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TITLE:

Coincubation Assay for Quantifying Competitive Interactions between *Vibrio fischeri* Isolates

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SUMMARY:

Bacteria encode diverse mechanisms for engaging in interbacterial competition. Here, we present a culture-based protocol for characterizing competitive interactions between bacterial isolates and how they impact the spatial structure of a mixed population.

ABSTRACT:

This manuscript describes a culture-based, coincubation assay for detecting and characterizing competitive interactions between two bacterial populations. This method employs stable plasmids that allow each population to be differentially tagged with distinct antibiotic resistance capabilities and fluorescent proteins for selection and visual discrimination of each population, respectively. Here, we describe the preparation and coincubation of competing *Vibrio fischeri* strains, fluorescence microscopy imaging, and quantitative data analysis. This approach is simple, yields quick results, and can be used to determine whether one population kills or inhibits the growth of another population, and whether competition is mediated through a diffusible molecule or requires direct cell-cell contact. Because each bacterial population expresses a different fluorescent protein, the assay permits the spatial discrimination of competing populations within a mixed colony. Although the described methods are performed with the symbiotic bacterium *V. fischeri* using conditions optimized for this species, the protocol can be adapted for most culturable bacterial isolates.

INTRODUCTION:

This manuscript outlines a culture-based method to determine whether two bacterial isolates are capable of competitive interactions. When studying mixed populations, it is important to assess the extent to which the bacterial isolates interact, particularly whether isolates are directly competing through interference mechanisms. Interference competition refers to interactions where one population directly inhibits the growth or kills a competitor population[1]. These

interactions are important to identify because they can have profound effects on a microbial community's structure and function[2,3].

Mechanisms for microbial competition have been discovered broadly in genomes of bacteria from diverse environments including both host-associated and free-living bacteria[4-9]. A variety of competition strategies have been described[10,11] including diffusible mechanisms, such as bactericidal chemicals[1,12] and secreted antimicrobial peptides[13], as well as contact-dependent mechanisms that require cell-cell contact to transfer an inhibitory effector into target cells[9, 14-18].

Although culture-based coincubations are commonly used in microbiology[5,8,19], this manuscript outlines how to use the assay to characterize the mechanism of competition, as well as suggestions for adapting the protocol for use with other bacterial species. Furthermore, this method describes multiple approaches for analyzing and presenting the data to answer different questions about the nature of the competitive interactions. Although the techniques described here were used previously to identify the interbacterial killing mechanism underlying intraspecific competition between symbiotic strains of coisolated *Vibrio fischeri* bacteria[19], they are suitable for many bacterial species including environmental isolates and human pathogens, and can be utilized to evaluate both contact-dependent and diffusible competitive mechanisms. Steps in the protocol may require optimization for other bacterial species. Given that more model systems are expanding their studies beyond the use of isogenic organisms to include different genotypes[10,16,20,21], this method will be a valuable resource for researchers seeking to understand how competition impacts multi-strain or multi-species systems.

PROTOCOL:

1. Prepare strains for coincubation

1.1. Choose an appropriate reference strain that will serve as the target for bacterial competition during the coincubation assay. See discussion for best practices when selecting a reference strain and how the reference strain will impact results. In this protocol, *V. fischeri* strain ES114 will serve as the reference strain.

1.2. Determining which selection and screening methods will be used to distinguish between the isolates in coincubation

1.2.1. Typically, transform strains with stable plasmids containing different antibiotic resistance genes (ex. kanamycin or chloramphenicol) to select for each strain, as well as genes encoding different fluorescent proteins (ex. GFP or RFP) for visually distinguishing strain types in coculture.

NOTE: Use of stable plasmids is required because if a strain loses the plasmid in the absence of selection, then this strain's numbers will be underestimated when quantified, and will not be visually detectable using fluorescence microscopy.

1.2.2. Tag coincubated *V. fischeri* strains differentially such that one strain contains the plasmid

pVSV102, which expresses a green fluorescent protein (GFP) and resistance to the antibiotic kanamycin (Kan^R), and the second strain contains plasmid pVSV208, which expresses a red fluorescent protein (dsRed) and resistance to the antibiotic chloramphenicol (Cm^R)[22].

NOTE: Other differential selections can be used to distinguish coincubating strains. See discussion for additional methods.

1.2.3. Include the following control during initial optimization of the coincubation assay to ensure the selection is robust. Plate each strain that is differentially tagged on agar plates containing the antibiotic that should select against it (i.e., plate strain 1 on media that selects for strain 2, and vice versa).

1.3. Two days prior to coincubation assay, streak reference strain pVSV102, reference strain pVSV208, and each competitor strain pVSV208 from -80 °C stocks onto LBS agar plates containing the appropriate antibiotic (e.g., 100 µg/mL kanamycin or 2 µg/mL chloramphenicol) and incubate overnight at 24 °C. Antibiotic selection is required to ensure all cells contain the plasmid at the beginning of the experiment.

1.4. One day prior to assay, pick and restreak four individual colonies per strain type (biological replicates) onto fresh LBS agar plates with appropriate antibiotic and incubated overnight at 24 °C.

NOTE: This step may require optimization for other bacterial species, as longer or shorter incubation times may be required to obtain sufficient cells depending on the growth rate of the organism of interest.

2. Coincubate bacterial strains

2.1. Preparation of each bacterial strain for incubation (Figure 1A)

2.1.1. Using a sterile toothpick, scrape the cells from the agar plate (as much as the size of a grain of rice) and re-suspend the cells in a 1.5 mL microcentrifuge tube containing 1 mL of LBS broth. Break up the cell clumps by pressing them into the side of the tube and pipetting up and down vigorously.

2.1.2. Cap the tube and vortex for 1-2 s. If the cell clumps are still visible, continue to vortex or pipette up and down until the sample is visually uniform.

2.1.3. Repeat steps 2.1.1-2.1.2 for all samples.

2.2. Measure and record the optical density at 600 nm (OD_{600}) for all samples. Samples will likely need to be diluted up to ten-fold using LBS broth to obtain an accurate OD measurement. Normalize each sample to desired OD_{600}. Strains are typically normalized to an OD_{600} ~ 1.0, which translates to ~10^6 bacteria/mL for *V. fischeri*.

NOTE: This step may require optimization for different bacterial species as the inoculum cell density impacts coincubation results depending on the mechanism of competition. See discussion for optimization.

2.3. Coincubating strains (Figure 1B, C)

2.3.1. Mix the reference strain and competitor strain in a 1:1 ratio (v/v) by adding 100 µL of each strain (normalized to OD 1.0) to a labeled 1.5 mL centrifuge tube. As a control, mix the reference strain with a differentially tagged version of itself (reference strain pVSV102 + reference strain pVSV208). This control is required for later statistical analysis to determine whether the presence of a competitor strain impacts the growth of the reference strain. Vortex the mixed-strain culture for 1-2 s.

NOTE: This step may require optimization as the starting ratio can significantly impact results and may need to be adjusted. See discussion for optimization.

2.3.2. Repeat step 2.3.1 for each biological replicate. After completing this step, there should be a total of eight mixed-strain tubes: four biological replicates with differentially-tagged reference strains (control), and four biological replicates with differentially-tagged reference and competitor strains (experimental).

2.3.3. Spot 10 µL of each control and experimental mixture onto Petri plates containing LBS agar; these culture spots will be used for fluorescence microscopy after the incubation.

2.3.4. Allow the spots to dry completely on the bench until all liquid has been absorbed into the agar and incubate the Petri plates at 24 °C for 24 h. A minimum of 15 h is required for *V. fischeri* strains tagged with pVSV102 and pVSV208 to grow to a high enough cell density to visualize GFP and RFP, respectively, at the population level using a fluorescence dissecting microscope. Here, we use a 24 h incubation for imaging mixed spots.

NOTE: It is important to use plates that are not too moist or too dry. If the plates are too moist, coincubation spots will not be absorbed into the agar plate; avoid using plates poured on the same day. If the plates are too dry, small waves or cracks may form on the agar surface, making coincubation spots irregular in shape.

2.3.5. Using the same bacterial suspensions as in step 2.3.3, spot 10 µL of each control and experimental mixtures into 24-well plates containing 1 mL of LBS agar per well. As in step 2.3.3, ensure the agar in 24-well plates has the appropriate moisture. Allow the spots to dry completely and incubate at 24 °C for 5 h. These culture spots will be used to quantify the colony forming units (CFUs) for each strain at the end of the experiment.

NOTE: Spotting bacterial suspensions for growth in individual wells allows for easier resuspension at the end time point. This step may be accomplished using square sterile filter pieces as a more

economical approach to using 24-well plates. Square sterile filter pieces can be placed on an agar plate and 10 µL of each experimental mixture can be spotted onto these filters, rather than onto 24-well plates. After the coincubation time, the filters can be transferred into a 1.5 mL centrifuge tube and the coincubation spot can be resuspended by vortexing or pipetting up and down. A 5 h incubation time is sufficient to detect interbacterial killing between *V. fischeri* isolates[19], however, this coincubation time may need to be shorter or longer for other species or competitive mechanisms. See discussion for more details.

2.4. Serial dilution for starting inoculum

2.4.1. To perform a serial dilution of starting coincubation mixtures, rotate a 96-well plate 90° so there are eight columns and twelve rows. Each row will contain a sample of the undiluted mixture in the first column followed by seven ten-fold serial dilutions across the remaining wells in the row (**Figure 2A**).

2.4.2. Label each row with the strain ID, the replicate number, and the time (starting inoculum = T0). Label the LBS agar plates supplemented with the appropriate antibiotic on which to spot the dilution series. Be sure to identify which strain each antibiotic plate is selecting for.

2.4.3. Using a multichannel pipette, add 180 µL of LBS broth to each well, leaving the first column empty for the undiluted coincubation mixture.

