenes,* c-di-AMP was found to be secreted outside of the cell through an efflux pump, which activates a host type I interferon response.

The detection of bacterial nucleic acids is critical for the activation of the innate immune system in humans and other animals. There is a transmembrane protein known as STING (stimulator of interferon genes) in humans that is important in viral or bacterial nucleic acid detection, and is found to bind c-di-GMP produced from bacterial infections (27, 30, 103). c-GAMP is also able to bind to STING and stimulate interferon gene expression (30). cGAS is a synthase enzyme that is used to synthesize c-GAMP when foreign DNA is present, and this production is what allows activation of STING (104). There are

numerous other ways in which bacteria utilize CDNs, and a better understanding of these processes can help researchers develop novel treatments and ways to manipulate bacterial signaling.

In this work, we aim to show that in addition to CAI-1, c-di-GMP is another chemoattractant that can be sensed by *C. elegans* by both AWC^{ON} and AWC^{OFF} neurons. Sensing c-di-GMP elicits a series of signal transduction pathways in the worms which lead to reduced innate immune response and a shortened lifespan through affecting the activity of two key stress response transcription factors, SKN-1 and HSF-1. Moving forward, it will be imperative to investigate how c-di-GMP and other CDNs are able to influence behavior of eukaryotes as well as modulate specific immune response and aging pathways.

Chapter 2

Materials and Methods

2.1 Bacterial Strains and *C. elegans* Strains

The plasmid pBAD33 (pACYC184 *araC* P_{araBAD}, Cm^r) (105) was obtained from Coli Genetic Stock center, pAT1568 (pBAD33::NTF3*vieA*-His_6) was provided by Dr. Andrew Camilli (Tufts University) (106). The *V. cholerae* strains used in this study were all derived from the wild-type C6706 strain (O1 serotype El Tor isolated from Peru) (107). The *cqsA*$^-$ (*cqsA* deletion), *luxS*$^-$ (*luxS* deletion), *cqsA*$^-$/*luxS*$^-$ (*cqsA* and *luxS* double deletion) strains were obtained from Dr. Jay Zhu (University of Pennsylvania) (108-110). *E. coli* OP50 came from the laboratory stocks. *E. coli* OP50 and *V. cholerae* were cultured in Lysogeny broth (LB) with 100 µg/mL of streptomycin. Chloramphenicol (5 µg/mL) was used for the selection of pBAD33 and pAT1568 in *V. cholerae*. L-arabinose was added at 0.2% final concentration to induce expression from P_{araBAD}..

N2 worms were acquired from the Caenorhabditis Genetics Center (CGC). Strains are listed in Table 2.2. Worms were maintained on nematode growth medium (NGM) seeded with *E. coli* OP50 at 25°C, and transferred to fresh plates every two days.

Table 2.1. Bacterial Strains used in this study

Strain Name	Relevant Characteristics	Source
V. cholerae **C6706 (O1 El Tor) Strains**		
CO-15 (10/26/2011)	Wild Type	Jun Zhu
CO-23	Δ*cqsA*	Jun Zhu (7/1/2013)
CO-24	Δ*luxS*	Jun Zhu (7/1/2013)
CO-25	Δ*luxS*/Δ*cqsA*	Jun Zhu (7/1/2013)
E. coli **(Laboratory Stock)**		
CE-71	OP50	Lab Stock

Table 2.2. *C. elegans* Strains used in this study

Strain Name	**Gene(s)**
Wild-type N2	
FK100/PR678	*tax-2*(ks10)/*tax-4*(p678)
FG125	*ocr-2*(ak47), *osm-9*(ky10); *ocr-1*(ak46)
FK311	*ceh-36*(ks86)
PR674	*che-1*(p674)
CX6161	*inx-19*(ky634) [previously *nsy-5*]
AU3	*nsy-1*(ag3) II
TJ1052	*age-1*(hx546) II
GR1352	*daf-16*(mgDf47) I; xrls87
GR1307	*daf-16*(mgDf50)
DR1572	*daf-2*(e1368) III
EU552	*glp-1*(or178) III
VC8	*jnk-1*(gk7) IV
KU25	*pmk-1*(km25) IV
PS3551	*hsf-1*(sy441) I

2.2 Cyclic di-nucleotides

Cyclic diadenosine monophosphate (c-di-AMP), cyclic diguanosine monophosphate (c-di-GMP), and cyclic adenosine monophosphate- guanine monophosphate (c-GAMP) were purchased from BioLog Life Science Institute. Their catalog numbers are C 088, C 057, and C 117, respectively. Stock concentrations of these sodium salt compounds were made following the manufacturer's protocol. The stock samples were stored in -20ºC freezer. Serial dilutions were performed from frozen stocks to obtain desired concentration before experiments were conducted.

2.3 Preparation of Media

NGM for maintenance was prepared via standard protocol in 60mm plates. *E. coli* OP50 was dropped on the center of the plates the night prior to transfer of worms. Bacterial strains were dropped onto the center of the plates 2 hours prior to worm transfer.

NGM-FUDR 35mm plates were used for lifespan assays. Fluorodeoxyuridine (FUDR) is an inhibitor of DNA synthesis, and at a concentration of 100µg/mL it prevents *C. elegans* from reproducing and doesn't interfere with development and aging post-maturation. Five times concentrated bacterial cultures were dropped in 100µL aliquots onto the center of plates 2 hours prior to worm transfer.

NGM or NGM-FUDR plates with 1nM c-di-GMP were created by added correct concentrations of stock c-di-GMP (1mM) to warm NGM mixtures after autoclaving. Media was allowed to cool to around 50°C before adding c-di-GMP to reach final concentration of 1nM. Media was mixed well and immediately poured.

2.4 Growth Curve

Overnight culture of *E. coli* OP50 was inoculated (1:100 dilution) in 50mL of LB medium in the presence or absence of 1nM c-di-GMP. Flasks were incubated at 37^{o}C incubator shaking at 150 RPM for 24 hours. Every hour 1mL of culture was taken and optical density measurements were taken at 600nm. Growth curve was plotted and the growth rate was calculated.

2.5 Surface Attachment (Biofilm) observation

Overnight culture of *E. coli* OP50 was inoculated (1:100 dilution) in 2mL of LB medium in the presence or absence of 1nM c-di-GMP. The polystyrene tubes were incubated statically at 37^{o}C for 24 hours to allow biofilm development. Biofilm development was visualized in each tube after the incubation period to determine if biofilm pellicle was present.

2.6 Sample Preparation for high performance liquid chromatography coupled with mass spectrometry (HPLC/MS)

V. cholerae C6706 was grown shaking at 37°C for 22 hours in 100mL of LB broth. After growth, samples are then centrifuged for 10 min at max g, to produce both a cell pellet and supernatant sample. The cell lysate and also the supernatant were tested for the production of CDNs, corresponding to the intracellular and extracellular concentrations respectively.

For the supernatant, it was initially treated with final concentration of 10mM EDTA for 10 min to inhibit bacteria, then passed through 0.45 micron filter to remove any remaining bacterial cells. Samples were then freeze-dried, resuspended in sterile ddH_2O, and centrifuged in Amicon Ultra-0.5 centrifugal filter devices. This was done to finally remove any remaining DNA or protein in the sample. Finally, the samples were lyophilized and sent off for LC-MS.

Cell pellets obtained after initial growth were treated with 10mM EDTA in 25mM of Tris-HCl. Cells were then lysed by sonication at 20W for 60 seconds (three 20 second pulses), and kept on ice to. Tricholoacetic acid (TCA) at a final concentration of 12% was used to precipitate cellular macromolecules and centrifugation was used to separate the precipitate. Samples were freeze-dried, resuspended in sterile ddH_2O, and centrifuged in Amicon Ultra- 0.5 centrifugal filter devices to remove any remaining DNA or protein in the sample. Finally, the samples were lyophilized and sent off for LC-MS.

2.7 HPLC/MS

This work was completed by Dr. Zeneng Wang at the Cleveland Clinic. C-di-GMP and cGAMP in supernatant and cell pellet were quantified by HPLC with online tandem mass spectrometry (MS). The CDNs were resolved on a C18 column (150 x 2 mm, Prodigy^{TM} 5 μm ODS-2 150 Å) (Phenomenx) using a gradient generated between 0.2% formic acid in water (A) and 0.2% formic acid in methanol (B) at a flow rate of 0.25 mL/min. Separation was performed using a gradient starting from 0% B over 2 min, then linearly to 20% B over 4 min, then to 100% B over 0.5 min, followed by this solution for 3 min, then back to 0%B. CDNs were analyzed on a Shimadzu 8050 triple-quadrupole mass spectrometer interfaced to a Shimadzu UHPLC multiplexing system using electrospray ionization in positive-ion mode with multiple reaction monitoring of parent and characteristic daughter ions m/z 675.1→136.1 for cGAMP and m/z 691.1→152.1 for c-di-GMP. Several other daughter ions were used as reference to confirm the defined CDNs. The MS parameters were optimized by using their respective standards. Series concentrations of the CDNs were monitored by the above UHPLC/MS to determine the retention time and prepare standard curves to calculate the concentrations in supernatant and cell pellet.

2.8 Choice Index (CI) Assays

C. elegans were grown at 25°C on *E. coli* OP50 under well-fed and un-crowded conditions. Chemotaxis assays were performed on standard LB agar

plates. The plates were then divided in half to reveal the center point in each plate. Bacteria strains that were used for this experiment were grown in a shaking incubator overnight in the proper growth medium and temperature at approximately 120 rpm. Overnight cultures were then seeded onto each end of the plate 6cm apart, and a 2cm radius is drawn around each lawn. Refer to Figure 2.1 to see experimental setup of chemotaxis assay plate. The lawns are allowed to dry for 2 hours before experiments were conducted. After the lawns were able to dry, 1µL of 10mM sodium azide (NaN_3) was dropped on the center of each lawn to paralyze *C. elegans* to make sure they did not change bacterial lawns once one was initially chosen. Between 50 and 150 well-fed N2 worms were then placed in the center of the assay plate to begin the choice experiment. Every hour for 2 to 4 hours, worms present within the 2cm radius of each bacterial lawn were counted. Each assay was performed independently and in at least triplicate. Choice index was calculated as follows:

***Choice Index = (# of worms on Test - # of worms on Control) / Total # of worms.**

***Positive values indicate preference towards Test strain, while negative values indicate preference towards Control**

Standard deviation is calculated for the experiments so variance can be observed. Graphs and calculations were performed in Microsoft Excel. Given the choice index calculation, if no preference is observed in the experimental procedure then the equation would yield a value of 0. To determine if preference

is significant towards the Test or Control lawn, choice index calculated from experimental procedure was compared to 0 (no preference) by conducting a t-test. If the choice index was to be analyzed further between bacterial strains or time points, then t-test were calculated further between the trials. After statistical analysis, a P-value <0.05 was accepted as statistically significant between variables.

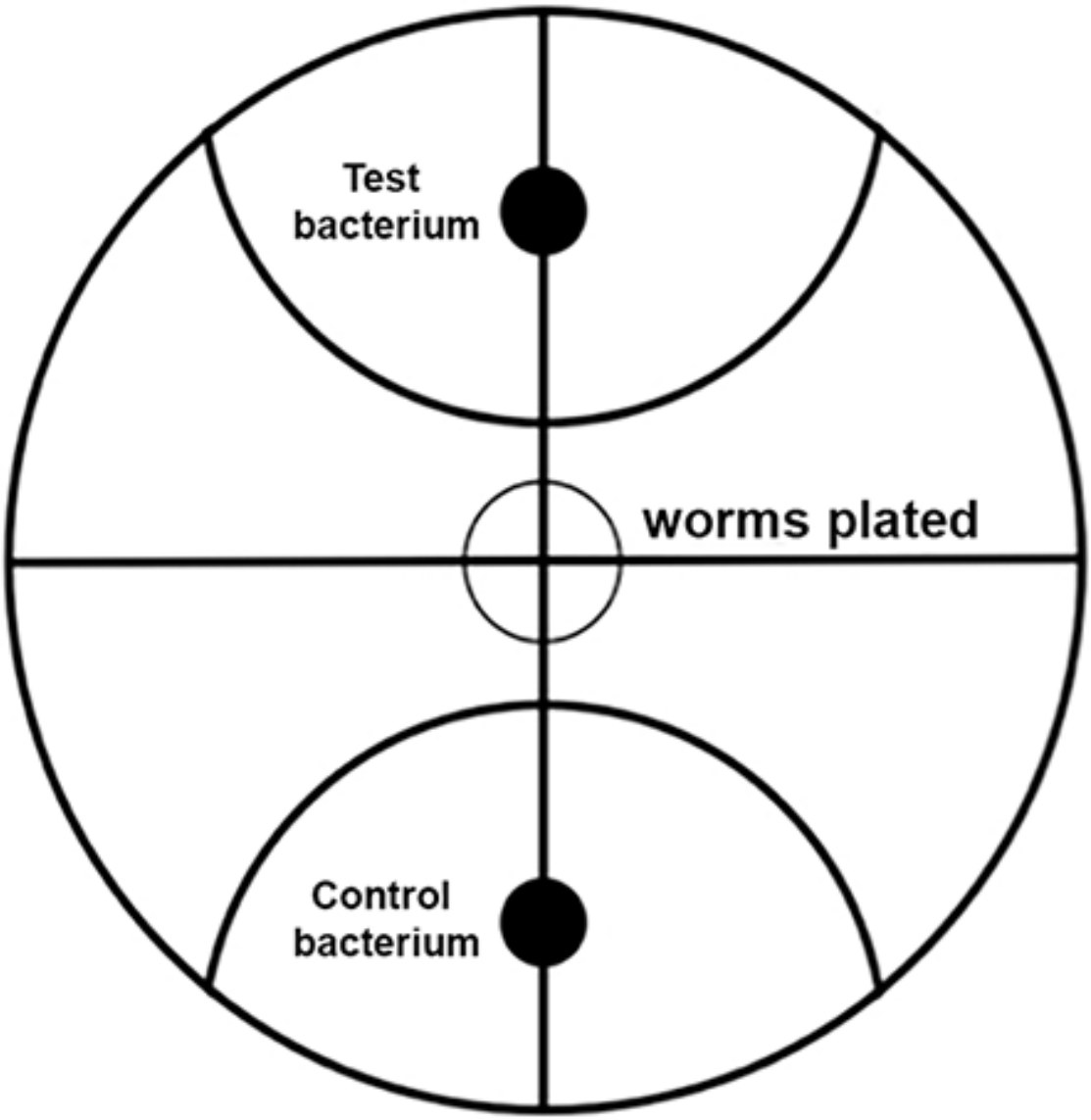


Figure 2.1. Visual representation of setup for CI Assays.

2.9 Brood Size of *C. elegans*

C. elegans strains were kept well-fed under non-crowded conditions during these experiments and synchronized precisely to yield populations that are all at the same stage of development. When the population reached a gravid stage of development adults were transferred to test plates containing 1nM c-di-GMP in the growth medium, or to control plates that did not have treatment with c-di-GMP. They were allowed to eat and lay eggs on these plates in the presence of *E. coli* OP50, and progeny was counted every 12h. The laid eggs were monitored until the next day to score hatching rate. At this time, the gravid parents were transferred to new plates. This process continued until the adult parents are no longer laying eggs. Tests were conducted in at least triplicate with at least 3 individual parents per treatment. Unpaired student's t-test (between two groups) was used for analysis, and a p-value < 0.05 was accepted as statistically significant.

2.10 Gut Colonization of *C. elegans*

E. coli OP50 was grown overnight in LB+streptomycin broth. 100µL of *E. coli* OP50 was dropped on NGM + 1nM c-di-GMP and control NGM plates. 5 N2 gravid worms were transferred onto each plate and incubated at 20^{o}C for 4h. Synchronized egg populations were allowed to grow to L4 stage (~40h), and then transferred to NGM-FUDR and NGM-FUDR + c-di-GMP plates with 100µL of *E. coli* OP50 (concentrated 5x) (this was seen as Day 0). Tests were then

conducted on Day 6 aged N2 worms. 10 worms were randomly chosen from each plate and transferred to LB+gentamycin plates for one hour to rid N2 worms of external *E. coli* OP50. Worms were then transferred to Eppendorf tubes containing 400mg of St. Si Carbide beads and 1mL of M9. Tubes were vortexed for 1min and then centrifuged at max rpm for 2 min. After, tubes were then quickly vortexed and serially diluted in M9 and plated on LB+streptomycin plates using the drop-plate method. CFU's were then determined after incubation at 37 ^{o}C for 12h. Tests were conducted in three independent trials, between three independent populations and CFU's were averaged per N2 worm. Unpaired student's t-test (between two groups) was used for analysis, and a p-value < 0.05 was accepted as statistically significant.

2.11 *C. elegans* Killing Assay

Lifespan assays were carried out at 25°C. Worms were synchronized by transferring 20 gravid worms to 60mm NGM plates seeded with *E. coli* OP50 2 days prior to the start of assays. Worms were allowed to lay eggs for 4 hours, and then parent worms were removed from plates, leaving only synchronized eggs. Worms were then incubated until L4 stage. Overnight bacterial cultures were concentrated by centrifugation and removal of 50% of supernatant. Cultures were then resuspended in remaining medium. Aliquots of 50µL of *V. cholerae* strains and 100µL of *E. coli* OP50 were dropped on the center of NGM-FUDR 35mm plates and allowed to dry for 2 hours. Worms in L4 stage were transferred

to assay plates; 20 worms per plate. Plates were subsequently checked daily and dead worms were counted and recorded. Day of transfer was defined as day zero. Statistical analysis was carried out through SPSS software under the Kaplan-Maier lifespan analysis. P-values were determined via log rank test, and $P<0.05$ was accepted as statistically significant.

2.12 RNA Interference

RNA interference (RNAi) clones were grown overnight at 37°C on Luria Broth plate in the presence of tetracycline (12.5 μg/ml) and carbenicillin (25 μg/ml). Bacterial colonies were inoculated and grown for 8-12 hours, then induced with 2mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours at 37°C. Ten-fold concentrated RNAi bacteria were seeded onto RNAi plates containing 25 μg/ml carbenicillin. The RNAi constructs targeting *mpk-1* was obtained from the *C. elegans*ORFeome RNAi library v1.1 and created by Dr. Yuqing Dong at Clemson University. Bacterial strain containing RNAi targeting *skn-1* was generously provided by Dr. T. Keith Blackwell at Harvard University (57).

2.13 Quantitative Real-Time PCR

All RNA samples were prepared using RNAzol RT reagent (Molecular Research Center, INC.) and stored at −80°C. Complementary DNA was synthesized using the BioRad iScript cDNA synthesis kit (Bio-Rad). For

C. elegans immune response genes, gravid worms were synchronized on NGM plates seeded with *E. coli* OP50 (OP50). 15 gravid worms to each NGM plate, 4 NGM plates total. Worms were allowed to lay eggs for 4 hours and then gravid adults were killed leaving only synchronous egg population. Once nematodes reached young adult stage, M9 buffer was used to wash collected worms. Worm populations were washed at least twice to remove any bacterium that was left in the media or on the worms. After this was completed, the population was split into 50ug populations into separate sterile tubes. This is done to ensure an appropriate amount of RNA in each sample, as well as a high purity. One sample was treated with appropriate final concentration of 1nM c-di-GMP, and the other sample served as a control. This sampling procedure was followed for every qRT-PCR conducted. qRT-PCR was performed using SensiFAST SYBR No-Rox Kit (Bioline) and the CFX96 real-time PCR detection system according to the manufacturer's suggested protocol (Bio-Rad). The qPCR conditions were: 95°C for 3 minutes, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. Relative fold changes are then to be calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method. Cycle thresholds of amplification were determined by Light Cycler software (Bio-Rad). Each qPCR experiment was repeated three times using independent RNA preparations. The immune response genes to be tested and that are known to be implicated in *V. cholerae* infection are listed in Table 2.3.

Table 2.3. Oligonucleotides used in quantitative real-time PCR.

Gene		Sequence of the oligonucleotide
act-1	Forward	5'-CCAGGAATTGCTGATCGTATGCAGAA-3'
	Reverse	5'-TGGAGAGGGAAGCGAGGATAGA-3'
C23G10.1	Forward	5'-CCATCCACTCTTGGTTGCTT-3'
	Reverse	5'-TCACGTGCTCCTTTTTCCTT-3'
clec-46	Forward	5'-CTTCCTCGGTTCTTGCACTT-3'
	Reverse	5'-GCGGTTTCCAACAAAAACAC-3'
clec-71	Forward	5'-TTGGCTGTTGTAGGCAATCA-3'
	Reverse	5'-TCACTGGGAATCCGTTATCC-3'
col-41	Forward	5'-CACCAGGAACTCCAGGAAAC-3'
	Reverse	5'-GTGGGGTTCTGTCGTCTTGT-3'
dct-5	Forward	5'-GCTGCAAAATGTGGAAATGA-3'
	Reverse	5'-AAGTTTTGGGCACAGTCCAG-3'
dod-22	Forward	5' –TTGTTGGTCCCAAGTTCACA- 3'
	Reverse	5' –AAGAACTTCGGCTGCTTCAG- 3'
fmo-2	Forward	5'-TGCTGTCATAGGAGCTGGTG-3'
	Reverse	5'-CATCTGACGCCTCAAAACAA-3'
pqn-5	Forward	5'-GCTCAGCCACAACAAACTCA-3'
	Reverse	5'-CTGGCACTGTTGCTGACATT-3'
dod-22	Forward	5'-TTGTTGGTCCCAAGTTCACA-3'
	Reverse	5'-AAGAACTTCGGCTGCTTCAG-3'
gst-4	Forward	5'-CTCTTGCTGAGCCAATCCGT-3'
	Reverse	5'-CTGGCCAAATGGAGTCGTTG-3'
gcs-1	Forward	5'-GGAATGCCTTACGGAGGTC-3'
	Reverse	5'-CGATAGACATGTTTCATCCTTC-3'
hsf-1	Forward	5'- CAGCCAACAGGGAATCAAAT -3'
	Reverse	5'- TGCTGCTCCAGAAACTGAAA -3'
hsp-16.2	Forward	5'-TTGCCATCAATCTCAACGTC-3'
	Reverse	5'-CTTTCTTTGGCGCTTCAATC-3'
hsp-70	Forward	5'-CGTTTCGAAGAACTGATCTATTCCGG-3'
	Reverse	5'-TTATCAACTTCCTAGGTCCTTGTGG-3'
pqn-5	Forward	5' - GCTCAGCCACAACAAACTCA -3'
	Reverse	5' - CTGGCACTGTTGCTGACATT -3'
sod-3	Forward	5′-CCAACCAGCGCTG-AAATT CAATGG-3′
	Reverse	5′-GGAACCGAAGTCGCGCTTAATAGT-3′

Chapter 3

C. elegans is attracted towards *Vibrio cholerae* C6706 as well as other *Vibrio* species through the production of autoinducers and other signaling molecules

3.1 Introduction

During initial lifespan experiments testing the potential beneficial effects of water-soluble cranberry extract (WSCE) on the health of *C. elegans* in the presence of pathogens, it was noticed that the worms seemed to be attracted to the *Vibrio cholerae* lawn and would readily feed on the bacterium. This seemed counterintuitive based on the lethality of this pathogen to *C. elegans*, and the behavior was not observed for the other pathogens tested. To test and see if the *C. elegans* were actually attracted to *V. cholerae*, choice index assays were designed to determine preference when worms were given the choice of *V. cholerae* or their standard laboratory food, *Escherichia coli* OP50. This peculiar communication pathway that could exist between a pathogen and a host could provide insight onto how infections arise, their underlying mechanisms of action, and how a host responds to such signals.

