A Tale of Two Coral Microbes: How motility and quorum sensing may play a role in competition between Vibrio species

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Introduction

Many microbial species interactions are of human and ecological importance (ex. plant biomass increase through rhizosphere communities[1]). A primary target for this research field are microbes that compete because they produce secondary metabolites that can be used in medicine or technology. These interactions are also important because they affect the whole microbial community and can sometimes even affect disease outcome in hosts (ex. E. coli strains competing in human gut[2]).

In particular, many species of coral depend on zooxanthellae and other interacting microbial symbionts to gain the nutrients they need or to provide a protective effect against disease or changing climate[3]. The coral symbiosis system has been used to develop the framework of the "holobiont", or the microbial community plus its host, which functions in a tightly co-evolved assemblage that can be perturbed by both biotic and abiotic threats[4]. In the first few weeks of enrichments, our group isolated two bacteria from unbleached corals living at 27ºC that appeared to be competing in culture. We observed that one microbe, a transparent, glossy colony, was growing right in the middle of a larger white, glossy colony, with what could be a zone of inhibition around it. It was unclear what direction this competition is occurring in (i.e. who was inhibiting who, or if there was truly inhibition at all) or the mechanisms behind the interaction. This provided a good opportunity to use culturing assays and microscopy to characterize the interaction of these two microbes.

One of the microbes (in this paper, referred to as Vibrio H) was previously identified via 16S rRNA sequencing as *Vibrio tubiashii*, which is a pathogen of bivalves[5] and closely related to the coral pathogen, *Vibrio coralliilyticus*[6]. The other microbe (referred to as Vibrio J) was identified as being either *V. tubiashii or Vibrio mytili*, which has been cultured from mussels[7]. Unfortunately, the 16S rRNA marker gene does not reliably provide enough resolution to tell us the exact species identification, as *Vibrio* species have high rates of homology between their DNA[5], so multiple variable region marker genes would be useful in further identifying the species and/or strains. Research has also shown that strains from the same *Vibrio* species can inhibit each other[8], which suggests our two *Vibrio* species could be interacting antagonistically as observed[9]. However, the ecological mechanisms behind these microbe-microbe interactions are not as well studied, and could help us understand the nature of the overall interaction better[9,10]. In particular, interactions can be context-dependent, for example only occurring when there is a specific nutrient source[10,11], or when they are with specific strains[11,12]. Characterizing the effects and ecological mechanisms behind the competition is an important piece of the puzzle in community-level interactions on corals and other hosts.

I chose to investigate the nature of this interaction by asking why these two microbes compete, who is the better competitor, and how the growth of each microbe is affected by the presence of the other. Based on the limited preliminary growth plates we had at the time, my working hypothesis was that Vibrio H was producing a metabolite that was triggering Vibrio J to

swim, swarm, or twitch away or lyse. I approached my questions using a combination of culture plate assays, swim/swarm/twitch agar assays, spent media experiments, competition assays, and microscopy.

Materials and Methods

Swim, swarm, twitch assay

Since I suspected that some of my microbes could be using motility when they were interacting, I wanted to test what types of motility they had. I made three types of plates and inoculated three different ways to test for swim, swarm, or twitch motility for Vibrio J and Vibrio H. The concentration of agar selects for a certain type of motility in each plate. Swim agar used 0.3% agar SWC media, and one of the two microbes was stabbed halfway through the agar in the center. Swarm agar used 0.5% agar SWC media, and 10 µL of each microbe was inoculated at the top of its own plate. Twitch agar used 1.5% agar SWC media, and one of the two microbes was stabbed all the way through to the bottom of the agar. These plates had to be inoculated the same day they were poured to avoid significant drying. They were checked after 24 hours and 48 hours, and were grown at room temperature. This assay yielded a swarming mutant of Vibrio J (Vibrio J_{mut}) that was re-plated onto SWC agar and used for later assays on whether this mutant, with its swarming phenotype, is a better competitor against Vibrio H than its wild type version.

Competition assays

I used a combination of plate and liquid culture assays to test whether competition occurs and observe any possible mechanisms behind it. All growth assays used Seawater Complete (SWC) media and were grown at room temperature. I spotted 10 µL of liquid culture of each microbe onto SWC plates 3 mm apart, and had controls in duplicate of each microbe spotted alone. I also spotted 10 µL of each microbe onto a lawn of either Vibrio H or Vibrio J, again in duplicate. The controls here for this assay were SWC spots and spots of the same species of microbe as the lawn. Each of the liquid cultures used for these plates were at 0.5 OD600. These were grown for 24 hours, at which point, I measured colony sizes and observed colony morphology and cell morphology (under the microscope at 100x, phase contrast). These culture assays tested whether the microbes had some change in morphology or behavior when grow together versus apart.

In another assay, I tested whether spatial distance on the plates was driving the interaction. I spotted 10 µL of Vibrio H and Vibrio J at increasing distances from each other on the same plate (3 mm, 5 mm, 7 mm, 9 mm, and 11 mm). I also did this with Vibrio H and Vibrio J_{mut} on another plate. The liquid cultures used here were at 0.5 OD600. I observed these plates after 24 and 48 hours. In the following days, I noted whether the spots moved towards or away from each other, or simply grew into each other over time.

To further test whether Vibrio H and J were interacting spatially, I also inoculated a streak of each microbe parallel to each other on a plate, about 5 mm apart. I observed these after 24 and 48 hours, noting whether they grew towards each other or not. At this point, I did not yet have J_{mut}, so it was not included in this assay.

I also wanted to gain further evidence of whether each microbe grows differently when it is alone as opposed to with the other, so I made a lawn on half a plate of either Vibrio H or J, and did a control streak of itself, a streak of the other, and a streak of J_{mut}. I also did each streak in duplicate on the same plate. All the liquid cultures used for the lawns and streaks were at 0.5

OD600. After 24 hours, I observed whether the streaks grew in the lawn at all and whether there was a visible difference between the part of the streak on its own versus in the lawn.