NOTE: Researchers may choose to use phosphate buffered saline (PBS) to resuspend coincubation spots and perform serial dilution if working with a particularly fast growing bacterial species or consistently performing large experiments where serial dilutions may take a long time to perform. Using PBS in these instances will prevent significant outgrowth of bacteria during the process of performing serial dilutions. However, it is important to be consistent with which solution is used (e.g., PBS or LBS) across all experiments, regardless of their size or duration.

2.4.4. Using a 200 µL single channel pipette, transfer 100 µL of the coincubation mixture from the tube to the first column well. Discard the tip and repeat for all coincubation mixtures.

2.4.5. Using a multichannel pipette, transfer 20 µL from column 1 to 2 and mix by pipetting up and down. Discard the tips and repeat for each column so that by the end, each row contains a ten-fold serial dilution of the initial coincubation mixture.

NOTE: bBe consistent with the number of times dilutions are pipetted up and down. For example, depress the pipette handle five times for each dilution to be consistent across the dilution series.

2.4.6. Using a multichannel pipette fitted with eight tips, aspirate 5 µL from each well in a dilution series (i.e., one row of eight wells) and spot it onto the LBS agar plate that selects for the reference strain (supplemented with kanamycin). Repeat this step spotting onto the plate selecting for the competitor strain (supplemented with chloramphenicol) (**Figure 2B**). Allow the spots to dry completely prior to placing in incubator.

NOTE: The same tips may be used to spot a replicate dilution series on plates selecting for the reference and competitor strain, but must be changed between replicates. Avoid touching the end of the pipette tip to the LBS agar plates as this can create a depression that can resemble a bacterial colony and skew results during plate counts. If the tip does touch the plate, make a note and do not include the depression in colony counts.

2.5. Taking T final measurements

2.5.1. After the coincubation spots in the 24-well plates have been incubated for 5 h at 24 °C, add 1 mL of LBS broth to each well and resuspend the coincubation spots by pipetting up and down until all cells are resuspended. Be consistent with the vigor in which the cells are resuspended across all replicates. For example, depress the pipette handle eight times to fully resuspend each sample.

2.5.2. Once each coincubation spot is resuspended, prepare a 96-well plate for a serial dilution and LBS agar plates with the appropriate antibiotics on which to spot the dilution in the same way as step 2.4.1.

2.5.3. Perform steps 2.4.2-2.4.6 to complete the serial dilution for T final measurements.

NOTE: An important control should be included during initial optimization of the coincubation assay. At the end of the coincubation period, plate the mixed strains onto media that includes both antibiotics. Neither strain should be able to grow unless 1) a spontaneous mutation occurs, or 2) selectable markers are exchanged between strains.

3. Visualizing coincubations using fluorescence microscopy

3.1. Image each coincubation spot on LBS agar petri plates from steps 2.3.3 and 2.3.4 using a stereo microscope equipped for green and red fluorescence detection, which correspond with the fluorescence proteins being expressed on the stable plasmids.

3.2. Begin by taking images of the control coincubation spot (reference strain pVSV102 + reference strain pVSV208).

3.2.1. Adjust magnification so the spot is in focus.

3.2.2. Using the appropriate excitation light and filter to observe GFP, adjust the exposure time so only fluorescence from the coincubation spot is detectable and any background fluorescence from the media is low or not visible.

3.2.3. Change the excitation light and filter to observe RFP, adjusting the exposure time to minimize background from the medium.

3.3. For each coincubation spot from the experimental treatments, image for the reference strain using the GFP filter and exposure time determined in step 3.2.2.

3.4. Image the competitor strain using the RFP filter, adjusting the exposure time so that fluorescence is only observed from the coincubation spot and background fluorescence from the medium is minimal. Save both images and name them to include both strain IDs, the replicate number, the incubation time when the image was taken (e.g., 24 h). Repeat for all replicates.

NOTE: Coincubation time may need to be adjusted for different plasmids or bacterial species. See discussion for optimization.

4. Data analysis

4.1. Calculating CFUs for each control and experimental treatment at each timepoint (T start and T final)

4.1.1. Once the colonies are visible on serial dilution plates (e.g., 24 h at 24 °C for *V. fischeri*), identify the dilution factor where individual colonies can be counted and have not grown together into large, multi-colony clusters (**Figure 2B**). For each replicate, count and record the number of individual colonies for a given dilution. This number represents the CFUs for that replicate.

4.1.2. Convert CFUs for the dilution to total CFUs for each strain in the coincubation using the formula below:

[CFUs ÷ (dilution factor x vol. of dilution spot)] x vol. of the undiluted sample = total CFUs

Example: T0: [(6 CFUs) ÷ (10^-6 x 0.005 mL)] x [0.01 mL] = 1.2E7 total CFUs of strain 1 in the mixed spot at the beginning of the experiment

T5: [(4 CFUs) ÷ (10^-3 x 0.005 mL)] x [1.0 mL] = 8.0E5 total CFUs of strain 1 in the mixed spot after 5 h coincubation

The CFU values for each strain at each time point are the raw data that can be used for several different analyses and statistical tests (see section 4.2 below).

4.2. Perform the following data transformations to determine whether a competitor strain outcompetes the reference strain by: (i) determining whether the proportion of the reference strain decreases in the presence of the competitor strain, or (ii) calculating the log relative competitive index (RCI). These analyses convert raw data into ratios and can be useful to determine the competitive outcome of an interaction, but cannot determine the mechanism of competition (i.e., killing vs growth inhibition).

4.2.1. Calculating the proportion of each strain for each time point during the coincubation

4.2.1.1. Convert CFU data to the proportion of each strain in a treatment. For the reference strain (RS) at T0, divide the total CFUs for the reference strain at T0 by the sum of total CFUs for the reference strain and the competitor (CS) at T0:

RS_{T0} CFUs ÷ (RS_{T0} CFUs + CS_{T0} CFUs) = Proportion of reference strain at T0.

Perform the same calculation to determine the proportion of the competitor strain at T0:

CS_{T0} CFUs ÷ (RS_{T0} CFUs + CS_{T0} CFUs) = Proportion of reference strain at T0

Repeat this process for each time point and replicate for both the experimental treatment and the control treatment (differentially-tagged reference strain coincubation).

4.2.1.2. Graph the average proportion of each strain type at T0 and T5 in a stacked bar graph (**Figure. 3A**).

4.2.1.3. Ensure that the desired starting ratio of strains was obtained. For coincubations where strains were initially mixed 1:1, each strain type should comprise ~0.5 of the population at T0 and should not be statistically different from each other using a Student's t-test ($P > 0.05$). If the proportion of one strain is significantly larger than that of the other strain at T0, then repeat the experiment until a 1:1 starting ratio is achieved.

4.2.1.4. Determine whether the competitor strain comprises a significantly larger proportion of the population after 5 h. Perform a Student's t-test comparing the proportion of each strain in the experimental treatment at T0 and T5. If the proportion of competitor strain is significantly different from that of the reference strain at T5 ($P < 0.05$), this result suggests strain competition may have occurred. Proceed to step 4.2.1.5.

4.2.1.5. To determine whether the presence of the competitor strain reduced the proportion of the reference strain after 5 h, perform a Student's t-test comparing the proportion of the reference strain in the control with the proportion of the reference strain in the experimental treatment (reference strain v competitor strain).

NOTE: If the proportion of the reference strain is statistically lower in the experimental treatment relative to the control ($P < 0.05$), then the competitor strain significantly reduced the proportion of the reference strain after 5 h and outcompeted the reference strain.

4.2.2. Calculating the log RCI value for each treatment (including the control)

NOTE: The relative competitive index, or RCI, is a single value that compares how the ending ratio of strain types differs from the starting ratio. The RCI value is log-transformed such that a log RCI value greater than zero indicates the competitor strain (CS) outcompeted the reference strain (RS), a log RCI value less than zero indicates the reference strain outcompeted the competitor strain, and a log RCI value of zero indicates that neither strain was outcompeted.

4.2.2.1. Calculate log RCI values for each control and experimental treatment using the following equation:

Log [(CS_{T5} CFU ÷ RS_{T5} CFUs) ÷ (CS_{T0} CFU ÷ RS_{T0} CFUs)] = log RCI

4.2.2.2. Graph log RCI values using a separated box & whiskers plot where data are graphed in the horizontal direction (**Figure 3B**).

4.2.2.3. Determine whether each treatment was significantly different from zero by performing a student's t-test comparing the log RCI values of each treatment (including the control) to a data set comparing the same number of replicates all with a zero value. If the log RCI values for the experimental treatment are statistically greater than zero ($P < 0.05$), then the competitor strain outcompeted the reference strain.

NOTE: The control should not be statistically different from zero ($P > 0.05$), because the differentially tagged reference strains are isogenic.

4.2.2.4. Perform a Student's t-test to determine whether log RCI values for the experimental treatment were significantly greater than the control *(*$P < 0.05$). If the log RCI values from the experimental treatment are statistically greater than the control treatment, then the competitor strain outcompeted the reference strain.

4.3. Perform the following statistical analysis to determine the mechanism by which the competitor strain outcompetes the reference strain: (i) analyze raw total CFU data, or (ii) examine percent recovery of the reference strain. These analyses can be more cumbersome to view relative to those from step 4.2, but will identify whether the competitor strain outcompetes the reference strain by outgrowing it, inhibiting reference strain growth, or actively eliminating the reference strain.

4.3.1. Analyzing raw total CFU data

4.3.1.1. Graph raw total CFU data for both strains using an interleaved scatter plot, where replicates are shown as individual data points (**Figure 4A**). Display data on a log scale, and add a horizontal line indicating the average T0 CFUs for both strains.

4.3.1.2. To determine whether the presence of the competitor strain negatively affected the reference strain, perform a Student's t-test comparing reference strain CFUs for the control and experimental treatments at T5. If the reference strain CFUs in the experimental treatment are statistically lower than in the control ($P < 0.05$), then the presence of the competitor strain significantly affected the reference strain.

