Vibrio fischeri: Laboratory Cultivation, Storage, and Common Phenotypic Assays

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Vibrio fischeri is a nonpathogenic organism related to pathogenic *Vibrio* species that can be readily grown and stored with common laboratory equipment. In this article, protocols for routine growth, storage, and phenotypic assessment of *V. fischeri*, as well as recipes for useful media, are included. Specifically, this article describes procedures and considerations for growth of this microbe in complex and minimal media. It also describes assays for biofilm formation, motility, and bioluminescence, three commonly assessed phenotypes of *V. fischeri*. © 2020 Wiley Periodicals LLC.

Keywords: bacterial cultivation • biofilm • luminescence • motility • symbiosis • vibrio • *Vibrio fischeri*

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INTRODUCTION

Vibrio fischeri (also classified as *Aliivibrio fischeri*) is a Gram-negative, comma-shaped, bioluminescent marine bacterium (Urbanczyk, Ast, Higgins, Carson, & Dunlap, 2007). The bacterial genome consists of two chromosomes with low GC content and often includes one large and/or one or more small plasmids (Boettcher & Ruby, 1994; Ruby et al., 2005). *V. fischeri* was originally studied for its bioluminescent properties and now is studied most intensively with respect to its symbiosis with the Hawaiian bobtail squid, *Euprymna scolopes*. *V. fischeri* is also capable of colonizing other squid species as well as fish species such as *Monocentris japonica* (Nishiguchi, 2001; Ruby & Nealson, 1976). In the squid symbioses, the bacteria produce bioluminescence, which protects their host from predation, while the host provides nutrients and a protected niche for the bacteria (Graf & Ruby, 1998; Jones & Nishiguchi, 2004).

Much work has been done to identify key factors that promote the interactions between *V. fischeri* and *E. scolopes*, from the perspectives of both the bacteria and their host. The

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squid hatch in an aposymbiotic state (i.e., lacking their symbiont) and acquire *V. fischeri* from the surrounding seawater (Nyholm, Stabb, Ruby, & McFall-Ngai, 2000; Stabb & Visick, 2013). Bacteria-containing seawater is flushed through the squid's mantle cavity and across the surface of the symbiotic light organ, where ciliary movements direct bacteria toward sheltered zones (McFall-Ngai & Ruby, 1991; Nawroth et al., 2017). There, *V. fischeri* cells form a bacterial aggregate or biofilm on the organ surface from which they subsequently disperse (Nyholm et al., 2000). Using motility and chemotaxis, the bacteria enter into pores and migrate through nonpermissive spaces to reach deep crypt spaces where they multiply and produce light (Aschtgen et al., 2019; Graf, Dunlap, & Ruby, 1994). Once symbiosis is established, the bacteria are maintained through a daily cyclic expulsion of ∼95% of the bacteria from the light organ into the surrounding seawater and a regrowth of the remaining ∼5% population (Lee & Ruby, 1994). In these different stages, the nutritional availability and therefore growth of *V. fischeri* is thought to shift (Wier et al., 2010). It is hypothesized that, during the day, the *V. fischeri* symbiont uses glycerol-3-phosphate from host-derived lipids, while at night and prior to expulsion host-derived chitin is the primary nutrient.

V. fischeri is a nonpathogenic, biosafety level 1 (BSL-1) organism that is related to pathogenic *Vibrio* species like *Vibrio cholerae*. This low safety level and a continually expanding genetic toolbox make *V. fischeri* an excellent bacterium to use at any level of research. *V. fischeri* serves as an important model organism to study a variety of processes including: requirements for symbiotic colonization, evolutionary adaptations between bacterium and host, quorum sensing, bioluminescence, host-associated biofilm formation, and motility.

STRATEGIC PLANNING

Strain Considerations

V. fischeri strains have been isolated from a variety of environmental locations. Historically, strain ES114 (Boettcher & Ruby, 1990), which was isolated from the *E. scolopes* light organ, has been used as an experimental wild-type strain for symbiosis experiments. Thus, most of the laboratory techniques for growth and genetic and phenotypic characterizations were developed and/or optimized for this strain.