Vibrio cholerae is a Gram-negative, rod shaped bacterium that is usually found in brackish water. Some strains are known to cause infections in humans when the bacterium enters the host and colonizes the intestine to cause infection. This action requires the toxin coregulated pilus (TCP) for attachment in the small intestine, and symptoms manifest when the bacterium secretes the cholera toxin (CT). In *C. elegans*, the TCP and CT of *V. cholerae* C6706 are not required for lethal infection of the model organism (111). In fact, an extracellular protease, PrtV, and the quorum sensing regulator, HapR, are required for killing of *C. elegans* through colonization of the intestine (111, 112). Hemolysin A, HlyA, produced by *V. cholerae* is also required for killing as it acts as a virulence factor in *C. elegans* and drives the severity of infection by forming intestinal vacuoles and growth delay (113). We have demonstrated the lethality of this pathogen, and *C. elegans* mean lifespan when exposed to this bacterium was around 8 days, significantly shorter than the 11 days for the control population (98). While we are becoming more aware of the mechanisms required for persistence and pathogenicity of this pathogen, there remains a gap in knowledge of how signaling not only allows for survival of the bacterium, but how these signals affect the health and behavior of a host.

Vibrio spp. have been of particular interest to researchers, and they are widely viewed as model organisms for studying quorum sensing (5, 18, 114). It is critical for this bacterium to communicate via quorum-sensing to persist and survive in the environment. Two autoinducers produced by *V. cholerae* have

been characterized so far. The first is CAI-1, which is an intra-species molecule, and the second is AI-2, which is inter-species (8). CqsA and LuxS produce these autoinducers, respectively. This, along with other second messengers, allows *V. cholerae* to control biofilm formation, virulence, and other traits (18-21). Numerous studies have been done studying the quorum-sensing mechanisms of *V. fischeri* and *V. harveyi* (24, 29, 115). These bacteria are known to have symbiotic relationships with various marine animals (24). Unlike *V. cholerae,* they do not pose a threat to health for *C. elegans* and do not significantly decrease lifespan (unpublished data). *Vibrio* spp. have some conserved autoinducers and chemicals that they produce, but some species are unique. *V. harveyi* produces HAI-1, AI-2, and Vh-CAI-1, whereas *V. fischeri* produces AI-1, AI-2, and C8-HSL (7, 18, 21, 25, 116). As one can see, the autoinducers produced by *V. cholerae, V. fischeri,* and *V. harveyi* differ, and could be unique in communication. It has been shown that autoinducers produced by *V. cholerae* (in particular CAI-1) play a role in *C. elegans* chemoattraction. While this phenomenon is also observed in this experiment, it also appears that other signaling molecules could be having an effect. It is possible that other second messengers, such as cyclic di-nucleotides (CDNs), could be affecting the chemotaxis behavior of *C. elegans.*

3.2 Results

3.2.1 *C. elegans* N2 prefer *V. cholera* wild-type over *E. coli* OP50

Through initial experiments conducted in the laboratory, it was noticed that *C. elegans* were strongly attracted to *V. cholerae* C6706. This was particularly interesting because *V. cholerae* was shown to kill *C. elegans* at a high rate, and significantly decreases lifespan. It is hypothesized that the production of AIs by *V. cholerae* aid in the lethality of this pathogen on a host by influencing behavior in the *C. elegans* model. For this reason, studies were completed to observe the chemotaxis behavior in *C. elegans* when exposed to *V. cholerae* wild-type (WT) strain.

Choice index assays were performed as described in the Materials and Methods section. This is a standard laboratory experiment to test preference in the nematode model. In this case, *E. coli* OP50 served as the control bacterium and *V. cholerae* was the test bacterium. The two cultures were grown at 37^{o}C in a shaking incubator at approximately 120 rpm overnight in LB + streptomycin (100ug/mL). Between 50 and 150 well-fed L4-stage N2 worms were scored on each assay plate after one and two hours. Each assay was performed independently and in at least triplicate. Average and standard error were calculated from three independent experiments. Unpaired Student's t-test (between two groups) and one-way balanced ANOVA (for more than two groups) were used for statistical analysis. A p-value < 0.05 was accepted as statistically significant.

Figure 3.1 shows that N2 worms readily attracted towards *V. cholerae* WT compared to its normal laboratory food, *E. coli* OP50. Choice index assays were conducted in which the behavior and preference of *C. elegans* was observed over the course of 2 hours. There was no significance difference in Choice Index between the hours studied.

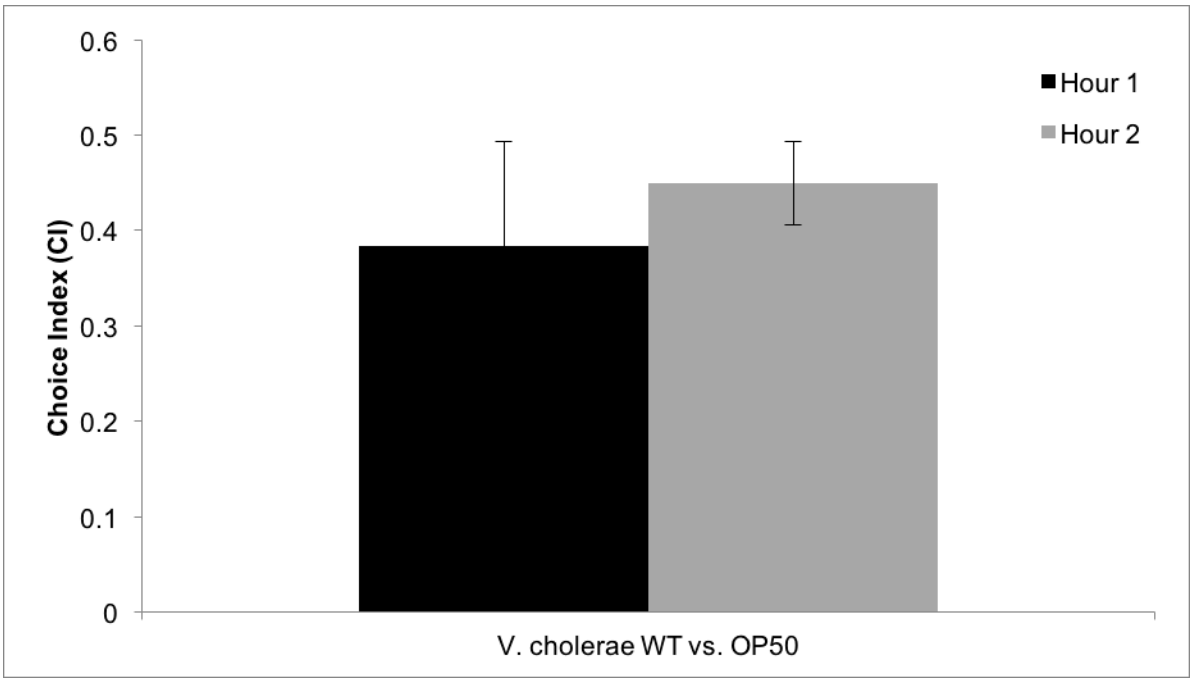


Figure 3.1. Choice Index calculations showing preference of *C. elegans* N2 towards *V. cholerae* C6706 WT over *E. coli* OP50 over the course of 2h. All choice indexes calculated had $p < 0.05$ when compared to CI = 0 (no preference to either strain).

3.2.2 Two AIs are involved in *C. elegans* chemotactic response towards *V. cholerae*

While conducting this research, a paper was published which demonstrated the role of *V. cholerae* AI production in attracting *C. elegans* (18). We sought to prove this was indeed the case since questions still remained in this communication pathway. Since the main autoinducers and genes responsible for their production in *V. cholerae* are known, their role in interkingdom communication can be determined. For this case, choice index assays were conducted using *V. cholerae* knockout mutant strains for the genes responsible for AI production (*ΔcqsA, ΔluxS,* and *ΔluxS /ΔcqsA*). *C. elegans* N2 were scored for their preference towards these mutant strains, and each mutant strain was tested individually against either *V. cholerae* WT or *E. coli* OP50. It is hypothesized that if the production of AIs by *V. cholerae* is playing a role in the attraction of *C. elegans*, then when given the choice between the various mutant strains or *V. cholerae* WT that the worms would choose the WT strain. If the production of AI over another is more important for the attractive behavior in *C. elegans*, the worms would choose one of the mutant lawns (*ΔcqsA* or *ΔluxS)* more readily than *E. coli* OP50. If the production of AIs by *V. cholerae* is the only thing driving attraction of *C. elegans*, then when given the choice between *V. cholerae ΔluxS /ΔcqsA* and *E. coli* OP50 the choice index of the worms would be approximately 0.

Based on the choice index calculated, it was found that *C. elegans* showed strong preference towards *V. cholerae* WT when compared to the other three mutant strains (Figure 3.3). Preference towards the WT over the double mutant strain, ***Δ****luxS* **/*Δ****cqsA*, which is unable to produce AI-2 and CAI-1, was significantly higher than preference observed towards the other two mutant strains.

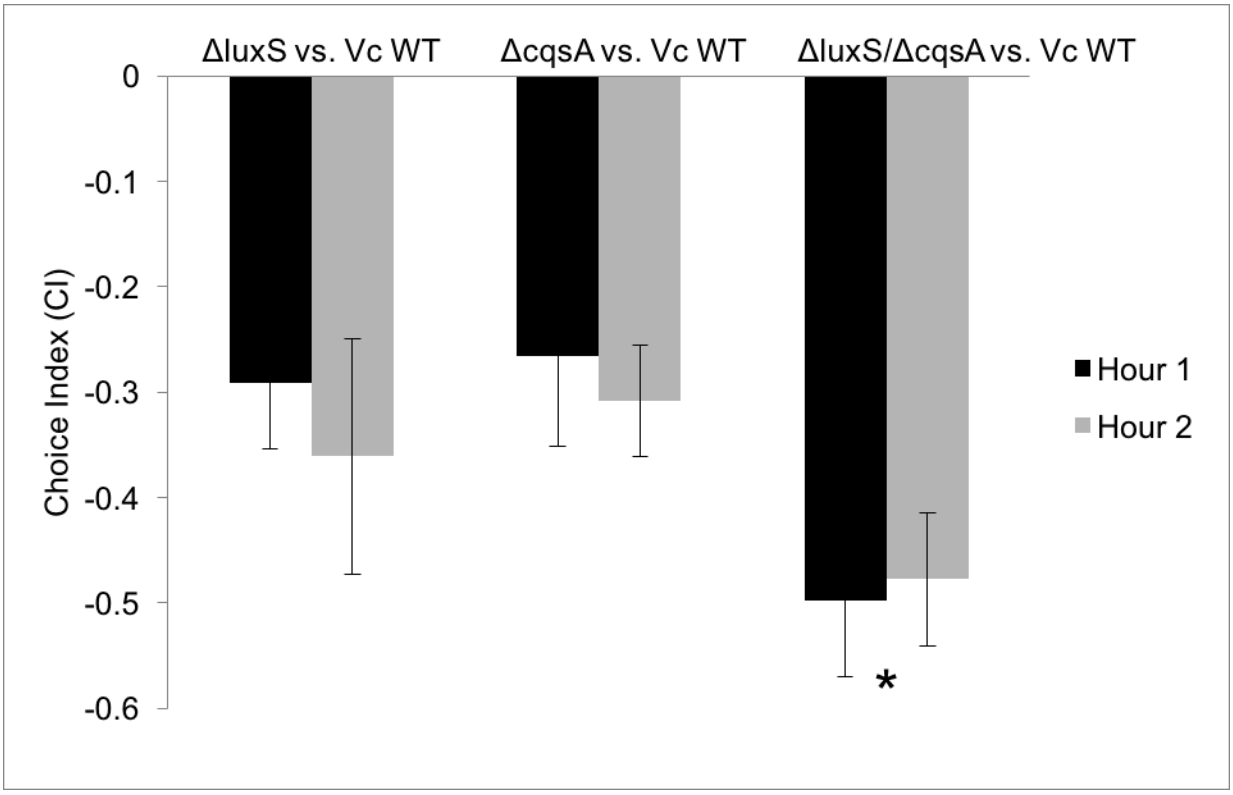


Figure 3.2. Choice Index calculations showing preference of *C. elegans* N2 towards *V. cholerae* C6706 WT (control) over deletion mutant strains of *V. cholerae* C6706, ***Δ****cqsA,* ***Δ****luxS,* ***ΔluxS /Δcqs****A* (test). All choice indexes calculated had $p < 0.05$ when compared to CI = 0 (no preference between test and control strain). * indicates $p < 0.05$ calculated between choice indexes for each trial (*ΔcqsA* v. Vc WT compared to *ΔluxS* v. Vc WT and *ΔluxS /ΔcqsA* v. Vc WT, etc.)

3.2.3 *C. elegans* can sense various AI molecules, with preferences

Knowing that AIs are playing a role in chemotaxis, it prompted investigation of the chemotaxis behavior of *C. elegans* when allowed to choose between two other non-pathogenic *Vibrio* strains when compared to the pathogenic *V. cholerae*. The autoinducers produced by *V. fischeri* and *V. harveyi* are different from *V. cholerae,* as well as each other. Through preliminary experiments, it was found that these *V. fischeri* and *V. harveyi* strains do not significantly decrease the lifespan of *C. elegans,* which is noted in the presence of *V. cholerae* (data not shown). Choice index assays were carried out, and preference was observed towards both *V. fischeri* and *V. harveyi* (Figure 3.3). The production of autoinducers in *V. fischeri* and *V. harveyi* is most likely playing a role in the health and behavior of *C. elegans*. Preference towards *V. fischeri* was evident, but the standard deviation was large, whereas preference of *C. elegans* towards *V. harveyi* was strong and conclusive.

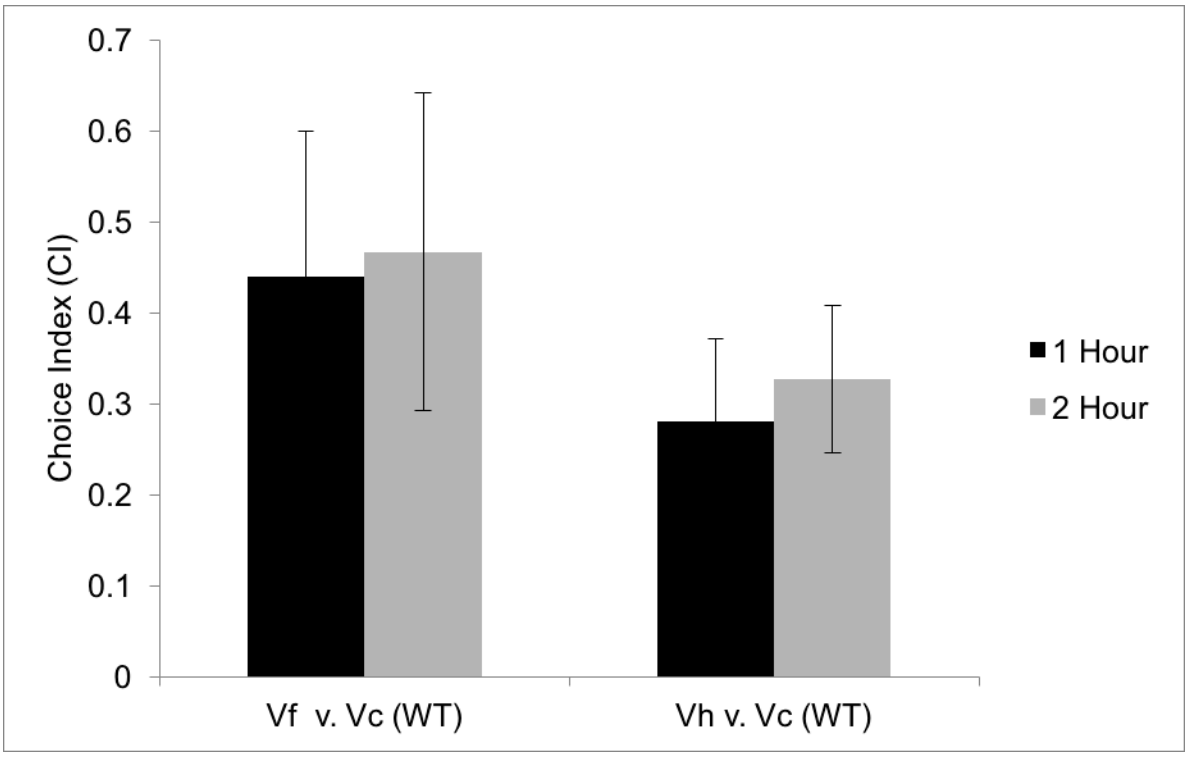


Figure 3.3. Choice Index calculations showing preference of *C. elegans* N2 towards *V. fischeri* and *V. harveyi* when tested against *V. cholerae* C6706 WT as a control. All choice indexes calculated had $p < 0.05$ when compared to CI = 0 (no preference between test and control strain).

3.2.4 Other signaling molecules appear to be involved in Chemotaxis.

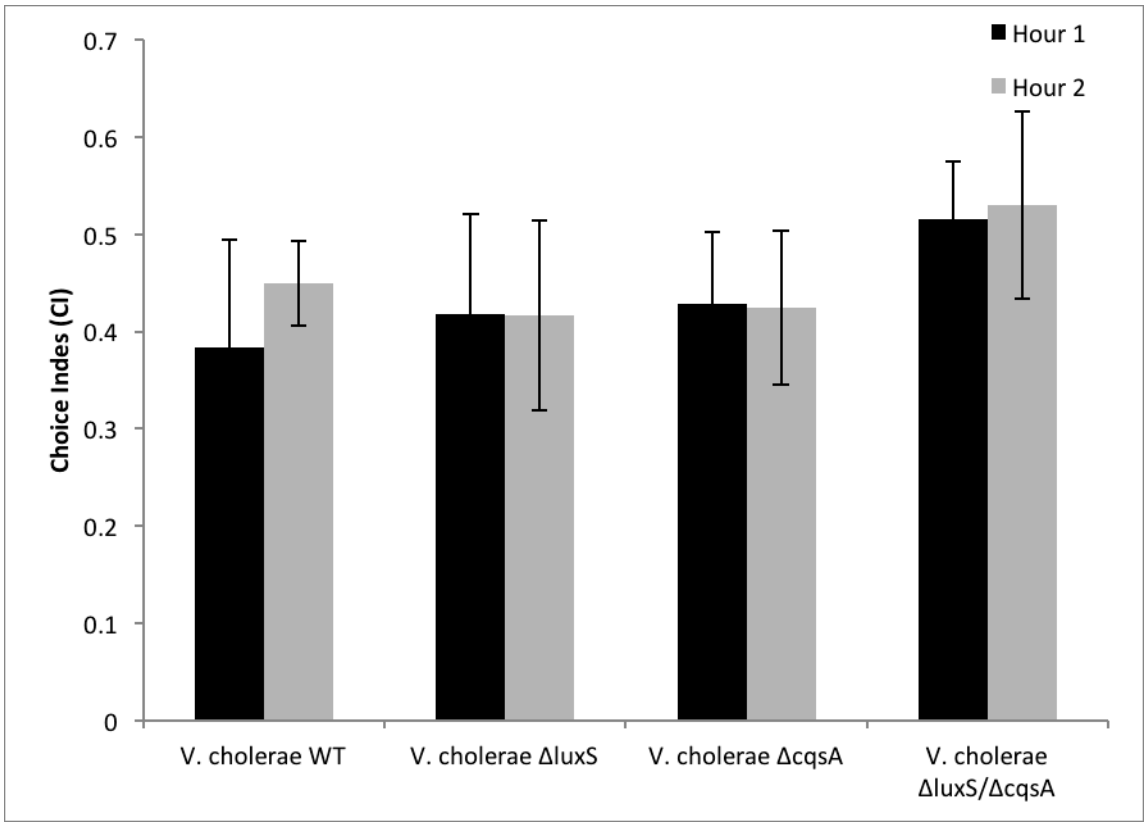
From the results obtained in Figure 3.3, it was seen that *C. elegans* was able to prefer *V. cholerae* WT over mutant strains that were unable to produce corresponding autoinducers. It was then of interest to test these mutant strains (***Δ****cqsA,* ***Δ****luxS,* ***Δ****luxS /****Δ****cqsA)* against *E. coli* OP50 to observe if preference was dependent on the production of autoinducers.

In Figure 3.5-A, the three mutant strains were tested against *E. coli* OP50 as a control. *C. elegans* N2 preference was observed towards all three mutant strains. Preference towards ***Δ****luxS* was higher when compared to the other two strains tested, while preference towards ***Δ****cqsA* was the lowest. On the other hand, preference is still observed towards the other two mutants conveying the notion that other molecules are also playing a role.

Of note in Figure 3.4-A, there is still strong preference towards *V. cholerae* C6706 ***Δ****luxS /****Δ****cqsA* strain (DM) over *E. coli* OP50 even though these two major autoinducers are not being produced. To determine if this preference was caused by something produced extracellularly by the pathogen, overnight cultures of the *V. cholerae* C6706 ***Δ****luxS /****Δ****cqsA* was centrifuged and the supernatant was then added to overnight inoculum of *E. coli* OP50. This bacterial solution was then seeded onto choice index assays plates, and tested against a normal overnight culture of *E. coli* OP50 as a control. The results can be found in Figure 3.4-B, and preference can still be seen towards *V. cholerae* ***Δ****luxS /****Δ****cqsA* strain over *E. coli* OP50. Preference towards ***Δ****luxS /****Δ****cqsA* leads

to the possibility that other signaling molecules produced by *V. cholerae* can be sensed by *C. elegans*.

