INVESTIGATING AMINO ACID-MODULATED MOTILITY OF THE ZEBRAFISH BACTERIAL ISOLATE, *AEROMONAS VERONII*

by

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A THESIS

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Title: Investigating Amino Acid-Modulated Motility of the Zebrafish Bacterial Isolate, *Aeromonas veronii*

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Primary Thesis Advisor

Animals are colonized by communities of microorganisms that influence the health and development of their host. However, the mechanisms of host colonization are still underexplored. To investigate this, previous work in the lab used experimental evolution to adapt a bacterial symbiont, *Aeromonas,* to the zebrafish gut. These experiments led to the identification of a novel gene, *spdE*, which significantly impacts host colonization. We found that evolved isolates with mutations in *spdE* had faster rates of motility and increased host immigration. Sequence analysis revealed that the protein, SpdE, has a domain for sensing extracellular signals and a diguanylate cyclase domain which produces an intercellular signaling molecule that regulates motility. Further biochemical investigation identified that the signal SpdE senses is hydrophobic amino acids, specifically proline, valine, and isoleucine. To further investigate the relationship between SpdE-dependent *Aeromonas* motility and environmental amino acids, we developed a new technique ("exploration assay") which is designed to measure differences in motility between strains or conditions. Using the exploration assay, we compared motility of wild type and *spdE* knockout strains in different amino

acid environments. From our results, we found that the wild type strain is more motile in the presence of these amino acids. However, even in the absence of amino acid signal, the *spdE* knockout is more motile than the wild type. From these data, we have created a model for how SpdE regulates motility in response to amino acids which offers novel insights into *Aeromonas* biology and the mechanisms of host colonization.

Acknowledgements

I would like to thank everyone in the Guillemin lab who have helped mentor me and answered any of the questions I had. The Guillemin lab has given me an amazing opportunity where I have learned so much about microbiology and what research entails. I would like to especially thank Cathy Robinson who has patiently guided me throughout my research experience, Emily Goers Sweeney for teaching me how to conduct a thermofluor assay as well as purify and crystalize proteins, and Elena Wall for teaching me about the germ-free process.

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Introduction

Host Microbe Interactions

According to scientists from the University of Georgia, the estimated number of bacteria on our planet is in the five million trillion trillion, which is more than the number of stars in the universe (BBC News | Sci/Tech | Planet bacteria, 2021). While many from this number can cause diseases, most bacteria are actually beneficial. An example of a beneficial bacteria is called *Lactobacilli acidophilus*, commonly found in the intestinal tracts of humans. They make lactic acid by breaking down carbohydrates and are used to treat lactose intolerance (Lactobacillus Acidophilus - Health Encyclopedia - University of Rochester Medical Center, 2021). This interaction between the bacteria and its host is termed host microbe interaction.

There are several forms of host microbe interactions. The relationship between *Lactobacilli* and humans would be a mutualistic relationship because both partners benefit from the other and also a symbiotic relationship because there is persistent contact between the two species. In this study, we used a bacterial symbiont called *Aeromonas veronii* because of its prevalence in aquatic environments, especially in the guts of zebrafish, which is the model organism of the study.

Aeromonas veronii is a gram-negative bacterium (Mencacci, 2003). *Aeromonas veronii* is found in both humans and in aquatic species. In humans certain *Aeromonas* species are linked with diseases such as gastroenteritis, septicemia, and skin diseases. Some motile species are food and waterborne pathogens that have been associated with food-borne outbreaks and are commonly isolated from human diarrhea (Igbinosa,

2012). On the other hand, in aquatic species such as zebrafish, *Aeromonas* help with immune defense, gut cell growth, and the development of the pancreas (Matos, 2018).

Previous Research

Previous work in the lab used experimental evolution to adapt a bacterial symbiont, *Aeromonas,* to the zebrafish gut. These experiments led to the identification of a novel gene, *spdE*, which significantly impacts host colonization. We found that evolved isolates with mutations in *spdE* had faster rates of motility and increased host immigration. Sequence analysis revealed that the protein, SpdE, has two domains, a domain for sensing extracellular signals and a diguanylate cyclase domain which produces an intercellular signaling molecule that regulates motility.

The sensory domain is called the PAS domain which stands for Per-Arnt-Sim. This domain is found in proteins from all kingdoms of life. In bacteria, the PAS domain is usually found at the amino terminus of signaling proteins of sensor histidine kinases, cyclic-di-GMP, and methyl-accepting chemotaxis proteins. While the structure of the PAS domain varies between proteins, the main functions of the domain are consistent throughout bacteria. Small-molecule metabolites bind to the PAS domain, which sends a signal for the PAS domain to initiate a cellular signaling response or allows it to respond to a secondary physical or chemical signal. The survival of bacteria lies in its ability to sense and adapt to changes in its environment. Therefore, the PAS domain is important since it plays a key role in sensory and can affect protein / protein interaction, and signal transfer (Henry et al., 2011). For the spdE protein, the PAS domain acts as a direct cellular sensor that also affects the bottom diguanylate cyclase domain.

The diguanylate cyclase domain synthesizes cyclic diguanosine monophosphate (c-di-GMP) which is a second messenger. Second messengers are intracellular signaling molecules that are released by the cell as a response mechanism to extracellular signaling molecules. Cyclic diguanosine monophosphate is found in a wide variety of bacteria and has been found to regulate cellular motility and also promotes adhesion to surfaces through the synthesis of organelles (Yang et al., 2011).

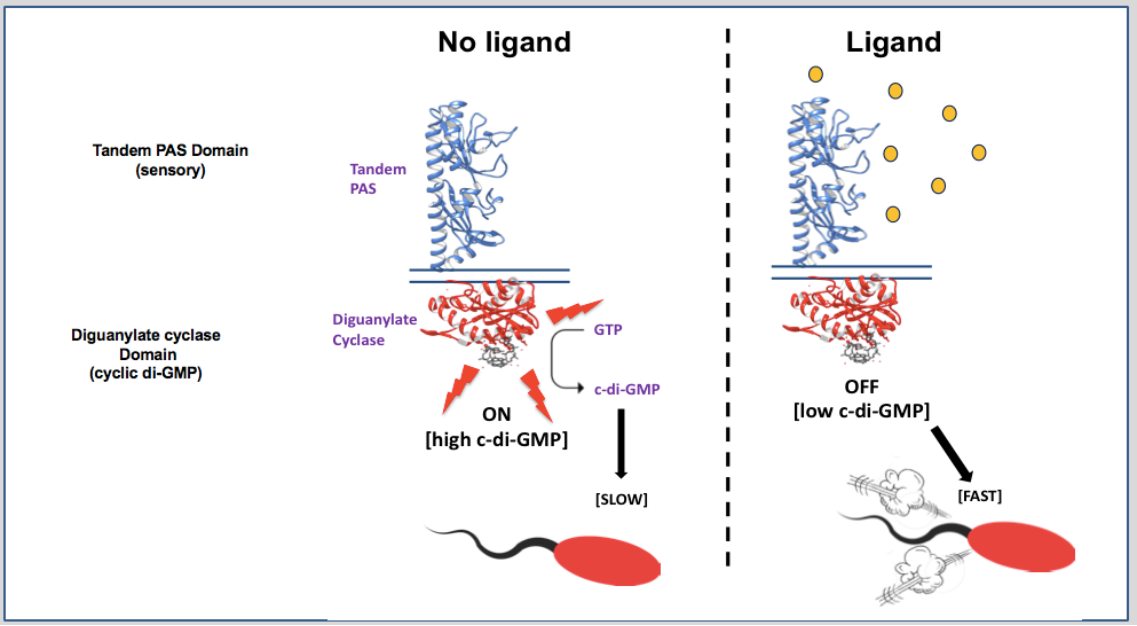

Figure 1. Model of SpdE Structure and Function. Note that c-di-GMP levels produced by the Diguanylate cyclase Domain shown in red decreases motility. Ligand binding turns off the Diguanylate cyclase Domain shown in red which increase motility. Image courtesy of Catherine Robinson.

