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Vibrio parahaemolyticus: Basic techniques for growth, genetic manipulation, and analysis of virulence factors

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Running Title: Methods for manipulation of *Vibrio parahaemolyticus*

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Abstract:

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium and opportunistic pathogen of humans and shrimp. Investigating the mechanisms of *V. parahaemolyticus* infection and the multifarious virulence factors it employs requires procedures for bacterial culture, genetic manipulation, and analysis of virulence phenotypes. Detailed protocols for growth assessment, generation of mutants, and phenotype assessment are included in this article.

Basic Protocol 1: Assessment of growth of *V. parahaemolyticus*

Alternate Protocol 1: Assessment of growth of *V. parahaemolyticus* using a plate reader

Basic Protocol 2: Swimming / Swarming motility assay

Basic Protocol 3: Genetic Manipulation

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Basic Protocol 4: Secretion Assay and Sample Preparation for Mass Spectrometry Analysis

Basic Protocol 5: Invasion assay (Gentamicin protection assay)

Basic Protocol 6: Immunofluorescence detection of intracellular *V. parahaemolyticus*

Basic Protocol 7: Cytotoxicity Assay for the T3SS2

Introduction:

Vibrio parahaemolyticus is a Gram-negative, halophilic gammaproteobacterial native to marine and estuarine environments. To date 49 pandemic serotypes have been identified, and isolates have been acquired from coastal waters worldwide, including those around North and South America, India, Southeast Asia, and Western Europe (CDC, 2019; Daniels et al., 2000; Han, Tang, Ren, Zhu, & Han, 2016). Although humans are not its primary reservoir, *V. parahaemolyticus* is nonetheless responsible for over 45000 cases of acute gastroenteritis annually in the United States (CDC, 2019). The life cycle of *V. parahaemolyticus* is facilitated by a vast array of virulence factors, including two

type VI secretion systems (T6SS), two type III secretion systems (T3SS), adhesins, quorum sensing, type II secretion system (T2SS) secreted hemolysins, and a newly reported GDSL lipase/esterase (Chimalapati et al., 2020; Chimalapati et al., 2018; de Souza Santos, Salomon, Li, Krachler, & Orth, 2015; Zhang & Orth, 2013). Both the first and second T3SS (T3SS1 and T3SS2, respectively) induce cytotoxicity *in vivo* by mediating an extracellular and intracellular infection respectively, however, only the latter is responsible for the enterotoxicity that drives acute gastroenteritis in human hosts (Kodama et al., 2015; K.-S. Park et al., 2004; K. S. Park et al., 2004; Zhang et al., 2012). *V. parahaemolyticus* strains carrying the plasmid encoding the Pir^{VP} toxin are also capable of inducing acute hepatopancreatic necrosis disease (AHPND) in shrimp, a disease responsible for the widespread devastation of aquaculture worldwide, including over \$20 billion in economic losses in Asia alone (Li et al., 2017; Undercurrentnews, 2016).

Given the clinical and economic impacts of *V. parahaemolyticus* infection, it is critical to understand the virulence factors the bacteria employ to facilitate their life cycle in and outside of the human host. Studying such a broad array of virulence factors requires a battery of experimental approaches. The most fundamental of these include *V. parahaemolyticus* culture techniques and maintenance, as well as evaluations of motility and growth rates under various culturing conditions. Cumulatively these techniques allow for the long-term propagation and storage of *V. parahaemolyticus* strains for study. In addition, they provide valuable insight into the impact of various nutritional sources, mutations, or other such stimuli on major cell-signaling pathways that govern swimming and swarming behavior, as well as on rates of replication.

Genetic manipulation represents a second core approach to investigating *V. parahaemolyticus*, as generating genomic knockouts and both chromosomal and plasmid complementation are critical for fulfilling molecular Koch's postulates when investigating the roles of specific genes or virulence factors in the *V. parahaemolyticus* life cycle (Falkow, 2004). Assessing the virulence phenotypes associated with the genetic manipulations and various infection models are critical for understanding the role of specific factors in *V. parahaemolyticus* pathogenesis. Microscopy of the fixed and live cell imaging permit direct visualization of *V. parahaemolyticus* and host tissue culture cells at discrete stages of infection and can reveal major changes to pathogenesis resulting from mutations, while phenotypic assays such as gentamicin protection assays and lactate dehydrogenase assays provide more quantitative measures of intracellular bacteria and cytotoxicity in the host, respectively. Western blots and mass spectrometry are also valuable techniques for evaluating the secretion of different virulence factors. The protocols are designed for analyzing various virulence factors of *V. parahaemolyticus* strains.

Importantly, when assessing *V. parahaemolyticus* phenotypes, virulence factors from one system can mask the activity of another system, especially in cell culture assays. Therefore, when assessing the phenotypes associated with the virulence factors, it is necessary to delete one system to uncover the activity of another system. For example, as the T3SS1 kills within 2-3 hours *in vitro*, when studying the T3SS2 associated phenotypes, T3SS1 system must be inactivated so that the cytotoxicity of the T3SS1 does not mask the activities of the T3SS2 which can extend up to 7-8h during *in vitro*

infections. *In vivo*, this is not as much a problem because of the different ways these two T3SSs are spatially activated.

In this article, Basic Protocol 1: Assessment of growth of *V. parahaemolyticus*; Basic Protocol 2: Swimming / Swarming motility assay; Basic Protocol 3: Genetic Manipulation; Basic Protocol 4: Virulence Factor and phenotype analyses are described in detail. Basic protocol 1 describes the growth curve protocol during which optical density of the culture is recorded at regular time intervals to assess the growth. The protocols for swimming and swarming motility patterns of *Vibrio parahaemolyticus* on motility agar plates are detailed in the basic protocol 2. Genetic manipulations such as generation of deletion and complementation mutants are described in the next section, basic protocol 3. Finally, for the assessment of various virulence factors and phenotype assessment, protocols for secretion assay, invasion assay, cytotoxicity assay and immunofluorescence detection of *Vibrio parahaemolyticus* are detailed in basic protocol 4.

Strategic planning:

Growth Conditions: *V. parahaemolyticus* will grow under aerobic conditions between 18°C and 37°C, but does so best at 30°C. Its preferred broth growth medium is MLB (Luria-Bertani broth with 30g/L NaCl). For plating, MMM agar (Minimal Marine Medium, see Reagents and Solutions section for recipe) is standard, and plates should be pre-warmed, as *V. parahaemolyticus* will not grow after prolonged exposure to temperatures around 4°C. A neutral pH is recommended for standard growth conditions, though *V. parahaemolyticus* can survive across a broad pH range. Typically, *V. parahaemolyticus* will double every 20 minutes in rich growth media, and an overnight culture at stationary phase can be expected to yield $> 1.5 \times 10^9$ CFU/mL. It is not recommended that ampicillin be used as for selection in *V. parahaemolyticus*, as most strains possess an innate resistance to the antibiotic.

Storage and Retrieval: For storage, *V. parahaemolyticus* overnight cultures may be mixed 1:3 v/v with sterile 50% glycerol and stored at -80°C; *V. parahaemolyticus* may be streaked onto room temperature plates or inoculated in liquid media directly when cultured from storage, and will grow at 30°C within 8-12 hours. Generally, it is encouraged that a single colony be obtained by streaking out an inoculum from a frozen glycerol stock for inoculation of liquid culture. If small volumes (>500µL) of bacterial culture are needed, a 0.5mL MLB culture of *V. parahaemolyticus* inoculated from a -80°C freezer stock will reach logarithmic phase within three hours when incubated shaking at 30°C.

Induction of the T3SS: The T3SS1 can be induced by incubating *V. parahaemolyticus* growing in logarithmic phase shaking at 37°C for one hour in a low calcium medium, such as high glucose DMEM (Sigma Aldrich, D5796) (Broberg, Zhang, Gonzalez, Laskowski-Arce, & Orth, 2010; "National

Enteric Disease Surveillance: COVIS Annual Summary, 2014," 2016). MLB with 10mM sodium oxalate and 10mM MgCl₂ can be used as an alternative to high glucose DMEM. The T3SS2 is induced in the presence of 0.05% bile salts (w/v prepared in deionized water) added to a logarithmic phase culture and incubation at 37°C for an hour (Kodama et al., 2015).

Basic Protocol 1: Assessment of growth of *V. parahaemolyticus*

In this protocol, *V. parahaemolyticus* is inoculated into growth medium at a very low density and growth pattern is followed by recording optical density at 600 nm (OD₆₀₀) at regular time intervals. *V. parahaemolyticus* follows a typical bacterial growth curve with an initial lag phase, followed by an exponential growth phase and then a stationary phase.

Note: *V. parahaemolyticus* is a Bio Safety Level 2 (BSL-2) pathogen and should be handled using appropriate safety precautions.