Since many of my assays are on solid medium and very spatially dependent, I also did a liquid culture competition assay to test how the populations of each microbe change when growing together in a homogeneous space. To start, I needed a way to differentiate between my two Vibrio species since I needed to count colonies of each microbe at each time point. I grew Vibrio H on SWC media with a Streptomycin concentration of 30 µg/mL. This selected for naturally resistant individuals in my pure culture, which I will refer to as H_r. Then, I grew a fresh overnight culture of each of H_r, J, and J_{mut}, subcultured at 0.05 OD600, and let them grow to 0.5 OD600. Initial "rough" growth curves of just the exponential phase were made as an aside in order to reliably culture cells at 0.5 OD600 that were already in the exponential phase. I combined each of the 0.5 OD600 cultures in 1:1 ratios between Vibrio H and J, and a separate treatment for Vibrio H and J_{mut}, in replicate. These cultures were grown in a shaker and I sampled from each tube at 0 hours, 3 hours, 5 hours, 8 hours, and 21 hours. At each time point, I sampled 100 µL of liquid, did a 1:10 serial dilution down to 10^{-8}, then plated 10 µL spots of each dilution onto SWC and Streptomycin SWC. After 24 hours, I counted how many CFU's were growing at the countable dilution. The SWC count gave me the total number of microbes, while the Streptomycin SWC gave me the number of Vibrio H_r cells, and I subtracted them to get the number of J or J_{mut} CFU's. The biggest caveat with this technique is that it was very hard to count on SWC because Vibrio J and J_{mut} colonies spread out and sometimes form a glossy film, so it is hard to differentiate between colonies. As such, some time-points are missing data that came from the SWC plates. All these data were analyzed and graphed in google sheets.

Inhibition testing

Some of the original plates used to isolate these microbes suggested that there might be a zone of inhibition, particularly when Vibrio H was spotted onto a lawn of Vibrio J. However, the "zone" was very small and could have been a result of very thin growth of Vibrio H spreading outward. To test whether there was an actual inhibitory compound, I did an assay wherein Vibrio H, Vibrio J, or a combination of both in a 1:1 ratio was inoculated at 0.05 OD600 into fresh SWC liquid medium. These cultures grew overnight (approximately 12 hours) and then filtered through 0.22 µm filters to remove cell mass. This spent media (three types: H, J, H and J together) was used as substrate for new inoculations of either Vibrio H or Vibrio J. There were controls with each microbe growing in its own spent media as well as fresh SWC media with each microbe. Each combination of spent media or SWC with H or J was done in duplicate. At 0 hours, 3 hours, 5.75 hours, 8.25 hours, and 24 hours I took OD measurements (OD600) of all the tubes as a proxy for growth. SWC was used as a standard every time I took OD measurements. All these data were analyzed and graphed in excel. As an added measure of whether the spent medias were inhibiting or lysing cells, I inoculated a lawn of H and a lawn of J with 10 µL each of the spent medias and observed whether they inhibited growth over 24 hours. I also spotted the same amount of each spent media onto separate already-grown lawns of H and J, then observed to see if any cells were lysed over the next 24 hours.

Microscopy

I used a Nikon Eclipse Ti2 inverted microscope system (Nikon Instruments Incorporated, Melville, NY) to create a video of my two different types of cells interacting. First, I created a 2% SWC agarose pad to make sure my microbes stayed in the same plane of focus throughout the video. After dissolving the agarose in SWC, 1 mL of liquid was pipetted into a glass bottom plate with a rubber seal to mold the shape of the pad. A coverslip was placed on top to make the

pad flat so it would not distort the view under the microscope. I stained Vibrio H with FM 4-64FX probe (Invitrogen, Carlsbad, CA), which stains the outer membrane, and took a photo of my field of view at 100x under a Cy5 filter before starting my video. This helped me differentiate between each of my two Vibrio species since they look the same under the microscope normally. I took snapshots every 1 minute for 4 hours, and processed these images in ImageJ afterwards to create a video. The main video (Video 1) reported here did not work with the stain, unfortunately, but is more compelling in terms of visualizing motility. The video where staining worked is included in the supplement.

Results and Discussion

Competition patterns

Spot plates show that the only difference between the microbes when grown together versus apart is that by the second day, Vibrio J starts to swarm away and around Vibrio H (Fig. 1). This growth pattern was replicated multiple times throughout various experiments and occurred every time the two microbes were grown together on solid medium. The parallel streaks of growth did not grow together (Appendix, Fig. A). Even as time passed and the sides of the streaks facing away from the other microbe grew outward, the streaks never grew toward each other. In assays where each microbe was streaked into a lawn of the other, I saw that although Vibrio H streaks grew fine on agar and with a lawn of itself, it could not grow in a lawn of Vibrio J (Appendix, Fig. B). This conflicts with the fact that Vibrio H did grow when spotted onto a lawn of Vibrio J (Appendix, Fig. C), suggesting that either the outcomes of this competition are stochastic or may be highly dependent on the density of the inoculum. Both streaks and spots were at the same OD and both used 10 µL of liquid, but a streak is spread over a larger area than what a spot covers.

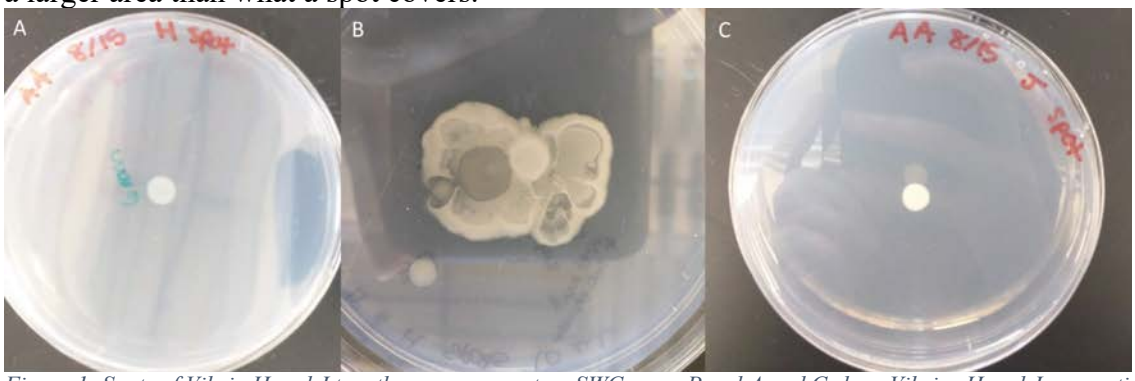


Figure 1: Spots of Vibrio H and J together versus apart on SWC agar. Panel A and C show Vibrios H and J, respectively. Panel B shows both growing together, with a spot of H on the left and a spot of J on the right. Of note, Vibrio J has different colony morphology in B than in C, and H seems to have other colonies forming with colony morphology that is similar to the spotted one. The plate in Panel B is after a few days of growth.