Further biochemical investigation identified that the signal SpdE senses is hydrophobic amino acids, specifically proline, valine, and isoleucine. This was tested through a series of thermofluor assays which found that certain amino acids that are bound to the tandem PAS domain stabilized the protein and increased its melting point. Mass spectrometer quantification of the intracellular cyclic di-GMP revealed that amino acids decreased the concentrations of c-di-GMP, which increased the motility of *Aeromonas*. Furthermore, when the *spdE* gene is knocked out or completely inactive,

the motility of the bacteria also increases due to the lack in concentration of c-di-GMP. The two-domain system is important because it allows for *Aeromonas* to conserve energy by decreasing its motility for survival purposes and increase its motility when it senses nutrients such as amino acids.

Further investigations into the motility of spdE

The goal of this paper is to measure differences in motility of the wild type strain of *Aeromonas* and its *spdE* knockout to understand how SpdE regulates motility in response to amino acids. While previous research reveals that certain hydrophobic amino acids increased the motility of SpdE, it is still not well understand whether differing concentrations has an influence on the motility of *Aeromonas* or if extracellular concentrations of amino acids affects the motility of *Aeromonas* since the thermofluor assay tested the binding affinity for specific amino acids with the SpdE domain. By investigating how SpdE regulates motility in response to amino acids, we are able to offer novel insights into *Aeromonas* biology and the mechanisms of host colonization.

Methods

Bacterial Strains

The bacterial zebrafish isolates used throughout the study include Aer01 (*Aeromonas veronii* ZOR0001; BioProject Accession PRJNA205571) and Aer02 (*Aeromonas caviae* ZOR0002; BioProject Accession PRJNA205572). The *E. coli* strain used for expression of the proteins used for the thermofluor assays was BL21 DE3 *Escherichia coli* (Robinson et al., 2021).

Thermofluor Assay

The tPAS/dCache portion of the SpdE protein was used for the assay. The protein was expressed and purified in *E. coli.* The thermofluor assay was set up on ice to prevent denaturing of the protein and the assay was performed using a Thermo Fisher Scientific StepOnePlus Real-time PCR instrument. A master mix solution was used for efficiency. The master mix solution was made in the following order: double distilled water, 5 M NaCl, 1 M TRIS pH 7.5, protein, and 4x SYPRO Orange dye. The ligand conditions to be tested consisted of free amino acid dissolved in double distilled water to a final concentration of 10 mM. To each well of a 96 well PCR plate, 18 µL of the master mix and 2 µL of the 10 mM ligand were added. All samples were replicated three times with at least three controls per trial. The temperature for the Thermo Fisher Scientific StepOnePlus Real-time PCR instrument started at a 25°C ramp and decreased at a maximum rate of 100% to 4°C (for 1 min) and then slowly increased at a 1% rate to 80°C. Fluorescent measurements were taken every 8.5 seconds (Robinson et al., 2021).

Exploration assay

Overnight 5 mL cultures of the wild type strain of *Aeromonas veronii* (ZOR0001; *BioProject Accession PRJNA205571*) and a *spdE* clean-deletion variant strain (spdE KO) were grown in filter sterilized TSB (rich media) at 30°C shaking. After 10-15 hours, 1 mL of each overnight bacteria culture were spun down in Eppendorf tubes at 7,000rpm for 1 minute. Supernatant was extracted and cell cultures were washed with 1 mL of filtered embryonic media solution. Small glass culture tubes containing either a ligand solution composed of 2mL embryonic media (EM) and 100μL of 100mM of the amino acid solution to equal a final amino acid concentration of 1 mM, or 2mL of the fish water collected from germ free or conventional fish, were added with 100μL of the washed bacteria culture. The cultures were then incubated for 3 hours with shaking at 30°C. After incubation, 800μL of each of the cultures are spun through a 0.2 µM filter (Corning® Costar® Spin-X® centrifuge tube filter at >10,000rpm for 1 minute to extract the cell-free supernatant. Using a 96-well plate round-bottom plate, 80μL of the supernant to a 96 well plate for 6 wells. Add 80μL of the culture solution to another 96 well plate for five wells. Add blank control for well 6. Using a Rainin Liquidator™ 96-channel benchtop pipettor pull up 5 μL of the supernatant into the pipette tips and submerge the pipette halfway into the 96-well plate containing the bacterial culture and blank control for 30 minutes at room temperature. Over time, bacteria "explore" into the supernatant in the submerged tips, as a measure of motility. Resulting supernatant containing bacteria was added into a sterile 96-well plate (Corning, flat-bottom, #3595) containing 195μL of sterile tryptic soy broth (TSB

medium). A FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany) was immediately used to calculate the growth curves by measuring the absorbance readings of the plate at 600 wavelength every 10 minutes for >8 hours at 30°C incubation with constant shaking.

The absorbance readings are later plotted against time to show the growth curves and the times shown in Figure 2 were calculated by finding the time at which the absorbance passed the 0.5 marker.

In order to ensure there was no significant difference between bacteria concentrations in the bacterial cultures, the 3 hour incubated bacteria cultures were diluted to 10^{-5} concentration in sterile embryonic media (EM) and plated on TSB plates. The plates were counted after incubating overnight at 30°C.

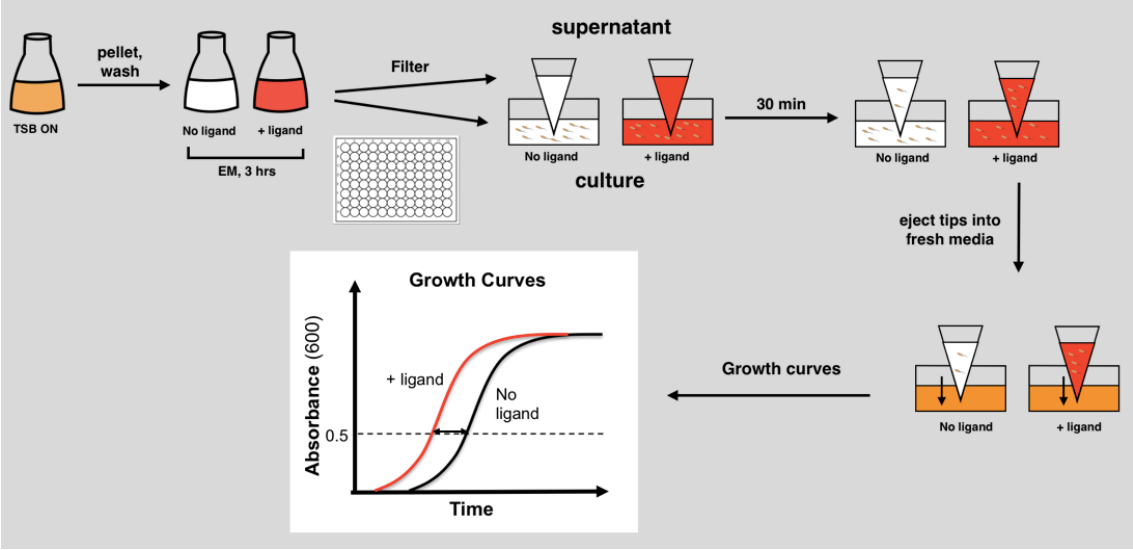

Figure 2. Exploration assay schematic that shows the procedural steps taken as well as the graphed points from the absorbance reading. Curves shifted to the left represents faster motility since the hours it takes to reach the 0.5 optimal density line that was used to compare the different conditions is less compared to the control shown in black. Thus, curves shifted to the right represent slower motility. Image courtesy of Catherine Robinson.