A


Hour 1
Hour 2
Choice Indes (CI)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
V. cholerae WT
V. cholerae ΔluxS
V. cholerae ΔcqsA
V. cholerae ΔluxS/ΔcqsA

B

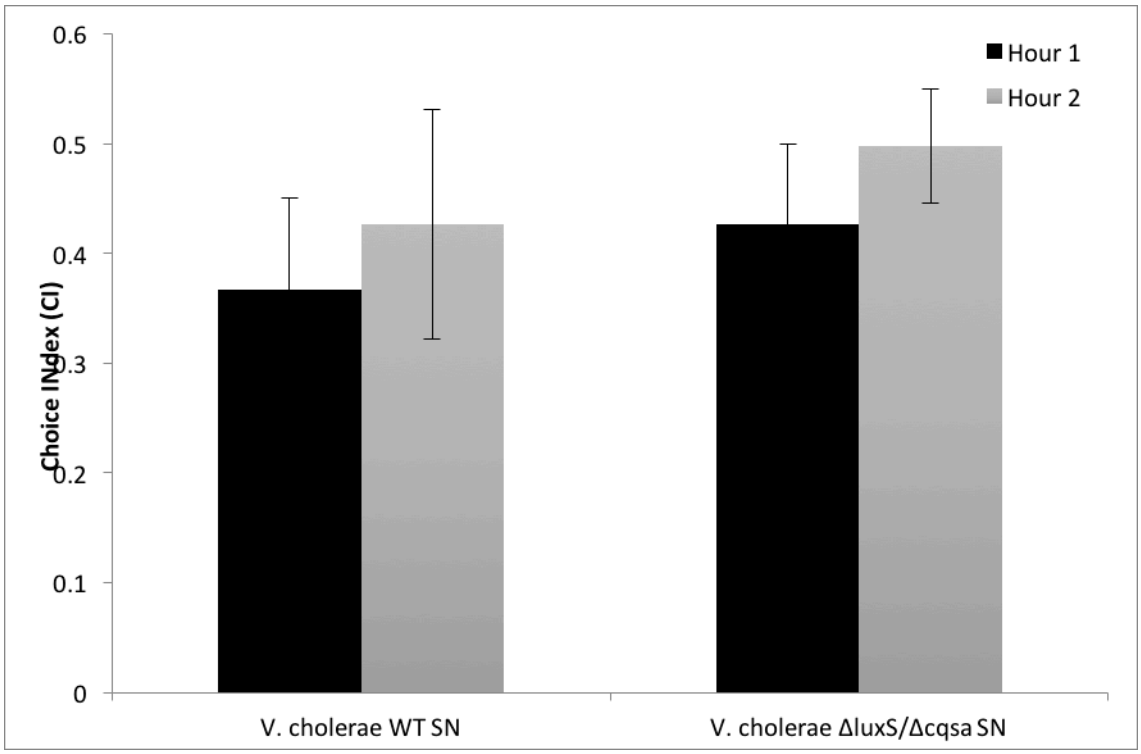

0.6
0.5
0.4
0.3
0.2
0.1
0
Choice INdex (CI)
Hour 1
Hour 2
V. cholerae WT SN
V. cholerae ΔluxS/Δcqsa SN

Figure 3.4. Choice Index calculations showing preference of *C. elegans* N2. **A**) Preference towards *V. cholerae* C6706 mutant strains (***Δ****cqsA, **Δ**luxS, **Δ**luxS /**Δ**cqsA*) over *E. coli* OP50 (control). **B**) Preference towards *E. coli* OP50 supplemented with supernatant of *V. cholerae C6706* overnight cultures (test) over standard *E. coli* OP50 (control). All choice indexes calculated had $p < 0.05$ when compared to CI = 0 (no preference between test and control strain). * indicates $p < 0.05$ calculated between choice indexes for each trial

3.3 Discussion

While initially studying this strange interaction that is occurring between *V. cholerae* and *C. elegans*, it was uncovered that AI production by this pathogen is indeed playing a role. During this time, a paper was published that described similar results (18). Interestingly, in both instances it was evident that while AIs are playing a role in the attraction of *C. elegans*, some other signaling molecules could also be involved. This area required more research and exploration.

Through this work it was demonstrated that *C. elegans* are able to respond and attract to a wide-range of quorum-sensing molecules produced by *Vibrio* spp. (Figures 3.3 and 3.4). The production of AIs by the *Vibrios* studied overlap in some cases (AI-2), while others are unique to certain species. Some are even very similar, structurally speaking (CAI-1, Vh-CAI-1, C8-HSL). It seems that although all of these AIs cause a behavioral response in *C. elegans*, our model organism has preference towards different ones. When *cqsA* is knocked out in *V. cholerae*, we see a diminished attractive response compared to the *luxS* knockout mutant (Figure 3.3). This response is even more significant when the production of both AIs are simultaneously knocked out as the worms prefer the wild-type strain at a much higher rate. *V. fischeri* and *V. harveyi* are not pathogenic to *C. elegans* (data not shown), and are often studied for their symbiotic relationships in marine organisms. When given the choice of these strains or the pathogenic *V. cholerae*, the worms readily choose *V. fischeri* or *V. harveyi*. The production of the unique AIs from these strains seem to outweigh

the AIs produced by *V. cholerae*. To reiterate, *V. harveyi* produces HAI-1, AI-2, and Vh-CAI-1, whereas *V. fischeri* produces AI-1, AI-2, and C8-HSL. These strains were all grown in similar conditions before testing, but the amounts of AIs produced from each strain was not determined. This could be a potential factor in the attraction we see towards these non-pathogenic *Vibrios* as concentration does play a role in chemotactic response.

While it is interesting that the production of specific AIs cause this attractive behavior in *C. elegans*, it seems there could be something else at play. When both AIs are knocked out in *V. cholerae* (***Δ****luxS* ***/Δ****cqsA*), we still notice strong, significant preference towards this strain over *E. coli* OP50 (Figure 3.5). This result is significant because it opens the possibility that other signaling molecules could be involved in this novel communication pathway. Even when the supernatant of *V. cholerae* ***Δ****luxS* ***/Δ****cqsA* was utilized, preference still occurred towards this strain signifying that these signaling molecules are present outside of the cells. Based on knowledge of the different signaling pathways utilized by *V. cholerae*, different avenues and molecules will be tested in the upcoming chapters.

Chapter 4

c-di-GMP is the major signaling molecule that attracts *C. elegans*, and it is sensed through the AWC neurons

4.1 Introduction

Over time, *V. cholerae* has developed intricate, specific signaling mechanisms that allow it to be successful in the environment and within a host. It often utilizes c-di-GMP to do so as it relays information from the environment. Within the cell, when c-di-GMP levels increase this leads to a more sessile state (92, 117, 118). c-di-GMP is synthesized by diguanylate cyclases (DGCs) that contain a GGDEF domain. Phosphodiesterases (PDEs) degrade the signaling molecule, and they possess either an EAL or HD-GYP domain (119). The genome of *V. cholerae* encodes 31 proteins with the GGDEF domain, 12 proteins with the EAL domain, nine proteins with the HD-GYP domain, and ten proteins with both GGDEF and EAL domains (120, 121). It has been shown that c-di-GMP production plays a crucial role in biofilm formation, which in turn leads to successful colonization of a host (99, 122-124).

Other CDNs have also been reported in other bacteria, specifically c-di-AMP. This molecule is known to function in Gram-positive bacteria by reporting

on DNA integrity and cell membrane stress (24, 27). To date, c-di-AMP production in *V. cholerae* has not been reported. Remarkably, *V. cholerae* is able to produce a hybrid molecule of the two, termed c-GAMP (125). ToxT is a gene that activates the transcription of the virulence genes that are necessary for the pathogenesis of *V. cholerae.* In previous works, it has been shown that ToxT activity, acting through the TarB-VspR pathway, can cause derepression of DncV, leading to increased levels of c-GAMP (3, 30, 125). DncV is the dinucleotide cyclase in *V. cholerae* that is responsible for the production of c-GAMP. It also possesses the ability to synthesize c-di-GMP and c-di-AMP, but it has a greater affinity towards catalyzing the hybrid molecule. An increase in c-GAMP levels is linked to an inhibition of chemotaxis for *V. cholerae* and could aid in the stimulation of colonization and increase infectivity (125).

From the results obtained previously (Chapter 3) highlighting the fact that there may be other signaling molecules causing the attractive behavior of *C. elegans* towards *V. cholerae*, I investigated whether CDNs were playing a role. Much work has been done studying CDN communication on an intracellular level, but research is lacking on whether these signals exist and function outside of cells. To determine how these small molecules are being sensed by the worms, different *C. elegans* strains were tested. Through CI assays, it was determined which neuronal pathways could be involved in sensation. If particular mutants are not attracted towards *V. cholerae* or the molecules it produces in a similar

manner as the N2 strain, then we know that these are required for attractive behavior.

4.2 Results

4.2.1 Possible role of CDNs in interkingdom communication

To initially test this hypothesis, focus was turned to ToxT, the master virulence regulator, and DncV, the diguanylate cyclase of c-GAMP, in *V. cholerae*. DncV and its ability to produce the novel CDN, 3'3' c-GAMP, was discovered in 2012 (125). More recently, its role has been demonstrated in biofilm formation through suppressing motility (126). DncV possesses the ability to produce c-di-GMP and c-di-AMP, but the dominant species in vivo is c-GAMP (125, 126). There are numerous genes in *V. cholerae* that have domains known to catalyze and degrade c-di-GMP. Studying DncV activity and its role in interkingdom communication would not only implicate the novel c-GAMP, but possibly the other CDNs as well. Figure 4.1-A depicts the model for regulation of DncV through the ToxT and VspR pathway (adapted from Davies et al.) (125).

V. cholerae Δ*toxT* and *V. cholerae* Δ*dncV* were obtained and choice index assays were conducted with *C. elegans* N2. In this case, each of the mutant strains were tested individually against the wild-type. If the production of c-GAMP is responsible for attraction of *C. elegans*, then the worms should choose the wild-type lawn over either of the two mutant strains.

The results in Figure 4.1-B show a strong preference of *C. elegans* N2 towards *V. cholerae* WT over *V. cholerae* Δ*toxT* (control). Preference appears to be dependent on the proper functioning of ToxT and production of c-GAMP,

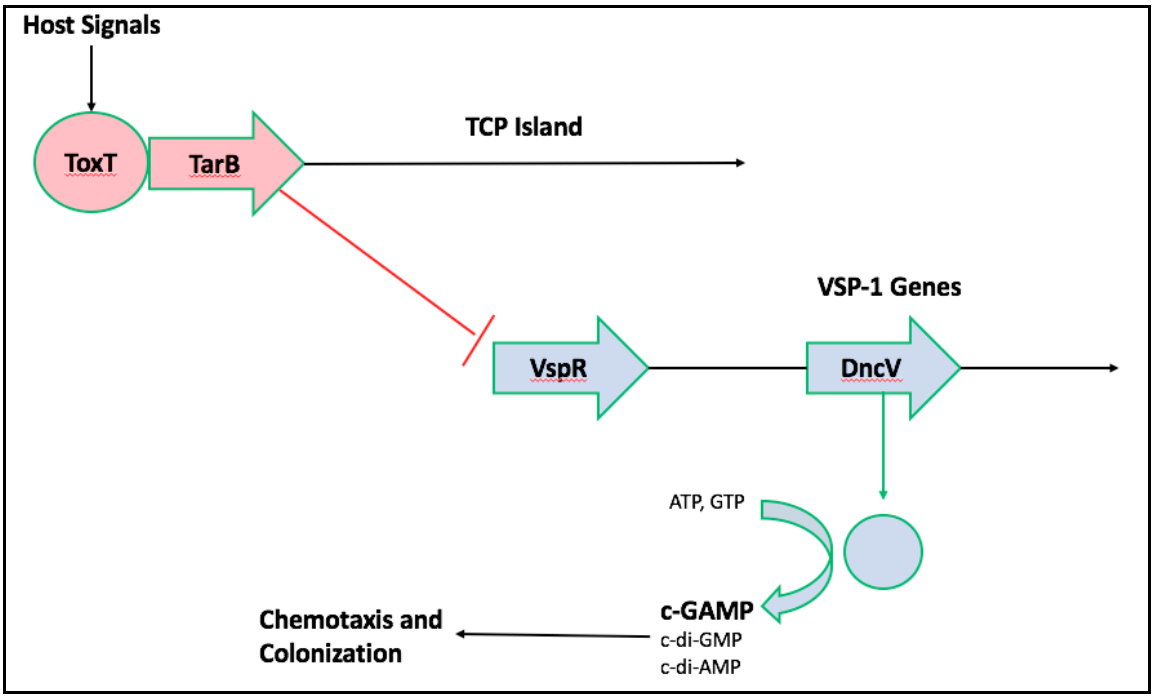
and possibly c-di-GMP and c-di-AMP. Since *C. elegans* preference toward *V. cholerae* seems to be dependent on ToxT, further tests were conducted to see if the di-nucleotide cyclase that is unique to *V. cholerae*, DncV, is also playing a role. This cyclase lies downstream of the ToxT cascade, in which de-repression of VSP-1 increases DncV activity. Choice index assays were conducted testing preference of *C. elegans* against *V. cholerae ΔdncV* and *V. cholerae* WT as a control. The results show a strong preference towards *V. cholerae* WT over *V. cholerae ΔdncV*. This CI was significantly greater when compared to the CI calculated for *V. cholerae* WT over *V. cholerae* Δ*toxT* . Based on these results, the hypothesis is further reaffirmed that *C. elegans* preference towards *V. cholerae* is dependent on these genes, as well as the production of CDNs.

As stated earlier, c-di-GMP is synthesized by the diguanylate cyclases containing a GGDEF domain and degraded by the phosphodiesterases containing either an EAL or HD-GYP domain (119). pAT1568, a plasmid that overexpresses the phosphodiesterase (EAL domain) of the *vieA* gene from the L-arabinose-inducible pBAD promoter (127), was introduced into the *V. cholerae* Δ*luxS/*Δ*cqsA* strain by electroporation. CI assays were conducted to determine if the activity level and production of c-di-GMP effected the behavior of *C. elegans*. The results can be seen in Figure 4.2.

When the *V. cholerae* Δ*luxS/*Δ*cqsA* strain is used as the background for the two plasmids, the behavior of *C. elegans* followed an interesting pattern. When the empty vector, pBAD33, was induced, worms readily chose this lawn

over its standard laboratory food, *E. coli* OP50. The CI here is comparable to that seen in Figure 3.2, where we confirmed that something other than AIs are involved in attractive chemotaxis. Interestingly, when pAT1568 is being induced in this strain no preference is noted in *C. elegans* between this lawn and *E. coli* OP50 (CI ≈ 0). Without induction, preference towards *V. cholerae* Δ*luxS*/Δ*cqsA* + pAT1568 is restored, to an extent, as the worms choose this lawn more often than *E. coli* OP50 (data not shown). This result shows us that ability for *V. cholerae* to produce and regulate c-di-GMP production is important for interkingdom communication. It also reaffirms that although the production of the major AIs aids in the ability of *V. cholerae* to attract a host, production of c-di-GMP seems to be indispensable.

A


Host Signals
ToxT
TarB
TCP Island
VSP-1 Genes
VspR
DncV
ATP, GTP
c-GAMP
c-di-GMP
c-di-AMP
Chemotaxis and
Colonization

B

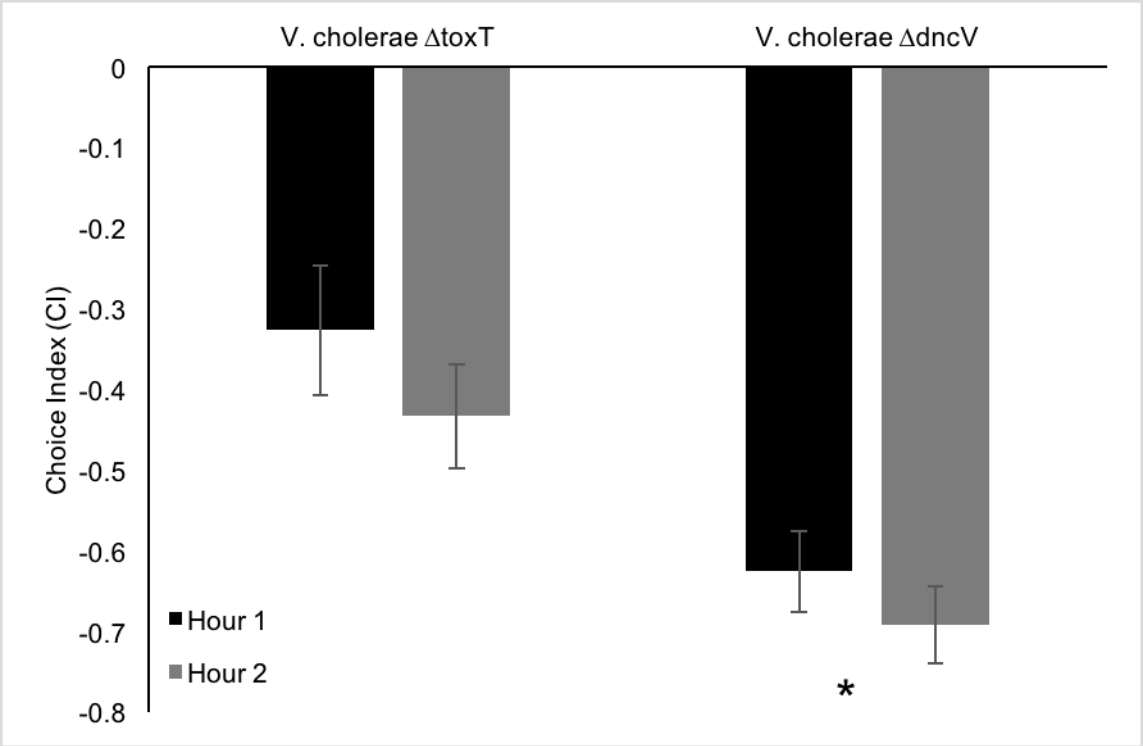

V. cholerae ΔtoxT
V. cholerae ΔdncV
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
Choice Index (CI)
Hour 1
Hour 2
*

Figure 4.1. A) Model for regulation of DncV through the ToxT and VspR pathway. Host signals induce ToxT activity and TarB is transcribed from the TCP Island. Activity of TarB downregulates the transcriptional repressor, VspR. This results in de-repression of VSP-1 genes, which includes DncV. DncV increases cellular concentration of c-GAMP, and possibly the other CDNs, resulting in inhibition of chemotaxis (125). **B**) Choice Index calculations showing preference of *C. elegans* N2. Preference towards *V. cholerae* C6706 WT when tested against *V. cholerae* Δ*toxT* and *V. cholerae* Δ*dncV*. All choice indexes calculated had $p < 0.05$ when compared to CI = 0. * represents $p < 0.05$ when CI for *Vc* WT v. *Vc* Δ*dncV* was compared to CI for *Vc* WT v. Δ*toxT*.

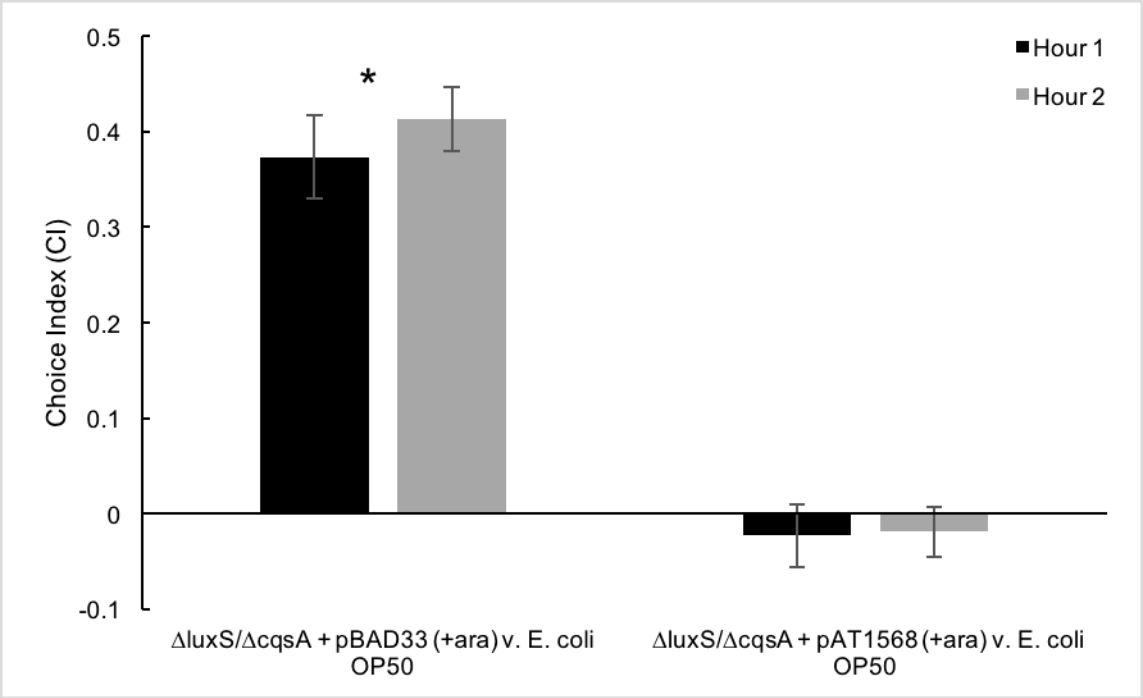


Figure 4.2. Choice indexes calculated using *V. cholerae* Δ*luxS*/Δ*cqsA* transformed with either pBAD33 or pAT1568 tested against *E. coli* OP50. Results are the average of three independent experiments, and error bars are standard error of the mean. *p* values were calculated by using the unpaired Student T-test (pBAD33 v. pAT1568). * indicates $p < 0.05$.