Materials

- Sterile culture tubes
 - Sterile 125 ml Erlenmeyer flasks
 - Sterile Eppendorf tubes
 - Sterile MLB culture medium
 - Temperature controlled incubator with shaking
 - Benchtop centrifuge
 - Spectrophotometer
1. Prepare 3mL cultures in triplicate of the *V. parahaemolyticus* strain by inoculating from a single colony or from a frozen glycerol stock in MLB medium and incubate overnight (O/N) at 30°C with shaking at 200 rpm.
 2. Measure the OD₆₀₀ of the O/N cultures and calculate the amount of culture needed to obtain a final OD₆₀₀ of 0.05 for 25mL culture medium using the formula $25 \times 0.05 / OD_{600}$ and transfer this volume aseptically into Eppendorf tubes.
 3. Centrifuge tubes at 16,000g for 5 min and wash the pellets once with 500 of sterile PBS.
 4. Under aseptic conditions resuspend these pellets in 100 µl of MLB medium and transfer to 25 mL of sterile culture medium in sterile 125 ml Erlenmeyer flasks.
 5. Immediately swirl the flasks and withdraw 1 ml sample from each flask.
 6. Immediately Incubate the flasks at 30°C and 200 rpm.
 7. Measure the OD₆₀₀ of the 1 ml sample withdrawn to obtain T₀ reading.

8. Aseptically withdraw 1 ml samples at 1 h intervals and measure OD₆₀₀. When the OD reaches 0.6 and higher, accurate OD readings can be obtained by using a 10-fold dilution of the culture to obtain OD measurement. Continue with measurements of OD up to 8 h of growth.
9. Growth curves can be obtained by plotting OD₆₀₀ readings against time intervals.
10. An example of *V. parahaemolyticus* CAB2 strain growth curve can be seen in Fig 1, with an initial lag phase up to 1 h, followed by exponential growth phase between 1-4h and an early stationary phase from 4-6h and late stationary phase between 6-8h. When the OD readings are taken using a 10-fold dilution, the reading should be multiplied by a factor of 10 to account for the dilution.

[*Place figure 1 near here.]

Alternate Protocol 1: Assessment of growth of *V. parahaemolyticus* using a plate reader

The growth curves can also be obtained by using a plate reader for automated measurement.

Materials

- Sterile culture tubes
 - Sterile Eppendorf tubes
 - Sterile culture medium (MLB /MMM)
 - Sterile 96 well flat bottom culture plate
 - Temperature controlled plate reader with provision for shaking
 - Benchtop centrifuge
 - Spectrophotometer
1. Prepare 3mL cultures in triplicate of the *V. parahaemolyticus* strain by inoculating from a single colony or from a frozen glycerol stock in MLB medium and incubate overnight (O/N) at 30°C with shaking at 200 rpm.
 2. For each of the triplicate O/N cultures, measure the OD₆₀₀ and calculate the amount of culture needed to obtain an OD₆₀₀ of 0.05 for 1mL culture medium using the formula $1 \times 0.05 / OD_{600}$ and transfer this volume aseptically into Eppendorf tubes.
 3. Microcentrifuge tubes at 16,000g for 5 min and wash the pellets once with 500 of sterile PBS.
 4. Under aseptic conditions, resuspend each of these pellets in 1 mL of MLB medium and aliquot 200 µl into 3 wells of a 96 well culture plate. Include 3 wells of 200 µl culture medium for blank measurement.

5. Immediately place the 96 well plate in the plate reader (set the plate reader temperature to 30°C with continuous shaking) and measure OD₆₀₀.
6. Set the plate reader to measure OD₆₀₀ at 1h time intervals up to 8h.
7. Growth curves can be obtained by plotting OD₆₀₀ readings against time intervals.

Basic Protocol 2: Swimming / Swarming motility assay:

V. parahaemolyticus strains use their polar and lateral flagella for motility in swimming and swarming modes. The following protocols describe the assessment of motility phenotype.

Materials

- Sterile culture tubes
- Sterile Eppendorf tubes
- Sterile swim and swarming agar plates (see 'Reagents and Solution' for recipes)
- Sterile MLB
- Benchtop centrifuge
- Spectrophotometer
- Temperature controlled incubator

1. Preparation of plates: Prepare swimming and swarming agar plates the day before the assay. (Follow the guidelines in the Reagents and Solutions to prepare these plates).
2. Use 150 mm plates so there is enough space to test multiple strains on the same plate.
3. Grow overnight cultures of bacteria in 3ml MLB as described in 1D.
4. Measure the OD₆₀₀ of the O/N cultures and re-inoculate in 3ml MLB for a final OD of 0.2. Grow at 30°C and 200 RPM for 2 h to obtain the cultures in log phase.
5. Dry the swim /swarm plates for 2hrs at 37°C.
6. Spin 1 ml culture in a sterile 1.5 ml Eppendorf tubes and wash 3 times with sterile PBS and resuspend the pellet in 1 ml PBS and adjust OD₆₀₀ to 0.1.
7. Spot inoculate 0.5 µl of each culture on the plates far enough from each other to swim / swarm. Up to 5 and 3 strains can be inoculated on 150 mm and 100mm plates respectively. Perform the spotting on 3 separate plates to obtain technical replicates.
8. Incubate plates upright at 30°C for 16h and measure the swimming / swarming diameter for each test strain.
9. An example of swimming and swarming motility of *V. parahaemolyticus* CAB2 strain can be seen in Fig 2. The diameter of the swimming and swarming zone may be determined using a ruler and used for comparison between strains.

[*Place figure 2 near here.]

Basic Protocol 3: Genetic Manipulation:

Generation of *V. parahaemolyticus* mutants via genetic manipulation is described in the following protocols.

Generation of chromosomal Knockouts/ Knock-ins: In this method an Ori6K/SacB suicide vector pDM4 is used to integrate required genomic change into the chromosome. The pDM4 vector containing the construct is introduced into *V. parahaemolyticus* via conjugation and, following recombination, the transconjugants are cured of the *sacB* containing pDM4 by counter selection on sucrose plates.

Materials

- Standard molecular biology reagents for PCR and cloning
- PCR Thermal Cycler
- Sterile culture tubes
- Temperature controlled incubator/shaker
- Sterile PBS
- LB – Chloramphenicol plates (LB Cm, refer to table 1 for concentration)
- MMM – Chloramphenicol plates (MMM Cm, refer to table 1 for concentration)
- MMM – Sucrose (15% w/v) plates
- Heart Infusion No salts agar plates (HI NS- see 'Reagents and Solution' for recipe)

[*Place table 1 near here.]

1. For gene deletion, as a first step, design PCR primers and amplify 1 kb upstream (-1kb) and downstream (+1kb) amplicons of the target gene such that 3' of the upstream and 5' of the downstream amplicons have same restriction endonuclease sites. Refer to Figure 3 for a schematic representation of these amplicons. Make sure that the amplicons do not contain the chosen restriction endonuclease sites, online tools such as NEB cutter may be used to visualize the restriction sites present in the amplicon. Complete genomic sequence of *V. parahaemolyticus* chromosome 1 and 2 are available via accession numbers NC_004613.1 and BA000032.2 respectively. Many online tools are available for designing primers such as NCBI Primer Blast or Primer3. Following the standard molecular cloning techniques clone the (-1kb and +1kb) amplicons into pDM4 vector and transform into S17 (λ pir) *E. coli* strain. Similarly, for a gene knock-in 1kb upstream and downstream homology arms are first fused to the amplicon containing the intended knock-in fragment as explained above. This construct is cloned into pDM4 using standard cloning techniques and transformed into S17 *E. coli*.

[*Place figure 3 near here.]

2. Day 1. Grow O/N cultures of the *V. parahaemolyticus* parental strain and the S17 *E. coli* strain harboring the amplicon containing pDM4 plasmid in 3mL of MLB and LB with chloramphenicol respectively.
3. Day 2. Transfer 100µl each of the above O/N cultures aseptically into a single sterile Eppendorf tube and mix by gentle vortexing.
4. Spot inoculate 10µl of the above mixture into 10 spots on to a single, pre warmed HI NS plate and incubate the plate at 37°C O/N taking care that the spots do not mix with each other (leave at room temperature until the spots are adsorbed on to the agar).
5. Day 3. Aseptically add 5mL of sterile PBS onto the O/N grown HI NS plate and gently scrape the growth into PBS using a sterile 1 mL pipette tip, re-suspend and collect the bacterial suspension into a sterile 15 ml culture tube. Gently vortex to break clumps of bacterial growth.
6. Aseptically transfer 10, 20 and 50 µl of the bacterial suspension onto 3 pre warmed MMM chloramphenicol plates containing a pool of 100 µl of sterile PBS and spread using sterile glass beads. Incubate the plates at 30°C for up to 48h until 1-1.5mm colonies become apparent.
7. Day 5. Aseptically patch 20 individual colonies on to MMM Cm plates using sterile toothpicks to confirm the integration of chloramphenicol marker in the *V. parahaemolyticus* parental strain. Incubate the plates at 30°C for 24 to 48h until 1-1.5mm colonies become apparent.
8. Day 6. Pick up to 8 colonies and streak aseptically on pre warmed MMM sucrose plates (up to 4 colonies can be streaked on to a single plate in a pie wedge format) in order to obtain individual colonies and incubate at 30°C for up to 48h until 1-1.5mm colonies become visible.
9. Grow O/N cultures in MLB of 2-3 colonies from each pie wedge and confirm the genomic edit by performing PCR and/or sequencing. Prepare glycerol stocks and store at -80°C.