Biofilm assay

Overnight >5 mL cultures of the wild type strain of *Aeromonas veronii* (WT) and its spdE knockout (spdE KO) were grown in filter sterilized TSB (rich media) at 30°C with shaking. After 10-15 hours, 500μL of each overnight bacteria culture are spun down in Eppendorf tubes at 7,000rpm for 2 minutes. Extract supernatant and add 500μL of amino acid solution. Amino acid solutions are composed of 1.5mL of EM and 15μL of 100mM amino acid solution. Plate 150μL of the final solutions on a 96 well plate (Corning, flat-bottom, #3595) for wells 1 through 5. Include a 6^{th} well for the blank control. Incubate at 30°C, stationary, for 48 hours.

For the *Aeromonas veronii* (Aer01 strain), the biofilm formation is mostly formed at the air-liquid interface (Robinson et al., 2021). After 48 hours, discard the liquid in the wells without touching the side of the well of the biofilm formation. Add 150 μL of filtered EM into each well and discard liquid. Do a total of three washes. Add 150µL of 0.1% crystal violet into each well and let sit for 10 minutes. Wash 5 times until the supernatant is clear looking. Add 150µL of 95% ethanol and let sit for 10 minutes. Mix well and transfer all of the solutions to a new 96-well plate. The end point absorbance at 570 nm was read on a FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany).

Chemotaxis assay

The chemotaxis set up is similar to the exploration assay. Overnight 5 mL cultures of the wild type strain of *Aeromonas veronii* (CR1), its spdE knockout (CR296), and the cheA-KO (CR344) were grown in filter sterilized TSB (rich media) at

30°C with shaking. After 10-15 hours, 1 mL of each overnight bacteria culture are spun down in Eppendorf tubes at 7,000rpm for 1 minute and are washed with embryonic media (EM). Add 100μL of the washed bacteria culture to small glass vials that contain 2mL of embryonic media (EM). Incubate for 3 hours with shaking at 30°C.

After incubation, add 80μL of the bacteria culture to a 96 well plate for 5 wells with the 6th well acting as the blank control. Add 80μL of the 100μM ligand solution (free amino acid dissolved double distilled water) that is to be tested to another 96 well plate for wells 1 through 5. Add a blank control for well 6. Using a Rainin Liquidator™ 96-channel benchtop pipettor, 5 μL of the ligand solution were transferred into pipette tips and halfway submerged in the bacterial culture for 30 minutes at room temperature. During this time, bacteria attracted to the ligand in the pipette tips will swim up the concentration gradient and accumulate in the pipette tips. After incubation, the contents of the pipette tips (containing bacteria) were added into a sterile 96-well plate containing 195μL of sterile tryptic soy broth. A FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany) was immediately used to calculate the growth curves by measuring the absorbance readings of the plate at 600 wavelength every 10 minutes for >8 hours at 30°C incubation with constant shaking. The absorbance readings are later plotted against time to show the growth curves.

In order to ensure there was no significant difference between concentrations of bacteria in the cultures, the 3-hour incubated bacterial cultures were diluted to 10^{-5} in sterile embryonic media (EM) and plated on TSB plates. The plates were counted after incubating overnight at 30°C.

Results + Discussion

Thermofluor assay results show specific ligand binding at the distal PAS/Cache domain

Previous research has shown that the *spdE* gene product consists of a 544 amino acid transmembrane protein that contains two functional domains - a N-terminal tandem PAS/dCache domain and a C-terminal diguanylate cyclase domain (Robinson et al., 2021). The tPAS/dCache domain is known to bind a variety of small molecules including amino acids, small organic acids, and urea **(**Upadhyay et al., 2016**).** Previous work in the lab (conducted by Emily Sweeney) used thermofluor assays to identify that SpdE from Aer01 interacts with a subset of hydrophobic amino acids (Figure 3A). For each of the curves shown in Figure 3, the melting temperature (T_m) was determined (blue circles), which corresponds to the inflection point of the curve, represented by the center of the exponential section of the curve. The ΔTm (difference between compound T_m and its water control T_m) was calculated for each ligand condition. As shown in Figure 3A, the largest thermal shift for SpdE was in the presence of proline. The average temperature difference after subtracting from the water control for each amino acid is shown in Figure 3A. The amino acids that resulted in the largest thermal shifts were proline, valine, and isoleucine, respectively. Leucine, alanine, methionine, threonine, and glycine had the smallest thermal shift difference.

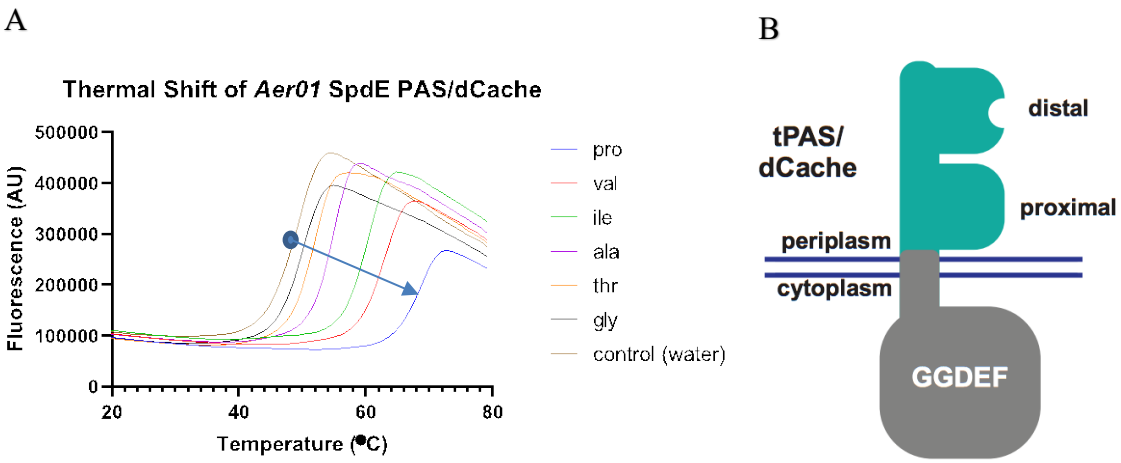

Figure 3A. Thermal shift assay curves of the tandem PAS/dCache region of Aer01 SpdE protein. The blue dots represent the approximate inflection points or melting temperatures (Tm) for the control and proline. The arrow shows the difference between Tm values for each amino acid with proline having the largest thermal shift difference. The amino acids included (pro = proline; val = valine; ile = isoleucine; leu = leucine; ala = alanine; met = methionine; thr = threonine; gly = glycine). Figure 3B (on the right) is a 2D model of SpdE within the inner cell membrane, which includes the distal and proximal domain binding pocket of the overall tPAS/dCache part of the protein. Image courtesy of Catherine Robinson.

Preliminary research conducted by Emily Sweeney showed that single acid residue changes in the distal PAS/Cache domain binding pocket led to a reduction in ligand binding, suggesting that the distal domain is important for stable ligand binding. Specifically, the tyrosine in the distal pocket disrupted ligand binding. This led to the conclusion that tyrosine is an important residue for binding. To determine which PAS/Cache domain, the distal or proximal, out of the two interacts with the hydrophobic ligands (Figure 3B), we picked residues within the ligand binding pocket that the ligands could be interacting with and mutated them to see if there would be any changes with the protein's thermal stability. Since the SpdE structure was unknown at the time, Emily Sweeney conducted some bioinformatic analysis to determine which residues might be important for binding. The residues that interact with the ligands within SpdE include those that have potential hydrogen bonding capabilities. Within SpdE's ligand binding pocket, both tryptophan and tyrosine can hydrogen bond and

hold ligands in place. By mutating one of these residues to alanine, which does not have the ability to hydrogen bond, there will be a change in ligand binding in that location. Therefore, the tyrosine (which has a hydroxyl group that can hydrogen bond) was mutated to alanine (which has a methyl group that is unable to hydrogen bond) in the proximal domain to determine which domain is involved in ligand binding. The mutation is labeled as Y211A. If the tyrosine in the proximal PAS/Cache domain was important for ligand binding, the mutation to alanine would disrupt ligand binding and decrease protein stabilization, resulting in a lower melting temperature (T_m) value. Figure 4 compares the WT version of *Aer*01 SpdE with the mutated Y211A version. The ΔTm (difference between compound Tm and its water control Tm) for the WT and mutated Y211A variant shows no significant difference. Thus, we can conclude that the proximal PAS/Cache domain is not important for stable ligand binding.