4.3.1.3. To determine whether the presence of the competitor strain either inhibited the growth of or eliminated the reference strain, perform a Student's t-test comparing the reference strain CFUs at T0 and T5 for the control and experimental treatments.

NOTE: In the absence of a competitor, the reference strain should show a significant increase in CFU when comparing T0 and T5 for the control treatment. When analyzing the experimental treatment, if reference strain CFUs at T5 are not statistically different compared to T0 ($P > 0.05$), then the competitor strain inhibited the growth of the reference strain. If reference strain T5 CFUs are significantly lower than T0 CFUs ($P < 0.05$), then the competitor strain killed the reference strain.

4.3.2. Calculating percent recovery of the reference strain

4.3.2.1. Transform total CFU data for the reference strain (RS) into percent recovery data using the below equation:

(RS_{T5} CFUs ÷ RS_{T0} CFUs) x 100 = % recovery of reference strain

4.3.2.2. Display the percent recovery of reference strain using a separated bar graph where each bar represents either the control or experimental treatment (**Figure 4B**). Add a dashed line at y = 100 to indicate 100% recovery of reference strain (no increase or decrease in reference strain CFUs from 0 h to 5 h).

4.3.2.3. To determine whether the competitor strain negatively affected the reference strain, perform a Student's t-test comparing reference strain percent recovery for the control treatment to the experimental treatment. If the percent recovery is significantly less than the control ($P < 0.05$), then the competitor strain negatively affected the reference strain.

4.3.2.4. To identify whether the competitor strain inhibited the growth of or eliminated the reference strain, perform a Student's t-test comparing reference strain percent recovery for the experimental treatment and a data set with the same number of replicates all with a value of 100.

NOTE: If the experimental treatment is not statistically different from 100 ($P > 0.05$), then the competitor strain inhibited the growth of the reference strain. If the reference strain percent recovery in the experimental treatment is significantly lower than 100 ($P < 0.05$), then the competitor strain killed the reference strain.

4.3.3. Interpreting fluorescence microscopy images

4.3.3.1. Once fluorescence microscopy images are taken, determine whether each strain is visibly detectable in the coincubation spot. For the control coincubation (reference strain pVSV102 + reference strain pVSV208), both GFP and RFP should be visible from one coincubation spot. If this is not the case, set up the experiment again ensuring strains are properly labeled and coincubated on antibiotic-free plates.

4.3.3.2. For each experimental coincubation spot, check for possible outcomes.

NOTE: If the competitor strain is visible, but the reference strain is not detected, that suggests the competitor strain killed or inhibited the growth of the reference strain. If both strains are present and uniformly mixed (GFP and RFP present throughout the coincubation spot), these data suggest the competitor strain does not compete with the reference strain and both strains coexist. If both strains are present but are spatially separated (microcolonies of either GFP or RFP are present throughout the coincubation spot), that suggests the reference strain and competitor strain may be engaging in competitive interactions and modifications to the reference strain or initial coincubation ratio may be required. See discussion for more details.

5. Determining whether interaction is contact-dependent

NOTE:If you find that one strain kills or inhibits the reference strain, the interaction may be diffusible or contact-dependent. To determine whether the interaction is dependent on cell-cell contact, perform a coincubation assay as described above for steps 1-2 with the following modifications.

5.1. Perform steps 1.1-2.2.

5.2. Once strains are normalized, physically separate strains using a nitrocellulose filter with a 0.22 μm pore size. This pore size allows for the diffusion of antimicrobials and small molecules but prevents physical contact between *V. fischeri* cells.

NOTE: If the interaction is dependent on cell-cell contact, separation of the reference strain from the competitor strain with the filter should abrogate the killing or inhibitory phenotype and the reference strain should not have reduced CFUs. If the interaction is not dependent on cell-cell contact, and is a diffusible mechanism, separating the strains should not prevent the competitor strain from killing the reference strain.

5.2.1. Spot 5 μL of the reference strain onto the center of a filter and spot 5 μL of the competitor strain onto the center of a different filter. Place the filter containing the reference strain onto the surface of an LBS agar plate. Place the filter containing the competitor strain directly on top of the filter with the reference strain.

NOTE: Each strain is spotted onto filters rather than the bottom strain spotted directly onto the agar plate so strains can be more easily placed directly on top of one another.

5.2.2. If there is concern that strains are not stacked directly on top of one another, decrease the volume of reference strain inoculum spotted onto the filter. This will ensure the area of the reference strain spot is smaller and fully above or beneath the competitor strain. Alternate which strain is on top and on bottom to account for differences in diffusion of nutrients from the agar medium.

5.2.3. To ensure the filter allows for diffusion of small molecules, include a control treatment where the reference strain is spotted onto a filter and an antibiotic that it is sensitive to

(chloramphenicol) is spotted onto the other. Stack the filters directly on top of one another and place on an LBS agar plate. The antibiotic should be able to diffuse through the filter and should kill the reference strain.

5.3. Incubate the plates with filters at 24 °C for 5 h. Do not invert the agar plate when incubating to avoid moving the filters.

5.4. Perform serial dilutions for the starting inoculum according to step 2.4.

5.5. Taking T final measurements

5.5.1. Using sterile tweezers, transfer each set of filters to a 50 mL Falcon tube containing 5 mL of LBS broth. Ensure that the filters are physically separated within the tube to allow for full resuspension of each strain type.

5.5.2. Resuspend the strains from filters by pipetting up and down until the filters are clear of all cells. Use tweezers to rotate the filters and clear cell material from both sides. Sterilize tweezers with ethanol between samples.

5.5.3. Perform serial dilution for T final according to step 2.5. When calculating total CFUs for T final, adjust "total volume of undiluted sample" from 1 mL to 5 mL.

REPRESENTATIVE RESULTS:

In order to assess competitive interactions between bacterial populations, a coincubation assay protocol was developed and optimized for *V. fischeri*. This method utilizes stable plasmids that encode antibiotic resistance genes and fluorescent proteins, allowing for differential selection and visual discrimination of each strain. By analyzing the data collected from the coincubation assay, the competitive outcome of an interaction and the mechanism of the interaction can be identified. As an example, the following experiments were performed using *V. fischeri* isolates. Coincubated strains harbored one of two stable plasmids: pVSV102 expressing kanamycin resistance and GFP+, or pVSV208 expressing chloramphenicol resistance and DsRed+. In the sample data, strains were mixed in a 1:1 ratio and incubated on LBS agar plates for 5 h. As a control treatment, differentially tagged versions of the reference strain were coincubated with each other. The experimental treatments were performed with the reference strain (harboring pVSV102) and either competitor strain 1 or competitor strain 2 (harboring pVSV208). Cultures of each strain were prepared and coincubated as described above and as shown in **Figures 1** and **Figure 2**.

In **Figure 3**, data analyzed to determine whether competitor strain 1 or 2 outcompeted the reference strain are presented in two ways. In **Figure 3A**, the proportion of each strain type for each time point during the experiment was calculated according to step 4.2.1. In the experimental treatments, the sample data show the reference strain and competitor strain 1 are present at a proportion of 0.5 at the beginning (0 h) and end (5 h) of the experiment, which is consistent with what is observed in the control treatment. These data show the proportion of

the strains did not change after a 5 h coincubation, and therefore no competition was observed. By contrast, when the reference strain was incubated with competitor strain 2, the reference strain was present at a proportion of 0.5 at the beginning (0 h), and a proportion <0.01 at the end (5 h) of the experiment, which was significantly lower than the control treatment (Student's t-test: $P < 0.001$). These data indicate that the proportion of the reference strain decreased in the presence of competitor strain 2, and therefore suggests competition between competitor strain 2 and the reference strain occurred. This type of analysis should be applied when determining how the proportion of strains within a community changes over time but cannot be used to determine the mechanism of the competitive interaction, and therefore should be combined with additional analysis. For example, the proportion of the reference strain decreasing in the presence of competitor strain 2 could be attributed to several types of interactions: (i) strain 2 grew more quickly than the reference strain, (ii) strain 2 inhibited the growth of the reference strain, or (iii) strain 2 eliminated the reference strain through killing.

In **Figure 3B**, the log relative competitive index (RCI) was calculated for each treatment according to step 4.2.2. When the reference strain was incubated with competitor strain 1, log RCI values were not statistically different from zero or from the control treatment ($P > 0.05$), suggesting competition between strains was not observed. When the reference strain was incubated with competitor strain 2, however, log RCI values were significantly greater than zero and the control treatment (Student's t-test: $P < 0.001$). These data suggest strain 2 outcompeted the reference strain. Analyzing log RCI values provides a simple method to determine whether one strain outcompeted the other during the incubation period. Because this analysis incorporates the ratio of strains at the end (5 h) and the beginning (0 h) of the experiment, the starting ratio can dramatically impact the result. Therefore, the starting ratio should be examined and considered when deriving conclusions from log RCI data. Furthermore, this analysis does not provide information about the competitive mechanism and simply reports how the ratio of strains change during the incubation.

Figure 4 displays two methods of data analysis to determine the mechanism of competition for a given interaction. In **Figure 4A**, total CFUs for each strain at each time point of the experiment are displayed. When the reference strain was incubated with competitor strain 1, CFUs of both strains increase over the course of 5 h and CFUs for the reference strain were not significantly different from strain 1 or the control at 5 h ($P > 0.05$). These data indicate that the reference strain grew in the presence of strain 1, and suggest no competition occurred. However, when the reference strain was incubated with competitor strain 2, strain 2 CFUs increased after 5 h but CFUs for the reference strain decreased. Reference strain CFUs were significantly lower than strain 2 CFUs and the control at 5 h (Student's t-test: $P < 0.002$). These data indicate that the reference strain CFUs decrease in the presence of strain 2, and suggest strain 2 kills reference strain cells. If the reference strain did not show a decrease in CFUs, but rather no change (no statistical difference between reference strain CFUs at 0 h and 5 h), these data would suggest strain 2 outcompeted the reference strain by inhibiting the growth of the reference strain. Analyzing untransformed total CFU data is particularly informative, as CFUs for both strains at each time point are displayed independently and can be used to identify the mechanism of competition.