Alternate protocol 2: Natural transformation. In this protocol *V. parahaemolyticus* strain expressing the competence factor *tfoX* is used to increase the natural competence for taking up extracellular DNA. In this method the target gene for deletion is replaced by a selectable antibiotic marker.

Materials

- Standard molecular biology reagents for PCR and cloning
- PCR Thermal Cycler
- Water bath
- Sterile culture tubes
- Temperature controlled incubator/shaker
- Sterile PBS
- MMM – Chloramphenicol plates (MMM Cm, refer to table 1 for concentration)
- MMM – Sucrose (15% w/v) plates

1. Design primers and amplify 3kb PCR fragments upstream and downstream of the target gene and generate a single construct containing the 3kb upstream and downstream amplicons fused to either side (upstream amplicon 5' and downstream amplicon on 3') of an antibiotic resistance marker such as chloramphenicol following standard molecular biology PCR techniques. This construct is referred to as the transforming DNA (tDNA).
2. Day 1. Grow O/N culture of *V. parahaemolyticus* expressing pMMB*sacBtfoX* plasmid in 3 mL of MLB+ kanamycin. pMMB*sacBtfoX* is a bacterial expression vector that can be induced with Isopropyl-beta-D-thiogalactoside (IPTG) and it contains a *sacB* gene for curing the plasmid after homologous recombination and integration of the tDNA into the genome. *tfoX* is a *Vibrio* competence factor that is used to increase the natural competence of *Vibrio* strains for taking up extracellular DNA.
3. Day 2. For each transformation, transfer 3.5 μ L of the overnight culture into an Eppendorf tube and dilute with 350 μ L of 2X Instant Ocean (2XIO, see appendix for recipe) +100 μ M IPTG (no antibiotic needed). Invert gently to mix. Include a "no DNA" control reaction.
4. Add tDNA (500 ng to 1 μ g) to each reaction and invert gently to mix. Incubate these reactions statically at 30°C for 4-6 hours.
5. Add 1 ml MLB to each reaction and incubate at 30°C with shaking for 2 hours.
6. Using glass beads spread plate all the reaction mixture on MMM antibiotic plates (MMM Cm plates if using chloramphenicol as selectable marker) from the tube containing the tDNA construct. Divide between 2-3 plates if necessary. For the control reaction 1 -2 plates are sufficient. Incubate the plates inverted at 30°C up to 2-3 days until colonies are visible.
7. Streak up to 10 colonies on MMM agar with 15% sucrose and antibiotic (MMM sucrose + Cm plates if using chloramphenicol as selectable marker) and incubate at 30°C up to 48 h or until isolated colonies appear.
8. Patch about 50 colonies on MMM plates with and without kanamycin (similar to replica plating, each colony will need to be patched onto MMM Kan plates first and then onto MMM plates). For each patched colony, select the colonies that grow on MMM but fail to grow on MMM Kan. These colonies denote *V. parahaemolyticus* strains that are cured of the pMMB*sacBtfoX* plasmid but have their target gene replaced by the antibiotic resistance marker.
9. Grow O/N cultures of 10 such colonies and confirm the gene deletion by performing PCR and/or sequencing. Prepare glycerol stocks and store at -80°C.

Basic Protocol 4: Secretion Assay and Sample Preparation for Mass Spectrometry Analysis

V. parahaemolyticus secretes either T3SS1 or T3SS2 mediated effectors depending on the type of induction. In this protocol, preparation of T3SS2 effectors secreted by *V. parahaemolyticus* CAB2 strain upon bile salts induction for mass spectrometry is described. T3SS1 effectors can be induced by growing *V. parahaemolyticus* in low calcium medium such as high glucose DMEM and incubation

at 37°C. Wear gloves for handling of all reagents and materials as collagen contamination can mask signals in final sample.

Materials:

- 5mL overnight culture of *V. parahaemolyticus* CAB2 strain grown in MLB
 - Growth media – MLB
 - Sterile 50 mL conical tubes
 - Sterile Eppendorf tubes
 - Ice bucket
 - 5% w/v Bile salts (Bile Bovine, Sigma Aldrich, B3883) solution
 - 150mg/mL Sodium deoxycholate (DOC)
 - 100% Trichloroacetic acid TCA (use within a month of preparation, as this reagent is labile)
 - 100% Acetone
 - 70% Methanol
1. From overnight cultures inoculate 50mL of growth media containing 0.05% bile salts to an $OD_{600} = 0.2$.
 2. Incubate the cultures shaking at 37°C for 3-5h.
 3. Measure the OD_{600} of all cultures. The final OD_{600} should be between 4 and 5, as all cultures should have reached stationary phase. Transfer cultures to a 50mL conical tube, normalizing the volumes of each culture so that an equivalent number of bacteria are added to each tube and all 50mL of the culture with the lowest OD_{600} is transferred.
 4. Pellet for 15 minutes at 4°C at 3200g.
 5. Filter the supernatants through a 0.22µm filter into a fresh 50mL conical tube.
 6. Add 150mg/mL DOC to each supernatant to a final concentration of 150µg/mL; vortex and incubate on ice 15 minutes.
 7. Add TCA to a final concentration of 8% (v/v) to each sample; vortex and incubate at 4°C overnight on ice in a cold room if possible.
 8. Resuspend the precipitated protein and transfer the suspensions to Nalgene centrifuge tubes that have been rinsed with MeOH and dried to remove collagen contamination
 9. Pellet the precipitated protein 1h at 4°C, 27000g.
 10. Discard the supernatant; apply 4mL -20°C 100% acetone and spin 40 minutes at 4°C, 27000g to rinse; discard the supernatant and repeat.
 11. Discard the supernatant and air dry each pellet 10 minutes.
 12. Resuspend each pellet in 1mL 10mM Tris pH 8 and transfer to a 1.5mL Eppendorf tube.
 13. Add 150mg/mL DOC to each supernatant to a final concentration of 150µg/mL; vortex and incubate on ice 15 minutes.
 14. Add TCA to a final concentration of 8% (v/v) to each sample; vortex and incubate at 4°C 1-5 h.
 15. Pellet the precipitated protein 1hr at 4°C, 21000g in a microfuge.

16. Discard the supernatant and apply 400 μL -20°C 100% acetone and spin 20 minutes at 4°C , 21000g in a microfuge to rinse; discard the supernatant and repeat.
17. Discard the supernatant and air dry each pellet 10 minutes.
18. Suspend each pellet in 20 μL 5x Sodium Dodecyl Sulfate (SDS) sample buffer (more may be used if needed, though the volume must be kept consistent for each sample). Ensure that the pH of each sample does not exceed the buffer capacity of the sample buffer by adding 10M NaOH in 0.2 μL increments as needed to keep sample neutral (blue).
19. Boil samples for 10 minutes.
20. Apply each sample to a MiniProtean Any kD precast SDS PAGE gel (Bio RAD, 4569033); run at 80V for 20 minutes or until the entire sample has permeated the gel
21. Excise each sample ($\sim 1\text{ cm}^2$) from the gel using the dye front as a guide and submit for mass spectrometry protein identification analysis.

Basic Protocol 5: Invasion assay (Gentamicin protection assay)

This protocol describes the HeLa cell invasion by *V. parahaemolyticus* strains and assessment of the invasion phenotype. When assessing *V. parahaemolyticus* invasion, the T3SS1 system must be genetically inactivated so that the cytotoxicity of the T3SS1 does not mask the invasion phenotype of the T3SS2.