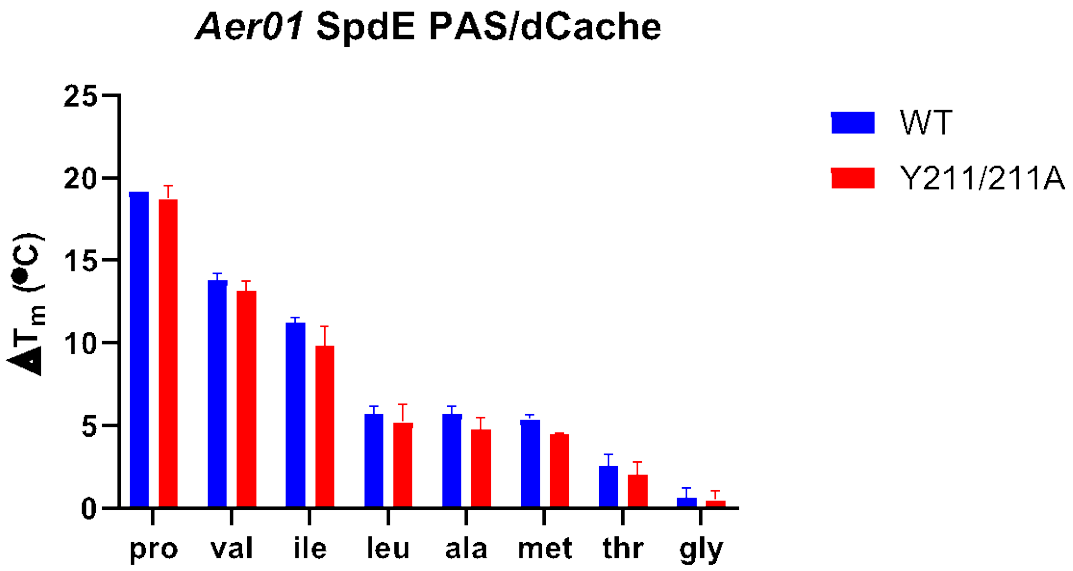

Figure 4. Graphed representation of the calculated delta Tm values (number of degrees shifted compared to the control) of the WT version of Aer01 SpdE PAS/dCache and the mutated Y211A version of Aer01 SpdE of the proximal

PAS/dCache domain for different L-amino acids (pro = proline; val = valine; ile = isoleucine; leu = leucine; ala = alanine; met = methionine; thr = threonine; gly = glycine)

Next, we looked at a closely related *Aeromonas* species (*Aeromonas caviae;* ZOR0002*)* within our zebrafish gut isolate culture collection, that also had the *spdE* gene. Gene neighborhoods surrounding *spdE* within the genomes of both the two *Aeromonas* species had high levels of genomic synteny, supporting that these genes are homologs. Furthermore, alignment of the amino acid sequences showed 52% identity across the SpdE tandem PAS/dCache region of the protein and 52.3% identity across the entire protein. Importantly, the key binding residues identified in the Aer01 SpdE were 100% conserved in Aer02 SpdE and are predicted to be in similar locations in the folded protein (Robinson et al., 2021). In order to confirm that ligand binding is also conserved for Aer02 SpdE, we performed the same thermal shift assay and saw the same pattern in the delta Tm values across the tested amino acid ligands as was the case for Aer01 SpdE (Figure 4). Although these proteins are only 52% identical, our biochemical analysis shows that they have similar ligand binding profiles and therefore have potentially similar functions.

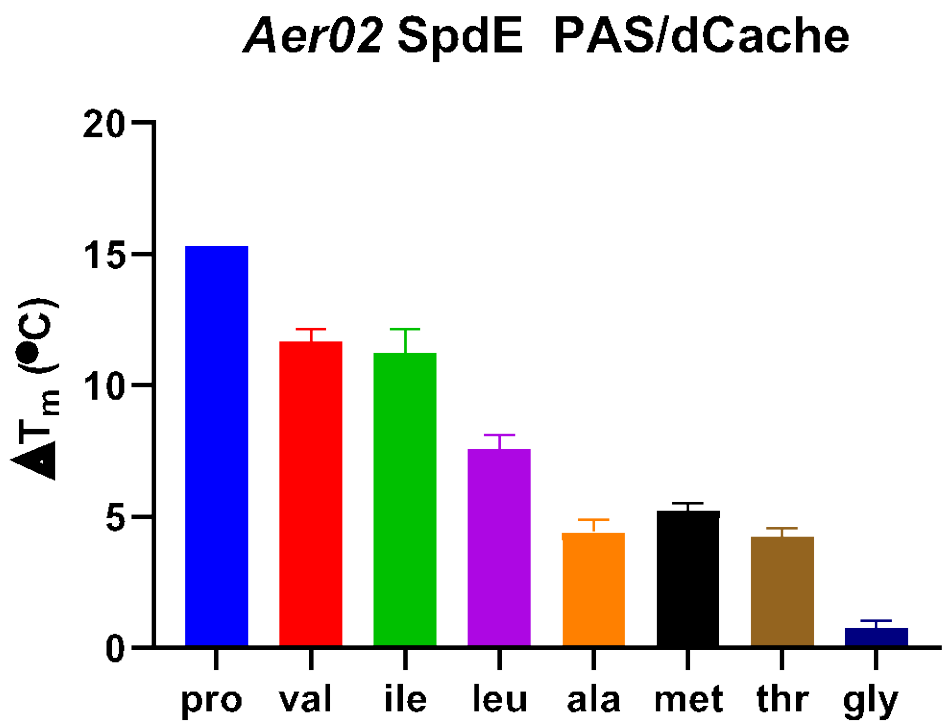

Figure 5. Graphed representation of the calculated delta Tm values (number of degrees shifted compared to the control) of Aer02 SpdE for different L-amino acids *(pro = proline; val = valine; ile = isoleucine; leu = leucine; ala = alanine; met = methionine; thr = threonine; gly = glycine)*

Exploration assay results show increase in motility is dependent on specific ligands for Aer01 and is dependent on c-di-GMP levels

The "exploration assay" technique was developed in the Guillemin lab and is designed to measure differences in motility between strains or conditions. This new technique was termed "exploration" because bacteria are allowed to explore their environment without a chemical gradient, so it is not dependent on chemotaxis but is a way to measure the motility. Bacteria in the wells of a 96-well plate are allowed to explore into submerged pipette tips containing culture supernatant, and after a short incubation the cells in the pipette tips are used to inoculate a 96-well plate containing

TSB and monitor growth curves . The more cells that explore into the pipette tips, the higher the inoculum and the earlier the growth curves reach a 0.5 OD value. The results from the growth curves are analyzed by calculating the time shift between curves. Curves shifted to the left represent faster motility, and vice versa. Using the exploration assay, we compared motility of Aer 01 wild type and *spdE* knockout strains in different amino acid environments. From our results, we found that the wild type strain is more motile in the presence of these amino acids. However, even in the absence of amino acid signal, the *spdE* knockout is more motile than the wild type.

In order to test how sensitive *Aeromonas veronii* motility was to the addition of SpdE's amino acid ligands, we tested it with a range of proline concentrations. The overall trend was that increasing the concentration of proline from 0 mM to 10 mM gradually increased the motility of the bacteria (Figure 6). However, as the concentration increases, the time difference between the rate of the growth curve decreased. Therefore, there is a saturation point where 1 mM and 10 mM of proline had the same effect on the wildtype (WT) strain. These investigations revealed that SpdE's response to ligand is dose-dependent and sensitive to very low concentrations. Even with a 0.0001mM concentration of proline, there was a substantial shift in the time difference, thus showing the dramatic impact certain amino acids have on the motility of these bacteria.