Figure 4B shows the percent recovery of the reference strain in order to determine how the presence of a competitor strain affects the reference strain. When the reference strain was incubated with competitor strain 1, a ~3,200 percent recovery was observed, which was not statistically different from the control and indicates strain 1 did not affect the percent recovery of the reference strain. When the reference strain was incubated with competitor strain 2, a ~4 percent recovery was observed, which was significantly lower than the control (Student's t-test: $P < 0.002$). The percent recovery was also significantly less than 100 (Student's t-test: $P < 0.002$), indicating strain 2 outcompeted the reference strain by killing reference strain cells. If the percent recovery was not statistically different from 100, those data would suggest strain 2 inhibited the growth of the reference strain. Percent recovery data provides a simplified way to characterize the mechanism of competition by examining how the reference strain population responds to the presence of a competitor strain. However, displaying the data in this way excludes information about the starting ratio as well as how the abundance of the competitor strain changed throughout the incubation.

FIGURE AND TABLE LEGENDS:

Figure 1. Flowchart illustrating the coincubation assay. (**A**) Bacterial strains harboring either pVSV102 (reference strain indicated Ref. Strain or R.S.) or pVSV208 (competitor strain indicated Comp. Strain or C.S.) are grown separately on media selective for either the reference strain (LBS Kan) or competitor strain (LBS Cam). Strains are then resuspended in LBS broth and normalized to an OD = 1.0. (**B**) The reference strain and competitor strain are mixed at a 1:1 ratio by volume. A serial dilution is performed with this mixture to determine CFUs for both strains at 0 h. (**C**) The strain mixture is then spotted onto 24-well plates containing LBS agar. Each replicate is spotted into its own well. Spots are allowed to dry and then incubated at 24 °C for 5 h. After 5 h, a serial dilution is performed to quantify CFUs for each strain. (**D**) The strain mixture from panel B is also spotted onto LBS agar Petri plates allowed to dry and incubated at 24 °C for 24 h. At 24 h, the coincubation spot is imaged using a fluorescence dissecting microscope that is adapted to detect green (reference strain) and red (competitor strain) fluorescence. Scale bar = 1 mm.

Figure 2. Representative images of plates required for a serial dilution. (**A**) 96-well plate used to perform serial dilution. The plate is rotated such that there are 12 rows and 8 columns. Descriptors of each treatment include the strains used and the plasmids they harbor (e.g., Reference strain with pVSV102 and Competitor strain with pVSV208) and the replicate number for each row (R1, R2, R3, or R4). The first column is the undiluted sample and each column to the right represents a 10-fold dilution from the previous column (dilution factor listed above). (**B**) LBS agar plate used to determine CFUs for the reference strain on LBS Kan plates (top) and competitor strain on LBS Cam plates (bottom) from the experimental treatment. Each row is one replicate in a treatment (e.g., R1) and the dilution factor of each spot is listed at the top of the plate. The number of CFUs counted for each replicate is listed to the right.

Figure 3. Sample data for assessing whether competitor strains outcompete the reference strain. (**A**) The proportion of coincubating strains harboring either pVSV102 (dark gray) or pVSV208 (light gray). R.S. indicates the reference strain and C.S. indicates the competitor strain.

Dashed horizontal line indicates a proportion of 0.5. Asterisk indicates the reference strain made up a statistically smaller proportion of the population than the competitor strain or reference strain in the control at 5 h (Student's t-test: $P < 0.001$); ns indicates not significant ($P > 0.05$). (**B**) Log relative competitive index (RCI) for coincubation assays. Dashed vertical line indicates log RCI = zero. Asterisk indicates the log RCI value is statistically greater than zero and the control (Student's t-test: $P < 0.001$). Error bars indicate SEM.

Figure 4. Sample data for determining the mechanism of competition. (**A**) Total CFU counts for coincubation assays performed with *V. fischeri* isolates that were differentially tagged with either pVSV102 or pVSV208. CFUs were collected at the beginning of the experiment (0 h) and after 5 h incubation. R.S. indicates reference strain and C.S. indicates competitor strain. Dashed horizontal line indicates the average 0 h CFUs for both strains; asterisk indicates reference strain CFUs were statistically lower in the experimental treatment relative to the control treatment at 5 h (Student's t-test: $P < 0.002$). (**B**) Percent recovery of the reference strain. Horizontal dashed line indicates 100% recovery (no increase or decrease in CFUs); asterisk indicates percent recovery was statistically lower than 100% and the control treatment (Student's t-test: $P < 0.002$). Error bars indicate SEM.

Figure 5. Sample data for incubation time and imaging optimization. (**A**) Total CFUs for coincubation assays where CFUs were collected at the beginning of the experiment (0 h), after 5, 12, and 24 h incubation. R.S. indicates reference strain and C.S.2 indicates competitor strain 2. Dashed horizontal line indicates the average 0 h CFUs for both strains; asterisks indicate reference strain CFUs were statistically lower in the experimental treatment relative to the control treatment at the given time point (Student's t-test: $P<0.002$). Error bars indicate SEM. (**B**) Fluorescent microscopy images corresponding with CFU data in panel A. Scale bar indicates 1 mm.

Figure 6. Sample data for coincubation ratio optimization. Coincubation experiments were performed between the reference strain (R.S.) harboring pVSV102 (green, top row) and competitor strain (C.S.) harboring pVSV208 (red, bottom row) and fluorescent microscopy images were taken at 24 h. (**A**) Strains were mixed in a 1:1 ratio or (**B**) a 1:5 ratio where the reference strain, containing pVSV102, was outnumbered by the competitor strain containing pVSV208. Scale bar indicates 1 mm.

DISCUSSION:

The coincubation assay described above provides a powerful method to discover interbacterial competition. This approach allowed for the identification of intraspecific competition among *V. fischeri* isolates and characterization of the competitive mechanism[19]. Although the method described was optimized for the marine bacterium *V. fischeri*, it can be easily modified to accommodate other bacterial species including clinical and environmental isolates. It is important to note that competitive mechanisms are often conditionally regulated[5,6,23-28], thus small differences in growth conditions (e.g., shaking vs standing culture, temperature, etc.) and media type (e.g., salt content) can dramatically affect the results. Therefore, optimizing coincubation conditions is likely necessary for different bacterial species as well as different

competitive mechanisms. It is best to choose culture conditions that closely reflect the natural environment of the isolates. For example, coincubation assays between *V. fischeri* strains were performed with LBS media at 24 °C, to reflect the salinity and temperature of the marine environment. However, some bacteria are naturally competent in their environment[27,28] and therefore could take up genetic material released by lysed cells during antagonistic interactions[29].To prevent such DNA transfer from impacting coincubation results, it is important to use conditions that do not promote competence or strains that are not competent, either naturally or through inactivation of DNA uptake machinery. Moreover, experimental parameters such as cell growth phase, culture density, incubation time, or starting strain ratio may also require optimization for different bacterial species or competitive mechanisms. For example, initial culture density will dictate the amount of cell-cell contact between strains, which can affect the ability of bacteria to deploy contact-dependent mechanisms of competition.

Figure 5A displays the process of optimization of coincubation assays with *V. fischeri* isolates. Here, a range of incubation times for CFU collection and fluorescent microscopy imaging were evaluated to determine the optimal time for each metric to be collected. CFUs were collected immediately after the mixture of the reference strain and competitor strain 2 was spotted onto LBS agar plates (0 h), and CFU measurements and fluorescent microscopy images were taken immediately and after 5, 12, and 24 h. These sample data highlight the importance of thorough optimization prior to drawing any conclusions about the interaction between two strains. For example, two different conclusions about the mechanism of interaction can be deduced based on when CFUs are collected: CFUs from 5 or 12 h indicate strain 2 killed the reference strain, while CFUs collected at 24 h suggest strain 2 inhibits the growth of the reference strain.

The optimal time for visualization of coincubation spots through fluorescent microscopy may be different than the optimal time for CFU collection. **Figure 5B** displays fluorescent microscopy images of coincubation spots at 0, 5, 12, and 24 h. At 0 and 5 h, the coincubation spots are not visible with fluorescent microscopy. For images taken at 12 h, both strains in the control treatment are visible, yet the RFP (reference strain harboring pVSV208) is notably dimmer. In the experimental treatment at 12 h, competitor strain 2 is visible (yet dim) and the reference strain is not detectable. Strain-specific differences between bacterial isolates can affect the expression of fluorescent proteins, and thus brightness of the cells in the mixed spot. Because RFP is notably dimmer than GFP in the control, the coincubation spots should continue to be incubated and be imaged again at a later time. In images taken at 24 h, both strains are visibly detectable and at a similar brightness in the control experiment. In the experimental treatment strain 2 is visible while the reference strain is not observed within the coincubation spot. 15 – 24 h incubation time is sufficient to visualize GFP and RFP for *V. fischeri* using stable plasmids pVSV102 and pVSV208, respectively, but the incubation time may need to be adjusted for different plasmids or bacterial species. Although the optimal time for visualization of coincubation spots and collecting CFU data are different, imaging at 24 h is a good way to quickly screen interactions for *V. fischeri*, because the result obtained from imaging at 24 h (target is visible or not) reflects the more time-intensive quantitative data obtained from plating CFUs at 5 or 12 h.

The starting ratio can significantly impact results, particularly when incubating two inhibitory strains, and may need to be adjusted to account for strain specific differences in killing efficiency or growth rate. For example, **Figure 6A** displays fluorescent microscopy images of experiments where the reference strain was coincubated with itself (control) and three other *V. fischeri* isolates starting at a 1:1 ratio. In these sample data, the reference strain is visibly detected when incubated with itself, competitor strain 1, and competitor strain 3 after 24 h. However, when the starting ratio was adjusted to 1:5 (i.e., 50 μL of reference strain mixed with 250 μL of competitor strain) the reference strain is only visibly detected when coincubated with itself and strain 1, indicating that both strain 2 and strain 3 outcompete the reference strain. This adjustment prevents the faster growth rate of the reference strain from obscuring the effect of any interference competition mechanisms exhibited by the competitor strains. Based on the results in **Figure 6A**, a ratio of 1:5 (reference strain : competitor strain) should be used to screen additional *V. fischeri* strains for the ability to kill the reference strain.