Materials:

- Tissue culture biosafety hood
- 24-well tissue culture plates
- Sterile culture tubes
- Spectrophotometer
- Temperature controlled incubator / Shaker
- CO_2 incubator for tissue culture (37°C , 5% CO_2)
- HeLa cells
- MLB
- 5% Bile salts solution (Bile Bovine (Sigma Aldrich, B3883) in deionized water, w/v and filter sterilized)
- DMEM complete medium (see 'Reagents and Solution' for recipe)
- DMEM infection medium (see 'Reagents and Solution' for recipe)
- Un-supplemented DMEM (see 'Reagents and Solution' for recipe)
- Gentamicin (100mg/mL stock solution)
- Sterile 0.5% Triton-X-100 prepared in PBS
- Sterile PBS

1. Day 1. Using the tissue culture biosafety hood, plate HeLa cells in complete DMEM medium at a concentration of 7×10^4 cell /mL (1 mL/well, in triplicate for each *V. parahaemolyticus* strain) in 24 well tissue culture plates. Plate one 24 well for each time point.
2. Grow O/N cultures of *V. parahaemolyticus* strains in MLB at 30°C and 200 RPM.
3. Day 2. Measure the OD₆₀₀ of diluted O/N grown cultures and dilute in 5 mL MLB containing 0.05% of bile salts solution to an initial OD₆₀₀ of 0.3 and incubate the cultures at 37°C and 200 RPM for 1.5h to induce T3SS2.
4. Warm up the MMM plates to room temperature.
5. Pre warm the infection medium and plain DMEM at 37°C.
6. Measure OD₆₀₀ of the cultures (dilute if necessary for accuracy) after 1.5h growth at 37°C. 1 mL of culture with an OD 600 of 1 is equivalent to 5×10^8 bacteria. Calculate the volume of bacteria needed to infect HeLa cells for an MOI of 10, following the equation:

$$\text{Volume of bacterial culture in } \mu\text{L per 1 mL of DMEM media} = (1 \text{OD}_{600} \text{ unit}) / (5 \times 10^8 \text{ bacteria/mL}) \times (10 \text{ bacteria/HeLa cell}) \times (1 \text{mL/OD}_{600} \text{ measured}) \times 7 \times 10^4 \text{ HeLa cells plated per well} \times (10^3 \mu\text{L/1mL})$$

7. Prepare adequate volume of bacterial infection medium for each strain (based on time course of infection) by adding the required volume of bacteria to prewarmed infection medium as calculated above.
8. Gently wash HeLa cells plated in 24 wells with un-supplemented DMEM twice and start the infection process by adding 1 mL of bacterial infection medium to HeLa cells plated in each well. There will be 3 replicates in total for each strain for every timepoint.
9. Synchronize the infection by centrifuging the 24 well plates at 1000 RPM (~200x g) for 5 minutes and incubate the plates at 37°C in TC incubator for 2 h.
10. Wash HeLa cells twice with un-supplemented DMEM and add 1 mL of infection medium containing 100 µg/ml of gentamicin to each well.
11. Return the plates to the TC incubator and further incubate each plate for the required length of invasion process, typically for 1, 3, 5 and 7h.
12. At the end of each time point, wash wells with sterile PBS twice (1mL/well).
13. Lyse the cells by adding 1 mL/well of sterile 0.5% TX-100 prepared in PBS and incubate for 10 minutes with shaking at room temperature.
14. Pipette the cell lysates vigorously 10 times using a 1 mL pipette tip to break any clumps of cells and to obtain a uniform cell lysate.
15. From each lysate sample, prepare 10-fold serial dilutions up to 10^{-3} using sterile PBS.
16. Spot inoculate 10 µl of each dilution and undiluted lysate onto room temperature MMM plates. Leave at room temperature undisturbed until the spotted lysates are adsorbed onto the MMM plates and incubate them at 30°C O/N or until colonies become visible.
17. Count the colonies and multiply by 100 (include the dilution factor if counting colonies from diluted samples) to determine CFU/mL. Invasion phenotype can be assessed by plotting CFU/mL at various time points of invasion.

18. An example of invasion phenotype of *V. parahaemolyticus* CAB2 strain during gentamicin protection assay has been shown in Fig 4. The number of intracellular bacteria during HeLa cell invasion assay increases with time up to 5 h which then reduces at 7h as the bacteria egress from the invaded HeLa cell and encounter the extracellular gentamicin.

[*Place figure 4 near here.]

Basic Protocol 6: Immunofluorescence detection of intracellular *V. parahaemolyticus*

In this protocol, HeLa cells are infected with *V. parahaemolyticus* strains that have been induced with bile (an activator of the T3SS2) and are treated with cell impermeable gentamicin antibiotic to assess the behavior of intracellular bacteria. This protocol can be modified to evaluate other cell types, organelles, and host cell proteins of interest. When assessing *V. parahaemolyticus* invasion, the T3SS1 system must be inactivated so that the cytotoxicity of the T3SS1 does not mask the invasion of the T3SS2. It is recommended that any *V. parahaemolyticus* strain used constitutively express GFP to visualize intracellular bacteria. Alternatively, an anti-*V. parahaemolyticus* antibody may also be used to detect *V. parahaemolyticus* by immunofluorescence.

Materials:

- Tissue culture biosafety hood
- Glass coverslips
- 6-well tissue culture plate
- DMEM complete medium (see 'Reagents and Solution' for recipe)
- DMEM infection medium (see 'Reagents and Solution' for recipe)
- Un-supplemented DMEM (see 'Reagents and Solution' for recipe) HeLa cells
- CO₂ incubator for tissue culture (37°C, 5% CO₂)
- GFP expressing *V. parahaemolyticus* glycerol stocks
- Luria-Bertani (LB) broth, supplemented with 3% NaCl (MLB)
- 5% Bile salts solution (Bile Bovine (Sigma Aldrich, B3883) in deionized water, w/v and filter sterilized)
- 30°C incubator
- 37°C incubator
- Spectrophotometer
- Gentamicin (100mg/mL stock solution)
- 1x phosphate buffered saline (PBS)
- Fixative: 4% paraformaldehyde (PFA) in 1x PBS (v/v)
- Permeabilization solution: 0.5% saponin in 1x PBS (w/v)
- Blocking solution: 1% bovine serum albumin (BSA) and 0.1% saponin in 1x PBS (w/v)
- Antibody dilution solution: 0.5% BSA and 0.1% saponin in 1x PBS (w/v)
- Wash solution: 0.1% saponin with 1x PBS (w/v)
- Hoechst 33342

- ProLong Gold Antifade Mountant
- Glass slides
- Zeiss LSM confocal microscope

1. Day 1. UV sterilize glass coverslips and place into sterile 6-well tissue culture plate(s) in the tissue culture biosafety hood.
2. For each well/coverslip, seed 1×10^5 HeLa cells in 2mL of complete DMEM. Incubate HeLa cell plate in CO₂ incubator at 37 °C with 5% CO₂.
3. Inoculate 5 mL of MLB with *V. parahaemolyticus* strains from glycerol stocks. Add spectinomycin at 50µg/mL to maintain expression of GFP and add other antibiotics if necessary. Incubate overnight at 30 °C and 200 rpm. If GFP expressing *V. parahaemolyticus* strains are not available, a commercially available anti *V. parahaemolyticus* O3 antibody (Abcam 78751) can be used to visualize the bacteria as detailed later in the protocol.
4. Day 2. Measure the OD₆₀₀ of the overnight cultures.
5. Start fresh cultures in 5 mL MLB for each strain from the overnight *Vibrio* cultures for an OD₆₀₀ of 0.3. Add spectinomycin at 50µg/mL to maintain expression of GFP and add other antibiotics if necessary.
6. To induce expression of the T3SS2, add 50 µL of 5% bile salt solution for a final concentration of 0.05%. Then incubate the cultures at 37 °C for 90 minutes at 200rpm.
7. After the incubation, check the OD₆₀₀ of each culture. 1 mL of culture with an OD 600 of 1 is equivalent to 5×10^8 bacteria.
8. Using the OD₆₀₀, calculate the volume of each bacterial sample needed for an approximate MOI of 10 according to the following equation:

$$\text{Volume of bacterial culture in } \mu\text{L per 1 mL of DMEM media} = (1\text{OD}_{600} \text{ unit}) / (5 \times 10^8 \text{ bacteria/mL}) \times (10 \text{ bacteria/HeLa cell}) \times (1\text{mL}/\text{OD}_{600} \text{ measured}) \times 1 \times 10^5 \text{ HeLa cells plated per well} \times (10^3 \mu\text{L}/1\text{mL})$$

9. Create the infection solution by adding calculated volume of bacterial cultures to pre-warmed DMEM infection media.
10. Wash HeLa cells 2x with un-supplemented DMEM.
11. Infect cells by adding 1mL of the infection solution to each well of HeLa cells.
12. Centrifuge the 6-well plate(s) at 1000 rpm (~200x g) for 5 min to synchronize the infection by forcing contact of the bacteria with the HeLa cells.
13. Incubate plate(s) in the CO₂ incubator at 37 °C with 5% CO₂ for 2 hours.
14. Wash cells 2x with un-supplemented DMEM.
15. Make a solution by adding gentamicin (100 µg/mL final concentration) to fresh DMEM infection media. Add 1 mL into each well and incubate the plate at 37 °C with 5% CO₂ for each time point (usually between 1-7h).