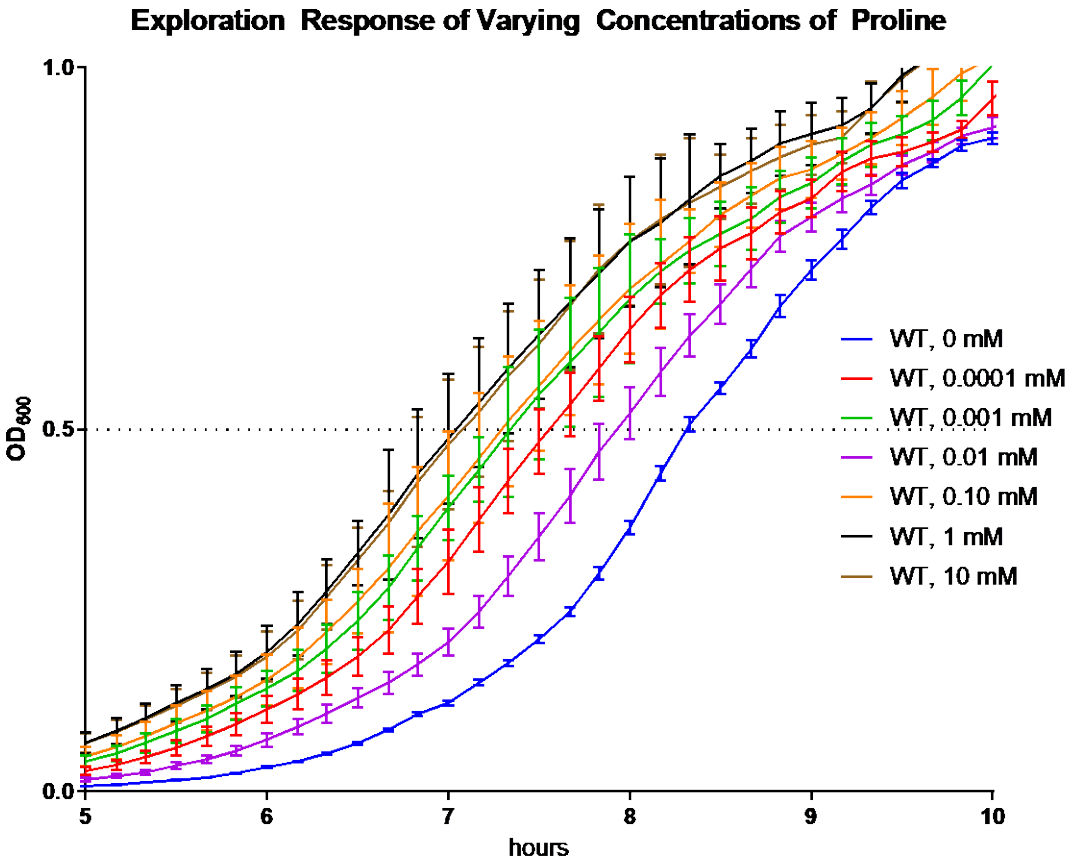

Figure 6. Graphed representation of the data points from measuring the absorbance readings from the exploration assay. The growth curves correspond to the absorbance readings taken at several time increments. The different curves represent the different conditions that were tested using increasing concentrations of proline.

According to the data collected from the thermofluor assay, the amino acids proline, valine, and isoleucine were stabilizing, while glycine had no effect on *Aeromonas* SpdE thermal stability. We tested if this trend would hold true for the impact of these amino acids on motility, by using the exploration assay. Additionally, we were interested to see if there were any differences between the motility of the WT and KO strains of *Aeromonas* since c-di-GMP levels are known to affect bacterial motility. Figure 7A is an example of one exploration assay that was conducted to test the effect the selected amino acids from the thermofluor assay had on the motility of the

WT and KO strains of *Aeromonas*. The blue arrow in Figure 7A represents the time difference between growth curve with ligand (WT EM + Proline) and growth curve with no ligand (WT EM) at OD 0.5 that are shown by the red and blue lines. Since the blue line (WT EM) comes up later in comparison to red line for WT EM + Proline, we can conclude that *Aeromonas* was faster with the addition of amino acids compared to the no amino acid control, because the growth curve crossed the OD 0.5 line sooner. The presence of proline significantly increased motility compared to the rest of the amino acids which is consistent with the thermal shift data. Similarly, the exploration assay data show that glycine does not increase the motility of *Aeromonas*, as was predicted from the thermofluor data. The orange, black, and purple lines are of the spdE knockout strain of *Aeromonas.* All three lines come up faster than the WT in EM. Based on the known function of diguanylate cyclase activity, since the *spdE* gene is knocked out, there is no production of c-di-GMP which will results in faster *Aeromonas* motility than the WT strain. Unlike the WT strain that had an increase with the addition of proline, there is no difference between adding amino acids to the KO strain. This is expected since the gene is deleted, *Aeromonas* can no longer sense and respond to amino acids. While the orange line representing KO EM + Proline may be seen a little slower than the other two KO conditions, it is still within the range of error of both KO conditions. While the KO strain should not respond to ligands, there could be other proteins that sense and respond to the addition of amino acids, which may cause slight differences. The main take away is that the KO EM was significantly faster than the WT EM which proves that the decrease in c-di-GMP levels as a result of the spdE KO strain increased bacterial motility.

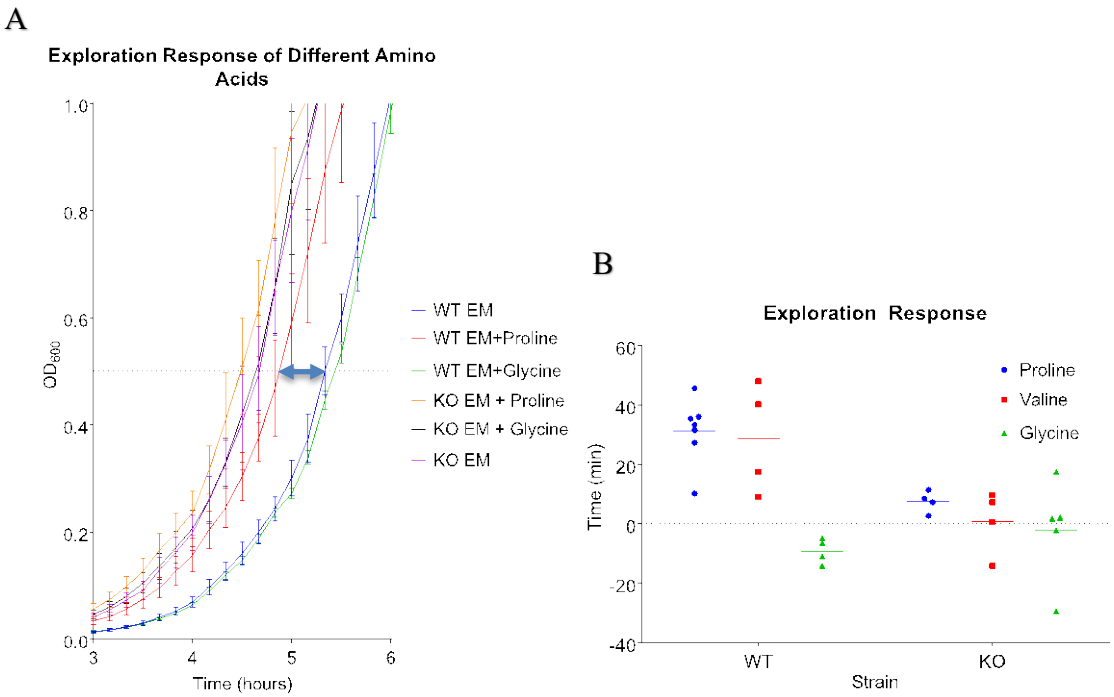

Figure 7A. Graphed representation of the data points collected from timed interval readings of the absorbance values of different bacterial conditions. The dotted line represents OD 0.5 that was used to compare the motility of each bacterial condition. The arrow represents the time difference in motility between the two conditions, showing that a shift to the left represents faster motility. Figure 7B (on the right). The time differences between amino acid conditions and its EM control were calculated by subtracting the time value at the OD 0.5 mark from the exploration assay. This collection includes all the data taken from multiple exploration assays. The horizontal line represents the median time difference values, not accounting for the outliers.