More recently, a number of additional symbiotic and environmental strains have been isolated and are beginning to be characterized (e.g., MB13B2 and FQ-A001; Bongrand & Ruby, 2019; Koehler et al., 2018; Sun et al., 2016). These strains exhibit substantial genotypic and phenotypic differences from ES114. For example, MB13B2 (Bongrand et al., 2016) and FQ-A001 (Bultman, Cecere, Miyashiro, Septer, & Mandel, 2019) contain additional sequences (250 kb and 54 kb, respectively) not present in ES114. In experiments designed to test superior symbiotic competence, MB13B2 or FQ-A001 outcompeted ES114, resulting in majority or exclusive colonization of the squid. As a result, they have been termed "dominant" strains. However, other recent isolates termed "sharing" will coexist with ES114 within a light organ (Bongrand et al., 2016), a result that indicates that ES114 remains intact as a wild-type strain and has not necessarily become "lab adapted."

Different isolates also vary in several other phenotypes. For example, dominant strain MB13B2 is vastly different relative to ES114 in its ability to form biofilms in symbiosis and in laboratory culture (Koehler et al., 2018). While ES114 can successfully form small aggregates on the squid light organ, it forms biofilms poorly in laboratory culture. Due to an incomplete understanding of signals present in the squid that promote these phenotypes, genetic manipulations are generally required to induce biofilm formation by ES114. In contrast, MB13B2 readily produces robust biofilm pellicles in static liquid

cultures in the test tube and both produces larger cellular aggregates on the light organ and migrates to the crypts earlier (Bongrand & Ruby, 2019; Koehler et al., 2018). Dominant strain FQ-A001 displays reduced migration in motility assays and increased bioluminescence relative to ES114 (Sun et al., 2016). This strain is also capable of attacking and killing ES114 via a type VI secretory apparatus that ES114 lacks (Speare et al., 2018).

Given the significant genotypic and phenotypic variation of these dominant and sharing strains, comparative analyses have tremendous power to address questions of the evolutionary advantages and trade-offs between bacteria with different colonization strategies. The specific study goal(s) will necessarily dictate the choice of strain(s) and the corresponding expectations for the outcome. One additional consideration for the choice of strain lies in the ease with which a given strain can be genetically manipulated. Our experience to date with MB13B2 suggests that this organism may prove challenging to manipulate. Thus, certain questions may require the genetic manipulability of a strain like ES114 to facilitate dissection of complex phenotypes.

Since much of the research investigating *V. fischeri* growth and behavior has been based on ES114, the experimental procedures described herein were developed and optimized using this isolate. However, it is likely that some *V. fischeri* isolates will exhibit different behaviors under the growth conditions described here, and thus these protocols may need alterations or may be wholly ineffective. For these strains, these protocols should serve as a first step but may require significant alterations to achieve the best results.

Growth Conditions

As *V. fischeri* is a bacterium found in the ocean, its optimum growth in the laboratory reflects those conditions. Optimal growth for *V. fischeri* occurs at 24°C to 28°C in a high-salt (∼20 g/L) environment. High temperatures (above 34°C for some strains) prove lethal for the bacterium (Cohen, Mashanova, Rosen, & Soto, 2019). Similarly, *V. fischeri* will readily lyse in fresh water. Traditionally, growth media for this organism have included glycerol, and many researchers continue to include this carbon source, even in complex media that do not require its addition for *V. fischeri* growth. However, *V. fischeri* readily metabolizes glycerol, resulting in acidification of the growth medium, which causes bacterial death. While buffering the medium can reduce this effect, medium acidification is a prime reason why overnight cultures of *V. fischeri* fail to regrow, or regrow poorly, following subculturing. Furthermore, recovery of a *V. fischeri* culture after overnight growth is reduced proportional to the duration of stationary phase. A shorter overnight incubation time (e.g., ∼14 hr) is optimal for the recovery of bacteria from frozen stocks and for use in experimental cultures.

Genetic manipulation of *V. fischeri* typically depends on antibiotics to select for strains with insertions or deletions of genetic material and to obtain and maintain plasmids of interest. Table 1 lists the antibiotic concentrations necessary to achieve selection for the common laboratory strain ES114. While this strain grows optimally on a high-salt medium such as LB salt (LBS), which contains 20 g/L NaCl, certain antibiotics do not work well under these high-salt conditions. Therefore, for some antibiotics, LB medium made with 10 g/L NaCl is used instead of LBS; the growth of ES114 is not substantially impaired under these lower-salt conditions. Alternatively, higher concentrations of antibiotics are required for selection in media like seawater tryptone (SWT) or Tris minimal medium (TMM) compared with LBS. Other *V. fischeri* strains may exhibit altered antibiotic resistance profiles and should be individually assessed to determine the optimal concentrations of antibiotics.