16. At the end of each time point, remove the 6-well plate from the incubator and protect from light for following steps.
17. Wash the coverslips with 1mL of 1x PBS and then fix the cells on the coverslip by adding 1mL of 1x PBS with 4% PFA for 10 minutes at room temperature.
18. Wash the coverslips three times with 1mL of 1x PBS for each well. If required 6 well plates containing coverslips may be stored at 4°C covered with aluminum foil up to 24 h.
19. If using anti *V. parahaemolyticus* antibody to detect extracellular bacteria, incubate the coverslips with diluted antibody (1: 500 in 1x PBS) and incubate at room temperature for 1 hour.
20. Wash the coverslips three times for 5 minutes with 1x PBS.
21. Incubate the coverslips with a fluorescent secondary antibody diluted in 1x PBS (see step 37 for fluorescent secondary antibody options) and incubate at room temperature for 1 hour.
22. Wash the coverslips three times for 5 minutes with 1x PBS.
23. Permeabilize the cells on the coverslips with 1mL of 1x PBS with 0.5% saponin for each well and incubate at room temperature for 10 minutes.
24. Wash the coverslips one time with 1mL of 1x PBS for each well.
25. If desiring to image other cellular proteins and markers, then block the cells on the coverslips using 1x PBS with 1% bovine serum albumin (BSA) and 0.1% saponin for 30 min at room temperature. If only assessing the bacterium, then skip to step 30.
26. Incubate the coverslips with primary antibody diluted in 1x PBS containing 0.5% BSA and 0.1% saponin at an appropriate dilution and incubate at room temperature for 1 hour.
27. Wash the coverslips three times for 5 minutes with 1x PBS containing 0.1% saponin.
28. Incubate the coverslips with secondary antibody diluted in 1x PBS containing 0.5% BSA and 0.1% saponin at an appropriate dilution and incubate at room temperature for 1 hour.
29. Wash the coverslips three times for 5 minutes with 1x PBS containing 0.1% saponin.
30. To visualize nuclei, incubate coverslips with 1x PBS containing 1µg/mL Hoechst 33342 for 10 minutes at room temperature.
31. To visualize actin, incubate coverslips with 1x PBS containing Rhodamine Phalloidin Reagent (Abcam ab235138) to a final concentration of 1:1000.
32. Wash the coverslips two times with 1x PBS.
33. Mount the coverslips on to glass slides with ProLong Gold Antifade Mountant.
34. Cure the slides overnight.
35. Day 3. Seal the edges of the coverslips with clear nail polish.
36. Image on Zeiss LSM confocal microscope.
37. Some common antibodies and dyes and their excitation / emission wavelength parameters used for imaging intracellular *V. parahaemolyticus* are described below.
38. Phalloidin binds F-actin and can be used to visualize the effects of the bacterium on the host actin cytoskeleton. This dye can be used to stain the coverslip when incubating with the Hoechst stain. 10 minutes at room temperature is sufficient for staining.
39. Fluorescent phalloidin is commercially available and examples are listed below:

- a. Alexa Fluor™ 488 phalloidin (green, excitation: 495nm, emission: 518nm)
 - b. Rhodamine-phalloidin (red, excitation: 540nm, emission: 565nm)
 - c. Alexa Fluor™ 647 phalloidin (red, excitation: 650nm, emission: 668nm)
40. Hoechst 33342 is cell permeable and can be used to stain the nucleus of host cells as well as bacterial DNA. (Blue, excitation: 361nm, emission: 497nm)
41. Cellular compartments can be visualized by staining with primary antibodies following secondary antibodies conjugated to different fluorophores (e.g. Alexa Fluor 488 - green, Alexa Fluor 555 - red, and Alexa Fluor 680 – far red). Examples of common markers for cellular compartments are EEA-1 (early endosome), Lamp-1 (late endosome/lysosome), KDEL and Calnexin (ER), and COX-4 (mitochondria).
42. Examples of confocal micrographs of HeLa cells invaded by *V. parahaemolyticus* GFP expressing CAB2 strain are given in Fig 5. A, B, C and D denote HeLa cells containing intracellular bacteria at 1, 3, 5 and 7h post gentamicin treatment respectively during a gentamicin protection assay. HeLa cell actin was stained with rhodamine phalloidin (red) and DNA was stained with Hoechst (blue). At 1 h, post gentamicin treatment *V. parahaemolyticus* are typically restricted to endosomes, hence their appearance is in small puncta. At 3h post gentamicin treatment, the bacteria that escaped from the endosome can be visualized in the cytoplasm where they replicate. This process typically continues up to 5 to 6h post gentamicin treatment. At 7h post gentamicin treatment, most bacteria have egressed from the host cell into the extracellular medium where they are killed by extracellular gentamicin.

[*Place figure 5 near here.]

Basic Protocol 7: Cytotoxicity Assay for the T3SS2

Lactate dehydrogenase (LDH) is an abundant metabolic enzyme found in nearly all living cells. When host cells undergo lysis and the plasma membrane is damaged, this enzyme is released into the extracellular environment with other intracellular contents. The amount of LDH released from a host cell population correlates with the proportion of lysed cells and thus serves as an indirect assessment of cytotoxicity. When induced, the type III secretion system 2 (T3SS2) of *V. parahaemolyticus* and its effectors cause nearly 100% HeLa cell lysis by 5-6 hours. By contrast, when the T3SS1 system is induced cytolysis can be detected in as little as two hours. Thus, when assessing *V. parahaemolyticus* invasion, the T3SS1 system must be genetically inactivated so that the cytotoxicity of the T3SS1 does not mask the invasion of the T3SS2. This protocol can be modified to test other cell types and other bacterial virulence factors.

Materials:

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- Tissue culture biosafety hood
 - DMEM complete medium (see 'Reagents and Solution' for recipe)
 - DMEM High glucose without phenol red (Sigma Aldrich)
 - HeLa cells
 - CO₂ incubator for tissue culture (37 °C, 5% CO₂)
 - *Vibrio parahaemolyticus* glycerol stocks
 - Luria-Bertani (LB) broth, supplemented with 3% NaCl (MLB)
 - 5% Bile salts solution (Bile Bovine (Sigma Aldrich B3883) in deionized water, w/v and filter sterilized)
 - 30°C incubator
 - 37°C incubator
 - 24-well tissue culture plates
 - 96-well clear bottom plates
 - OD600 Spectrophotometer
 - Multichannel pipet
 - LDH Cytotoxicity Detection Kit (Takara Bio)
 - 10% Triton X-100 solution
1. Day 1. In the tissue culture biosafety hood, seed 7×10^4 HeLa cells in 1 mL complete DMEM for each well of the 24-well plate. For each sample at each time point, cells should be plated in triplicate. In addition to experimental strains, include a *Vibrio parahaemolyticus* strain with intact T3SS2 as positive control and a *Vibrio parahaemolyticus* strain with inactivated T3SS2 as negative control.
 2. Separate plates should be used for each time point and each plate should include 3 extra wells of HeLa cells (total lysis control).
 3. Inoculate 5 mL of MLB with *V. parahaemolyticus* strains from glycerol stocks. Add antibiotics if necessary. Incubate overnight at 30 °C and 200rpm.
 4. Day 2. Measure the OD₆₀₀ of the overnight cultures.
 5. Start fresh cultures in 5 mL MLB for each strain and from the overnight *Vibrio* cultures for an OD₆₀₀ of 0.3. Add antibiotics if necessary.
 6. To induce expression of the T3SS2, add 50 µL of 5% bile salt solution for a final concentration of 0.05%. Then incubate the cultures at 37 °C for 90 minutes at 200rpm.
 7. After the incubation, check the OD₆₀₀ of each culture. 1 mL of culture with an OD 600 of 1 is equivalent to 5×10^8 bacteria.
 8. Using the OD₆₀₀, calculate the volume of each bacterial sample needed for an approximate MOI of 10. For 1 mL of cells:

Volume of bacterial culture in µL per 1 mL of DMEM media = $(10 \text{OD}_{600} \text{ unit}) / (5 \times 10^8 \text{ bacteria/mL}) \times (10 \text{ bacteria/HeLa cell}) \times (1 \text{ mL} / \text{OD}_{600} \text{ measured}) \times 7 \times 10^4 \text{ HeLa cells plated per well} \times (10^3 \text{ µL/1mL})$