The data collected from multiple Exploration Assay experiments such as those from are shown in figure 7B. Each point on the graph corresponds to the time difference between the *Aeromonas* strain (WT or KO) when in the presence of ligand compared to the no ligand control (EM). Positive values mean that *Aeromonas* was faster in that amino acid compared to no amino acid control. The dotted horizontal line shows no time difference. The short horizontal line shows the average of all the experimental values collected for the certain condition. Since the *spde* KO's average values for all three ligand conditions are close to zero, compared to the about 30-minute difference with the WT strain for proline and valine, the WT strain is more motile in response to

ligands, while the *spdE* KO is not. Again, glycine is shown to be a ligand that does not affect the motility of the WT strain.

The gene *spdE* was discovered from evolution experiments where there was a huge advantage for mutation. Since Aer01 still maintains a functional *spdE,* it must have a function in the natural system. Because the evolution experiments were conducted in germ free (GF) fish, we wanted to test if a loss of *spdE* would still have an advantage in conventional (CV) fish. The conventional setting is more relevant because of the presence of microbiota which is biologically relevant. The concentrations of amino acids in this fish host-microbe system are unknown. In order to explore the relevance of SpdE in a more natural setting, we compared motility of *Aeromonas* in water collected from flasks of larval zebrafish that were raised germ free (GF; without microbes) or conventionally (CV; with a complex microbiota). Since we were unable to determine the concentration and types of amino acids in the GF and CV fish water via analytical techniques (e.g., mass spectrometry, enzymatic methods; data not shown) because the ligand concentrations were below the limit of detection for the quantification assays, we tested motility using the exploration assay because we know that even a 0.0001mM concentration of proline has a major effect on the motility of SpdE (Figure 6). Therefore, we wanted to compare Aer01 motility in the GF and CV fish water since we hypothesize that there are amino acids present in the environment of conventional zebrafish, while there would be much less or none in the germ-free conditions. We collected the flask water from fish that were 4-7 dpf (days post fertilization), filter-sterilized it, and used it for the exploration assays. We hypothesized that conventional conditions would contain more ligands compared to germ free

conditions and thus there would be a larger exploration response for Aeromonas in CV versus GF fish water, which is what we saw (Figure 8). Further analysis on whether or not the developmental time point (days post fertilization) accounted for any time differences between motility were inconclusive as there seemed to be no consistent pattern found (data shown as the colored dots in Figure 8). While the variation is high, there is still a consistent trend of an increase in motility in CV fish water compared to GF fish water with the average time difference of 21 minutes. This average time difference is lower than the average time difference found when comparing the WT strain to proline and valine, seen in Figure 8. Without knowing the ligand concentration or types of ligands present in the CV fish water, the exploration response difference between CV and GF fish water can help us conclude that there must be at least one of the amino acid ligands, specifically proline, valine, or isoleucine present in the solution. Due to variability shown in Figure 8, we can assume that the types and concentrations of free amino acids vary depending on the microbial communities within the flasks that

were tested.

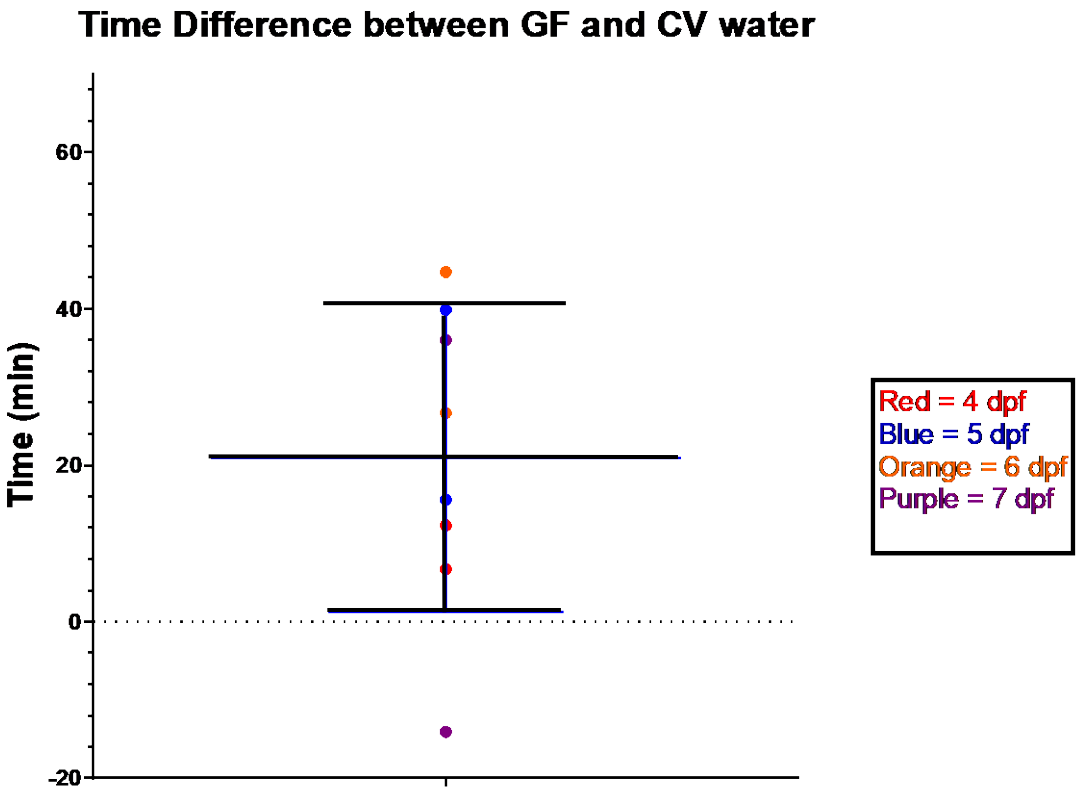

Figure 8. From the exploration assay, we are able to calculate the time differences between conventional and germ free flask water by subtracting the time points at the 0.5 OD line. This is a compilation of several exploration assay experiments that tested the motility of 4-7 days post fertilization GF and CV water. A one sample t test found that the p-value (two tailed) is 0.0196. The p-value suggests that the time difference between CV and GF water is significantly different.

Biofilm assay results show increase in motility is dependent on specific ligands for Aer01 and is dependent on c-di-GMP levels

Another way to measure the motility of the *Aeromonas* strains is by measuring their ability to form biofilms. Biofilms are formed when microbes attach to the surface of an object[10]. If a bacterium is more motile, we would except there to be less biofilm growth. In a sense, biofilm formation is the opposite of motility. Past studies have shown that cyclic diguanosine monophosphate (cyclic di-GMP) is found in a wide variety of bacteria and has been found to regulate cellular motility and also promote

adhesion to surfaces. Therefore, cyclic di-GMP inversely regulates motility and biofilm formation, so cells that are more motile should have less biofilm formation. By staining the biofilm growth with a crystal violet solution, we are able to measure biofilm formation by the optical density (absorbance) readings at 570 nm. Like we see in the exploration assay where increasing proline concentrations increased motility, here we saw that increasing the concentration of proline resulted in less biofilm growth (Figure 9). However, compared to the exploration assay there was a smaller difference between increasing the proline concentration from 1μM to 100μM. An ordinary one-way ANOVA test analysis concluded that when the control (WT, EM) was compared to the differing concentrations of proline, only the EM vs proline with the highest concentration (1mM) was shown to be significant. All other proline concentrations were not significant when compared to the control. Because the slight differences between changes in proline concentrations were not big enough to be significant, we can conclude that the biofilm assay is not as sensitive as the exploration assay.