This protocol discriminates between coincubating strains by differentially labeling strains with plasmids containing either kanamycin or chloramphenicol resistance genes. However, different antibiotics or other selection methods may be better suited for different bacterial species. Other methods for differential selection could include: 1) exploiting a strain/species-specific auxotrophy for specific growth factors (e.g., DAP or thymidine), 2) conditional growth requirements (e.g., one strain grows at 37 °C while the other does not), or 3) counterselection markers that eliminate or inhibit the growth of the tagged strain when grown under appropriate conditions to express a "kill" gene (e.g., *ccdB* or *sacB*).

Selecting the appropriate reference strain is critical for obtaining and interpreting reproducible results from the coincubation assay. A reference strain should be well-studied (i.e., have a broad body of scientific literature), have no apparent killing or inhibitory ability, and ideally have a sequenced genome. For example, certain strains of *Escherichia coli* are common reference strains for many bacterial coincubation experiments[30,31]. However, *E. coli* may not be ecologically relevant for a given competitive mechanism or competitor, which can affect results. For example, some bacteria may have evolved mechanisms specifically targeting closely-related species or competitors for the same ecological niche and their competitive mechanism would not be effective against an *E. coli* reference strain.

In summary, the method described here aims to provide an easily modified and robust approach to evaluate interbacterial interactions and competition. This method can be applied to bacterial isolates relevant to environmental or clinical research, and can be used to explore diverse mechanisms of microbial interaction that have been previously unknown or difficult to investigate.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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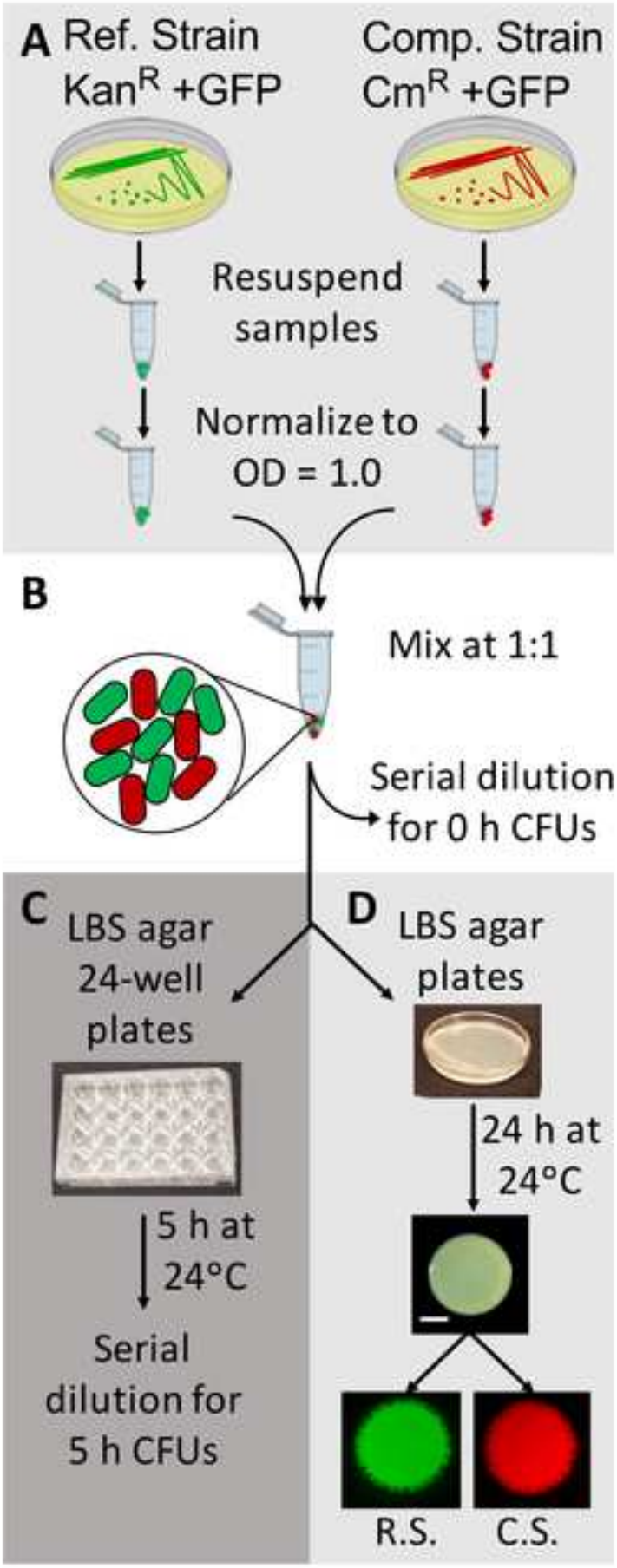

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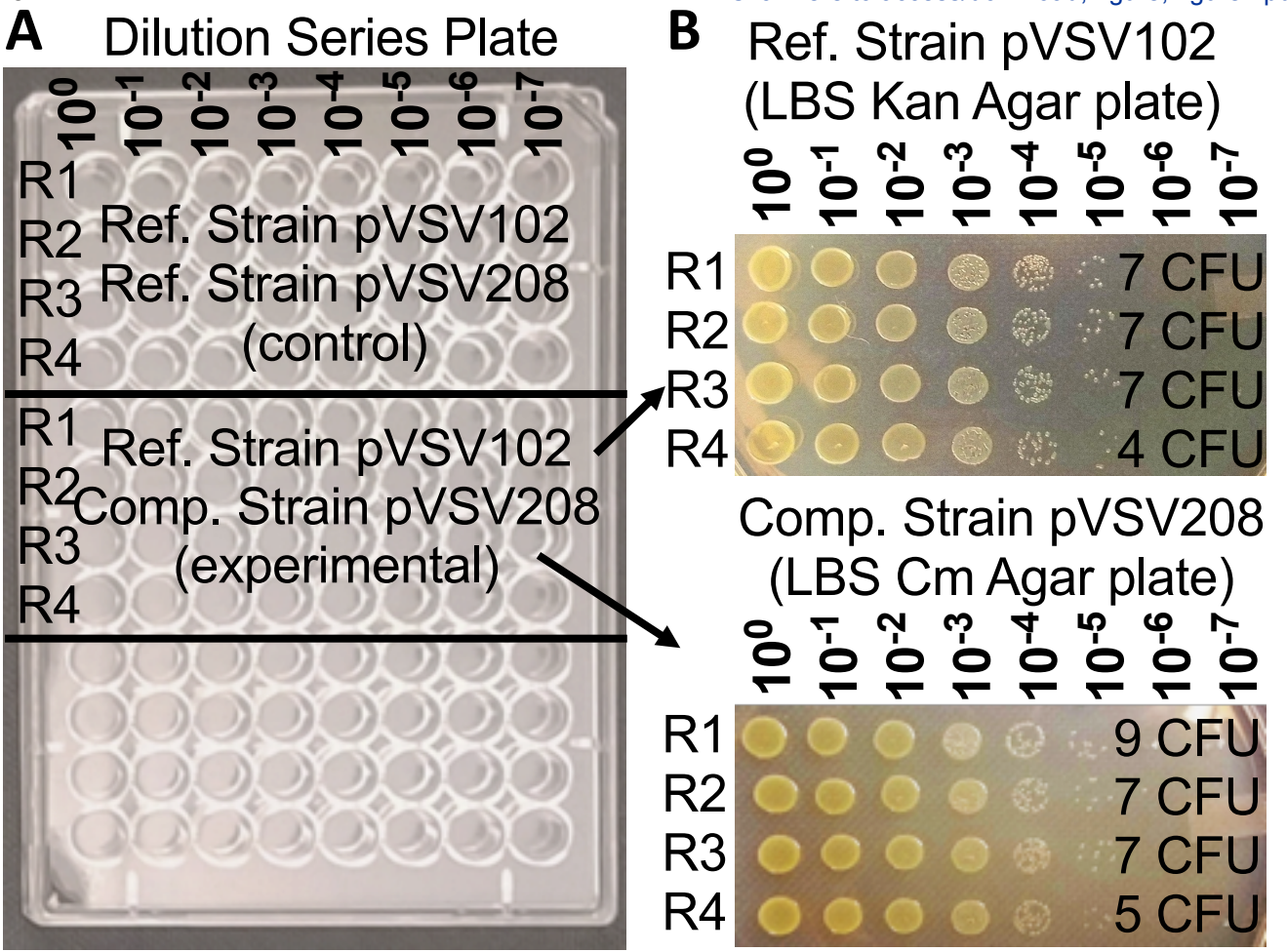