9. 1mL of media is required for each well of HeLa cells. Make the infection solution by adding the calculated volume of bacteria to pre-warmed DMEM without phenol red.
10. Wash HeLa cells twice with DMEM without phenol red to remove residual phenol red and antibiotics.
11. To each well of HeLa cells, add 1 mL/well of the bacteria infection solution. For the lysis control wells, add 1mL/well of the DMEM without phenol red (no bacteria). Add 1 mL/well of DMEM without phenol red to three empty wells as blanks.
12. Centrifuge the plate(s) at 1000 rpm (~200x g) for 5 minutes to synchronize the infection by forcing contact of the bacteria with the HeLa cells.
13. Incubate plate(s) in the CO₂ incubator at 37 °C with 5% CO₂.
14. 10 minutes prior to each time point, remove 500 µL from the total lysis control wells and add 500 µL of DMEM without phenol red + 2% Triton X-100.
15. At the end of each time point, remove 200 µL of media in triplicate from each well and transfer to a 96-well plate. Spin this plate down for 5 min at 1000 rpm to remove any debris.
16. Carefully take out 100 µL of supernatant and transfer to a new 96-well plate. Place the new plate at 4 °C.
17. LDH can be measured by kits from many manufacturers. Here, we use the LDH Cytotoxicity Detection Kit from Takara Bio.
18. For 50 reactions/wells, prepare the working solution by mixing 125 µL of the catalyst solution + 5.625 mL of the dye solution. Scale up as necessary.
19. To each well of the 96 well plate(s), add 100 µL of the working solution with a multichannel pipettor.
20. If desired, add 50 µL of 1N HCl to stop the reaction (final concentration = 0.2N HCl). If not, the reaction product can be measured without a stop solution.
21. Measure the absorbance at 490nm using a 96 well plate reader.
22. Determine the average absorbance of technical replicates and then calculate the percent of lysis by the equation below:

$$\frac{((\text{absorbance } x - \text{absorbance blank}) / (\text{absorbance total lysis} - \text{absorbance blank})) \times 100}{}$$

23. Results can be plotted as a percentage of cytotoxicity relative to the total lysis control.
24. Representative results of LDH assay are presented in Fig 5, from an infection of HeLa cells with *Vibrio parahaemolyticus* strain CAB2 (T3SS2 only). LDH levels were measured at 1.5, 3, 4.5, and 6 hours post infection with a MOI of 10. The percent cytotoxicity is calculated by comparing the LDH levels in the sample wells to the total lysis control. Cytotoxicity is used as a proxy for infection-mediated cell death, and is measured based levels LDH, a stable enzyme released into the media upon disruption of tissue culture cells' plasma

membranes. LDH reduces NAD^+ to NADH/H^+ in the process of converting lactic acid to pyruvate. Cell-free supernatant from the infection at each time point is mixed with a lactic acid reaction mixture provided by the LDH assay kit for a set period of time, and the resulting accumulated NADH/H^+ is then allowed to react with a colorimetric dye that turns red when reduced by NADH/H^+ . More cytotoxicity results in increased release of LDH into the media, which when applied to the kit produces more pyruvate and NADH/H^+ , in turn reacting with more of the dye to produce a stronger absorbance measurement in the red spectrum. Absorbance levels recorded experimentally are normalized to an LDH standard to generate the percent cytotoxicity shown on the Y-axis. The cytotoxicity level increases during the infection of HeLa cells with *Vibrio parahaemolyticus* CAB2 strain and reaches maximal levels between 5 and 6h of infection as depicted in Fig 6.

[*Place figure 6 near here.]

Reagents and Solutions:

1. MLB (LB broth with 3% NaCl (w/v)): Tryptone 10g, Yeast extract 5g, NaCl 30g / liter of Milli-Q deionized (Mq) H_2O . Adjust pH to 7.4 with NaOH and sterilize by autoclaving at 15 psi for 20 min.
2. Swimming agar plates: Tryptone 10g, NaCl 20g, Agar 3.25g / liter of Mq H_2O , pH 7.4. Autoclave at 15 psi for 20 min. Do not invert plates, store them upright.
3. Swarming agar plates: HI (Heart infusion broth) 25g, NaCl 20g, Agar 15g / liter of Mq H_2O , pH 7.4. Autoclave at 15 psi for 20 min. These plates can be inverted.
4. MMM agar: NaCl 20g, Agar 15g / 925 mL of Mq H_2O , Autoclave at 15 psi for 20 min and cool to 55°C . Add 50 mL sterile 20X Salts, 20 mL sterile 20% galactose, 5 mL sterile 1 M MgSO_4 . Mix well and pour plates.
5. 20X Salts: K_2SO_4 20g, K_2HPO_4 270g, KH_2PO_4 94g, NH_4Cl 22g / Liter of Mq H_2O . Autoclave at 15 psi for 20 min.
6. 2XIO (2X Instant Ocean): Instant Ocean (Instant Ocean Sea Salts for aquarium) 28g / Liter of Mq H_2O . Autoclave at 15 psi for 20 min.
7. SDS Sample Buffer: 250mM Tris pH6.8, 50% glycerol v/v, 5% SDS, 0.25% Bromophenol blue m/v, 1:1000 2-mercaptoethanol.
8. DMEM complete medium: High glucose DMEM with phenol red (Sigma Aldrich, D5796) Add the following supplements to the final concentrations as listed. Fetal bovine serum (FBS): 10% FBS, Sodium pyruvate (100mM, Sigma Aldrich S8636): 1mM, Penicillin/streptomycin/l-glutamine (Sigma Aldrich G1146): 1%.

9. DMEM infection medium: High glucose DMEM with phenol red (Sigma Aldrich, D5796) Add the following supplements to the final concentrations as listed. Fetal bovine serum (FBS): 10% FBS, Sodium pyruvate (100mM, Sigma Aldrich S8636): 1mM.
10. Un-supplemented DMEM: High glucose DMEM with phenol red (Sigma Aldrich, D5796), without any additives.
11. 5% Bile salts solution: Dissolve 0.5 g of Bile Bovine (Sigma Aldrich B3883) in 10 mL deionized water, and filter sterilize by passing through 0.22 μ m filters. This solution can be stored at room temperature and should be used within one month.

Commentary

Background information

V. parahaemolyticus was first isolated from a food poisoning outbreak in Japan in 1950 and was originally categorized as *Pasteurella parahaemolytica*, which was later re-classified into the genus *Vibrio* based on advances in taxonomy and after its halophilic nature was discovered in 1955 (Shinoda, 2011). Prior to the emergence of the pandemic strain O3:K6 serotype in 1996 from India, *V. parahaemolyticus* was generally confined to localized outbreaks and linked to various serotypes such as O2:K3, O3:K6 and O4:K8 (Okuda et al., 1997; Wong et al., 2000). The alphabets O and K refer to the somatic and capsular antigens of *V. parahaemolyticus* respectively. The pandemic O3:K6 strain quickly spread globally and lead to several outbreaks throughout the world. Recently 22 new serotypes (e.g. O4:K68, O1:K25, O1:KUT (untypable)) have been discovered with genotyping profiles identical to O3:K6 and referred to as serovariants of the pandemic strain O3:K6. Based on MLST (multilocus sequence typing), a recent study reported a total of 49 pandemic serotypes that grouped into 14 sequence types (Han et al., 2016).

Multiple laboratory strains have been developed to study the virulence factors of *V. parahaemolyticus*. The clinical isolate RIMD **2210633** was collected from a patient from Osaka, Japan in 1996 and is the parent strain of many such laboratory mutants, most notably the CAB and POR strains (Makino et al., 2003; Ono, Park, Ueta, Iida, & Honda, 2006). POR1 features a deletion of the two hemolysin-encoding genes *tdhA* and *tdhS* and is typically utilized as a wild type control in T3SS studies because it permits study of the cytotoxic effects of both T3SS without interference from the cytotoxic properties of the hemolysins. From this strain were derived additional POR strains, POR2, which lacks a functional T3SS1 due to deletion of export apparatus *vcrD1*, POR3, which lacks a functional T3SS2 due to deletion of export apparatus gene *vcrD2*, and POR4, which contains both mutations and no functional T3SS (De Nisco et al., 2017; K. S. Park et al., 2004). Also derived from POR1 were the CAB strains – CAB2, which lacks a functional T3SS1 due to a deletion of the

transcription regulatory gene *exsA*, CAB3, which lacks a functional T3SS2 due to a deletion of the transcription regulatory gene *vtrA*, and CAB4, which lacks any functional T3SS due to deletions in both aforementioned transcriptional regulatory genes (Calder et al., 2014; Zhang et al., 2012).

Critical parameters and trouble shooting

Genetics: If no or slow growth is observed in liquid cultures inoculated from freezer stocks, it is possible that too much bacteria has been used for inoculation in limited volume; it is recommended that only very small volume of glycerol stocks be used for inoculation for optimal growth. Moreover, if no colonies appear after transconjugation matings after 72 hours of incubation, adjusting the ratio of bacteria from 1:1 to 1:2 *Vibrio* to *E. coli* donor/helper strains may rectify the issue.