ANOVA tests the null hypothesis. If the results from the ANOVA test show a statistically significant result, the alternative hypothesis is accepted which means that there are at least two group means that are statistically significantly different from each other. Since the one-way ANOVA is an omnibus test statistic, "it cannot tell which specific groups are statistically significantly different from each other, only that at least two groups were" (One-way). If the p-value is greater than 0.05, there are no statistically significant differences between group means. On the other hand, if the p-value is less than 0.05, there is a statistically significant differences between groups.

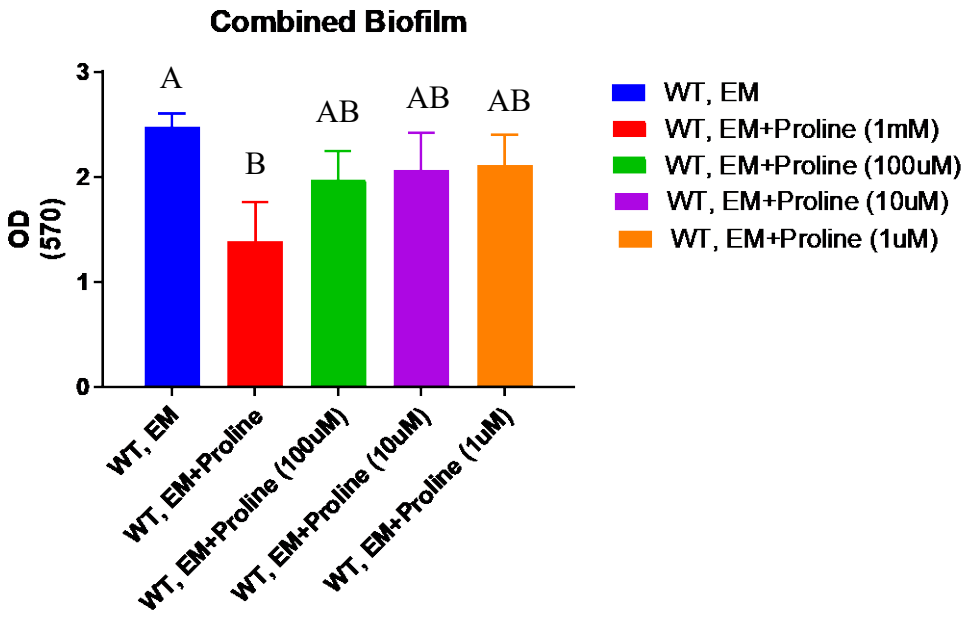

Figure 9. The absorbance readings at 570 nm were graphed for each biofilm condition. The calculated range of error is shown by the horizontal line above the bar graphs. The biofilm growth shows a difference with increasing concentrations of ligand. Letters correspond to the one-way Anova test. Groups that do not share a letter have a mean difference that is stastically significant. Data and image courtesy of Peter Shen.

The biofilm assay was also used to confirm the results obtained from the exploration assay for Figure 6 and 7. The results again confirmed that *spdE* KO had the fastest motility even when WT *spdE* had the addition of proline (Figure 10). Overall, the biofilm growth for *spdE* KO stayed the same despite the addition of amino acids and WT was most affected by proline, valine, and leucine (Figure 10). Again, there is a difference between proline and valine similar to the exploration and thermofluor results (Figure 10). The WT control and all the ligands were found to be significantly different. Importantly, the p value of WT proline and WT valine was 0.0104, which shows a significant difference. While we would have predicted the KO control and the KO ligands to have no difference, proline and isoleucine were found to be significantly

different. However, when compared to the differences between the WT control and the tested ligands, the p values are considerately higher for those of the KO.

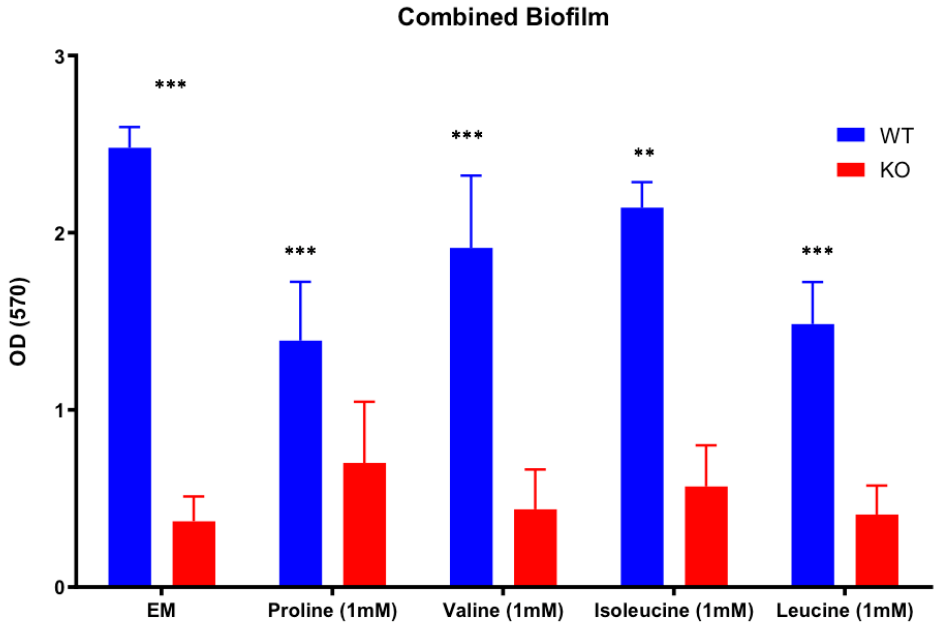

Figure 10. The absorbance readings at 570 nm were graphed for each biofilm condition. The calculated range of error is shown by the horizontal line above the bar graphs. The biofilm growth shows a difference with certain ligands. Unpaired T test results are shown with asterisks (= P < 0.05; ** = P < 0.01; *** = P < 0.001). Asterisks represent comparison to WT EM. Data and image courtesy of Peter Shen.*

From these data, we have created a model for how SpdE regulates motility in response to amino acids which offers novel insights into *Aeromonas* biology and the mechanisms of host colonization shown in the introduction. By understanding the forces that affect motility, we may find new approaches for preventing the spread of pathogenic bacteria and increase the colonization of mutualistic bacteria.

Chemotaxis assay results show SpdE ligands act as chemoattractants

From the results above, we learned that Aer01 uses SpdE to sense and respond to amino acids by modulating motility and biofilm formation. Another way that bacteria

navigate their environment is through chemotaxis, which also facilitates host colonization, so we decided to test if *spdE* ligands are also chemotaxis signals. A chemotaxis assay is used to test the response of cells to a chemical stimulus. In this experiment, certain amino acids and different strains of *Aeromonas* were used to test what ligands acted as chemoattractants. The three bacteria strains tested include the wild type strain of Aeromonas veronii*, spdE* KO*,* and the cheA-KO. CheA is a histidine protein kinase that is a central regulator of bacterial chemotaxis and acts to control gene expression. Unlike most histidine kinases, CheA is not part of the integral membrane protein and instead is associated with and regulated by several different transmembrane chemotaxis receptors that detect different classes of attractant and repellent chemicals (Stock). For the chemotaxis assay, serine acted as a positive control and is a chemoattractant. Figure 11 is an example of one chemotaxis assay that was conducted to test if spdE ligands are chemotactic signals for WT *Aeromonas*. As predicted the lines for all the ligands come up earlier in comparison to the black line that represents WT EM. Therefore, we can conclude that all the ligands tested are chemoattractants. The time differences where each line comes up shows that different ligands have different strengths of attraction. However, since it is difficult to separate whether the growth curves came up earlier was due to chemoattraction or that the ligands affected the motility of WT and caused the growth curve to come up earlier, a separate chemotaxis was conducted with the CheA-KO and also with the spdE knockout.