4.2.2 *C. elegans* can sense and respond to c-GAMP and c-di-GMP at an optimal concentration of 1nM

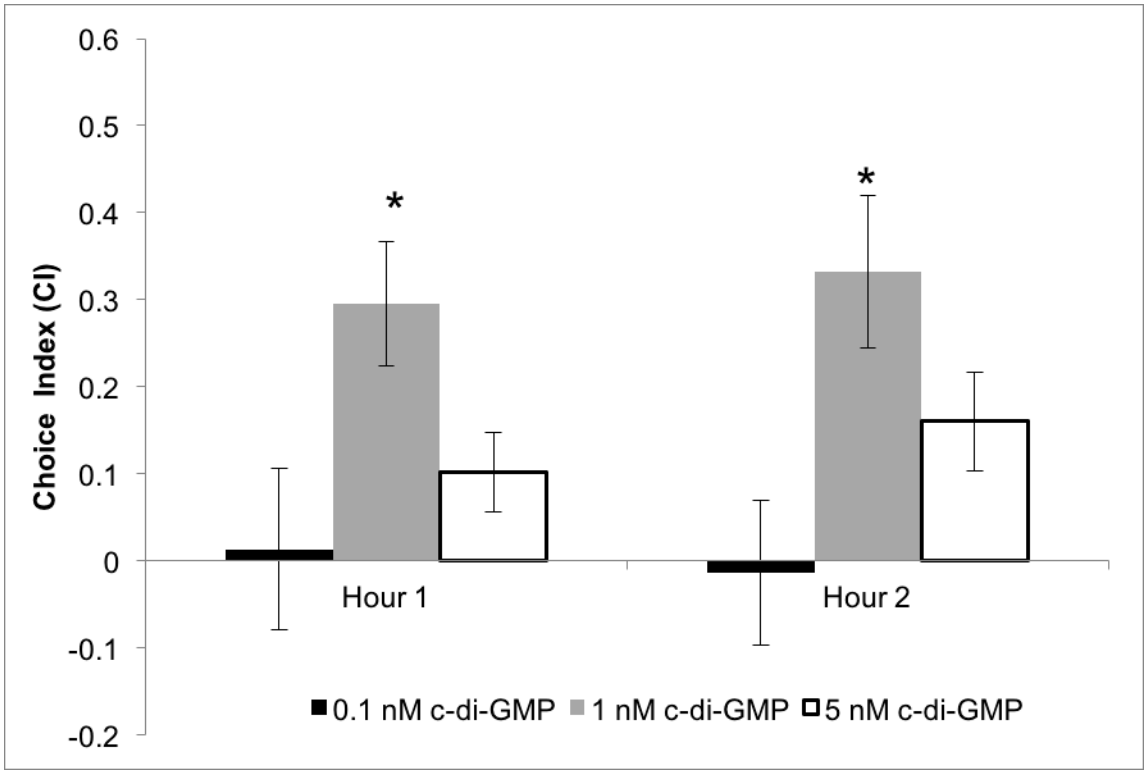
To get a better understanding of the role of all three known CDNs, pure solutions of each molecule was tested. Based on previous studies, a range of concentrations was tested (0.1nM – 5nM). These concentrations were added to overnight cultures of *E. coli* OP50 after growth. Choice index assays were conducted with *E. coli* OP50 supplemented with various concentrations of CDNs (serving as the test strain) and standard *E. coli* OP50 (serving as the control strain). If these molecules can be sensed by *C. elegans* then preference will be noted towards OP50 supplemented with the CDN. This also allows minimum concentration of each CDN that can be sensed by *C. elegans* to be determined. In accordance with the fact that ToxT and DncV function to produce c-GAMP in *V. cholerae*, if we supplement the proper CDNs to these mutant strains it should restore chemoattraction in *C. elegans*.

Figure 4.3-A and B shows that 1nM of both c-di-GMP and c-GAMP are able to cause a strong attractive behavior in *C. elegans*. There was a stronger chemotactic response seen toward the *E. coli* OP50 supplemented with c-GAMP. However, as shown in Figure 4.3-C, this same preference isn't noticed when tested with c-di-AMP, and the worms seem to be repulsed by the presence of this molecule. This is interesting in that preference seems to be influenced by both c-di-GMP and c-GAMP, and not c-di-AMP ruling out the possibility that maybe preference by *C. elegans* could just be influenced by the presence of small DNA

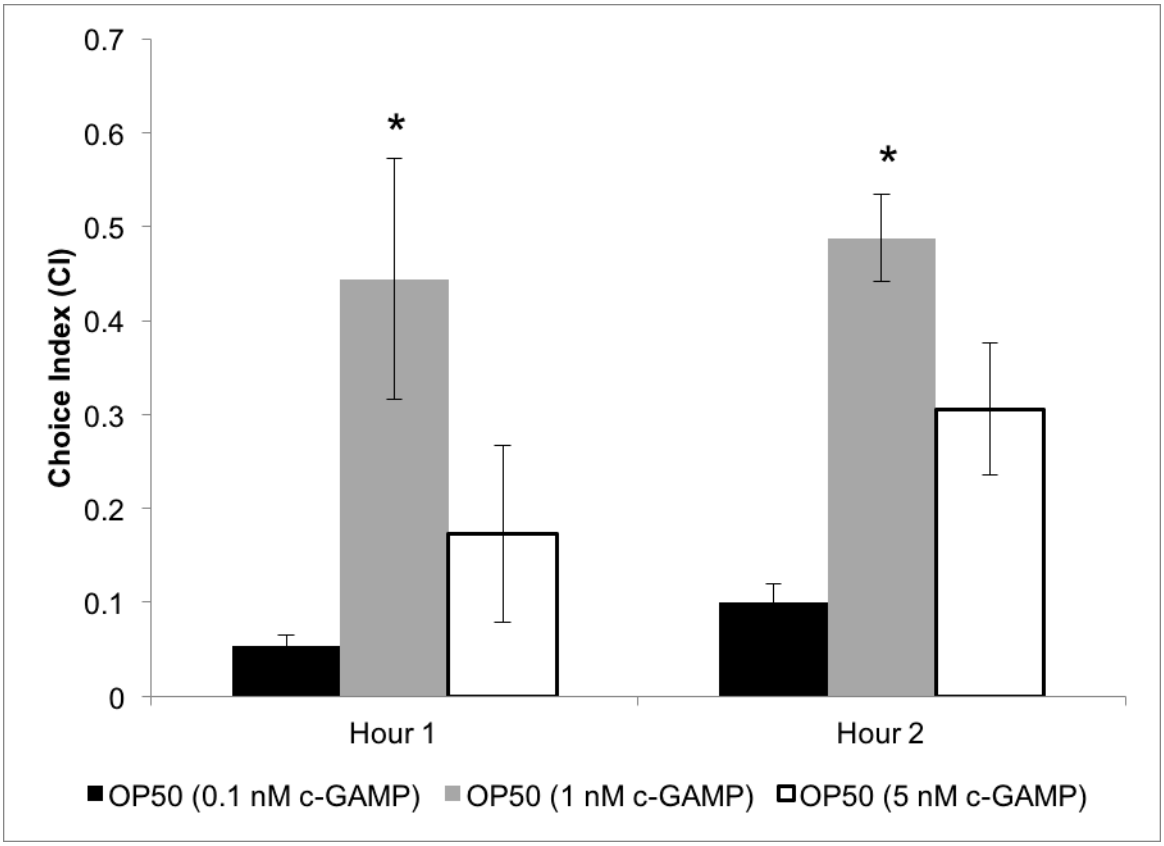
molecules. 0.1nM concentration of c-di-AMP was not tested because this concentration did not produce much of a response based on the results from Figure 4.3-A and B. While there was still preference noticed towards both c-di-GMP and c-GAMP at a concentration of 5nM, this was not as great as the 1nM concentration for each molecule. Saturation of these molecules could explain as why we do not see similar preference at this concentration. Perhaps greater diffusion into the CI plates is occurring which would affect *C. elegans* ability to respond in a distinct, directional manner.

Referring back to Figure 4.1, it is noted that when ToxT and DncV are not functioning in *V. cholerae*, *C. elegans* is not able to sense these bacterial lawns and readily prefer the normal *V. cholerae* WT strain. To test if this response could be reversed, supplementation of c-di-GMP and c-GAMP at a concentration of 1nM were added to Δ*toxT* and Δ*dncV* overnight cultures and chemotaxis assays were conducted. What was found is very interesting in that when these mutants were supplemented with 1nM concentrations of both c-di-GMP and c-GAMP, chemoattraction of *C. elegans* was seen towards these lawns (Figure 4.4-A, B).

A


0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
Choice Index (CI)
*
*
Hour 1
Hour 2
0.1 nM c-di-GMP
1 nM c-di-GMP
5 nM c-di-GMP

B


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Choice Index (CI)
*
*
Hour 1
Hour 2
OP50 (0.1 nM c-GAMP)
OP50 (1 nM c-GAMP)
OP50 (5 nM c-GAMP)

C

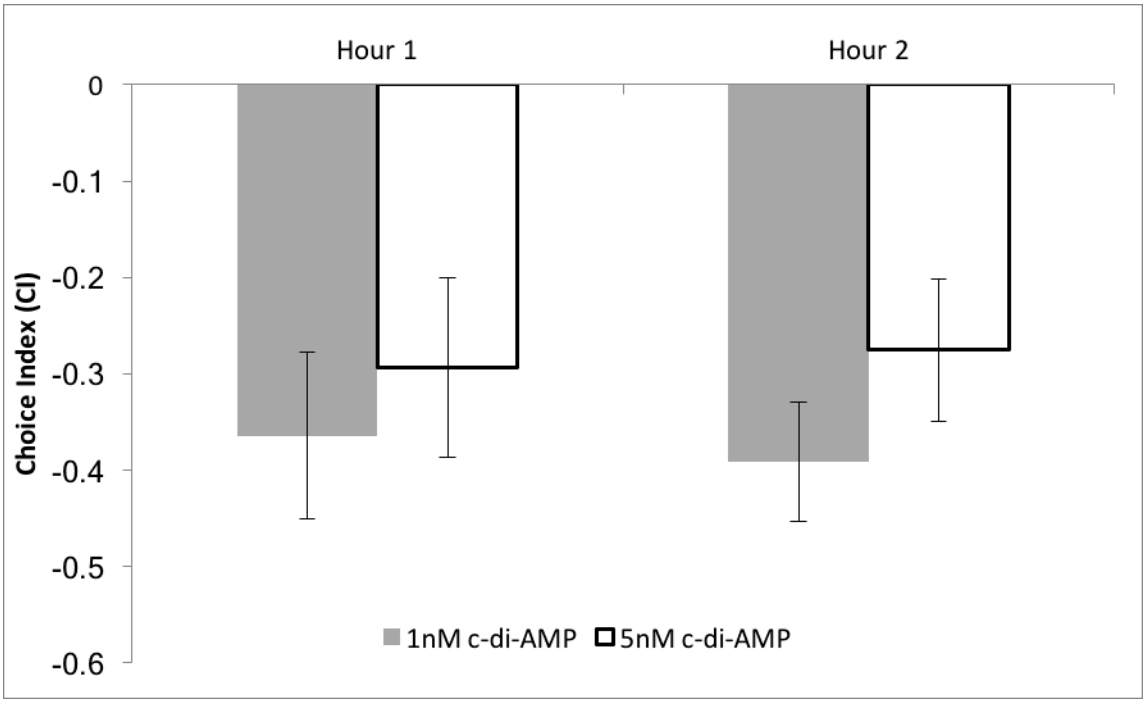
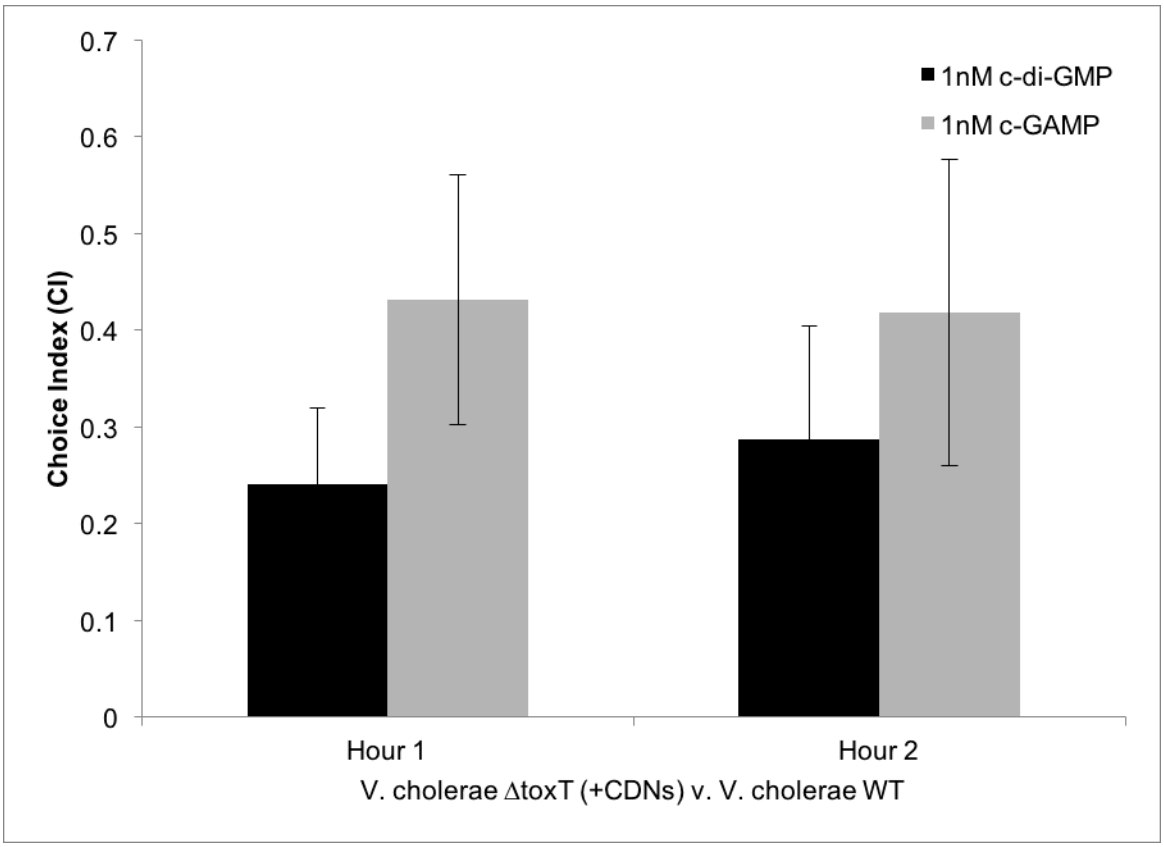


Figure 4.3. Chemotaxis index showing preference of *C. elegans* N2. A) Preference towards *E. coli* OP50 supplemented with c-di-GMP at an optimal concentration of 1nM. B) Preference towards *E. coli* OP50 supplemented with c-GAMP at an optimal concentration of 1nM. C) Preference towards *E. coli* OP50 (control) over *E. coli* OP50 supplemented with c-di-AMP. CI's calculated for 0.1nM concentrations of c-di-GMP and c-GAMP did not yield significance. All other CI's calculated had $p < 0.001$ when compared to CI = 0. * indicates $p < 0.05$ calculated between different concentrations of CDNs.

A


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Choice Index (CI)
1nM c-di-GMP
1nM c-GAMP
Hour 1
Hour 2
V. cholerae ΔtoxT (+CDNs) v. V. cholerae WT

B

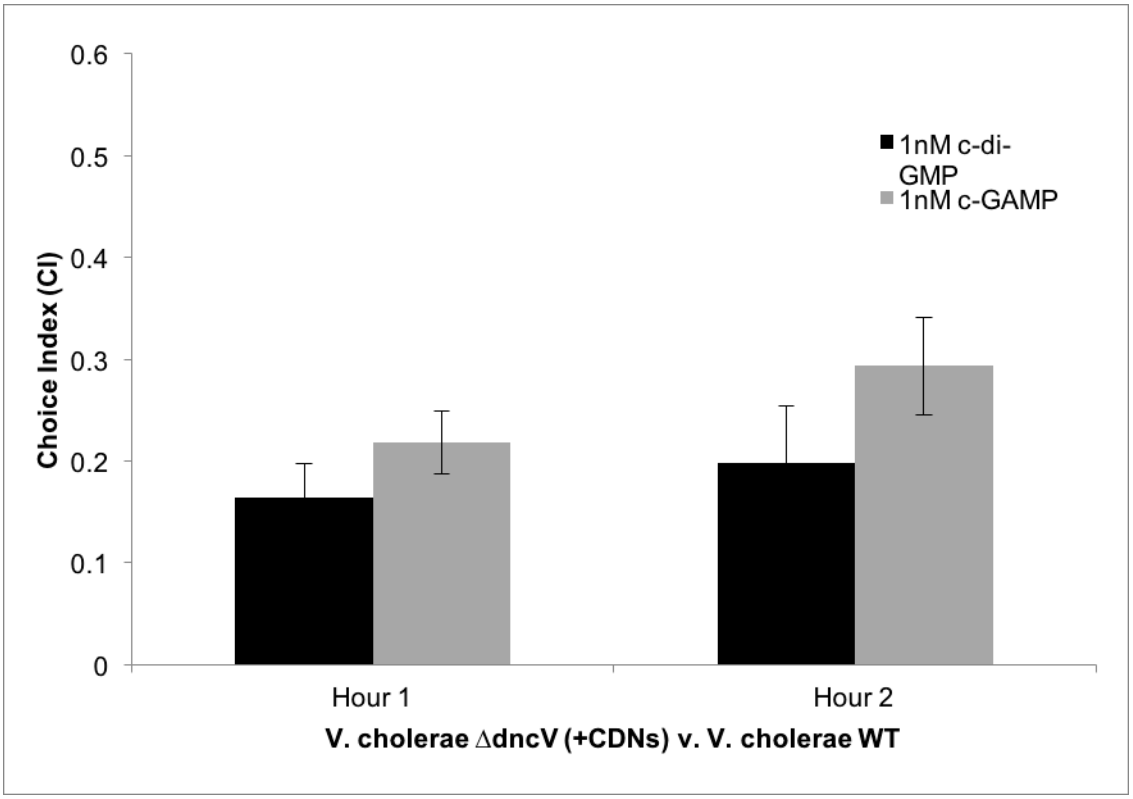


Figure 4.4. Chemotaxis index showing preference of *C. elegans* N2. **A**) Preference towards *V. cholerae* Δ*toxT* supplemented with 1nM c-di-GMP and 1nM c-GAMP over *V. cholerae* WT. **B**) Preference towards *V. cholerae* Δ*dncV* supplemented with 1nM c-di-GMP and 1nM c-GAMP over *V. cholerae* WT. All choice indexes calculated had $p < 0.05$ when compared to CI = 0.

4.2.3 c-di-GMP is the major signaling molecule that attracts *C. elegans*

If the different CDNs are able to attract *C. elegans*, then these molecules must exist in the environment outside of the bacterial cell. To investigate this possibility, we harvested the supernatant and the cell pellet from the wild type *V. cholerae* overnight culture (~ 10^9 CFU/ml) by centrifugation and measured the concentration of c-di-GMP, c-di-AMP, and cGAMP (if they were present) from the two portions using LC-MS assay. Pure CDN solutions were used as standards for the assay.

As shown in Figure 4.4, c-di-GMP was detected in both the cell lysate and the supernatant. The concentration was calculated as 177.7±49.6 nM in the cell lysate and 19.9±4.3 nM in the supernatant. No c-GAMP was found in the cell lysate or the supernatant. These results suggest that c-di-GMP may be the additional signal that attracts *C. elegans* towards *V. cholerae*. Taking this result into account, we consider that c-di-GMP is the major signaling molecule produced by *V. cholerae* that attracts *C. elegans*.

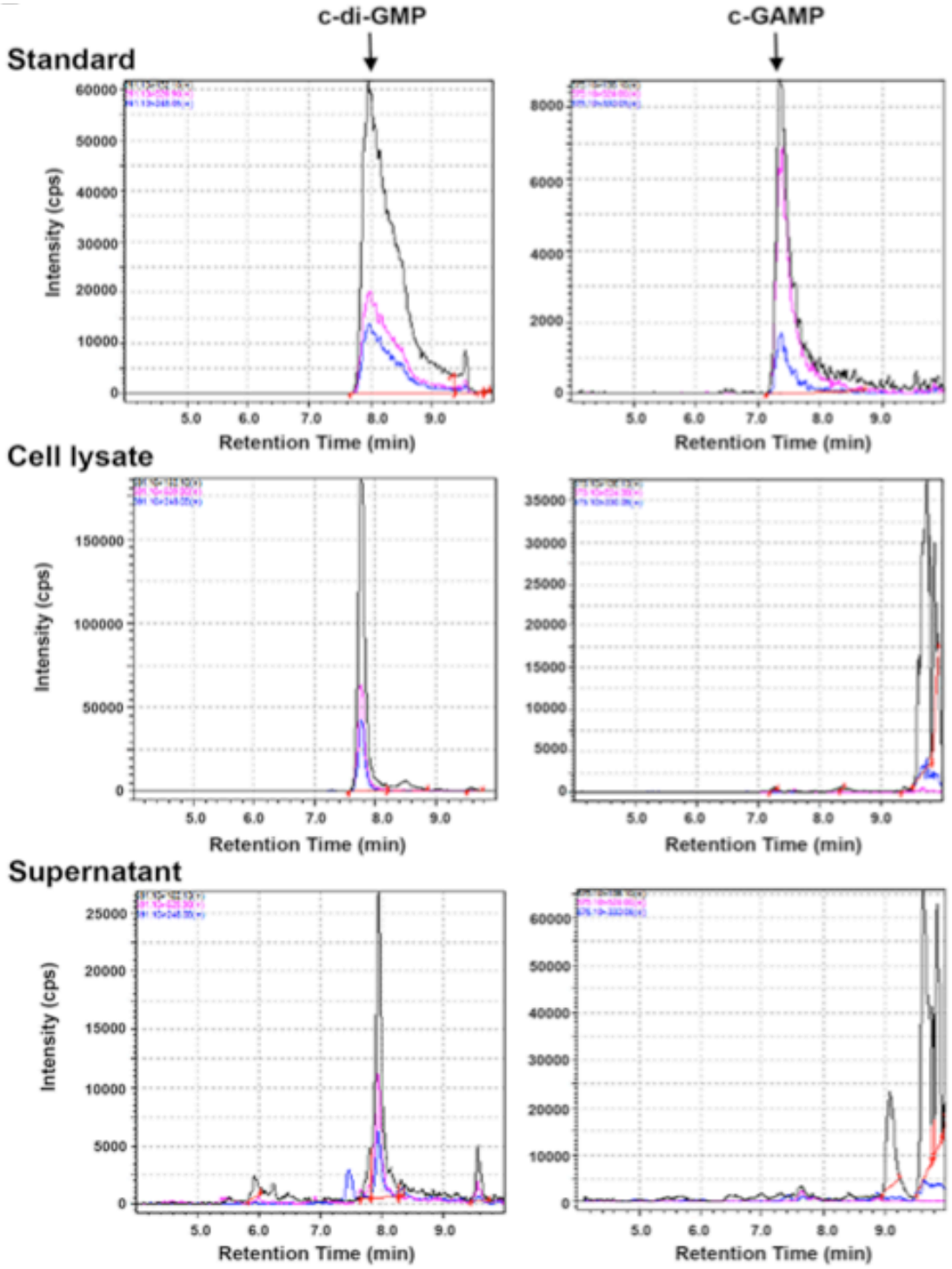

c-di-GMP
c-GAMP
Standard
Cell lysate
Supernatant
Intensity (cps)
Retention Time (min)

Figure 4.5. LC-MS assay of c-di-GMP and cGAMP from the cell lysate and supernatant of wild type *V. cholerae* overnight culture. c-di-GMP and cGAMP were monitored by electrospray ionization mass spectrometry in positive mode with multiple reaction monitoring (MRM) at the transitions of m/z 691.10→152.10, 691.10→539.90, 691.10→248.05 and m/z 675.10→136.10, 675.10→524.00, 675.10→330.05, respectively.