For natural transformations, issues associated with the ligation of fragments via PCR may be rectified by shortening the homologous amplicons from 3kb up and downstream of the target gene to 2kb, resulting in a shorter overall nucleotide strand that is easier to amplify (note however that shortening the regions of homology may impact the efficiency of recombination). Gibson assembly may also be used to increase the efficiency of ligating PCR amplicons to the antibiotic marker gene.

Proteomics: Whenever analyzing secretion profiles for mass spectrometry, it is critical that gloves be worn at all times and that all tubes be washed with 70% methanol to minimize collagen contamination, as this can significantly decrease the sensitivity of the assay. Use of a positive pressure hood for sample preparation is recommended when possible.

Cell biology: For visualization of *V. parahaemolyticus* via confocal microscopy, it is important to minimize exposure of slides to light even before staining if the bacteria express GFP, as the GFP signal is often weak in comparison to that of many phalloidin stains, and photobleaching will further compromise the resolution of images taken. If you use a fluorescent reporter to label the bacteria (e.g. GFP), be aware that using methanol for tissue fixation may result in loss of signal and may require an anti-GFP antibody for visualization. If there is difficulty seeing the bacteria through the microscope lens due to dim signal from the antibody staining/fluorescent reporter, it is possible to find them with the Hoechst stain with the blue channel (Hoechst will stain the bacterial DNA). Different antibody markers may necessitate testing different types of fixation and permeabilization methods for the best staining. A list of some more common problems encountered during these assays and possible remedies are presented in Table 2.

[*Place table 2 near here.]

Understanding Results:

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See the figures and text within each protocol for expected results and interpretation. Expected results for each assay are shown at the end of the respective protocol.

Time Considerations:

Bacterial growth: Bacterial cultures typically require 8 hours to reach stationary phase and are best prepared the night before they are needed. Inoculation generally takes at most 1-2 minutes, though additional time should be allocated based on the number of cultures needed. For transconjugation matings, colonies will typically appear within 2-3 days after plating on selection media, though up to 4 may be necessary depending on the gene being modified.

Phenotype assays: During gentamicin protection assay or invasion assay, particularly when extended time points are required e.g. 7h and later, It is recommended to perform the 7h time point of infection first followed by 5, 3 and 1h time points to complete the experiment in a timely manner. It is recommended that infections be conducted in reverse chronological order. Application of bacteria to HeLa cells during infection should be carried out in pairs to ensure that the centrifuge is balanced during centrifugation to synchronize infections. Also, one should allot 5 minutes for washing and infecting each pair of tissue culture plates in addition to 5 minutes required for centrifugation, culminating in 40-50 minutes of prep for a standard 1/3/5/7-hour gentamicin protection or invasion assay.

Acknowledgements:

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Figure Legends:

Fig 1. Growth curve for *V. parahaemolyticus* CAB2 strain in MLB medium (OD readings in triplicate).

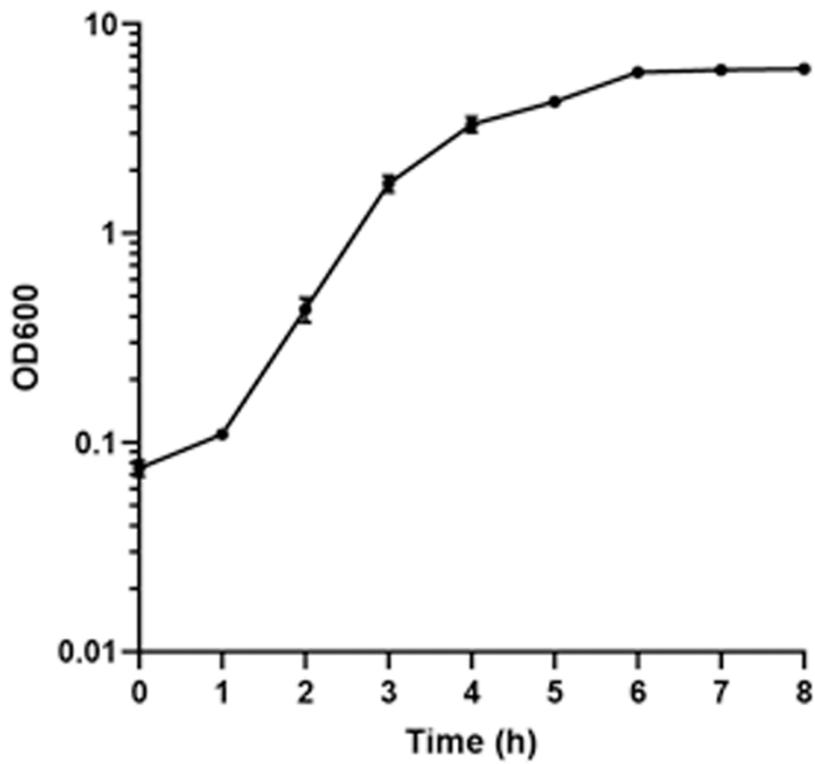


Fig 2. Examples of A) swimming and B) swarming patterns of *V. parahaemolyticus* CAB2 strain.

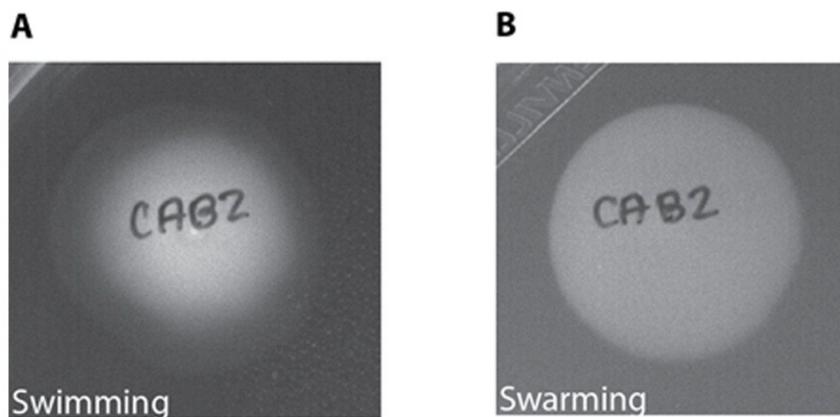


Fig 3. Schematic representation of upstream (-1kb) and downstream (+1kb) amplicons for the deletion of gene of interest.

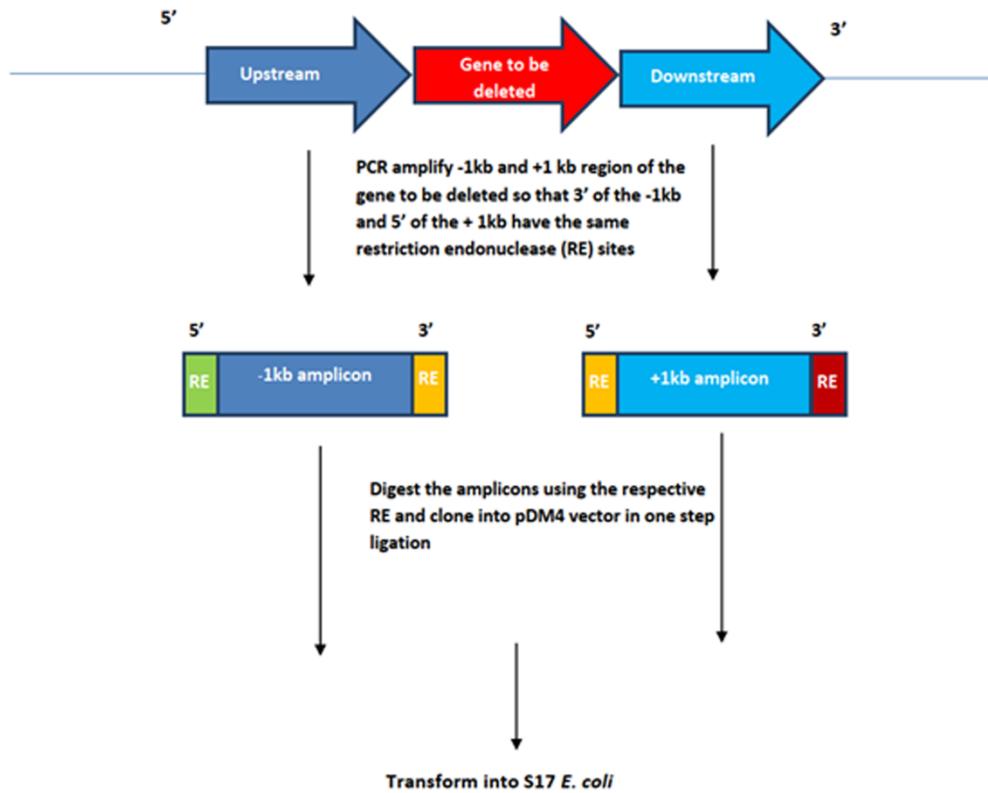


Fig 4. Invasion phenotype of *V. parahaemolyticus* CAB2 strain as assessed by gentamicin protection assay.