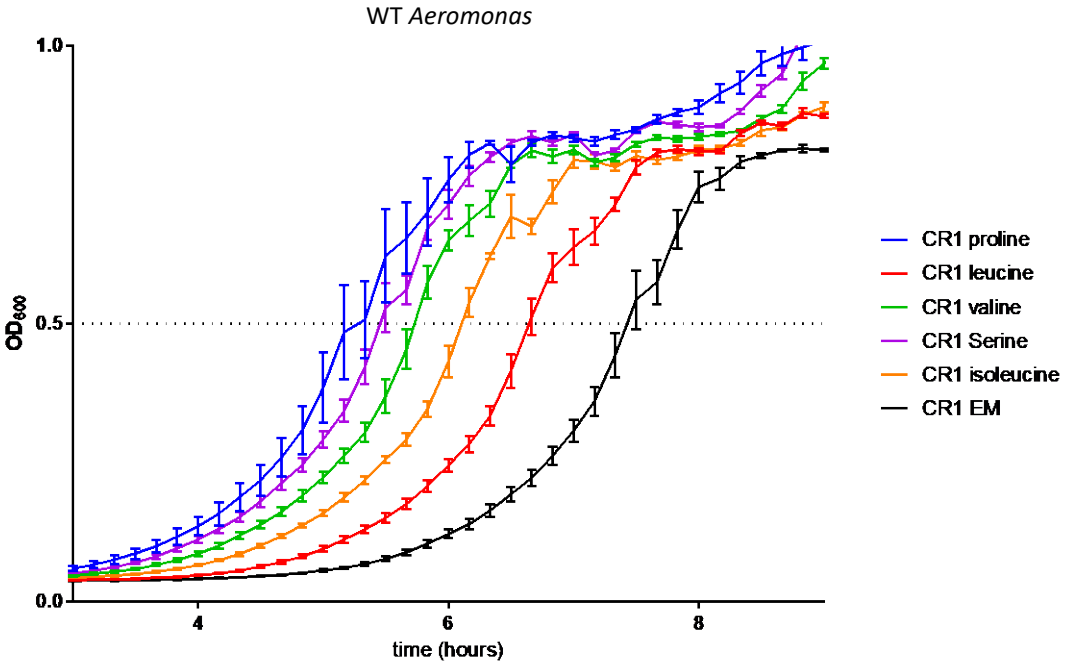

Figure 11. Graphed representation of the data points collected from timed interval readings of the absorbance values of different bacterial conditions for CR1 using a chemotaxis assay. The dotted line represents OD 0.5 that was used to compare the motility of each bacterial condition.

Figure 12 is an example of one chemotaxis assay that was conducted to test the chemoattractants of the spdE KO strain of *Aeromonas*. All of the tested ligands came up earlier in comparison to the black line that represents spdE KO EM. However, in comparison to WT, there is no time difference between the different ligands as all of them are clustered together before the EM condition. Since it is known that the motility of spdE KO is faster than the WT, it is understandable that the curves would be shifted more to the left.

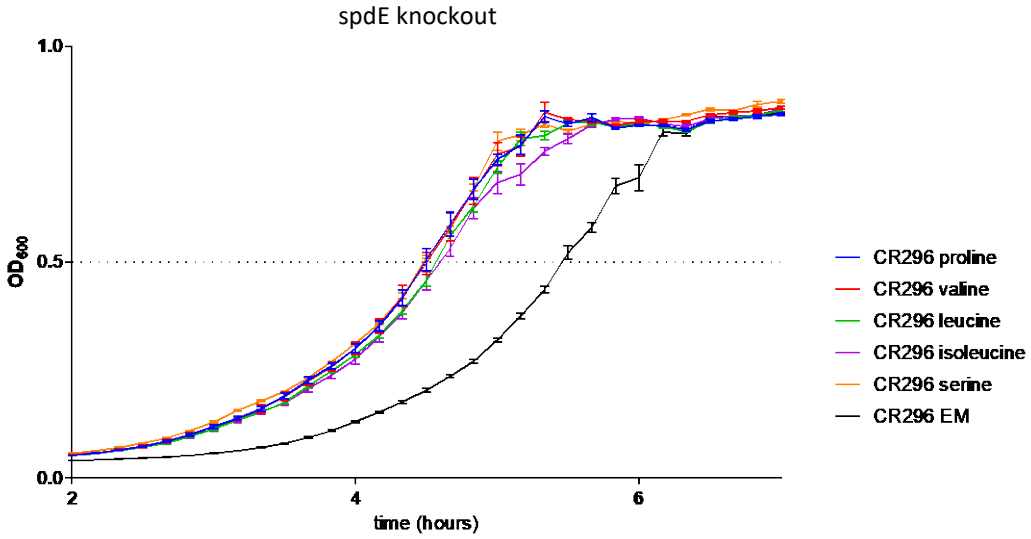

Figure 12. Graphed representation of the data points collected from timed interval readings of the absorbance values of different bacterial conditions for CR296 using a chemotaxis assay. The dotted line represents OD 0.5 that was used to compare the motility of each bacterial condition.

Figure 13 is an example of one chemotaxis assay that was conducted to test the chemoattractants of the CheA-KO strain of *Aeromonas*. Since the CheA-KO strain and CheA is a central regulator of bacterial chemotaxis that acts to control gene expression, we would except CheA-KO to be similar to WT but have smaller shifts.

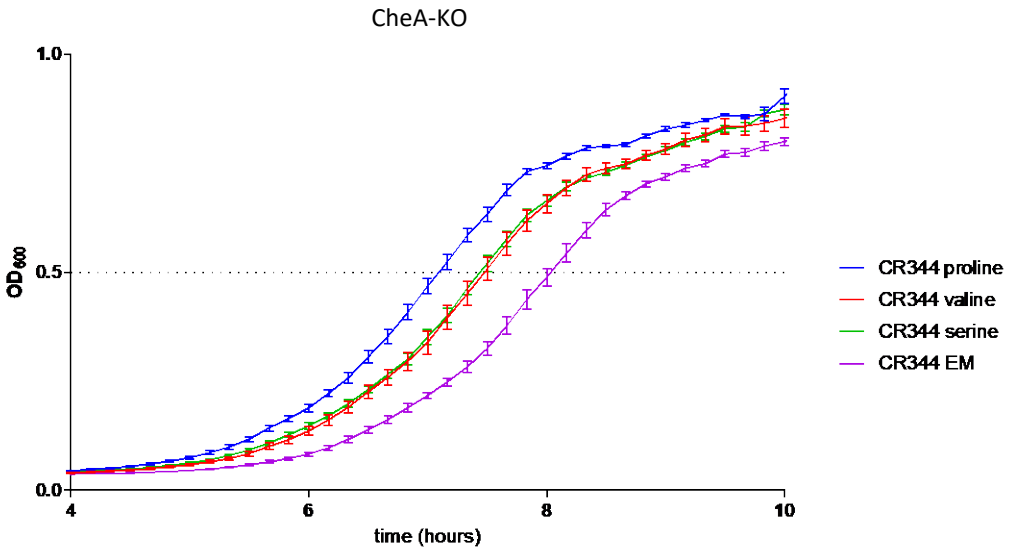

Figure 13 Graphed representation of the data points collected from timed interval readings of the absorbance values of different bacterial conditions for the CheA-KO using a chemotaxis assay. The dotted line represents OD 0.5 that was used to compare the motility of each bacterial condition.

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