4.2.4 *C. elegans* is able to sense bacterial c-di-GMP through the AWC^{ON} and AWC^{OFF} neurons

The chemosensory system in *C. elegans* allows the organism to detect food, develop, avoid danger, mate, etc. There are 11 pairs of amphid chemosensory neurons in *C. elegans*. Of these, the ASE gustatory neurons are known to sense salts and water- soluble attractants, and the AWA and AWC olfactory neurons are required to sense attractants with volatile odors. Different chemical signals are sensed by different G protein-coupled chemoreceptors in the chemosensory neurons and then passed to two major signal transduction sensory channels, the cGMP-gated TAX-2/TAX-4 channel and the lipid-sensing OSM-9/OCR-2 TRPV channel (82).

Since the neuronal network has been extensively studied and documented in *C. elegans*, it will be of particular interest for us to determine how they are able to sense different CDNs. To accomplish this task we particularly looked at c-di-GMP because this molecule was shown to be present extracellularly from *V. cholerae* and elicited strong chemoattraction of *C. elegans*.

To conduct this part of the experiment, CI assays were used to determine how *C. elegans* neuronal mutants will respond to *V. cholerae* as well as c-di-GMP. It has previously been shown that *C. elegans* is able to sense different autoinducers produced by *V. cholerae* through the AWC^{ON} neuron (18). With this information in hand, we can first study if this is the case for the CDNs. To do

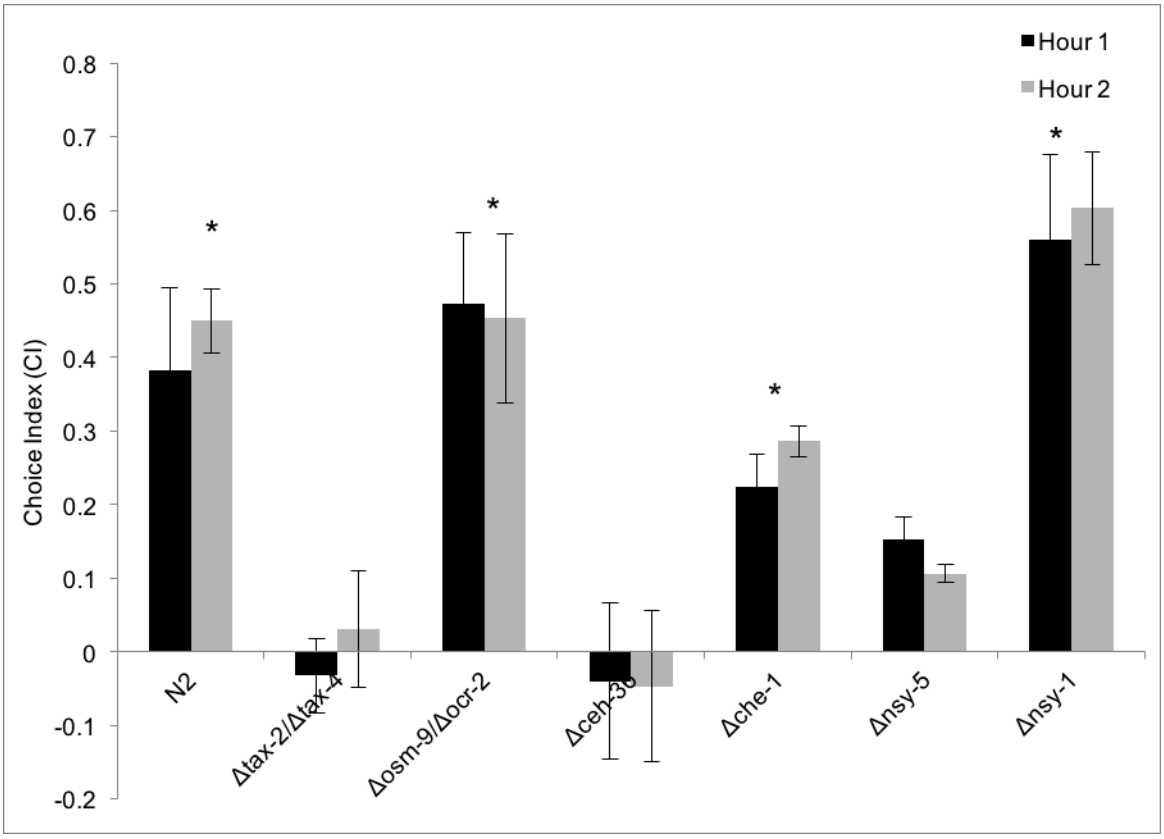
this, a series of neuronal mutants will be tested that are known to play a role in chemosensation. These mutants range from pathways that start more general, with the sensory channels, to more specific, testing individual neurons. The *C. elegans* strains used are listed in Table 4.1.

First, previous work was confirmed in Figure 4.6-A showing that *C. elegans* does in fact need a functional AWC^{ON} neuron for chemoattraction towards *V. cholerae*. In Figure 4.6-B, we investigated to see if the same held true when exposed to c-di-GMP. The *tax-2/tax-4* mutant was on longer attracted to c-di-GMP, while the *osm-9/ocr-2* mutant was still attracted, signifying that the cGMP-gated TAX-2/TAX-4 channel is required (Fig 4.6-B). Mutation to *ceh-36* causes defections in developing functional AWC and ASEL neurons, and mutation to *che-1* causes inability to develop functional ASEL and ASER neurons. Mutations to *nsy-5* and *nsy-1* cause inability to develop the AWC^{ON} and AWC^{OFF} neuron, respectively. Figure 4 shows that loss of functional AWC or ASEL neuron (*ceh-36*) kept the worms from the attraction, and worms without the functional ASEL and ASER neuron (*che-1*) were still attracted, indicating the AWC neuron is involved. Further assay with the *nsy-5* and *nsy-1* mutants revealed that the AWC^{ON} and AWC^{OFF} neurons are both necessary because no preference was observed when studying these mutants. Based on these results, it can be concluded that *C. elegans* senses c-di-GMP through the AWC^{ON} and AWC^{OFF} neurons and the cGMP-gated TAX-2/TAX-4 channel.

Table 4.1. *C. elegans* mutant strains to be tested to elucidate neuronal networks sensing CDNs.

***C. elegans* strain**	**Functional mutation**
tax-2/tax-4	Defective in developing cGMP gated channels
osm-9/ ocr-2	Defective in developing TRPV gated channels
ceh-36	Defective in developing functional AWC + ASEL Neurons
che-1	Defecting in developing functional ASEL + ASER Neurons
nsy-5	Fail to develop AWC^{ON} Neuron
nsy-1	Fail to develop AWC^{OFF} Neuron

A


Hour 1
Hour 2
Choice Index (CI)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
*
*
*
*
N2
Δtax-2/Δtax-4
Δosm-9/Δocr-2
Δceh-36
Δche-1
Δnsy-5
Δnsy-1

B

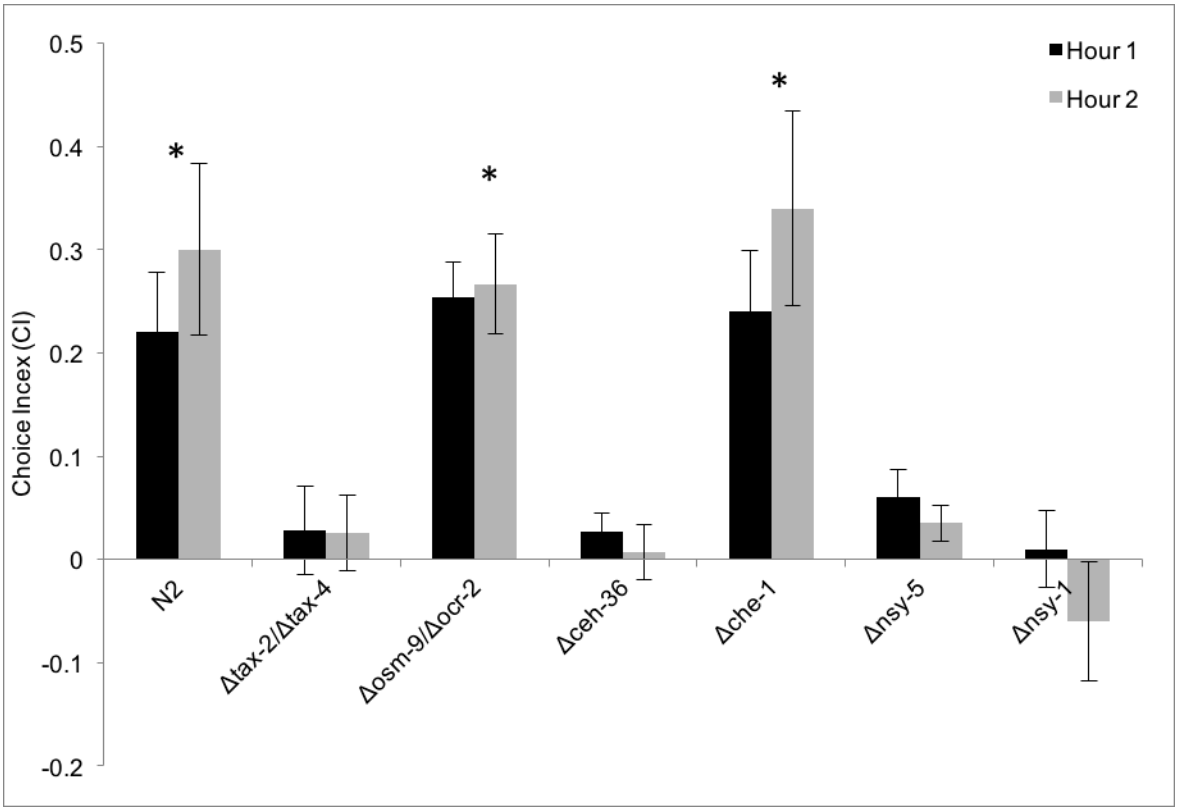


Figure 4.6. A) Choice Index showing preference N2 and different neuronal deficient *C. elegans* strains and their preference towards *V. cholerae* WT. B) Choice Index showing preference of N2 and different neuronal deficient *C. elegans* strains and their preference towards 1nM c-di-GMP. The choice index of those strains that show significant preference (*) are those genes that are not involved in sensation of the pathogen. Results are the average of three independent experiments, and error bars are standard error of the mean. *p* values were calculated by using the unpaired Student T-test.

4.4 Discussion

To be successful, *V. cholerae* must be able to establish within the gut of a host and correctly utilize many transcription factors to influence virulence (11, 13). CDNs are important second messengers, and as research advances new discoveries are being made about how they affect and influence many cellular pathways (14, 128-131). CDNs have been studied extensively in bacteria, but new roles are being observed and studied in eukaryotes (27). c-di-GMP and c-di-AMP have been an area of interest for a number of years, and have different roles in Gram-negative and Gram-positive bacteria respectively (81). In *V. cholerae*, c-di-GMP has been shown to regulate things such as virulence factors, flagellum biosynthesis, and biofilm formation (127, 132, 133). C-di-AMP is more prevalent in Gram-positive bacteria and can function to report on DNA integrity, cell membrane stress, and can play a role in establishing bacterial infection within a host (134, 135). Recently, a novel cyclase has been identified in *V. cholerae*, DncV, that is able to synthesize the hybrid molecule c-GAMP. In this study, it was hypothesized that CDNs produced by *V. cholerae* are the underlying molecules that are aiding in the communication between this bacterium and a eukaryotic host.

It was shown through choice index assays that DncV and ToxT activity are required in *V. cholerae* for attractive chemotaxis in *C. elegans* (Figure 4.1). In a study done by Davies et al., in vitro studies revealed that DncV is able to produce c-di-GMP and c-di-AMP, but preferentially produces more of the hybrid molecule,

c-GAMP (125). Using pure solutions of the known CDNs and testing different concentrations, it was seen that c-di-GMP and c-GAMP were able to sensed and responded to by *C. elegans*. Interestingly enough the opposite behavior was observed for c-di-AMP, which could be explained because this is typically utilized in Gram-positive bacteria (Figure 4.2). It could be possible that *C. elegans* is using this sensation mechanism to distinguish between viable food sources (136). The sensation and attraction towards the CDNs, specifically c-di-GMP and c-GAMP, occur in a concentration dependent manner. When these concentrations of c-di-GMP and c-GAMP were introduced externally to the *V. cholerae* Δ*toxT* and Δ*dncV* mutant strains, attraction was restored in *C. elegans* (Figure 4.3). These findings together demonstrate that the production of these CDNs are crucial in the *C. elegans* chemotactic response.

To date, tests done on DncV, c-GAMP, and the other CDNs have been focused mainly on the production and cellular response that takes place inside the cell. In this study, it was important to determine that these CDNs exist outside of *V. cholerae* so that they can be sensed by *C. elegans*. To answer this question, *V. cholerae* was grown in the same manner as previous tests in which chemoattraction was observed, and samples were sent off for LC-MS. Under these conditions, only c-di-GMP was found to be present in the supernatant of samples, and no c-GAMP or c-di-AMP was detected. This does not take away from the fact that *C. elegans* elicits a behavioral response to these molecules, just that under these experimental settings it seems that c-di-GMP is the major

molecule being sensed by the host in vivo. Different growth parameters would have to be checked and analyzed to gain further insight on the production of the hybrid molecule in vivo. c-di-AMP is the only one of the three CDNs that has been reported to be secreted (102). Our result suggests c-di-GMP may also be secreted and the mechanism is waiting to be explored.

Figure 4.5 further shows that c-di-GMP production in *V. cholerae* is vital in this interkingdom communication pathway. Introduction of plasmids to *V. cholerae* that have been constructed to increase or decrease intracellular levels of c-di-GMP proved a valuable tool in determining the importance of c-di-GMP. When AI production was abolished in *V. cholerae* (Δ*cqsA* / Δ*luxS*) and pAT1568 was introduced (decrease in intracellular c-di-GMP), there was strong preference towards the strain expressing the empty vector (pBAD33).

With this knowledge in hand, we next wanted to delve into the topic of how *C. elegans* was able to sense c-di-GMP. Through the results presented in Figure 4.6, it was found that sensation of c-di-GMP is through the AWC neurons and the cGMP-gated TAX-2/TAX-4 channel. *C. elegans* use 16 pairs of chemosensory neurons to find food, avoid dangerous conditions, develop properly, and mate. The AWC neurons are a pair of asymmetric amphid olfactory neurons (AWC^{ON} and AWC^{OFF}) that sense volatile odors and are linked to attractive behavior (82). The two neurons express different chemoreceptors to discriminate diverse odors, however, it was reported that both ON and OFF neurons are required to sense

benzaldehyde (137). Here we showed that both AWC^{ON} and AWC^{OFF} neurons are required to sense c-di-GMP. This is different from the case of sensing *V. cholerae* CAI-1, which only requires the AWC^{ON} neuron (18). Unlike the gustatory neurons, olfactory neurons are able to detect long-range signals usually at nanomolar concentrations. In consensus with this, the concentration of c-di-GMP from the overnight *V. cholerae* culture was measured to be around 20 nM. Pure solution of c- di-GMP, as low as 1 nM, could also be sensed and cause attraction. These findings together provide an overview of the mechanisms of action that occur during *C. elegans* attraction towards *V. cholerae*. We know these signaling molecules, specifically c-di-GMP, are involved, but does this communication have an effect on the host and how?

Chapter 5

c-di-GMP exposure causes physiological changes in *C. elegans* that affects its health by suppressing its innate immune response

5.1 Introduction

Knowing that *C. elegans* is able to respond and attract towards c-di-GMP produced by *V. cholerae*, we wanted to determine how this communication affects the host. The hypothesis is that attractive behavior towards a stimulus must result in underlying physiological changes in *C. elegans*. Many attributes of *C. elegans* were tested in response to c-di-GMP, over both short and long term exposure to the signaling molecule.

Being a bacteriovore, *C. elegans* often encounters pathogens in the environment and has developed different methods to protect itself. These methods include behavioral responses, physical barriers, and an innate physiological defense. I have touched base previously on the behavioral responses of *C. elegans* towards pathogens, and this is its first line of defense. Through the olfactory senses, the worms are able to avoid certain pathogens

through a naïve or learned response. This has been shown through innate ability of *C. elegans* to avoid *Serratia marcescens* by detecting Serrawettin W2, and in a learned ability to avoid *Pseudomonas aeruginosa* after initial exposure (138, 139). There are also conserved regions on pathogens known as Microbe Assisted Molecular Patterns (MAMPS) that are able to bind to Pattern Recognition Receptors (PRRs) of the host. *C. elegans* possess the Toll Like Receptors, a known PRR, and TOL-1 was found to be involved in the aversion behavior towards *S. marcescens*. DAF-2, the Insulin receptor homolog of *C. elegans,* is also known to be involved in pathogen avoidance, as well as aging (140-142).

Aging occurs when an organism undergoes many different physiological changes, and this process has been well studied in *C. elegans* (143, 144). Numerous genes (AGE genes) have been identified that affect the lifespan of *C. elegans*, and these genes also play a role in host defenses (145-147). Aging is associated with the decline of innate immunity. As the worms age, the pharynx and intestine become distended with bacterial accumulation, and this can ultimately lead to death (148). Gut immunity has been shown to be less efficient in older worms, which leads to greater bacterial accumulation in the gut and a shorter lifespan (149). Many pathogens have been shown to cause increased bacterial load in the gut, which leads to the demise of the host.

The innate immune response in *C. elegans* is highly conserved, and the specific response differs based on the environment. Genomic analysis of *C.*

elegans has been completed studying the effects of *V. cholerae* pathogenesis and the hosts innate immune response (150). The cytolysin of *V. cholerae*, encoded by the aforementioned *hlyA* gene, is a pore-forming toxin that is required for killing of *C. elegans*. In response to *V. cholerae* infection, many genes involved in innate immunity are up-regulated. These include C-type lectins, genes involved in the blocked unfolded protein response, and those downstream of DAF-16. Work has also been completed to uncovering the underlying mechanisms in *C. elegans* immunity in response to other gut pathogens, like *P. aeruginosa* and *S. aureus* (151). Interestingly, a subset of genes that were up-regulated upon *V. cholerae* infection were also up-regulated in response to *P. aeruginosa* and *S. aureus*. While unique genes and pathways are utilized in response to different pathogens, it is of note that some responses in *C. elegans* overlap, possibly for a more generalized response to certain infections.

5.2 Results

5.2.1 The presence of c-di-GMP significantly decreases the lifespan of *C. elegans*.

We next wanted to determine the effects of CDNs, specifically c-di-GMP, and see if this molecule was having an effect on the lifespan of *C. elegans.* Since preference towards c-di-GMP happened over the course of a short amount of time, we needed to determine a way to provide constant exposure of this signaling molecule to *C. elegans*. Supplementing c-di-GMP into bacteria culture might prove effective for experiments like choice index assays because we are only observing over a short amount of time, but this might not be the most effective way to determine effects on lifespan. CDNs are produced and utilized by most bacteria studied and they potentially have mechanisms to degrade these molecules over time. The bacteria can utilize enzymes such as phosphodiesterase that would negate c-di-GMP over the course of treatment. So, for lifespan assays, supplementation of CDNs will be added into the growth medium prior to plating and conducting experiments.

Lifespan assays were carried out at 25°C. Worms were synchronized and incubated until L4 stage. Overnight bacterial cultures of *E. coli* OP50 were used as the food source on the center of NGM-FUDR plates or NGM-FUDR with 1nM CDNs and allowed to dry for 2 hours. Worms in L4 stage are transferred to assay plates; 20 worms per plate. Plates will subsequently be checked daily and dead

worms were counted and recorded. Day of transfer is defined as day zero. Statistical analysis was carried out through SPSS software under the Kaplan-Maier lifespan analysis. P-values were determined via log rank test, and $P<0.05$ was accepted as statistically significant.

Based on the results illustrated in Figure 5.1 and Table 5.1, it seems that c-di-GMP is having a significant effect on the lifespan of *C. elegans*. When exposed to this molecule over the course of its life, *C. elegans* N2 have a mean lifespan of 11.30±0.48 days while the control group lived an average of 13.89±0.27 days. Supplementation of c-di-AMP and c-GAMP did not have a significant effect on the lifespan of *C. elegans* N2.

With the knowledge in hand from previous experiments, it was critical to determine if c-di-GMP was causing any changes to the food source of *C. elegans*, *E. coli* OP50. We investigated this possibility because there is a chance that supplementation of c-di-GMP could bring about changes to the bacterial food source that could result in these physiological changes observed in the host. c-di-GMP has been shown to play a role in bacteria that can allow some species to form robust biofilms, regulate virulence factors, and establish in a host more effectively (152). To the best of our knowledge, these systems are not in place within *E. coli* OP50, but it is still possible that other pathways could be utilized from the presence of this second messenger that prove detrimental to *C. elegans*.

Lifespan assays were conducted to see if supplementation of c-di-GMP prior to growth of *E. coli* OP50 would cause killing of *C. elegans* as noted in Figure 5.2. It is very possible that c-di-GMP can be degraded over time within a bacterial culture, and thus the effects of this signaling molecule would be difficult to determine. It is critical for this research that c-di-GMP be present over the course of the lifespan to gain accurate insights on to what changes this molecule is causing. Based on the results in Figure 5.2, supplementation of 1nM c-di-GMP into culture media prior to growth of *E. coli* OP50 did not have any significant impact on the lifespan of *C. elegans*. The worms followed a typical lifespan and did not exhibit behavioral or physiological changes that were noted previously.