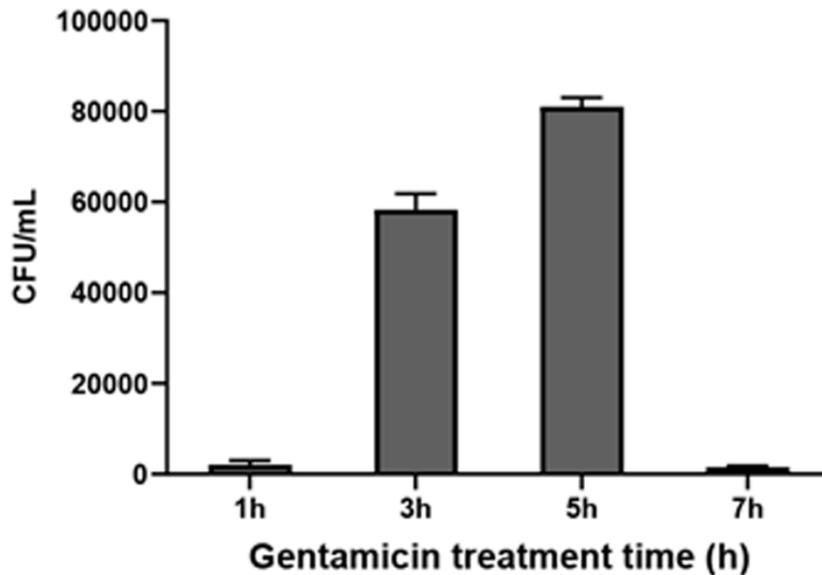
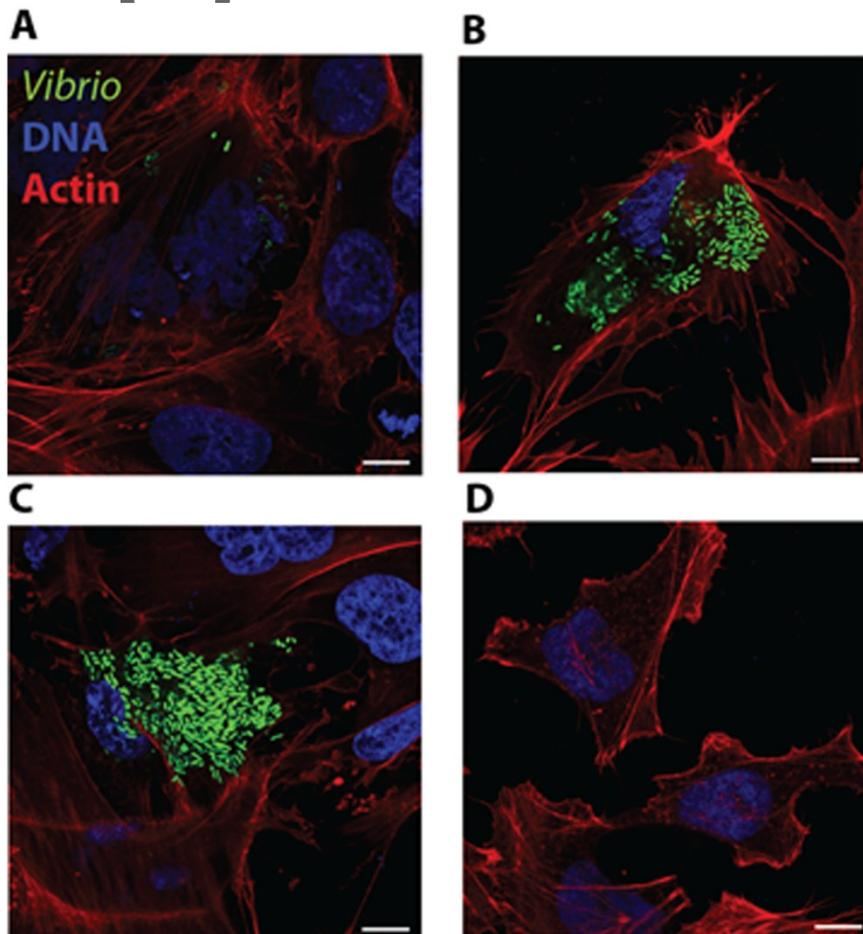


Fig 5. Confocal micrographs of HeLa cells invaded by *V. parahaemolyticus* GFP expressing CAB2 strain at A)1, B)3, C)5 and D)7h post gentamicin treatment during a gentamicin protection assay.

HeLa cell actin was stained with rhodamine phalloidin (red) and DNA was stained with Hoechst (blue). Scale bars = 10 μ m.



Autho

Fig 6. Representative LDH assay results from infection with *Vibrio parahaemolyticus* strain CAB2 (T3SS2 only). LDH levels were measured at 1.5, 3, 4.5, and 6 hours post infection with a MOI of 10. The percent cytotoxicity is calculated by comparing the LDH levels in the sample wells to the total lysis control.

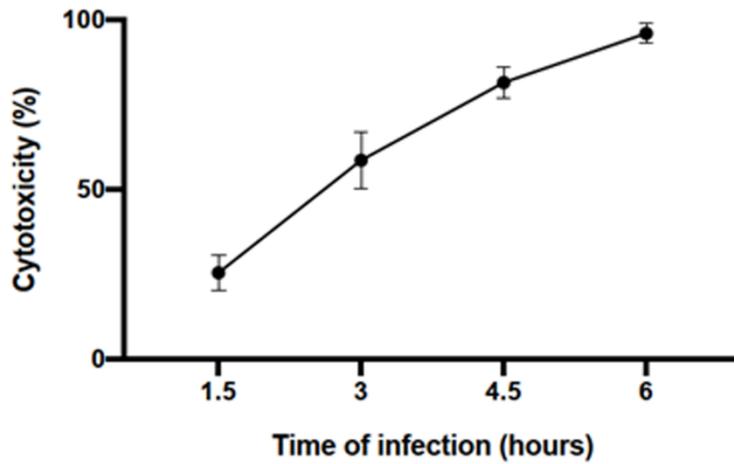


Table 1. Generally used antibiotics and their concentrations for *V. parahaemolyticus* strains^a

Antibiotic	Solvent	Stock concentration (mg/mL)	Final concentration (μ g/mL)
Spectinomycin	Deionized water	50	50
Chloramphenicol	100% Ethanol	25	25
Kanamycin	Deionized water	100	250
Gentamicin	Deionized water	50	100

^a All stock solutions should be sterilized by passing through 0.22 μ M filters and stored at -20°C as aliquots.

Table 2. List of commonly encountered problems and possible remedies

Problem	Possible reason	Possible remedy
Low or no growth in overnight <i>Vibrio</i> cultures and during regular growth cycle	High inoculum from the frozen glycerol stocks; Contaminated culture tubes; Low ratio of growth flask to culture	Inoculate very low volume from frozen glycerol stocks, make sure to use clean and sterile culture tubes; for growth curves, make sure that the ratio of culture / vessel volume is at least 1:5.

Contamination in motility agar plates	Moisture accumulation	Use freshly prepared plates, as the swimming plates cannot be stored inverted causing moisture-based contamination issues. Make sure to dry the plates at 37°C prior to inoculation.
No colonies with the intended genetic change (gene deletion or complementation) after genetic manipulation, only wild type colonies	Lack of homologous recombination	For streaking colonies on to MMM sucrose plates, use a very low initial inoculum from the MMM Cm plates. Giving enough time (24-48h) to obtain well, isolated colonies from low inoculum is crucial thus encouraging double crossover and homologous recombination providing the intended mutant colonies.
No colonies after gentamicin protection assay/ No intracellular bacteria in invasion assay / No cytotoxicity in LDH assay	T3SS2 not induced	Use freshly prepared bile salts solution and make sure the <i>Vibrio</i> cultures are grown at 37°C which is crucial for T3SS2 induction. It is also important to use fresh MMM plates (use within a month of preparation) as the salts precipitate in the agar after prolonged storage at 4°C.
Emulsion/ brown coloration in sample buffer after attempted resuspension of precipitated protein	Too much NaOH	Be mindful of 10M NaOH droplets that cling to the outside of the pipette tip before adding NaOH, as the base is highly concentrated and too much can prevent the protein precipitate from properly resuspending. Supplementation of 10M HCl in 0.2µL increments or increasing the volume of SDS sample buffer may help, but often it is better to retry the experiment. Diluting NaOH to 5M in MilliQ deionized water may help prevent this outcome as well.
Low hit count of proteins on mass-spectrometry analysis of	Collagen contamination, Not enough precipitated protein	Use clean PPE, rinse gloves and all tubes with 70% MeOH before use, and prepare samples in a positive pressure hood;

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