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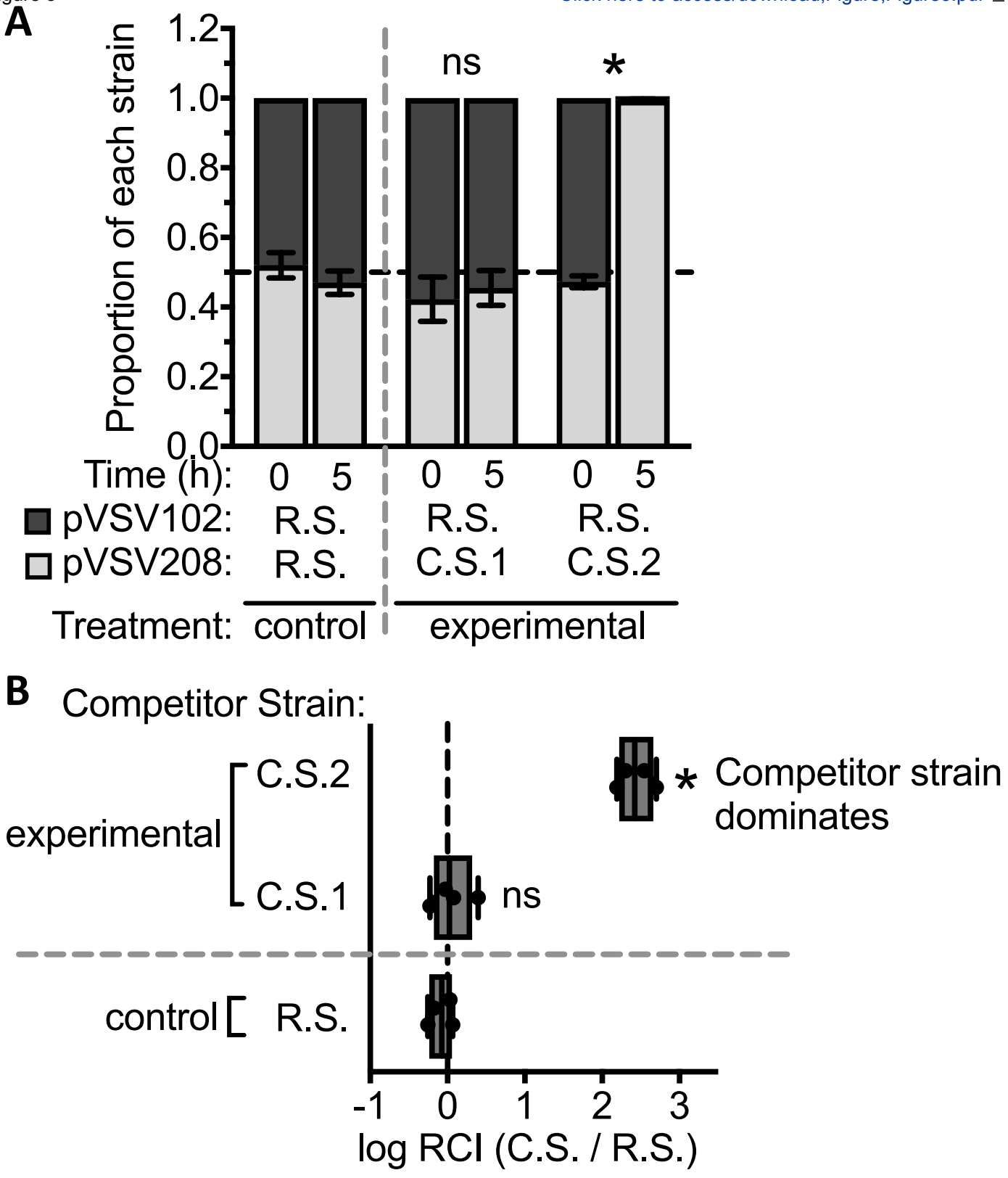

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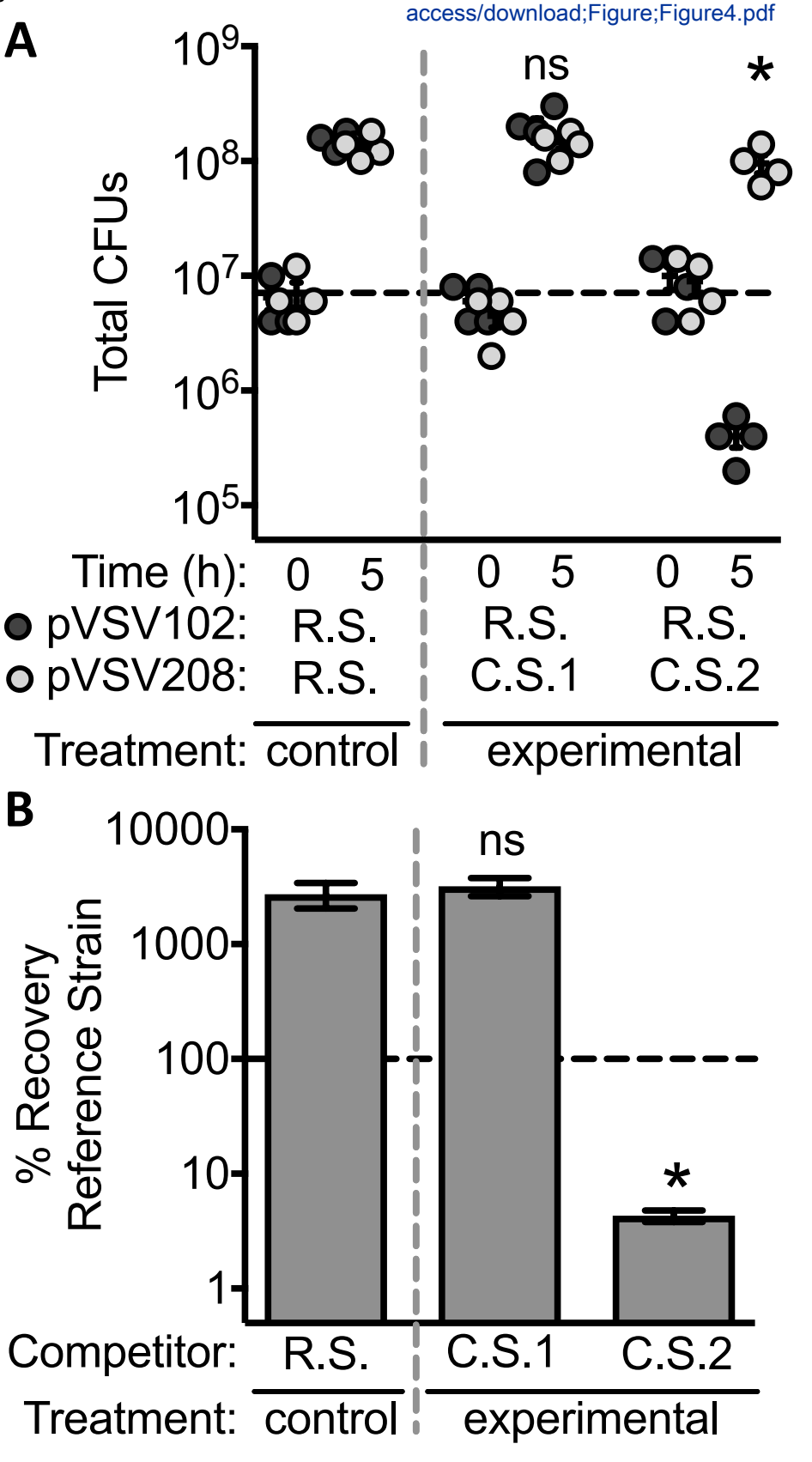

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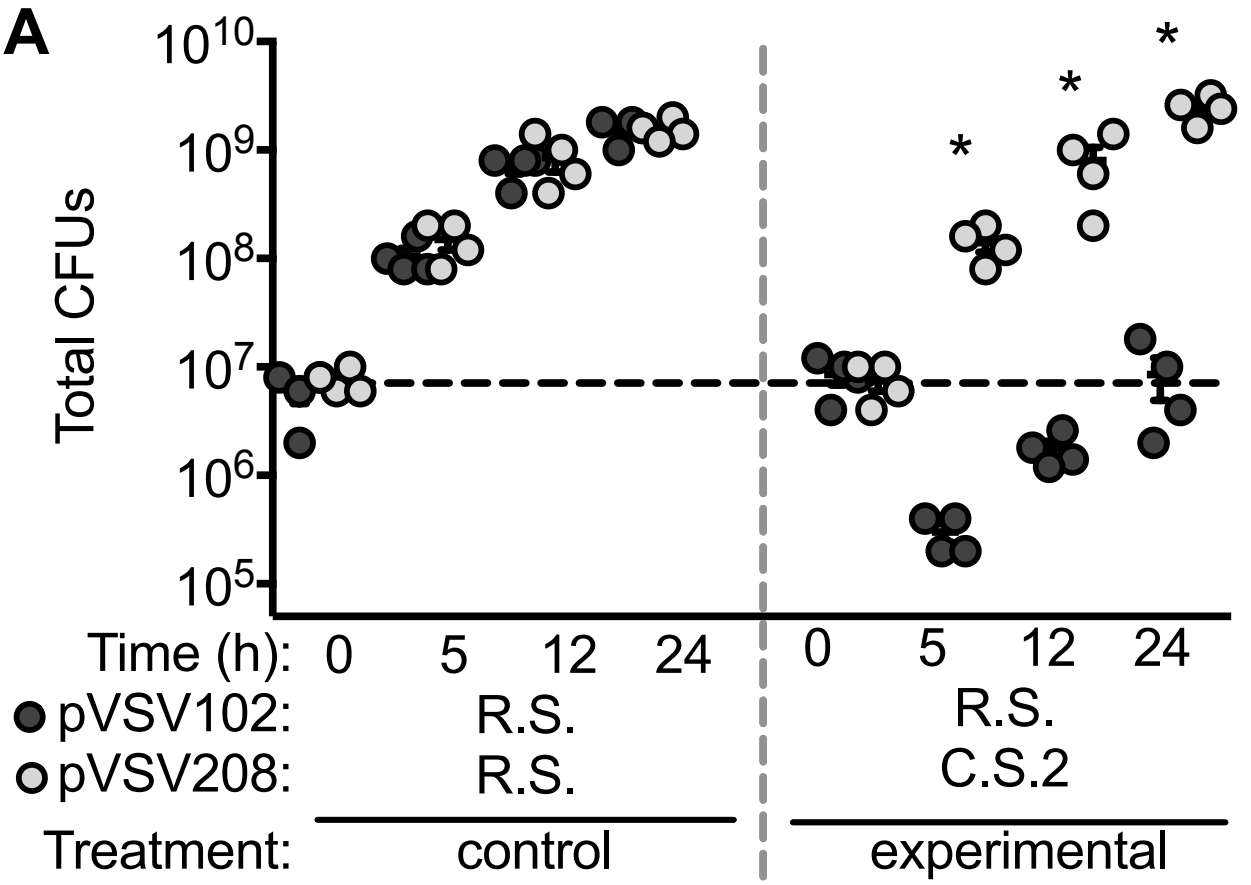