The competition liquid cultures indicate that Vibrio H does increase in growth initially, while Vibrio J plummets (Fig. 2). Vibrio J_{mut} showed a similar growth pattern with Vibrio H (Supplemental, Fig. D), so the hypothesis that J_{mut} is a better competitor against H than J is not supported. A caveat to this is that some of the colony counting was difficult with J_{mut} because its colonies spread out and merge, so counts could have been underestimated and some data points are missing from the graphs (hence the lack of continuity in the line). If we focus only on the trial competing Vibrio J and H, we see that Vibrio J recovers over time, after some back-and-forth in the number of cells between the two microbes. The "rough" growth curves that were

made in the process of setting up this competition only go through the exponential phase, but not the lag or stationary phase. They showed that Vibrio J actually has a faster rate of exponential growth than Vibrio H. Vibrio H could have a shorter lag phase, which would explain its initial increase in growth, while it takes Vibrio J some time to reach exponential growth. Once it does, it is able to win out in terms of growth levels. Future work on this system needs full growth curves of both Vibrio H and Vibrio J in order to further determine how growth rates affect this competition.

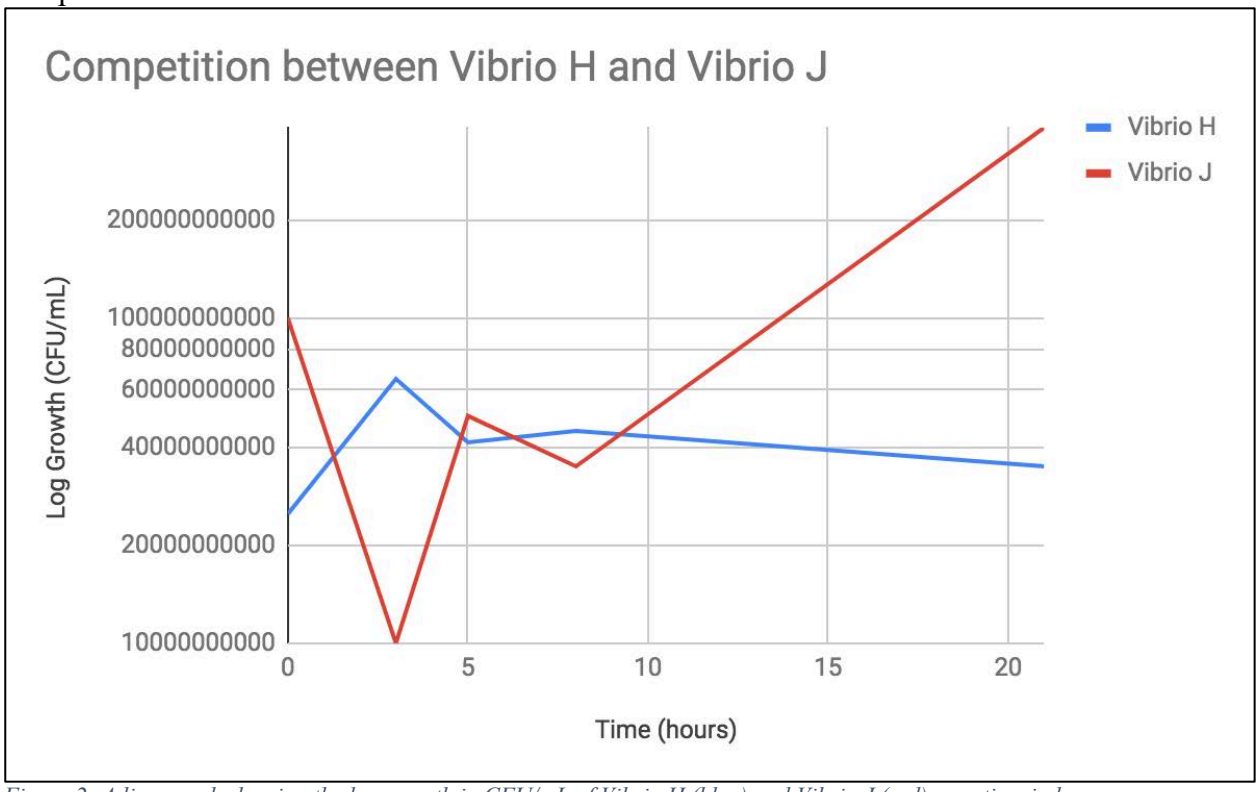


Figure 2: A line graph showing the log growth in CFU/mL of Vibrio H (blue) and Vibrio J (red) over time in hours.

Inhibition

The spent media assay indicated that it is unlikely that inhibition is part of the interaction between these two microbes. These trials did not include added media after the media was spent, so it is difficult to use the controls as a true baseline for the growth seen in the tubes. I can still at least compare between the trials, however. Most of the trials grew similarly to each other, with only Vibrio J in spent media from H growing at a faster rate (Fig. 3). To further confirm that there was no effect of inhibition in the spent media, I also spotted 10 µL of each spent media onto a lawn of H and a lawn of J, one assay where the lawns were not grown before spotting, and one where they were grown and then spotted. This assay did not show any inhibition or lysis of

cells in the lawn (Appendix, Fig. F). The results of the inhibition experiment could be explained if Vibrio H is producing something to stimulate Vibrio J growth as opposed to inhibiting it, which could also account for Vibrio J's faster growth on the second day of plate culturing.