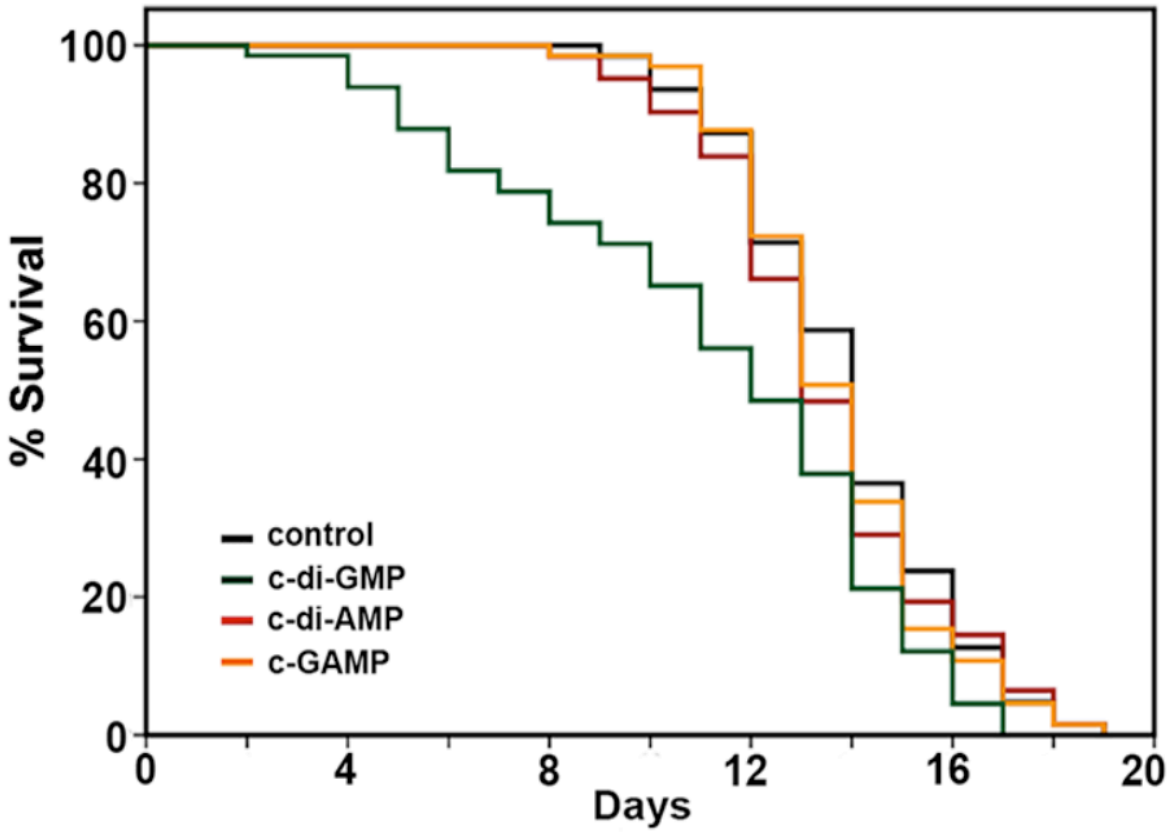


Figure 5.1. Survival curve depicting the lifespan of *C. elegans* N2 when exposed to c-di-GMP, c-di-AMP, or c-GAMP over the course of their entire life. *C. elegans* N2 were fed *E. coli* OP50 and CDNs (1nM concentration for all) were supplemented into the NGM Agar plates. Lifespans carried out at 25^{o}C. Each lifespan experiment was repeated in at least three independent trials with similar results. Statistical analysis is shown in Table 6.1

Table 5.1. Statistical analysis of killing assay of *C. elegans* in the presence of different CDNs.

Strain	**Mean ± SE (Day)**	**Median (Day)**	**# of worms**	***p*-value**
N2	13.89±0.27	14.00	63	
N2 + c-di-GMP	11.30±0.48	12.00	66	<0.001
N2 + c-di-AMP	13.53±0.30	13.00	62	0.583
N2 + c-GAMP	13.71±0.26	14.00	65	0.577

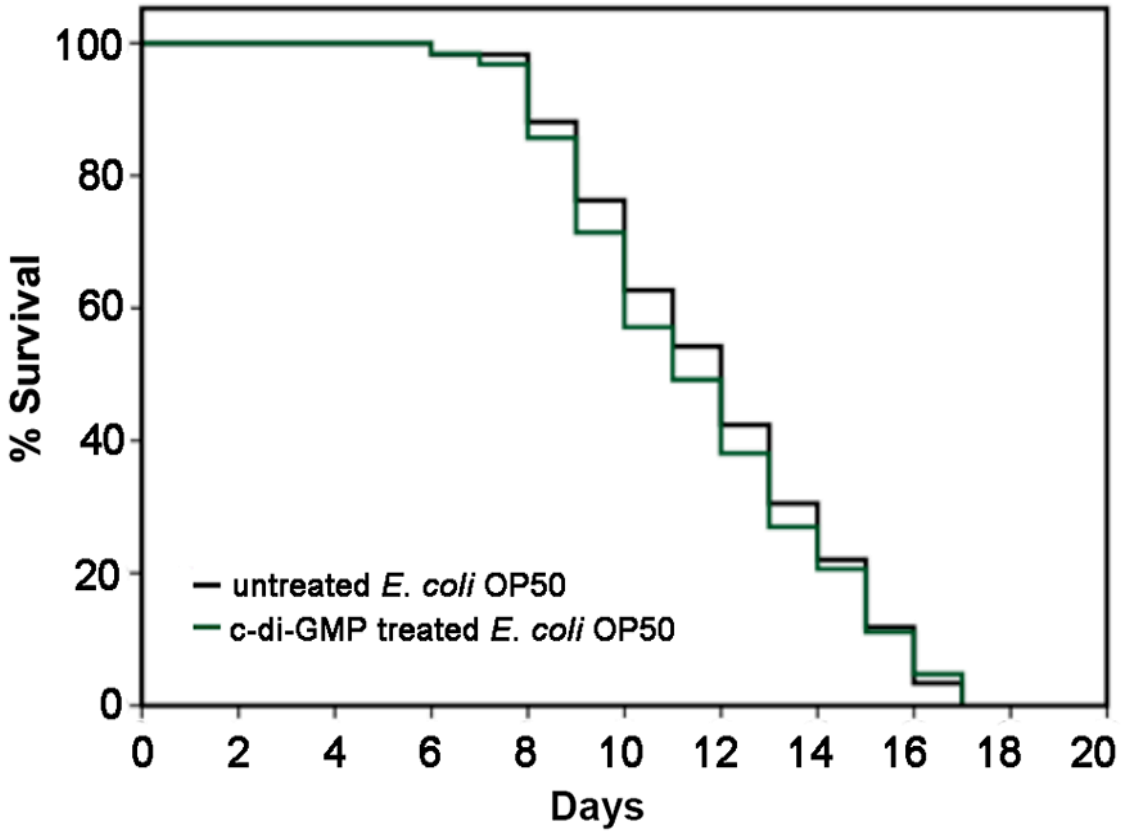


Figure 5.2. Lifespan of N2 worms fed on untreated *E. coli* OP50 overnight culture (Black line) and treated *E. coli* OP50 that was grown overnight with 1 nM c-di-GMP (Green line). No c-di-GMP was added to the lifespan assay plates. The assay was repeated in at least three independent trials with similar results. The data shown in the figure are representatives from one of the trials.

5.2.2 c-di-GMP allows for *E. coli* OP50 to establish more readily in the gut of *C. elegans*

Aging is a result of many physiological changes in an organism. As stated previously, this phenomenon is well studied in *C. elegans*. Accompanied with aging, *C. elegans* become more susceptible to bacterial accumulation in the gut as they get older (148). This is associated with a decrease in gut immunity, and the response of bacterial accumulation within the intestine can ultimately result in the death of the worm (149).

Gut colonization assays were carried out to see the effects of c-di-GMP on gut immunity in *C. elegans*. Based on previous lifespan experiments, it was noticed that during the experimental procedures the worms living in the presence of c-di-GMP had a darker intestinal lumen under the microscope at a younger age than the control population. This could be indicative of bacteria establishing in the gut of the worms. To complete the gut colonization assays, a synchronized population of worms were allowed to develop to the L4 stage before being individually transferred onto NGM-FUDR or NGM-FUDR + 1nM c-di-GMP plates seeded with *E. coli* OP50 (Day 0). The adults were then harvested on Day 6 (based on previous results), and completely obliterated to release any of the bacteria that was able to establish in the gut. It was hypothesized that the presence of c-di-GMP would result in greater bacterial accumulation in the intestine of *C. elegans* N2.

Based on the results from Figure 6.7, this does in fact seem to be the case. Worms that were fed on *E. coli* OP50 in the presence of 1nM c-di-GMP had a significantly higher amount of *E. coli* OP50 within the gut on Day 6. On average, each adult worm fed in the presence of 1nM c-di-GMP had a bacterial load of 2.97×10^3 CFU of *E. coli* OP50, whereas the control parents had 383 CFU of *E. coli* OP50.

Given these results, we also wanted to check and see if c-di-GMP supplementation had an effect on the growth of *E. coli* OP50. To test this issue, a standard growth curve of the bacterium was employed with optical density readings at 600nm taken every hour. When the bacterium was inoculated into culture flasks, c-di-GMP was also added at a final concentration of 1nM. Figure 5.4 shows that supplementation of 1nM c-di-GMP did not have any sort of effect on the growth of *E. coli* OP50 over the course of 24h.

Since it is known that c-di-GMP is crucial for biofilm formation in different bacterial species, we also wanted to visually address this possible concern. We thought it may be possible that supplementation of this molecule in culture, without a host present, could stimulate biofilm formation. A simple biofilm assay was conducted where *E. coli* OP50 was allowed to grow statically with or without the presence of c-di-GMP in the culture media. Figure 5.5 depicts the growth of these two cultures after incubation at 37^{o}C for 24 hours. If biofilm formation did occur, there would be a noticeable bacterial pellet on the surface of the culture media and on the inside of the sterile glass culture tubes. Supplementation of c-

di-GMP did not result in any biofilm formation in *E. coli* OP50. Culture tubes were also allowed to incubate for 72h, and even after this longer time no noticeable biofilm formation was observed (data not shown).

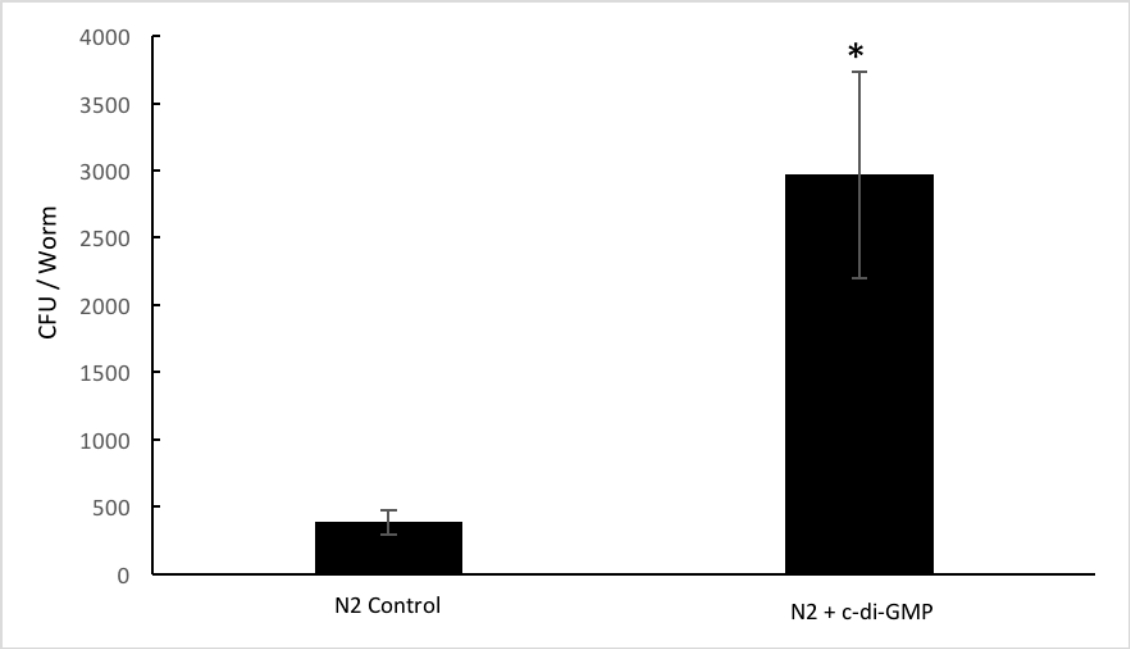


Figure 5.3. Average CFU/N2 worm (aged to Day 6) when fed with *E. coli* OP50 at 25°C in the presence (N2 + c-di- GMP) or absence (N2 Control) of c-di-GMP. Results are the average of three independent experiments, and error bars are standard error of the mean. *p* values were calculated by using the unpaired Student T-test (N2 vs. N2+c-di-GMP). * indicates $p < 0.05$.

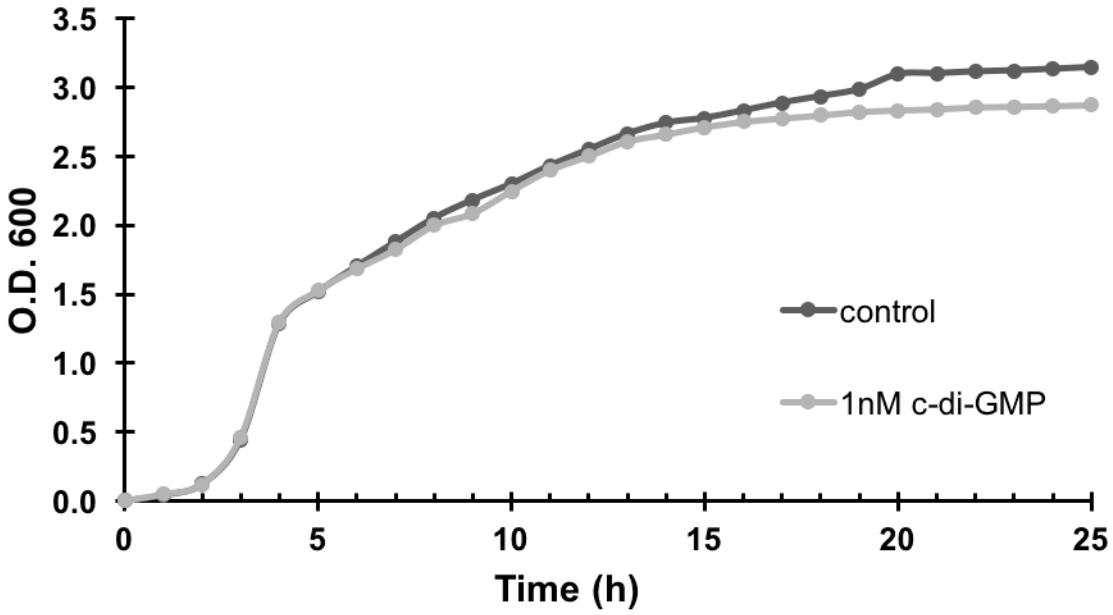


Figure 5.4 C-di-GMP does not affect the growth of *E. coli* OP50. Overnight culture of *E. coli* OP50 was inoculated (1:100 dilution) in 50 mL of LB medium in the presence (grey line) or absence (black line) of 1nM c-di-GMP. Flasks were incubated at 37^{o}C incubator shaking at 150 RPM for 24 hours. Every hour 1 mL of culture was taken, and optical density measurements were taken at 600 nm.

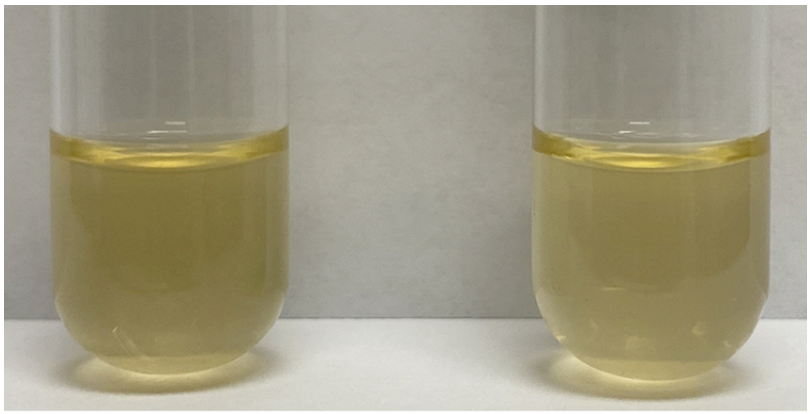


Figure 5.5. C-di-GMP does not enhance the surface attachment of *E. coli* OP50. Overnight culture of *E. coli* OP50 was inoculated (1:100 dilution) in 2 mL of LB medium in the presence or absence of 1 nM c-di-GMP. The tubes were incubated statically at 37^{o}C for 24 hours.

5.2.3 c-di-GMP suppresses innate immunity in *C. elegans* through downregulating genes involved in infection response

In previous studies, it has been well documented which innate immune response genes are differentially expressed in *C. elegans* in response to *V. cholerae* infection (150). c-di-GMP plays an extensive role in many key processes in *V. cholerae* as well as other Gram-negative bacteria. Since *C. elegans* is able to respond and sense towards c-di-GMP, it would be beneficial to further elucidate its specific effect on the immune response in our model organism.

We speculated that expression of *C. elegans* innate immune response genes may be inhibited by c-di-GMP. To examine this, we selected a few innate immune genes (*C23G10.*1, *clec-46*, *clec-71, col-41*, *dct-5*, *fmo-2*, *pqn-5,* and *dod-22)* that are reported to be upregulated during bacterial infections and analyzed their expression by qRT-PCR (150, 151). In consistence with our speculation, as shown in Figure 6.8, when synchronized L4-stage N2 worms were exposed to 1 nM of c-di-GMP for only 10 min, expression of these genes was generally reduced by 2- to 5-fold except for *dod-22*, which decreased by 1.6-fold.

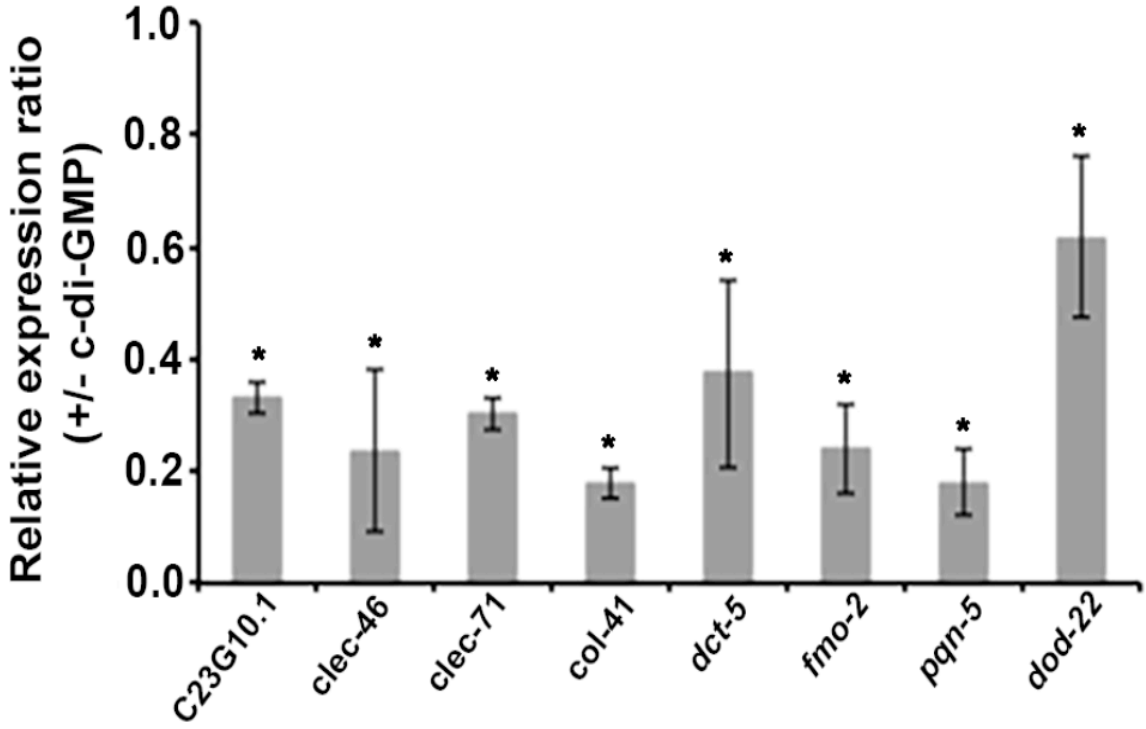


Figure 5.6. qRT-PCR analysis of *C. elegans* innate immunity genes in response to 1nM c-di-GMP (normalized to *act-1*). Results are the average of three independent experiments, and error bars are standard error of the mean. *p* values were calculated by using the unpaired Student T-test (each gene vs. *act-1*). * indicates $p < 0.05$.

5.3 Discussion

Although all three CDNs can be sensed by *C. elegans*, their impacts on host's physiology may be quite dissimilar. It was determined that c-di-GMP is actually present outside of *V. cholerae* and that this CDN is the main molecule in attractive chemotaxis behavior in *C. elegans*. To understand the overall effects of this molecule, and the other CDNs, tests were completed to determine how the host responds through this novel communication pathway.

While c-di-GMP shortened *C. elegans* lifespan, c-GAMP and c-di-AMP did not have any effect on the lifespan. This was quite interesting for the hybrid c-GAMP molecule because up until this point, behavior from *C. elegans* was remarkably similar to that noticed in response to c-di-GMP. c-di-AMP, on the other hand, did not cause attraction from *C. elegans* thus the differences in lifespan with treatment of this molecule made sense from a theoretical standpoint. While there may be only small differences in the make-up of these small CDNs, it is not safe to assume that they operate through similar mechanisms once detected by a host. Further study with c-di-GMP uncovered its role in immunosuppression. *C. elegans* supplemented with c-di-GMP had an increased number of bacterial colonies in the intestine and decreased expression of genes responsible for innate immune response.

This observation seems contradictory to the current knowledge that CDNs trigger the innate immune system by activating the expression of interferon

genes, however, we don't think it's a contradiction. *C. elegans* lack the c-di-GMP receptor STING and the interferon system (30). Therefore, c-di-GMP won't trigger the canonic STING pathway in *C. elegans*, and the pathways involved in c-di-GMP detection are still a mystery. Through this work we have also demonstrated that this signaling molecule is in fact acting on the host, and is not responsible for any changes in the bacterium being studied. Possible reasoning for such changes that were observed need to be addressed by studying the specific pathways in *C. elegans* that play a role in communication via c-di-GMP.

Chapter 6

p38 MAPK (PMK-1) and RTK-Ras-ERK MAPK (MPK-1) are required in the c-di-GMP-elicited signal transduction pathway, which acts through SKN-1 and HSF-1 to impact immunity and lifespan

6.1 Introduction

To fully understand the big picture of what is happening through sensation of c-di-GMP, specific pathways must be addressed to see exactly how c-di-GMP treatment is affecting *C. elegans*. As said earlier, since *C. elegans* genome has been regularly studied, many mutant strains are available, and RNA interference is a powerful tool in this model organism, these methods provide the most succinct way to tackle this question. Since it is hypothesized that c-di-GMP treatment may be able to cause an overall decrease in the immune response pathways, it is possible that these known pathways are converging and acting in unison with one another.

6.1.1 Downstream major transcription factors in the innate immune response

The FOXO family protein DAF-16, the SKN-1 protein, and the heat shock protein HSF-1 are the three major stress response transcription regulators in *C. elegans*. These transcription factors are conserved from *C. elegans* to humans. DAF-16, the downstream transcription factor of the *insulin/insulin-like growth factor-1 signaling (IIS) pathway,* plays a key role in modulating longevity and immunity (55, 153, 154). The FOXO family proteins are found in the cytoplasm and translocate to the nucleus upon activation. The activity of DAF-16 is modulated by its association with different proteins and it is regulated by certain serine threonine kinases. Some of the key genes found to be involved downstream of this transcription factor that play a role in modulating immunity are the superoxide dismutase (*sod-3*), metaothionine (*mtl-1*), heat shock proteins of the *hsp-16* family, and a CUB like protein encoded by *dod-22* (58, 79).