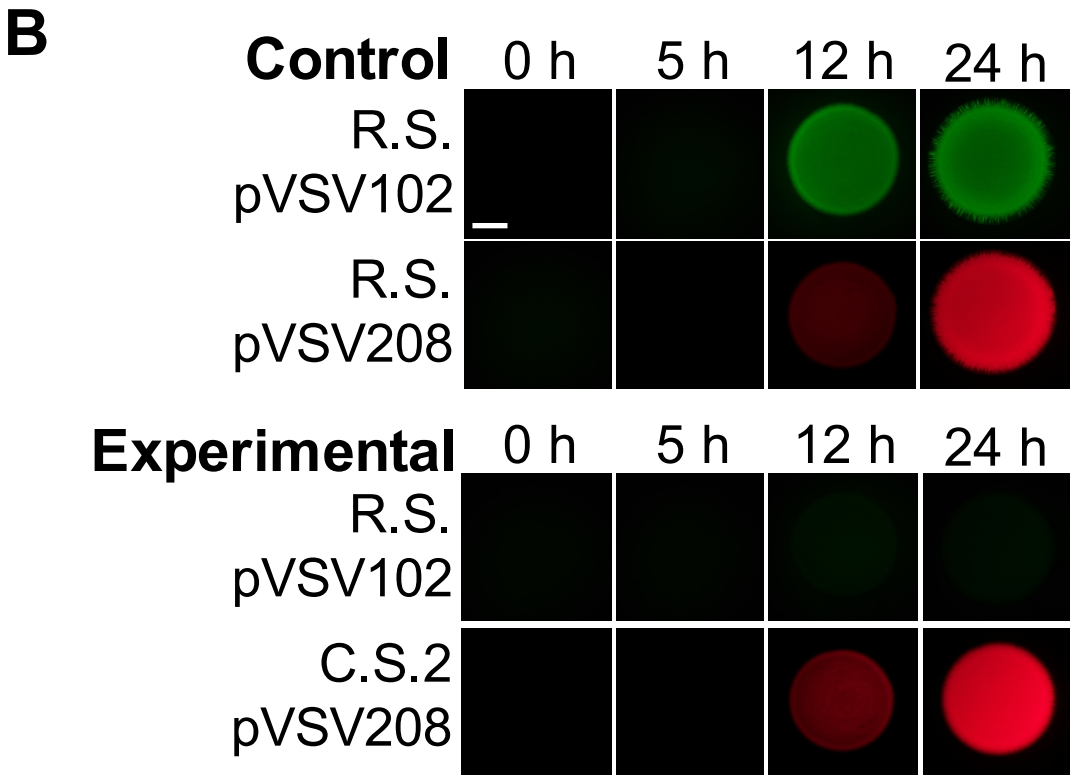

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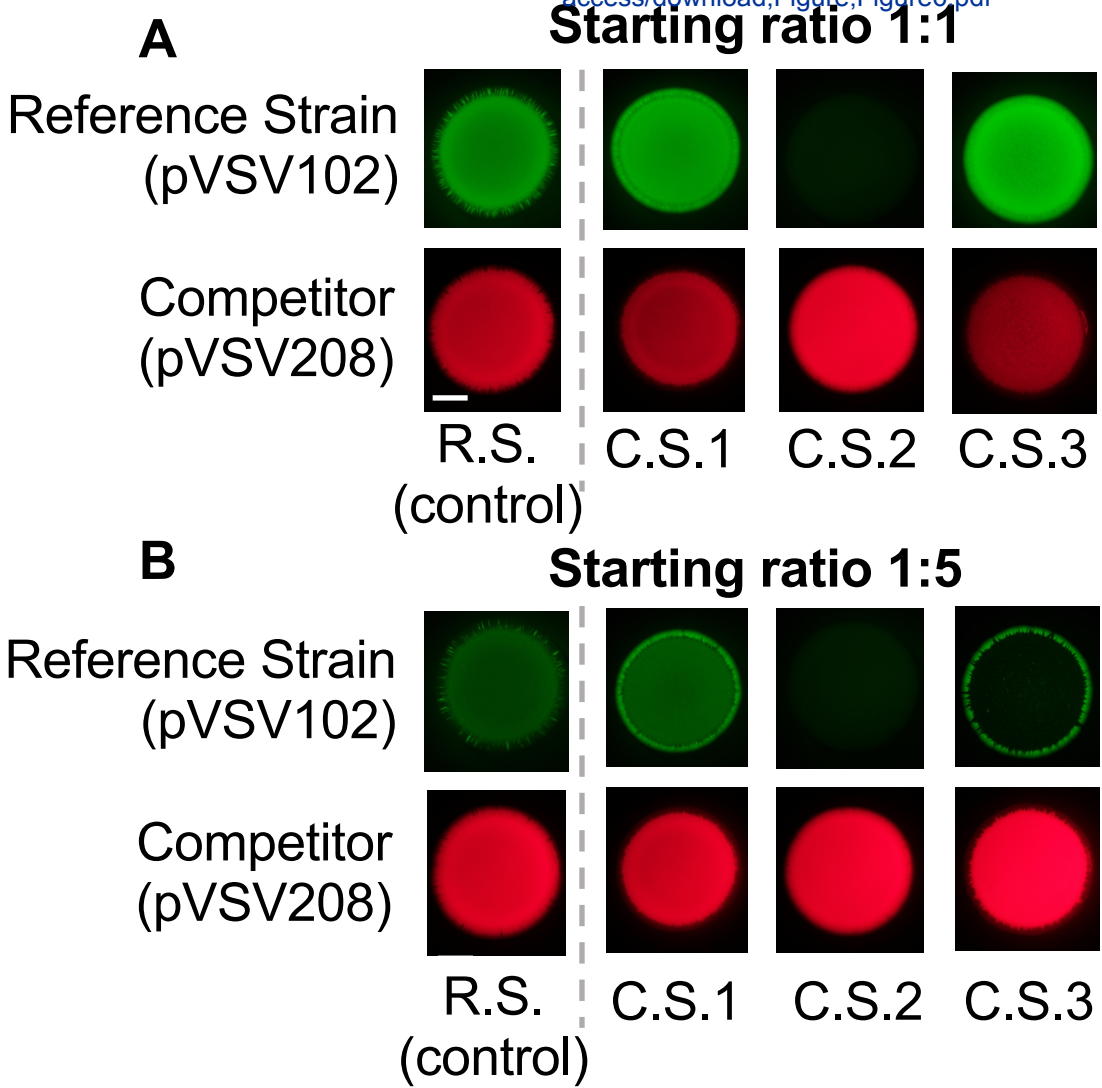

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL Microcentrifuge Tubes	Fisher	05-408-129	
10 µL multichannel pipette			
100 µL multichannel pipette			
300 µL multichannel pipette			
10 µL single channel pipette			
20 µL single channel pipette			
200 µL single channel pipette			
1000 µL single channel pipette			
24-well plates	Fisher	07-200-84	sterile with lid
96-well plates	VWR	10062-900	sterile with lid
Calculator			
Chloramphenicol	Sigma	C0378	stock (20 mg/mL in Ethanol
Fluorescence dissecting microscope with camera and imaging software			
forceps	Fisher	08-880	
Kanamycin Sulfate	Fisher	BP906-5	stock (100 mg/mL in water
Nitrocellulose membrane (FS MCE, 25MM, NS	Fisher	SA1J788H5	0.22 µm nitrocellulose
petri plates	Fisher	FB0875713	sterile with lid
Spectrophotometer			
Semi-micro cuvettes	VWR	97000-586	
TipOne 0.1-10 µL starter system	USA Scientific	1111-3500	10 racks
TipOne 200 µL starter system	USA Scientific	1111-500	10 racks
TipOne 1000 µL starter system	USA Scientific	1111-2520	10 racks
Vortex			
LBS media			
1M Tris Buffer (pH ~7.5)			50 mL 1 M stock buffer (62
Agar Technical	Fisher	DF0812-17-9	15 g (Add only for plates)
DI water			950 mL
Sodium Chloride	Fisher	S640-3	20 g
Tryptone	Fisher	BP97265	10 g
Yeast Extract	Fisher	BP9727-2	5 g

); final concentration in media (2 µg /mL LBS)

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mL HCl, 938 mL DI water, 121 g Trizma Base)

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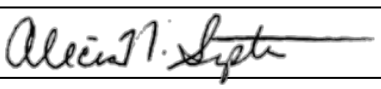
Signature:	
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Response to Reviewers for manuscript JoVE59759 "Coincubation assay for quantifying competitive interactions between Vibrio fischeri isolates."

_____

Reviewer #1:

Manuscript Summary:

The manuscript describes culture-based approaches to characterize competitive interactions between bacterial isolates, along with approaches for analyzing and presenting the data obtained. Although the manuscript focuses on Vibrio fischeri, there are comments related to how these approaches could be applied to other non-related bacterial isolates.

Major Concerns:

I don't have any major concerns. It seems that the procedures, controls, and supplies/equipment are explained as needed in a manner that would allow someone to implement the procedure with Vibrio fischeri or modify the procedure for other bacteria. I have a few suggestions in the "minor concerns" section for ways the procedure might be clarified.

Minor Concerns:

1. Since this technique could be modified for other types of bacteria, maybe changing the title to suggest this possibility would be good.

Response: We agree that it is important to convey to readers that this technique can be applied to other bacteria, which is why we use more general language in both the summary and abstract. However, we decided to name the species (*Vibrio fischeri*) in the title because JoVE instructions to authors indicate that the model organism name should be included.

2. section 1.4: Why incubate overnight?