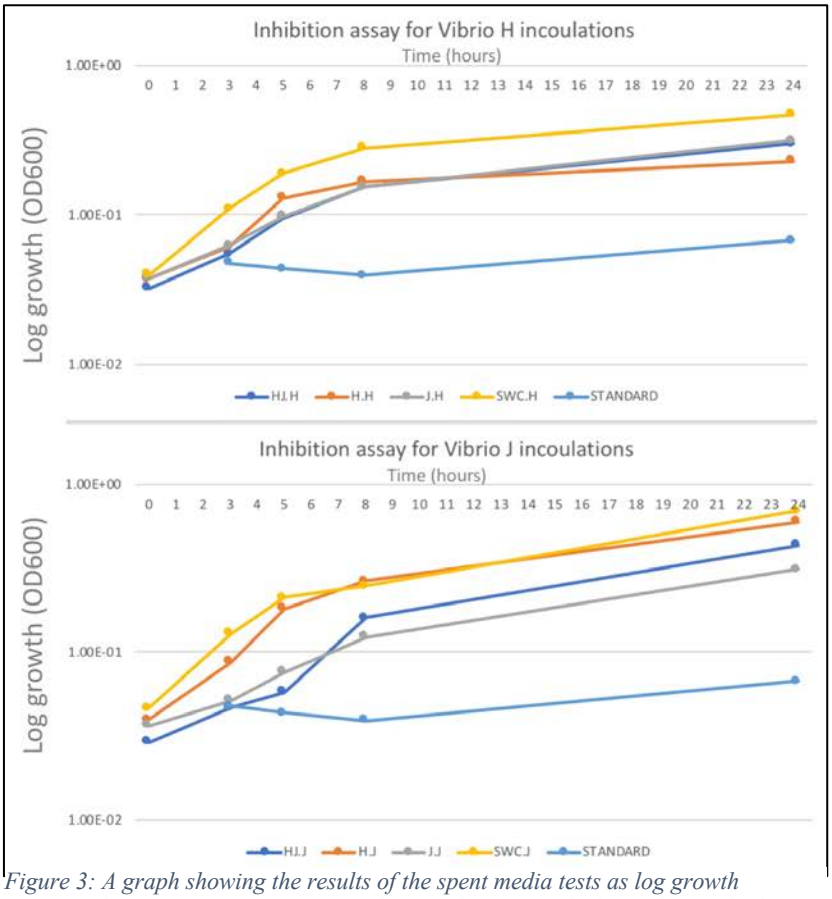


Figure 3: A graph showing the results of the spent media tests as log growth (measured using OD600) over time in hours. The top graph shows the results for Vibrio H inoculations into spent media, while the bottom graph shows the same for Vibrio J. The names of the trials are written as the type of medium-period-inoculation, and there is an SWC standard included for the OD measurements.

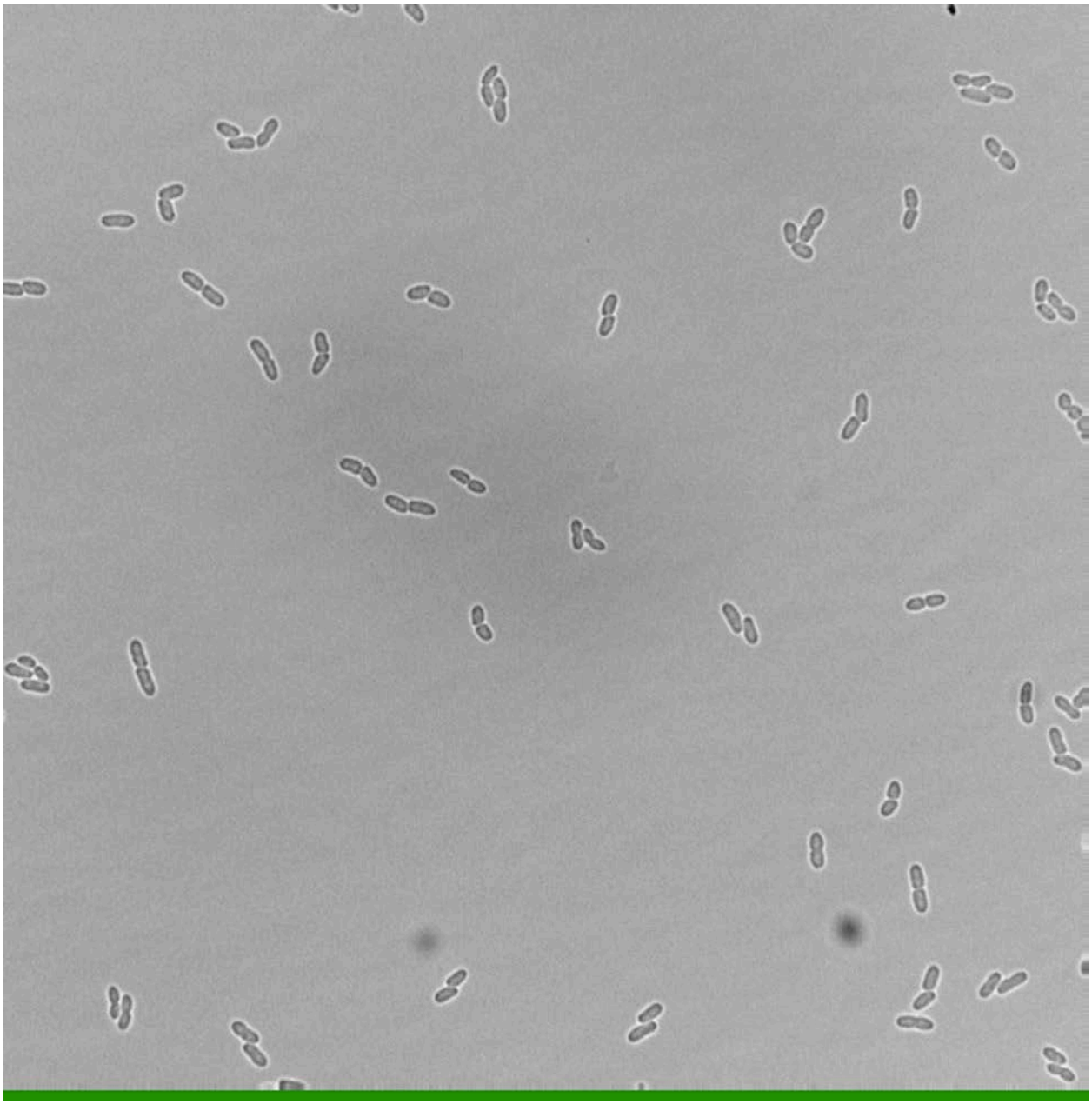
The role of motility and quorum sensing

The swim, swarm, and twitch plates showed that Vibrio H can swim and swarm, while Vibrio J can swim at a slower rate than Vibrio H can (Appendix, Fig. G). However, Vibrio J had the ability to mutate so that it could swarm (Fig. 4). This occurred after more than 24 hours of growth, suggesting that perhaps as nutrients at that particular spot on the minimal media plate depleted, Vibrio J mutated to deal with the conditions of starvation. I found that there were no differences in colony size, colony morphology, or cell morphology after 24 hours of growth when the microbes were grown together, but after 48 hours of growth, I always observed Vibrio J changing its colony morphology, only in the presence of Vibrio H (Fig. 1). The colony morphology looks as if Vibrio J is trying to swim or swarm away from Vibrio H, and microscopy at 100x phase contrast does show fast motile rods. Over the course of many days, Vibrio J grows around Vibrio H and slowly takes over the whole plate. It is possible that the same mutation that leads to Vibrio J swarming suddenly in the swarm plates is driving this sudden change in colony growth on plates, and that Vibrio H being in the space and taking up nutrients is what induces this mutation. To test this further, both species could be grown on swarm agar and observed over time to see how being in the presence of H affects J.