SKN-1 is the downstream effector of the major immune-signaling p38 mitogen-activated protein kinase (MAPK) pathway and controls numerous genes involved in stress response and lifespan regulation (155). In *C. elegans*, SKN-1 is the Nrf ortholog. Its transcription has been studied pertaining to its response to oxidative stress via reactive oxygen species (ROS). In response to stress SKN-1 accumulates in the intestinal nuclei, but under normal conditions it has been shown to be constitutively inhibited to do so by the glycogen synthase kinase-3 (GSK-3) (56). PMK-1, the evolutionarily conserved mitogen-activated protein

kinase (MAPK), phosphorylates SKN-1, leading to its accumulation in intestine nuclei, where SKN-1 activates transcription of *gcs-1*, a phase II detoxification enzyme gene (156).

HSF-1 is another versatile transcription factor that regulates a multitude of genes involved not only in stress response but also in development, metabolism, as well as lifespan and immunity modulation (157-159). In *C. elegans*, the homolog of the heat shock factor, *hsf-1*, was found to be involved in lifespan extension mediated by *daf-2* (160, 161). HSF-1 can be regulated by the IIS pathway, and under situations of stress HSF-1 undergoes phosphorylation, enters the nucleus, and regulates transcription of its downstream proteins (162). Regarding immunity, HSF-1 has also been shown to be required for protection against both Gram-negative and Gram-positive bacteria (159). This is mediated through the insulin signaling pathway, and the role of HSF-1 on maintaining proteostasis is important for aging related diseases. The activity of this transcription factor declines as the worms increase in age (158).

6.1.2 Role of MAPKs in innate immune response

MAP kinases are central components of a series of signal transduction pathways that control many vital cellular processes. MAP kinases in mammals are grouped into three families: p38/SAPKs (stress-activated protein kinases), JNKs (Jun amino-terminal kinases), and ERKs (extracellular-signal-regulated kinases) (163). These MAPKs act in the cytoplasm or are translocated to the

nucleus where they can activate transcription factors for downstream cellular response. The three corresponding MAPKs in *C. elegans* are PMK-1, JNK-1, and MPK-1. The p38 MAPK and cJun MAPK are commonly referred to as stress-activated because their activity is initiated in response to pathogens, toxins, and other environmental stressors (33, 61, 164, 165). In *C. elegans* the p38 MAPK has been shown to regulate specific responses to toxins produced by pathogens such as *S. aureus* and *V. cholerae,* among others (166).

The Receptor Tyrosine Kinase (RTK)-Ras-ERK pathway in *C. elegans* controls many elements when it comes to development and behavior (167). This pathway has been implicated in many important biological roles including germline development, vulval cell fates, aging, and olfaction (168-171). To go along with the many roles of this pathway, it also shares interplay with the Wnt signaling and Notch signaling pathways to control downstream responses (172, 173), mainly in developmental events.

Activity and cellular localization of SKN-1, HSF-1, and DAF-16 are modified by phosphorylation. Phosphorylated SKN-1 and HSF-1 are translocated into the nucleolus to activate their target genes. Previous studies reported that either PMK-1 (174) or MPK-1 (175) can directly phosphorylate SKN-1 at the same sites. Kinases that phosphorylate HSF-1 are still unidentified.

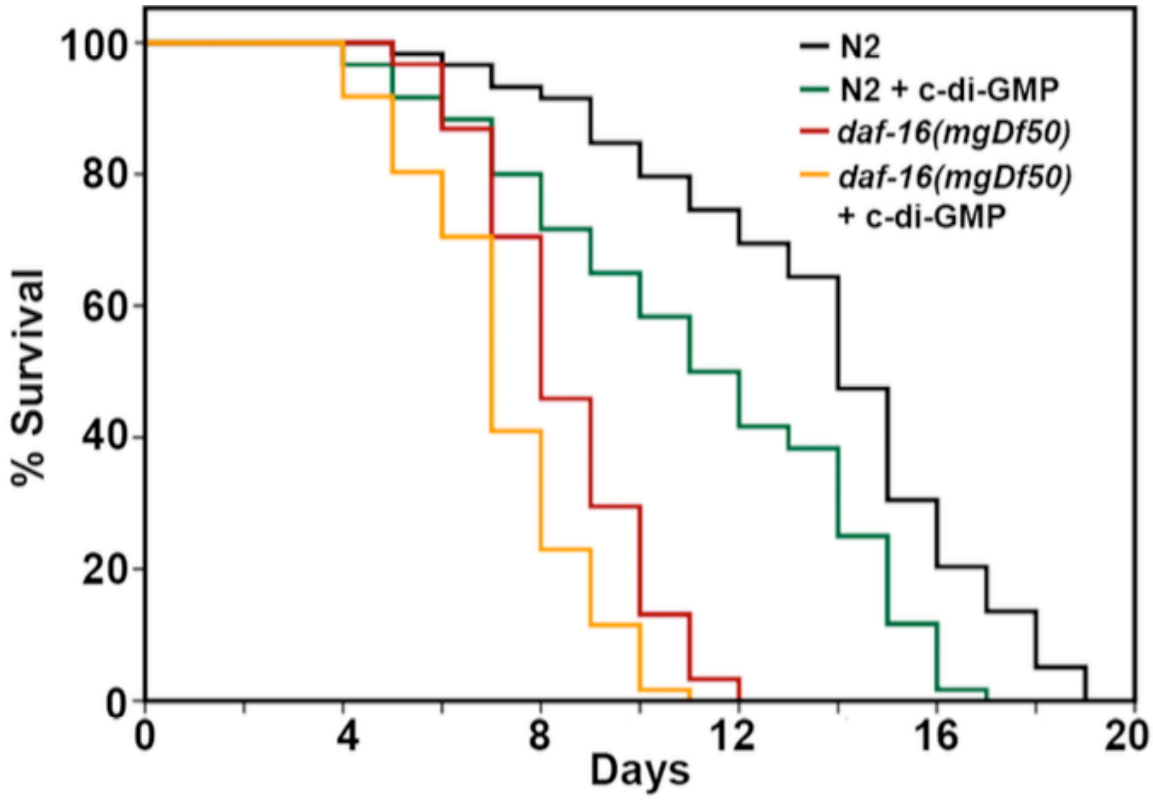
6.2 Results

6.2.1 c-di-GMP acts through two major stress response transcription regulators in *C. elegans*, SKN-1 and HSF-1

To test whether c-di-GMP acts through these regulators, lifespan assays in the presence and absence of c-di-GMP were conducted in mutant *daf-16* and *hsf-1* strains. In the case of *skn-1*, RNA interference (RNAi) was applied to knock down the gene expression, and the empty vector pL4440 was used in parallel to serve as the control. We reasoned that if c-di-GMP acts through a particular transcriptional regulator to shorten the lifespan, mutating or knocking down this gene will eliminate the effect.

Figure 6.1A-C and Table 6.1 show the results. Supplementation of 1nM of c-di-GMP still reduced the lifespan of the *daf-16* mutant strain, and the percentage of decrease is comparable to that in the wild type N2. Lifespan was not affected by c-di-GMP in the *hsf-1* mutant and the *skn-1* RNAi knock down strains, meaning that SKN-1 and HSF-1 are required for c-di-GMP to exert its effect. Next, two representative downstream target genes of SKN-1 (*gst-4* and *gcs-1*) and two of HSF-1 (*hsp-16.2* and *hsp-70*) were measured for their expression levels in the presence and absence of c-di-GMP. As shown in Fig 6.1D, c-di-GMP triggered down-regulation of these genes. These results suggest that sensing c-di-GMP by the chemoreceptors in the sensory neuron elicited signal transduction that eventually led to reduced activity of SKN-1 and HSF-1.

A


100
80
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% Survival
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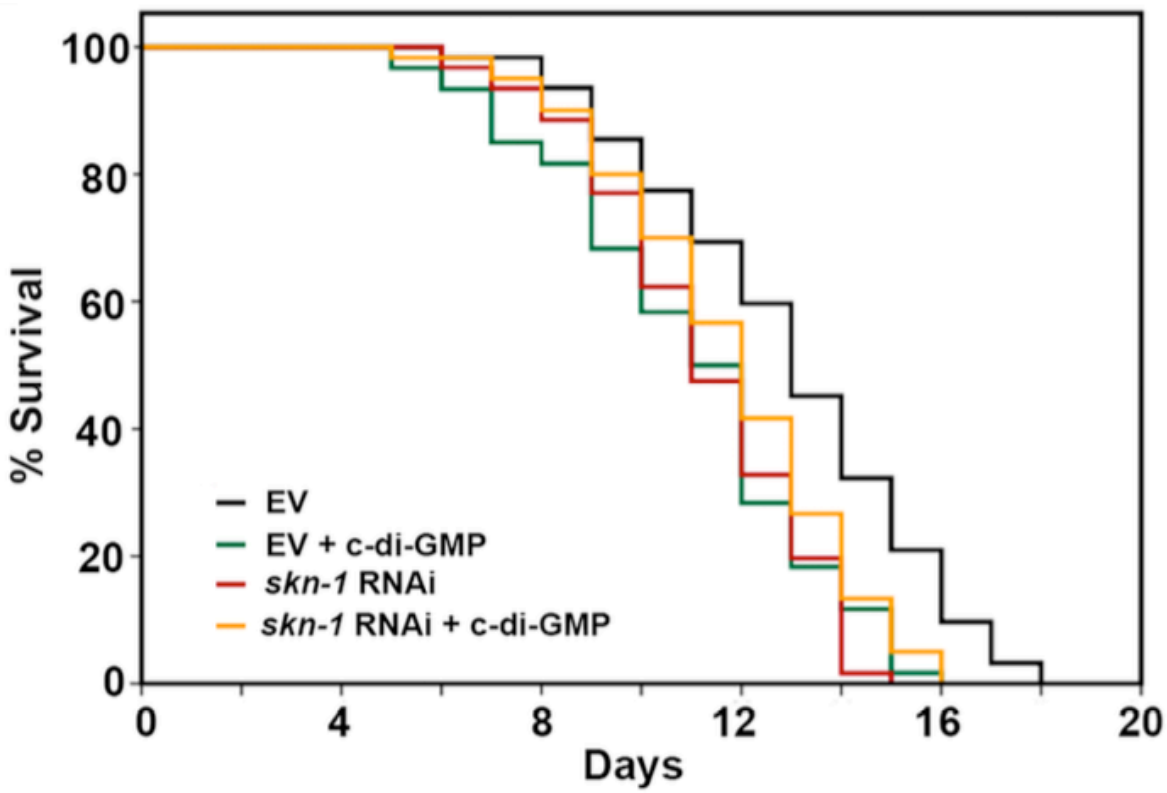
4
8
12
16
20
Days
N2
N2 + c-di-GMP
daf-16(mgDf50)
daf-16(mgDf50)
+ c-di-GMP

B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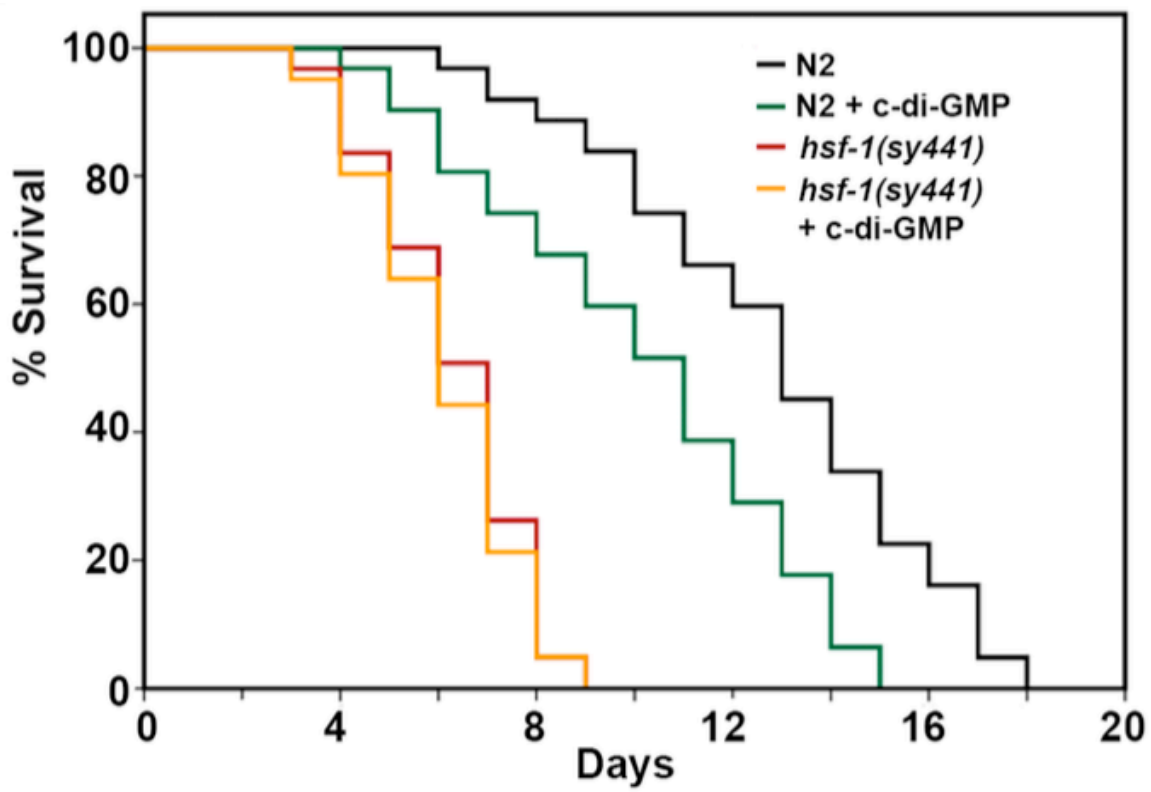
Days
EV
EV + c-di-GMP
skn-1 RNAi
skn-1 RNAi + c-di-GMP

C


100
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% Survival
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4
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12
16
20
Days
N2
N2 + c-di-GMP
hsf-1(sy441)
hsf-1(sy441)
+ c-di-GMP

D

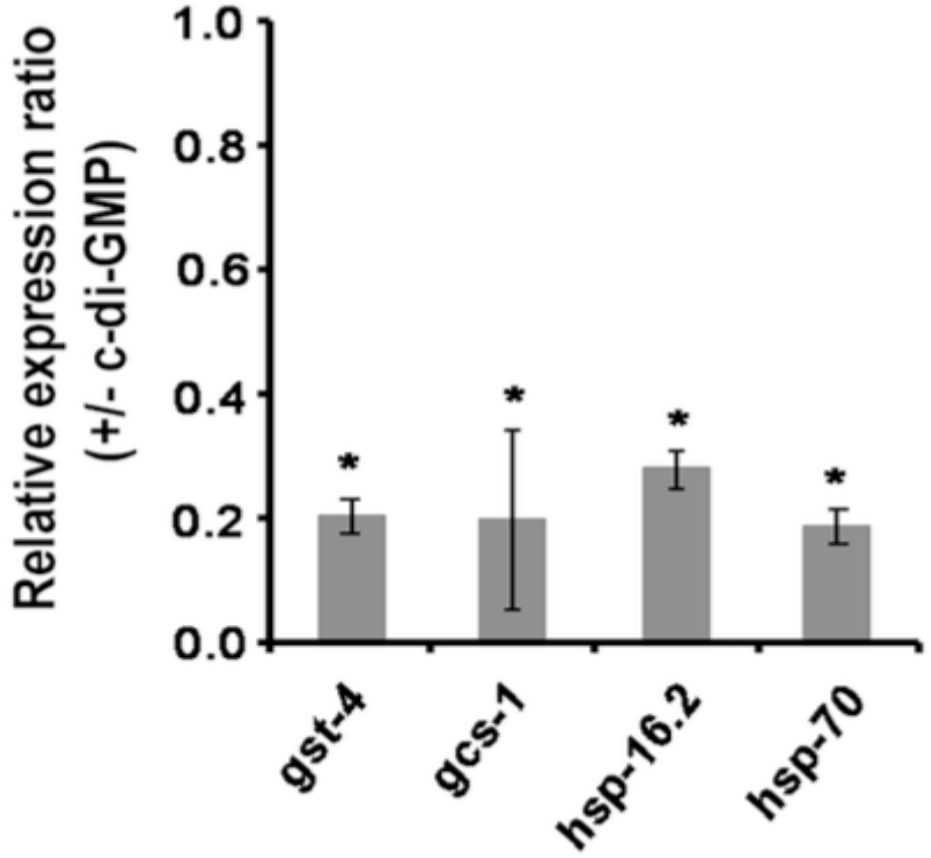

Relative expression ratio
(+/- c-di-GMP)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
gst-4
gcs-1
hsp-16.2
hsp-70

Figure 6.1 C-di-GMP acts through SKN-1 and HSF-1, but not DAF-16. Lifespan of (A) *daf-16(mgDf50)*, (B) *skn-1* RNAi, and (C) *hsf-1(sy441)* worms when supplemented with 1 nM c-di-GMP. Each lifespan experiment was repeated in at least three independent trials with similar results. Quantitative data and statistical analyses for all trails are included in Table 2. EV stands for "empty vector pL4440". (D) qRT-PCR analysis of the representative target genes of SKN-1 (*gst-4* and *gcs-1*) and HSF-1 (*hsp-16.2* and *hsp-70*) in response to 1nM c-di-GMP (normalized to *act-1*). Results are the average of three independent experiments, and error bars are standard error of the mean. *p* values were calculated by using the unpaired Student T-test (each gene vs. *act-1*). * indicates $p < 0.05$.

Table 6.1 The lifespan (days) of N2 *C. elegans* and various mutant strains at 25°C in the absence and presence of 1nM of c-di-GMP.

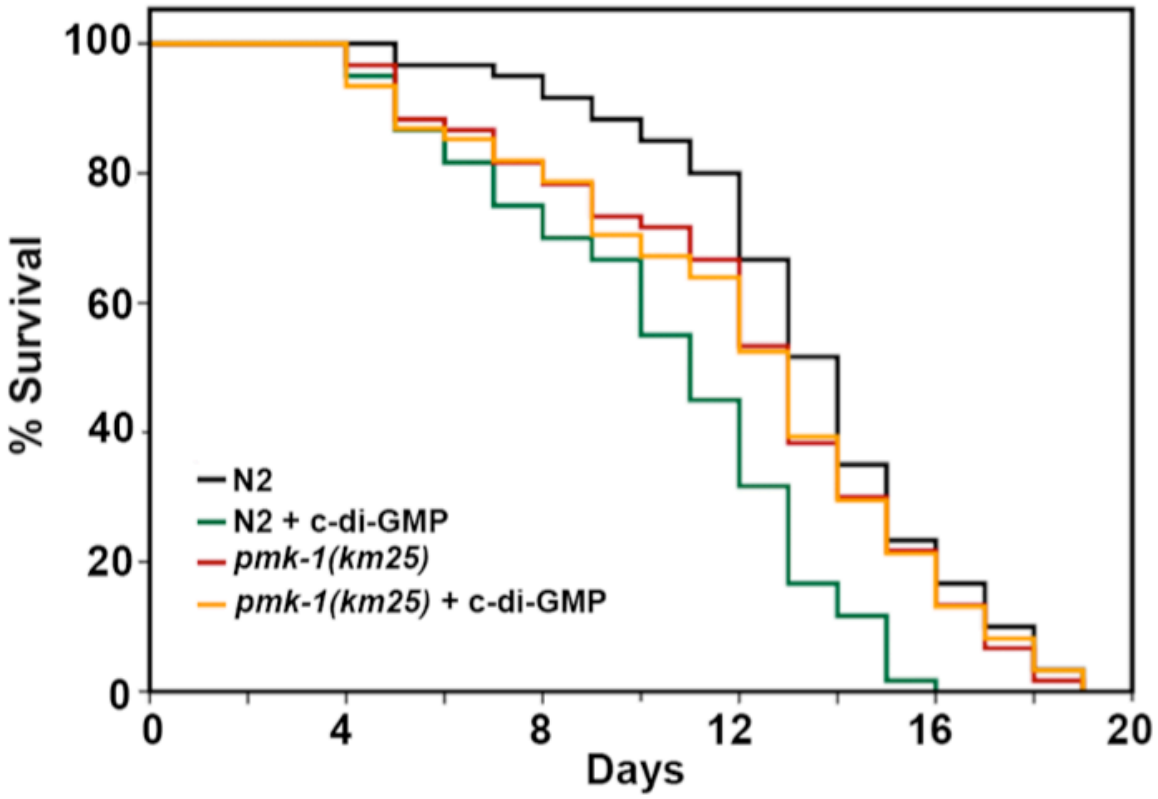
Strain	Mean ± SE (Day)	Median (Day)	# of worms	*p*-value	*% change*
N2 [a]	13.70±0.45	14.00	59		
N2 + c-di-GMP [a]	11.20±0.48	11.00	60	<0.001	-18.2
daf-16(mgDf50) [a]	8.46±0.22	8.00	61		
daf-16(mgDf50) + c-di-GMP [a]	7.20±0.23	7.00	61	<0.001	-14.9
EV [b]	12.94±0.36	13.00	62		
EV + c-di-GMP [b]	10.93±0.36	11.00	60	<0.001	-15.5
skn-1 RNAi [b]	11.20±0.29	11.00	61		
skn-1 RNAi + c-di-GMP [b]	11.75±0.32	12.00	60	0.080	4.9
N2 [c]	12.84±0.41	13.00	62		
N2 + c-di-GMP [c]	10.13±0.41	11.00	62	<0.001	-21.1
hsf-1(sy441) [c]	6.31±0.20	7.00	61		
hsf-1(sy441) + c-di-GMP [c]	6.10±0.21	6.00	61	0.495	-3.3

The lifespan experiments were repeated at least three times with similar results, and the data for representative experiments are shown. The lifespan data were analyzed using the log-rank test and *p*-values for each individual experiment are shown. The *p*-value was calculated by comparing c-di-GMP-treated to the untreated worms.
[a]Results presented in Figure 6.1A. [b] Results presented in Figure 6.1B. [c] Results presented in Figure 6.1C.

6.2.2 p38 MAPK(PMK-1) and RTK-Ras-ERK MAPK (MPK-1) are required in the c-di-GMP-elicited signal transduction pathway

Using the *pmk-1* and *jnk-1* mutant strains as well as the *mpk-1* RNAi, we examined whether these MAPKs are involved in signal transduction elicited by c-di-GMP. Lifespans of these mutant worms were compared in the presence and absence of 1nM c-di-GMP and shown in Fig 6.2A-C and Table 6.2. In the *pmk-1* mutant and the *mpk-1* RNAi worms, adding c-di-GMP in the medium could no long reduce the lifespan as compared to that in the N2 or N2/pL4440 worms. While in the *jnk-1* mutant, a 10% decrease of lifespan was observed with the addition of c-di-GMP. Our data suggest that the two MAP kinases, PMK-1 from the p38 MAPK pathway and MPK-1 from the RTK-Ras-ERK pathway, are required in the c-di-GMP-elicited signal transduction.