Response: Incubating overnight allows cultures to grow to a high enough density on plates to easily obtain enough cells to set up the coincubation assay. Because this incubation time is optimal for *V. fischeri,* we made a note that this step can be optimized and more or less time may be required to obtain sufficient cells depending on the growth rate of the organism of interest. See lines 124 and 126.

3. section 2.1.1: Are the cells gathered from individual colonies or the heavy streak?

Response: For each biological replicate, all cells scraped from the agar plate have grown up from a single original colony picked in step 1.4, and are therefore clonal. At this point cells gathered from either individual colonies or the heavy streak would be appropriate as they should be clonal.

4. section 2.1.2: How long can you vortex or pipette before the cells are damaged? In

other sections it is indicated that the pipetting action should be normalized between samples. Maybe including that information here as well would be useful.

Response: The cells should not be damaged by pipetting or vortexing in these assays. In lines 233 and 234, we note that pipetting should be normalized between samples to ensure the sample is a homogenous mix of cells and each sample is processed in the same way.

5. section 2.2.1: With the normalization of an OD of 1.0 equaling a certain number of CFU or bacteria/ml (which?), is this consistent between all strains of V. fischeri? The bolded text is usually highlighted as "Note:…" in other sections, it might be helpful to do that here as well? Also, indicating that the discussion holds more information about cell density might be helpful and conform to the structure of other sections.

Response: An OD of 1.0 translates to ~10^6 bacterial cells / mL for all of the *V. fischeri* strains tested (33 strains). However, this same OD value may not result in the same CFU for other species, which is why it is important to plate the T0.
We thank the reviewer for pointing out this discrepancy in the format in this section. We intended to bold only notes that indicate a step may require optimization. We have reformatted this section to be consistent with the rest of the document and clarified why some notes were bolded by adding a sentence in the introduction. See line 70.
.

6. sections 2.3.3 and 2.3.4. If the spots for visualization and those for CFU/ml are not incubated for the same amount of time, how are these results reconciled? It would seem that they are measuring different things, but maybe clarifying in the text would be helpful.

Response: The reviewer makes an excellent point, that although visual and CFU data assess competition between bacterial strains, these data are collected at separate times and are fundamentally measuring different things. Fluorescent microscopy images are used to determine whether each strain is visibly detectable within the coincubation spot and if strains are spatially separated, while CFUs are used to determine whether one strain outcompetes the other and the mechanism used. Because of these two types of data are assessing different things, using different optimized times to collect these data is appropriate. To clarify this point, we have added a statement in the discussion describing why imaging after 24 h is a good way to screen interactions that reflect results obtained from CFU measurements. See lines 738 - 741.

7. section 2.3.4: is there anything special about how the 24-well plates are prepared, is it similar to the larger petri plates where one needs to be careful about agar moisture, etc.? Are there any special tips for dealing with the 24-well plates?

Response: 24-well plates are prepared in the same manner as standard petri plates, and the same consideration of agar moisture applies to both types of plates. We agree with the reviewer that it would be helpful to state this in the manuscript and have added a statement addressing this point and referencing step 2.3.3, which discusses appropriate agar moisture in detail. See lines 181-184 and 187-188.

8. Lines 308-309: It might be helpful to the reader to indicate that the reference strain in the control incubation labeled with the same marker as the reference strain in the experimental? I know it seems obvious, but stating in some way might be useful.

Response: If the reviewer is referring to the sentence stating “Repeat this process for each time point and replicate for both the experimental treatment and the control treatment (reference strain vs reference strain)”, then we have edited this statement to say (differentially-tagged reference strain coincubation). See line 339.

9. section 4.2.2 and later sections with equations: Earlier in the manuscript RS and CS are defined. I know it is typical to define abbreviations the first time and then not after in a manuscript, but for a methods-type paper it might be helpful to put the definition in each section where it appears as people may just skip around for the most relevant analysis and then have to search for the definition.

Response: We agree with the reviewer that defining RS and CS in each section in which they appear would be helpful for readers, in particular those that may use only one of the data analysis methods included here. This change has been included throughout the manuscript. See lines 363-378 and line 430.

10. Lines 411-412: modifications/modified used twice in the sentence.

Response: We thank the reviewer for pointing out this grammatical issue and have changed the wording of this sentence. See lines 466-470.

Reviewer #2:

Manuscript Summary:

The manuscript JoVE 59759 written by Spear et al,. describes an efficient and simple method to investigate the competition between two different species. The author uses two different strains of Vibrio fischeri to illustrate the protocol. Based on different antibiotic cassette (kanamycin vs chloramphenicol) and different fluorescent proteins (GFP vs dsRed) they are able to show, by quantitative (CFU) and visual (fluorescence) approaches, the antagonistic interactions between both strains. I appreciate that the authors distinguished between contact-dependent and contact-independent competition. They also provide some clear advices to represent data by using graphical tools and statistical analysis.

The overall manuscript is well written, the cited literature is supporting the author's explanations and the advices given are judicious.

The Title : "Coincubation assay for quantifying competitive interactions between Vibrio fischeri isolates" is in agreement with the information provide in the abstract and describe well the purpose of this protocol.

The procedure is clear and well explained. Even laboratories that are not familiar with bacterial competition should be able to apply this protocol.

Minor Concerns:

I have few comments regarding the manuscript:

1. Step 2.3.1: The authors should add a control of each strains (reference and competitor) alone to test at T=0 (Step 2.4) and T=5 (Step 2.5) if each strain has not acquired any mutation that could confer spontaneous antibiotic resistance.

Response: We thank the reviewer for pointing out this control that we had not considered adding to the protocol. We agree that this is an important control to include during initial optimization of the coincubation assay and should be included in the protocol. We have added a description of this control, see lines 109-112 and 263-266.

2. Step 2.3.3 NOTE: authors could also advice not to use plate dried for too long to avoid the formation of small waves at the plate surface. These structures make difficult to get a nice circular spot (may be important for the visual representation).

Response: We agree with the reviewer that moisture of agar plates, whether they are too moist or too dry, is important to discuss. We have added further detail describing why using plates that are too dry should be avoided. See lines 181-184.

3. Step 2.3.4: To facilitate the resuspension, and to avoid 24-well plates, square sterile filter pieces (1cm x 1cm) can be deposited on an agar plate and strains can be spotted on the filter. Then the filter can be removed after 5h of coincubation and transferred into an Eppendorf tube to resuspend the colony by vortexing or pipetting up and down.

Response: We thank the reviewer for pointing out this optional modification and agree that using sterile filter pieces is a more economical approach. We have added an optional modification step describing the use of sterile filters in place of 24-well plates. See lines 199-203.

4. Step 2.4.2: To prevent overgrowth of the bacteria during the time to perform serial dilution (especially during long experiment), authors should advice to use phosphate buffer saline (PBS) instead of LB or LB supplemented with a bacteriostatic antibiotic.

Response: We agree with the reviewer that this would be an appropriate recommendation for researchers using particularly fast growing bacterial species or performing large experiments. In our experience, performing a serial dilution does not tend to be a particularly long process, typically taking between ~10 and 30 minutes. This duration of time is less than a doubling time for *V. fischeri* at room temperature. Because of this we find LBS is an appropriate medium with which to resuspend the coincubation spots. However, we agree using PBS may be beneficial when working with other, faster growing bacteria, and added a statement reflecting this in the text. We also included a statement recommending that researchers be consistent between experiments, using either PBS or LB / LBS in all experiments and not switching between the two. See lines 219-224.

5. NOTE L142-143: The authors use a 1:1 ratio between reference strain and competitor. They mention that this choice may need optimization. Even if this ratio is sufficient to records antagonistic interaction between V. fischeri isolates, in my opinion is better to use a higher amount of predator strain (4:1 or 10:1) to be sure that each prey is in contact with predator strain. Thanks to these ratios the killing observed should be

more important.

We agree with the reviewer that a higher ratio of predator / inhibitor strain to prey / target strain ratio would result in a more dramatic killing phenotype and have included data and a description of the significance from such a ratio in Figure 6A and lines 748-758. We typically use a 1:1 ratio to account for killing as well as other ecologically relevant interactions between strains, such as growth rate. For example, if the killer is a faster grower than the target, using a higher ratio of killer to target can make it difficult to differentiate competitive outcomes due to killing vs growth rate. Ideally researchers will test multiple starting ratios to optimize the assay for answering their specific question. We have added text to the discussion section on starting ratio to emphasize when a higher ratio of inhibitor to target is ideal to use. See lines 756-758.

6. L627-628: As mentioned by the authors, the optimal culture conditions should mimic the ones found in the natural environment of the isolate (L627-628). Nevertheless some strains are naturally competent in their environment (e.g Vibrio fischeri, Vibrio cholerae when they are in chitinous surfaces (Pollack-Berti et al., 2010)(Meibom et al., 2005)). During antagonistic interaction performed in media from the environment (e.g sea water that could contain chitin particles e.g copepods) some genetic material can be released in the environment and incorporated in the genome of the competent species (Borgeaud et al., 2015). These phenomena should at least be cited, so the exchange of antibiotic resistance gene can be prevented during long contact experiment between two strains. Best would be to use mutants that have inactivate DNA uptake machinery.

Response: We thank the reviewer for pointing out how DNA transfer might affect experimental outcomes, and papers addressing it. We have included this point and the suggested references in the text and recommended using conditions that do not promote competence or strains that are not competent to avoid this potential issue. See lines 703-707.

7. L669: There is a mistake. 50 μL reference strain mixed with 500 μL strain 1 is not a 1: 5 ratio but a 1: 10 ratio.

Response: We thank the reviewer for pointing out this mistake and have edited the volumes included in line 669 (now line 752) to accurately describe a 1:5 ratio (50 μl reference strain mixed with 250 μl strain 1).

In my opinion this protocol is highly useful and describe well all the steps to investigate antibacterial behavior between two different species / isolates. I would recommend to published this work in JoVE.