Figure 4: A close up of the four replicate colonies on swarm agar plates that show swarming mutants of Vibrio J.

In addition, microscopy under the inverted microscope showed that at a certain density, the cells all start becoming motile (Video 1), suggesting that density-dependent motility could be a mechanism behind this interaction. In bacteria, density-dependent motility is usually accomplished via quorum sensing, a system that you do see commonly in Vibrio species and that has been found to

occur even across different Vibrio species interacting in the same environment[13–15]. Quorum sensing is also part of self-self recognition between cells, which could also be part of the interactions between these two closely related species. One hypothesis would be that Vibrio H has a shorter lag phase, so it is able to start growing and taking up nutrients first. This sudden growth of H might induce a mutation for swarming due to starvation, allowing it to locate new nutrients. It could also be that Vibrio J swarms because it recognizes the inducer from Vibrio H, since they are so closely related. Either way, once Vibrio J exits lag phase, it has a faster growth rate, and is able to take over the culture. This hypothesis would require measuring a full growth curve and would benefit from optimizing the microscopy done in this paper, as well as more chemical-driven analyses to determine whether this interaction relies on underlying quorum sensing systems. It would also be beneficial to do genomic sequencing or mutational genetic studies of the microbes, especially the swarming mutant, to better understand the genetic mechanism behind this sudden change. Overall, these microbes' competition provides many new directions and possibilities for studying the interactions between closely related species or strains, which can further benefit our understanding of microbial ecology and communities.



Video 1: A video of a mixed culture of Vibrio H and J growing over time.

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Supplement:

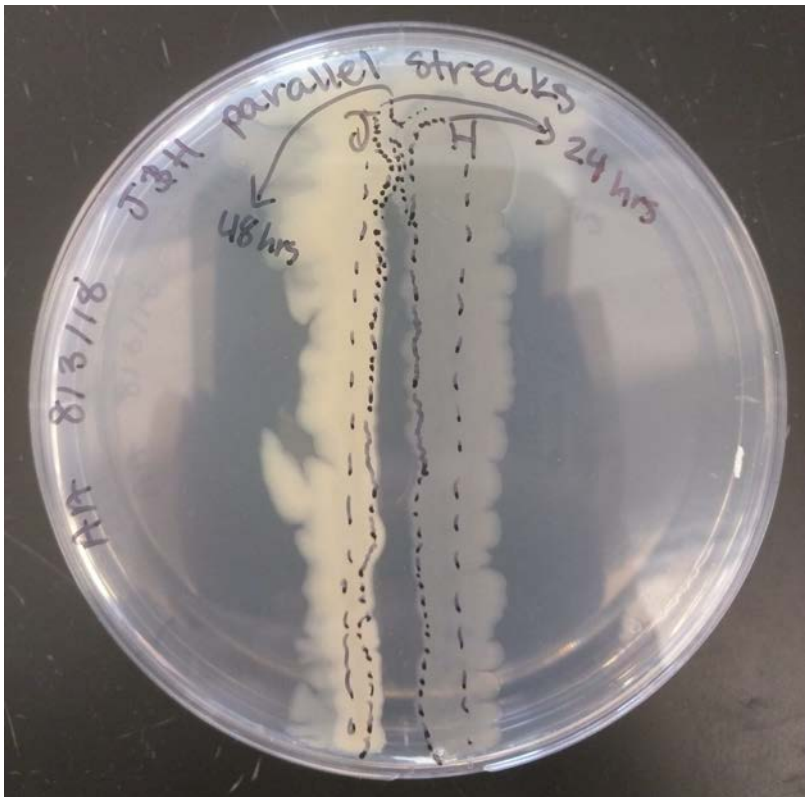


Figure A. Parallel streaks assay. To the left is Vibrio J, and to the right is Vibrio H. Dotted lines indicate boundary of growth on the colony interfaces that are facing each other (at 24 and 48 hours).

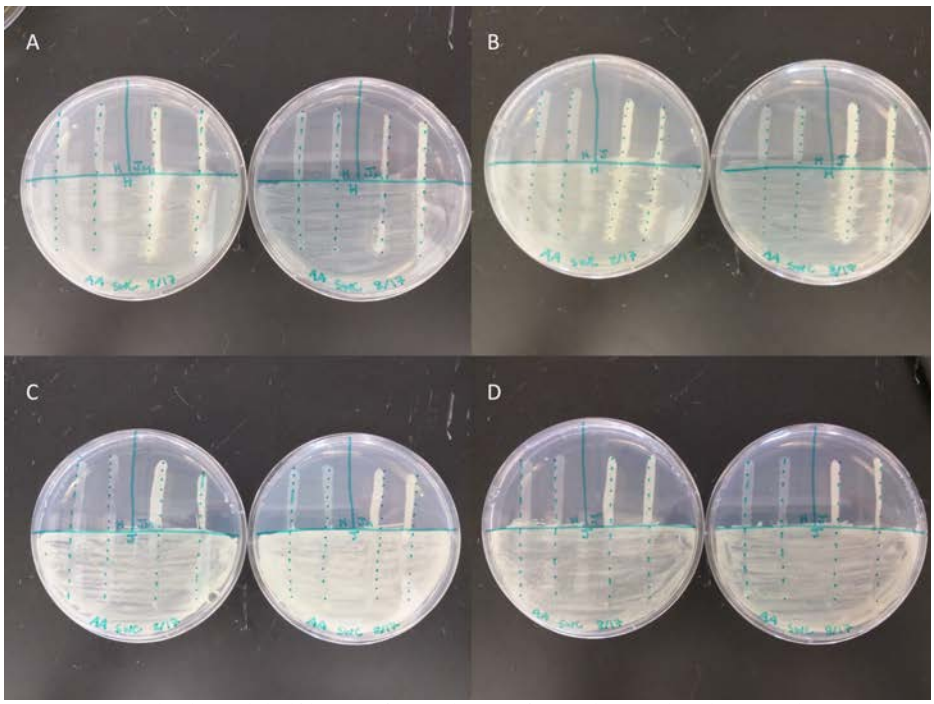


Figure B. Streaks through half lawns after 24 hours of growth. Panel A shows replicate plates of J_m streaks with controls on a lawn of H. Panel B shows streaks of J on a lawn of H. Panel C shows streaks of J_m on a lawn of J. Panel D shows streaks of H on a lawn of J. All plates have replicate streaks of each type of streak, and controls testing self on self growth are included.