A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% Survival
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4
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16
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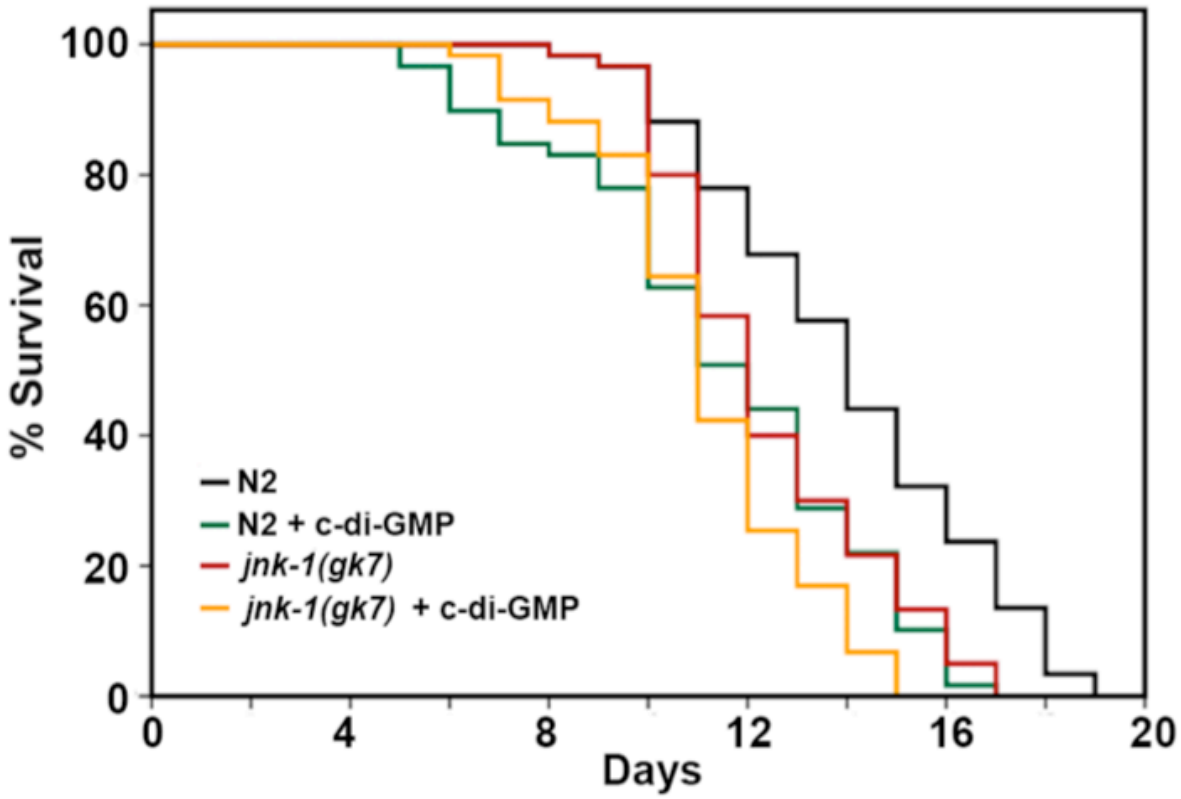
Days
N2
N2 + c-di-GMP
pmk-1(km25)
pmk-1(km25) + c-di-GMP

B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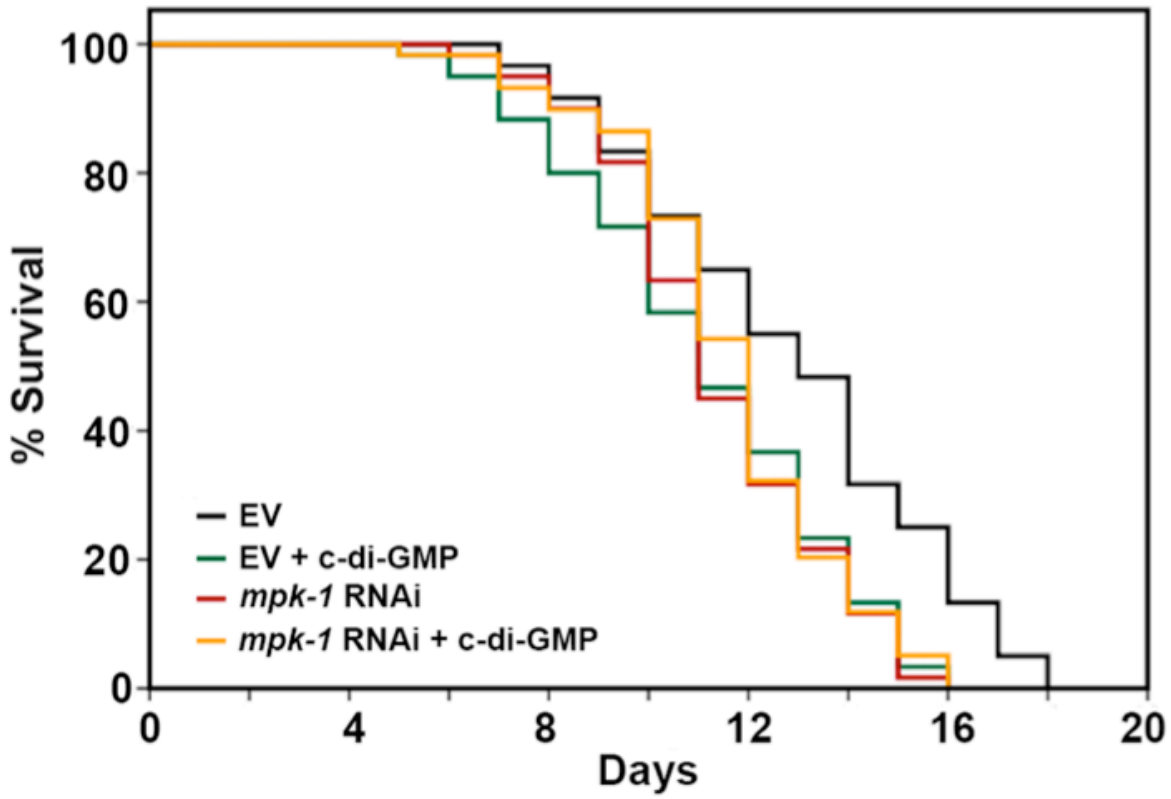
Days
N2
N2 + c-di-GMP
jnk-1(gk7)
jnk-1(gk7) + c-di-GMP

C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% Survival
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16
20
Days
EV
EV + c-di-GMP
mpk-1 RNAi
mpk-1 RNAi + c-di-GMP

Figure 6.2. PMK-1 and MPK-1 are required in the c-di-GMP-elicited signal transduction pathway. Lifespan of (A) *pmk-1(km25)*, (B) *jnk-1(gk7)*, and (C) *mpk-1* RNAi worms when supplemented with 1 nM c-di-GMP. Each lifespan experiment was repeated in at least three independent trials with similar results. Quantitative data and statistical analyses for all trails are included in Table 2. EV stands for “empty vector pL4440”.

Table 6.2. The lifespan (days) of N2 *C. elegans* and various mutant strains at 25°C in the absence and presence of 1nM of c-di-GMP.

Strain	Mean ± SE (Day)	Median (Day)	# of worms	*p*-value	*% change*
N2 [a]	13.40±0.41	14.00	60		
N2 + c-di-GMP [a]	10.37±0.44	11.00	60	<0.001	-22.6
pmk-1(km25) [a]	12.08±0.52	13.00	60		
pmk-1(km25) + c-di-GMP [a]	11.95±0.54	13.00	61	0.950	-1.1
N2 [b]	14.03±0.37	14.00	59		
N2 + c-di-GMP [b]	11.53±0.41	12.00	59	<0.001	-17.8
jnk-1(gk7) [b]	12.43±0.29	12.00	60		
jnk-1(gk7) + c-di-GMP [b]	11.17±0.28	11.00	59	0.003	-10.1
EV [c]	12.88±0.40	13.00	60		
EV + c-di-GMP [c]	11.15±0.36	11.00	60	<0.001	-13.4
mpk-1 RNAi [c]	11.40±0.30	11.00	60		
mpk-1 RNAi + c-di-GMP [c]	11.63±0.31	12.00	59	0.518	2.0

The lifespan experiments were repeated at least three times with similar results, and the data for representative experiments are shown. The lifespan data were analyzed using the log-rank test and *p*-values for each individual experiment are shown. The *p*-value was calculated by comparing c-di-GMP-treated to the untreated worms.
[a]Results presented in Figure 6.2A.[b] Results presented in Figure 6.2B. [c] Results presented in Figure 6.2C.

6.4 Discussion

As described in the previous chapter, *C. elegans* lacks the canonical STING receptor known to be present in humans that binds c-di-GMP. The pathway and mode of action of this small signaling molecule is unknown in our model organism, and it is important to understand this pathway as it pertains to innate immunity.

We showed that c-di-GMP instead acts through two key stress response transcription factors, SKN-1 and HSF-1. SKN-1 is the downstream effector of the major immune-signaling p38 MAPK pathway and controls numerous genes involved in stress response and lifespan regulation (36). HSF-1 is a multifaceted transcription factor involved in stress response, development, metabolism, as well as lifespan and immunity modulation (37-39). We propose that c-di-GMP causes reduced activity of SKN-1 and HSF-1, and results in downregulation of their target genes that are responsible for innate immune response and longevity. The possible MAP kinases (PMK-1, JNK-1, and MPK-1) that regulate SKN-1 and HSF-1 activity and nucleus localization were investigated. PMK-1 from the p38 MAPK pathway and MPK-1 from the RTK-Ras-ERK pathway are required in the c-di-GMP-elicited signal transduction. It is known that both PMK-1 and MPK-1 can phosphorylate SKN-1 to modulate its function (41, 42), while the kinase in *C. elegans* to phosphorylate HSF-1 is still not clear. Our data propose the possibility that PMK-1 or MPK-1 might be the upstream kinase for HSF-1. Of course, further studies are needed to address this interesting question.

There is a striking benefit for the c-di-GMP- producing bacteria, as this mechanism promotes bacterial invasion and establishment in their hosts. As for the hosts, the innate immunity suppressed by bacterial signals might be an essential step in the process of co-evolution with the bacteria. With the emergence of the c-di-GMP receptor STING, the hosts will learn to fight back.

Chapter 7

Conclusion and Future Directions

7.1 Conclusion

In this study, based on a simple observation, we uncovered a novel pathway of communication that exists between bacteria and eukaryotes. It was first noticed that our model organism, *C. elegans*, seemed to readily feed and spend time grazing on *V. cholerae*, a known pathogen to the worm and the cause of severe gastrointestinal illness in humans. We first sought to uncover if this attraction was indeed happening, and what was causing this behavioral change towards something that was so harmful. Being a bacterivore, *C. elegans* provides a good model to study interkingdom communication. This nematode possesses many attractive features such as a short lifespan, easy cultivation and maintenance, and genetic tractability (46). Using the *V. cholerae-C. elegans* interaction model, we demonstrated the role of bacterial c-di-GMP in interkingdom communication, *i.e.* chemoattracting host animals and impairing their immune response.

While studying the protective benefits that water-soluble cranberry extract provides to *C. elegans* when exposed to known pathogens, a counterintuitive behavior was observed. Something was happening that resulted in attractive behavior of *C. elegans* to a bacterium that caused fatal infection, *V. cholerae*. To first delve into this topic, a review of the literature was completed, and choice index assays were employed to see if this was indeed the case. Our hypothesis was validated as we saw a strong choice preference of our model organism towards *V. cholerae* over its standard laboratory food, *E. coli* OP50. Finding out what was causing this interaction was the next step in the search for answers regarding this peculiar phenomenon.

We found that the AIs produced by *V. cholerae* were playing a role in the chemotactic response of the worms. During this time in my research, it seems as though someone was studying this interaction as well, and a paper was published highlighting the role of CAI-1 produced by *V. cholerae* in interkingdom communication (18). An interesting note that was found in this research and my own was that this communication occurs with many AIs produced by different bacterial species, with preferences. Of note, it was also observed in *V. cholerae* that when AI production is knocked out, there still seems to be attraction to this pathogen. We succeeded in identifying the role of CDNs, specifically c-di-GMP, as the major signal in this previously undescribed communication pathway.

The role of these CDNs has been ever expanding since their discovery just over 30 years ago. They are known to regulate a wide range of physiological processes in bacteria, and in *V. cholerae* they are fundamental for controlling motility, biofilm formation, and virulence gene expression (176). Through work previously completed in our lab, it was found that c-di-GMP is possibly acting independently from AIs and HapR (quorum-sensing master regulator) to control biofilm formation (99). For these reasons, we turned our attention to these second messengers, and identified c-di-GMP and c-GAMP as attractive signaling molecules of *C. elegans*. c-di-AMP, on the other hand, caused a repulsive behavior. However, using LC-MS we were only able to detect c-di-GMP outside of *V. cholerae* cell cultures under our experimental settings. This led us to believe that c-di-GMP, even at small concentrations, was the major chemoattractant of *C. elegans* in vivo. By using genetic knockout mutants, we identified that sensation of c-di-GMP was through the AWC neurons and the cGMP-gated TAX-2/TAX-4 channel. These neurons sense volatile odors and are linked to attractive behavior. With this novel chemoattractant identified, we shifted our focus to reveal any physiological changes this molecule may cause in our model organism.

Through numerous experiments, it was evident that exposure to c-di-GMP was affecting the health of *C. elegans*. When exposed to this molecule over the course of its entire adult life, worms lived a significantly shorter amount of time.

They also had significantly higher amounts of bacteria present in the gut, which has been linked to a decline in overall health and gut immunity (148, 149, 177). Initial tests were also conducted testing the effects of c-di-GMP on brood size. While it seems c-di-GMP exposure is causing an increase in egg-laying overall, I think this area regarding its role in the developmental process should be studied in more detail. With this overall decline in health, we looked closer to see if there were any changes in gene expression levels related to innate immunity. We chose to study a set of genes whose expression levels are significantly up-regulated during infection by *V. cholerae*, as well as *S. aureus* and *P. aeruginosa* (150, 151). Short-term exposure to c-di-GMP, with no pathogen present, caused expression levels of these innate immune response genes to be generally reduced 2- to 5-fold. Based on our findings, this may seem contradictory because studies have shown CDNs are able to trigger the expression of interferon genes through binding of the STING receptor in mammals (30). However, we do not think this is a contradiction because *C. elegans* lacks this receptor, and they also do not possess the powerful IFN system. So, naturally we do not expect to see a similar innate immune response in our model organism, and in fact we see the opposite.

C. elegans has coevolved with bacteria over the years, and being a bacterivore they have developed a particularly intimate relationship. I believe that production of c-di-GMP by bacteria, or its presence outside of the bacterial

cell, acts as a cue attracting *C. elegans* towards a food source. While this attraction may decrease immune response initially, degradation and weakening of this signal over time could lead to a return to baseline immunity under non-infectious conditions in vivo. However, when this signal is produced by a pathogen, such as *V. cholerae*, this initial attraction could be all it takes for *C. elegans* to begin feeding and thus become infected. This would allow *V. cholerae* to colonize the gut of a new host, cause infection, and finally be released into the environment to start the cycle again.

There are numerous conserved pathways in *C. elegans* that play a role in the innate immune response to pathogens, and some of these pathways can become intertwined depending on the response needed. We sought to uncover the pathways and key immune response regulators to see which were being targeted for this overall decline in health. This feat was accomplished by observing the effect of c-di-GMP exposure over the course of the lifespan of *C. elegans* knock-out mutants for genes integral to each pathway. Downstream gene expression analysis was also used to further confirm our findings. Based on our results, we propose that exposure to c-di-GMP causes reduced activity of SKN-1 and HSF-1, two key stress response transcription factors. This results in downregulation of their target genes responsible for innate immunity and longevity (*gst-4, gcs-1, hsp-16.2* and *hsp-70*). Through lifespan analysis, we also determined that PMK-1 from the p38 MAPK pathway and MPK-1 from the RTK-

Ras-ERK pathway were required for signal transduction elicited in the presence of c-di-GMP. In previous work, it was demonstrated that both PMK-1 and MPK-1 can phosphorylate SKN-1 to modulate its function (66, 156). The kinase that is responsible for phosphorylation of HSF-1 in *C. elegans* is unclear at this time. Based on our results we propose that either PMK-1 or MPK-1 is the upstream kinase for HSF-1. Further studies need to be conducted to address this possibility, however.

The bacterivore *C. elegans* provides a good model to study interkingdom communication. This nematode possesses many attractive features such as a short lifespan, easy cultivation and maintenance, and genetic tractability (37). Numerous human and animal microbial pathogens have been shown to infect and kill *C. elegans* (178, 179). Using the *V. cholerae-C. elegans* interaction model, we demonstrated the role of bacterial c-di-GMP in interkingdom communication, *i.e.* chemoattracting host animals and impairing their immune response. There is a striking benefit for the c-di-GMP- producing bacteria, as this mechanism promotes bacterial invasion and establishment in their hosts. As for the hosts, the innate immunity suppressed by bacterial signals might be an essential step in the process of co-evolution with the bacteria. With the emergence of the c-di-GMP receptor STING, the hosts will learn to fight back.

7.2 Future Directions

The utilization of the CDNs by bacteria has wide ranging impacts on the cell and are fundamental to the success of the organism. It has only recently been noticed that these messengers are involved in interkingdom communication, as humans and other animals can detect and respond to these signals. While more research is being conducted in this field, there still remains a lack of knowledge concerning the effects this signaling has on a host and the CDN-elicited signal transduction.

Only within the last decade has significant research been conducted investigating the binding of bacterial CDNs to the human STING receptor. The activation of the STING receptor by these small DNA molecules stimulates the production of type I interferon (IFN) (180). Initially this was implicated in the innate immune response to pathogens, but more recently it has been shown to detect tumor-derived DNA and directs antitumor immunity through the versatile type I IFN (181). The human STING receptor has a higher binding affinity with c-GAMP and c-di-GMP than it does to c-di-AMP (182). Its affinity is even greater when binding endogenous mammalian c-GAMP, which possesses a 2'-5' phosphodiester linkage rather than the microbial 3'-5' linkage (183). Through these studies, it was found that STING has the affinity to bind to the bacterial c-di-GMP at a concentration of about 4.4uM, which initiates the downstream signal transduction. Efforts have been made in attempts to modify STING for higher affinity, and while this proved successful, the concentration of c-di-GMP needed

for binding was still significantly higher, 0.461 ± 0.053 μM (182).

In our study, we have found that 1nM concentration of c-di-GMP is being sensed by *C. elegans* and this decreases its immune response. We also found that this behavioral response occurred at similar concentrations towards microbial c-GAMP. As mentioned previously, *C. elegans* does not possess this evolutionarily conserved STING receptor. Although not uncovered in this work, there is a possibility that *C. elegans* may possess a receptor with higher affinity for CDNs than the mammalian STING. If this were the case, humans or higher organisms may also have a similar receptor that can be initiated at lower concentrations of CDNs. This would be highly beneficial for the advancement of innate and anti-cancer immunity. The ability to bind these molecules at lower concentrations would make the response to foreign and cancer cell-derived DNA even that much more precise. In 2017, researchers at the University of Bristol were even remarkably able to successfully design a synthetic receptor that was able to mimic the response of G-protein coupled receptors (184). This is an exciting new area of research, and it has the possibility to be far-reaching, especially in the fight against cancer and improving immunotherapies.

The activity of HSF-1 has been shown to induce the transcription of cytoprotective genes, including molecular chaperones, DNA damage repair components, and metabolic enzymes (185). Together, these play a vital role in human physiology and ageing, as well as in pathological processes such as

cardiovascular disease, neurodegeneration, and cancer (185, 186). Increased levels of HSF-1 support malignant transformation and can result in poor prognosis of different cancers, making it a viable target for cancer treatment (187, 188). In humans, the involvement of MAPK signaling has been reported to activate HSF-1, and this hypothesis also holds true for *C. elegans*. More recently, in vitro studies have been completed demonstrating that the p38 MAPK is able to phosphorylate mammalian HSF-1 (186). In our study, we have demonstrated that the MAPK from either p38 MAPK or RTK-Ras-ERK pathway are able to phosphorylate HSF-1. This would be a new avenue of signaling in understanding the activity of HSF-1, and more research should be done in this area. Phosphorylation of this transcription factor could be carried out by different kinases, depending on the environment. A better understanding of this pathway would allow a much thorough understanding of both acute stress response and a more general, integrative response considers the overall stress status of the organism. *C. elegans* provides a good model to study this network.

Another interesting area to study in this field of work would be the effects of bacterial signaling on host developmental processes. We know that CDNs play a role in regulating immunity in higher organisms. In these model systems, if immunity or other pathways are affected, there is a higher probability that reproduction or development could be as well. Fecundity has been measured and observed in *C. elegans* to determine how the worms behave under both

favorable and unfavorable conditions. When exposed to harmful substances in the environment, such as pathogens, the worms will retain their eggs in an effort to protect their progeny (189). Egg-laying in *C. elegans* is regulated by a small neuronal circuit, and when these neurons are in an active state serotonin is released and egg-laying ensues (190, 191). In the absence of a food source, egg-laying is halted, but when food is reintroduced normal behavior and egg-laying is restarted (192). In this study, the effect of c-di-GMP exposure on the reproductive ability of *C. elegans* was briefly investigated (data not shown). It seemed that the presence of this molecule increased the egg-laying ability of *C. elegans* initially, and leveled out over time. I believe this should be looked at with finer detail, to uncover what is truly happening and the potential pathways that could be involved. The importance of bacteria in reproductive health and function is a growing area of research, and the more we understand

Quorum sensing and the cyclic di-nucleotide second messengers are vital signaling systems used by many bacteria, and we have come a long way in understanding the mechanisms behind such communication. To date, no known mechanism has been identified that allows for the secretion of c-di-GMP outside of bacterial cells. Although we have not identified a mechanism in this study, we provide supporting evidence that this could be the case in *V. cholerae* and could aid in the success of the pathogen. The study of extracellular production of c-di-GMP is extremely limited, and should be further studied given the implications of

this molecule in diverse processes. Of note, many quorum sensing inhibitors have been identified (131, 193), while only a small number of compounds have been identified as inhibitors of cyclic di-nucleotides. To date, there are also no known inhibitors of c-GAMP. Inhibition of c-di-GMP has proven to be difficult given the fact that bacteria seem to have a wide distribution of DGCs and PDEs (responsible for synthesis and degradation, respectively) (194). Still, some advancements have been made and more researchers are getting involved in this area (195). Given our understanding of these molecules so far, inhibitors of CDNs have the potential to positively impact modern medicine or be used in industrial settings (control of biofilm formation).

Although I believe that we have successfully defined this new role for bacterial second messengers and their effect on eukaryotes, more work still needs to be done. The interplay that exists between bacteria and a host organism can be ever changing, with continuous back-and-forth modifications being made by both organisms for optimal survival. The coordination between multiple signal transduction pathways is complex. While signaling is better understood in single-celled organisms, there remains gaps in knowledge in regards to multicellular organisms and hierarchical response mechanisms.

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