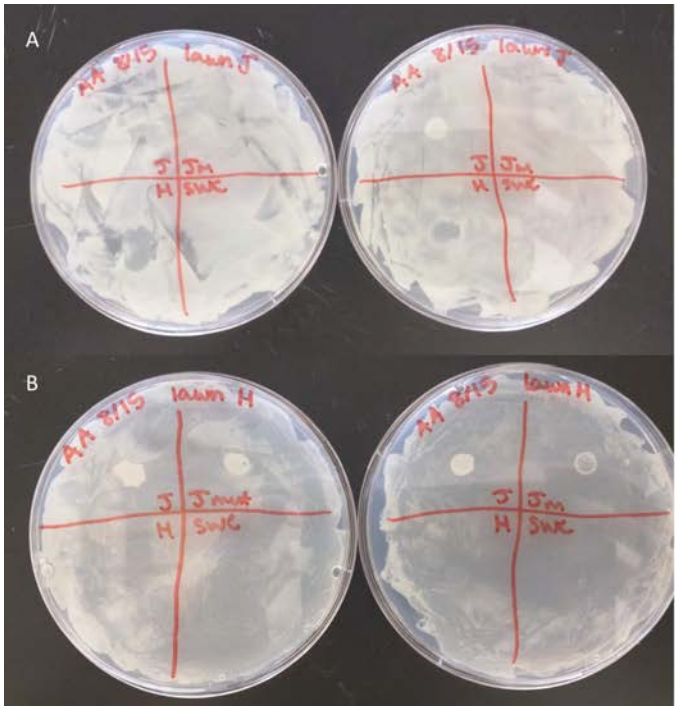


Figure C. Spot plates on lawns. Each plate is done in replicate, and controls of self on self growth and SWC are included in each trial. Panel A shows a lawn of J with H and J_m spots. Panel B shows a lawn of H with J and J_m spots. Some spots did not drop and grow in a perfect circle, so their oddly shaped colonies are likely not due to motility.

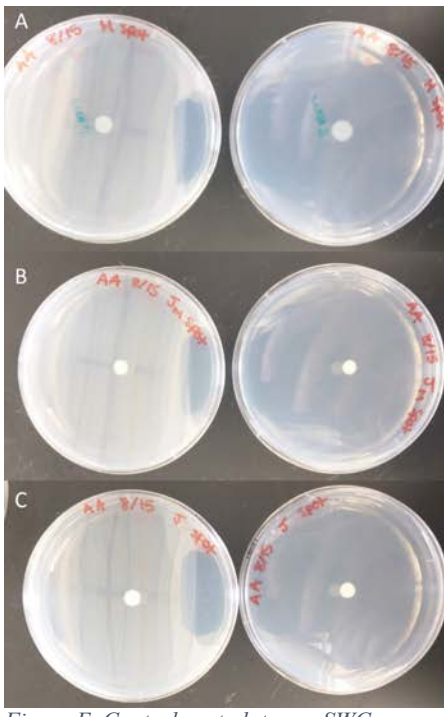


Figure E. Control spot plates on SWC agar media, in duplicate. These show spots of growth of Vibrio H (A), J_m (B), and J (C).

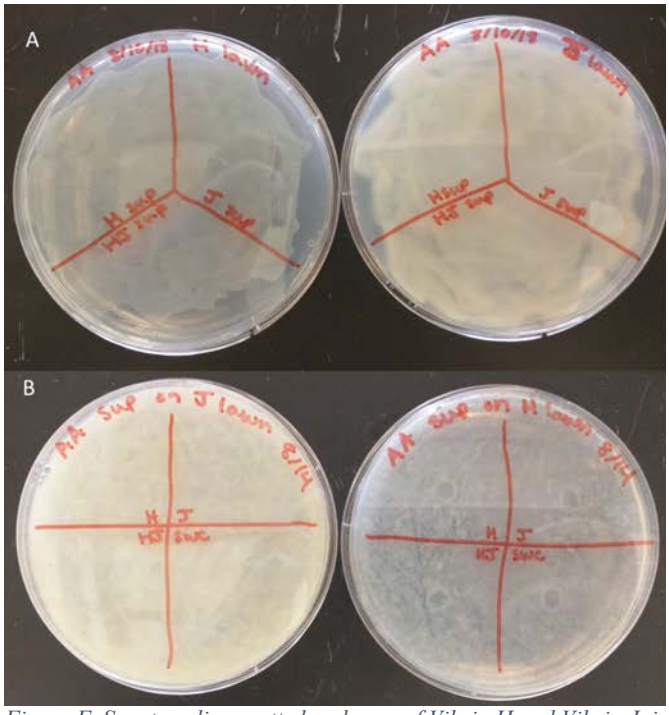


Figure F. Spent medias spotted on lawns of Vibrio H and Vibrio J, in duplicate. Panel A shows the spent media from H, J, and H+J overnight cultures (filtered of cells) spotted onto a lawn of H (right) or J (left), with inoculation of lawns and spots occurring at the same time. Panel B shows the spent media from each of the overnight cultures and an SWC control spotted onto a lawn of J (right) or H (left), with lawns inoculated and grown first, then spots added 24 hours later.

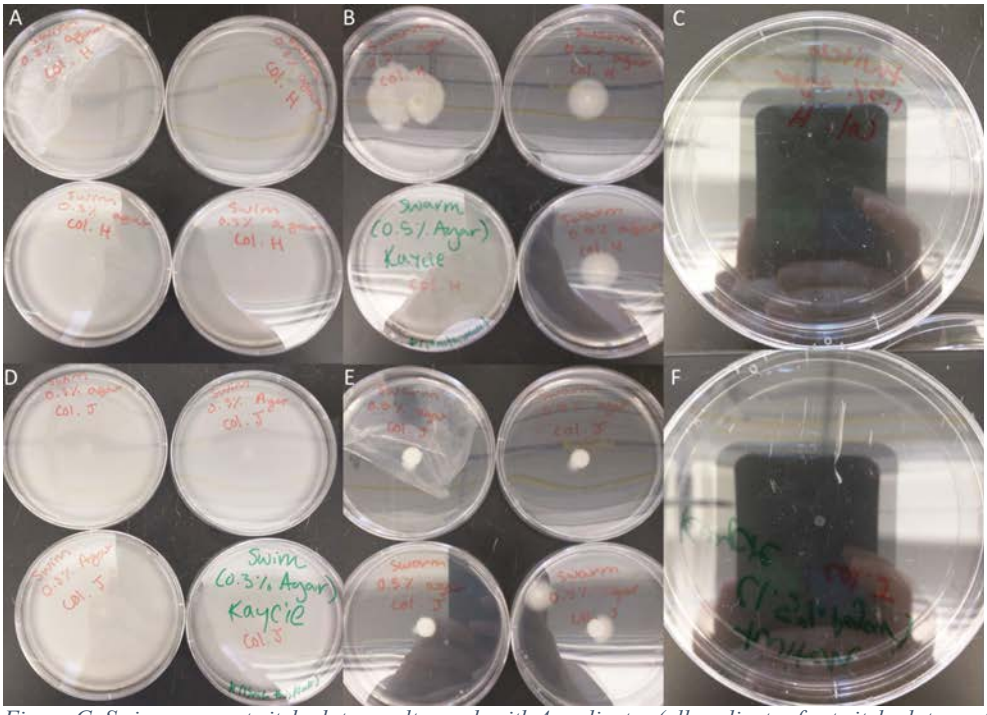


Figure G. Swim, swarm, twitch plate results, each with 4 replicates (all replicates for twitch plates not shown since they were negative). Across the top, A-C, show results for colony H. D-E shows colony J. Panels A and D show swim media, B and E show swarm media, and panel E has some visible J mutants forming. C and F show twitch media growth with the agar removed to show any potential growth.

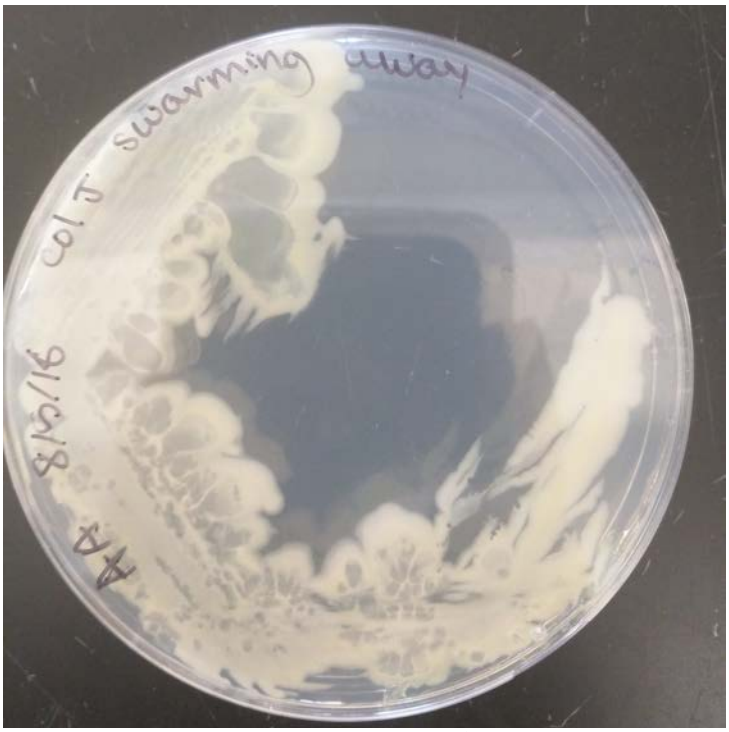


Figure H. Mutant J growing on an SWC agar plate.

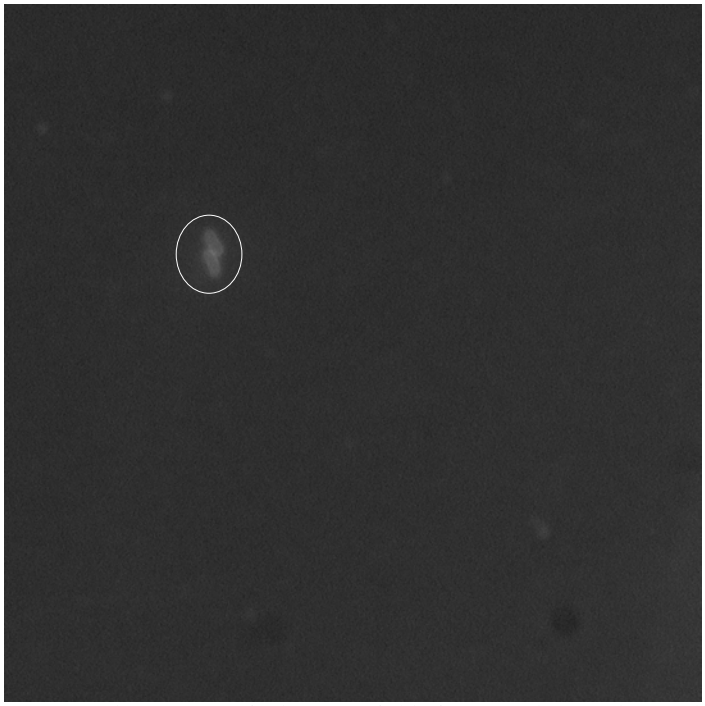
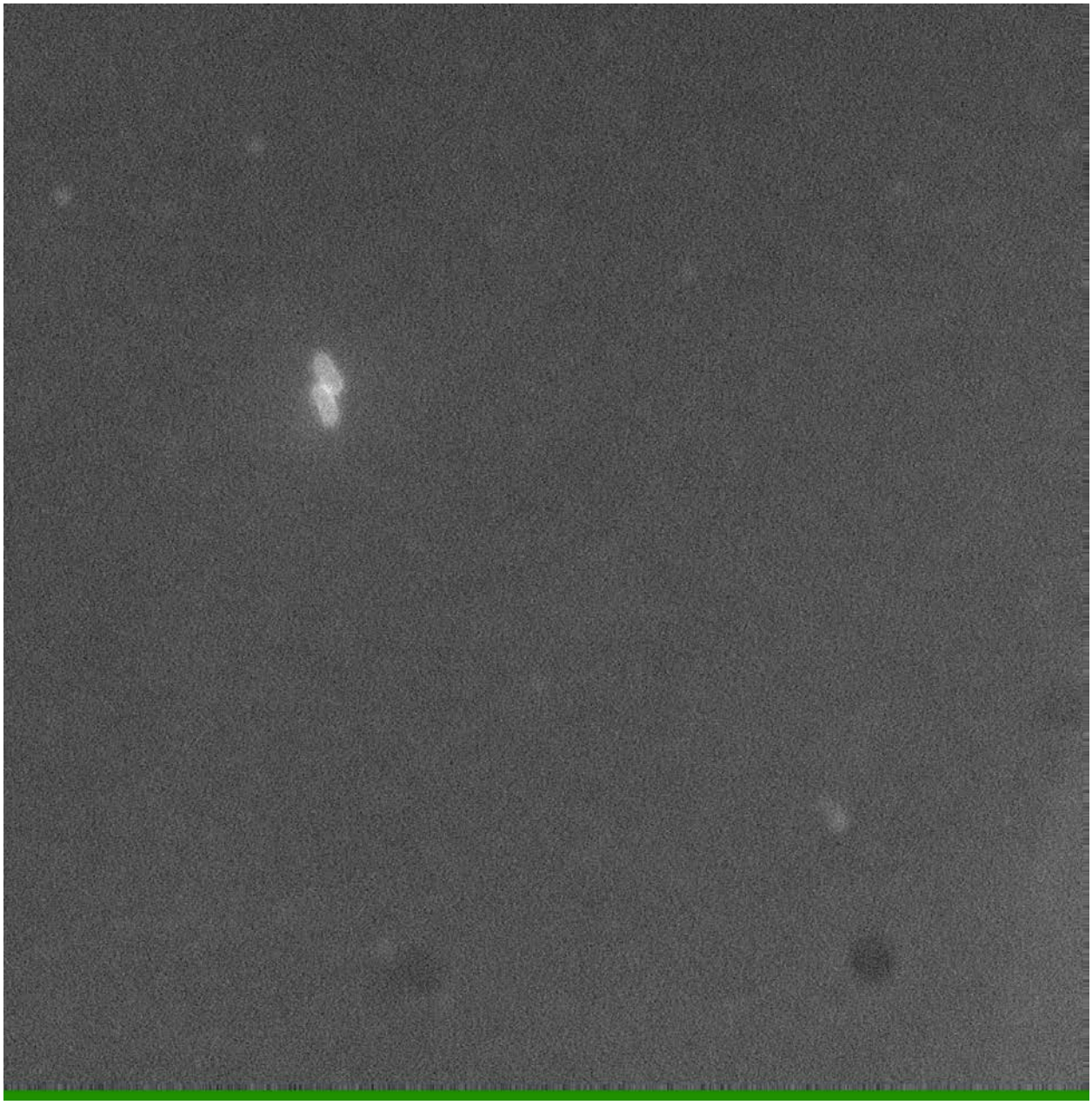


Figure I. Fluorescence of Vibrio H in field of view used in supplemental Video 1.



Video A: A second video of Vibrio rods dividing where the cells are differentiated using a membrane stain.

Table 1. Results of all competition growth experiments

Experiment	Measure	Measure details	Date of observation	Results	Notes
Spot together and apart	Colony morphology H		8/16/18	Thick white, glossy colony, circular, raised, entire edge	

	Colony morphology J		8/16/18	Glossy, clear colony, circular, raised, entire edge	
	Colony morphology Jmut		8/16/18	same as J WT	
	Colony morphology HJ	For H	8/5/18		No change, but does seem to excrete some kind of metabolite (clear liquid, could also be col J running away though), and maybe produces satellite colonies??
	Colony morphology HJ	For J	8/5/18		Might be producing some small satellite colonies but less than Col H; edges suddenly become lobate, move around col H, and have clear sections as if "running away", when restruck onto new plate, has mixed growth with col H and J and by second day has same thinned out, lobate growth as if running away from certain spots.
	Colony H on lawn J		8/16/18	Same as alone and as H on H	
	Colony J on lawn H		8/16/18	Same as alone and as J on J	
	Colony J mut on lawn H		8/16/18	Not as thick as alone, in one repl it is starting to spread out; this is also true of the Jmut on wtJ though	
	Colony size H		8/16/18	7 mm	6 mm, 8 mm

	Colony size J		8/16/18	6 mm	6 mm, 6 mm
	Colony size Jmut		8/16/18	5.5 mm	5.5 mm, 5.5 mm
	Colony size H on lawn J		8/16/18	Spots were not measurable because some of them moved	
	Colony size J on lawn H		8/16/18	Spots were not measurable because some of them moved	
	Colony size Jmut on lawn H		8/16/18	Spots were not measurable because some of them moved	
	Wet mount H (100x, Ph3)		8/16/18	No difference between wet mounts together v. apart	
	Wet mount J (100x, Ph3)		8/16/18		
	Wet mount H on lawn J (100x, Ph3)		8/16/18		
	Wet mount J on lawn H (100x, Ph3)		8/16/18		
	Wet mount Jmut on lawn H (100x, Ph3)		8/16/18		
Distance spots	Colony size lvl 1	3 mm	8/17/18	Grew into each other, J started to swarm away as usual	
	Colony size lvl 2	5 mm	8/17/18	Nothing	
	Colony size lvl 3	7 mm	8/17/18	Nothing	
	Colony size lvl 4	9 mm	8/17/18	Nothing	
	Colony size lvl 5	11 mm	8/17/18	Nothing	

Parallel growth plates	Colony growth pattern		8/9/18	Tops of lines where they were thickest when inoculated eventually grew together and saw "swarming" growth in col J. Rest of the parallel lines did not meet and in fact grew in the opposite direction away from each other	
Half lawn	Colonies streaked heavy on each half of plate		8/9/18	Do not grow into each other. Col J tries to grow around Col H to avoid it.	
Lawn of J with H, J, HJ sup	Measure inhibition zone	Control = self on self	8/16/18	no inhibition and no lysing	
Lawn of H with H, J, HJ sup	Measure inhibition zone	Control = self on self	8/16/18	no inhibition and no lysing	
Half lawn H, line Jmut	Observe inhibition	Control = self on self	8/18/18	Growing into lawn, slightly more swarmy pattern in lawn than by itself	After first trial that had few controls, one replicate swarmed and took over a space in the H lawn, although not when it is growing on not-lawn; other replicate had H grow up and into the line; WT J grew along the streak line in the lawn but did not swarm.
Half lawn H, line J	Observe inhibition	Control = self on self	8/18/18	Growing into lawn, same as Jmut	
Half lawn J, line H	Observe inhibition	Control = self on self	8/18/18	J grew solidly, blocking out H growth; H grew fine on agar alone	

Half lawn J, line Jmut	Observe inhibition	Control = self on self	8/18/18	Jmut grew solidly, no H growth; H on agar grew but thinned out as it approached the Jmut lawn	
Swim, swarm, twitch	Observe behavior	Controls = each on its own on the diff types of plates	8/10/18	H swarms, J swarms under starvation (prob) mutation; both swim but J swims faster; neither twitch, but J might twitch (ambiguously small reaction)	