Table 1 Antibiotic Concentrations and Medium for Selection of *Vibrio fischeri*

Antibiotic[a,b]	Solvent	Stock concentration (mg/ml)	Working concentration (μg/ml)	Medium[c]
Chloramphenicol	100% ethanol	1	1	LBS
			5	TMM or high copy number plasmid in LBS
Erythromycin	100% ethanol	2.5	2.5	LBS
Gentamicin	distilled deionized water	10	10	LB
Kanamycin	distilled deionized water	20	100	LBS or TMM
Spectinomycin	distilled deionized water	40	200	LB
Tetracycline	100% ethanol	5	2.5	LBS
			30	SWT or TMM
Trimethoprim	90% ethanol	2	10	LBS

[a] *V. fischeri* is highly resistant to ampicillin.
[b] Zeocin has been used at a working concentration of 10 μg/ml in LB, but further optimization is necessary to enhance selectivity (Visick, Hodge-Hanson, Tischler, Bennett, & Mastrodomenico, 2018).
[c] Optimal growth medium for different antibiotics was determined empirically.

BASIC PROTOCOL 1

GROWTH OF *V. fischeri* FROM FROZEN STOCKS

V. fischeri can be recovered from a frozen stock in a glycerol solution (see Basic Protocol 3) at −80°C and remains viable on petri plates for about 1 week. The bacteria can be streaked onto LBS plates, and single colonies can then be used to inoculate liquid cultures. See Figure 1 for an example of a streak plate and expected colony appearance.

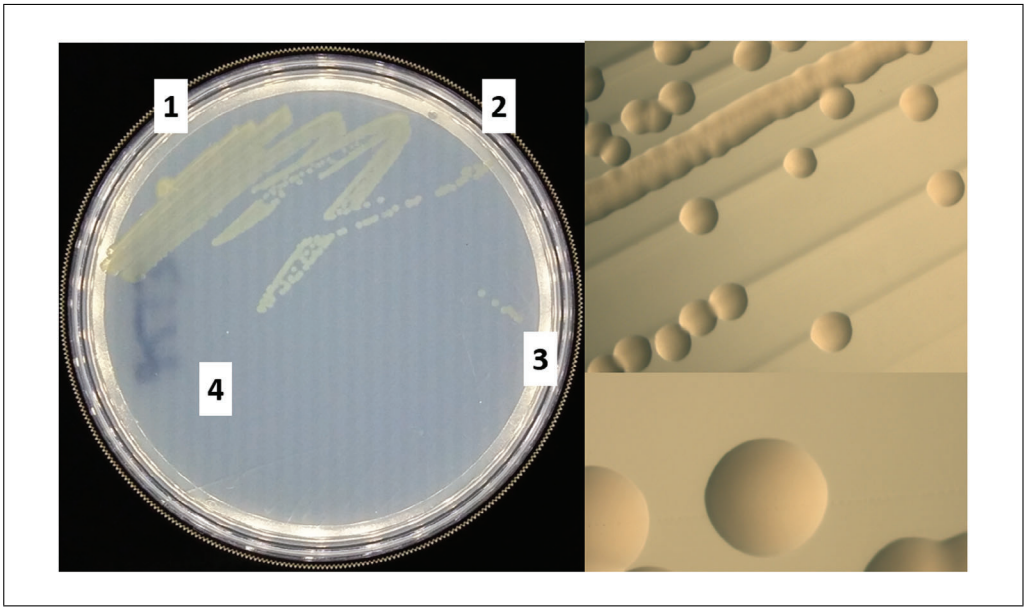

Figure 1 Wild-type *Vibrio fischeri* strain ES114 streak plate. Left: cells were streaked from a frozen culture with four independent streaks and incubated at 28°C for 20 hr. Top right: magnified image of single colonies from a streak. Bottom right: further magnified image of a single *V. fischeri* colony.

Materials

V. fischeri frozen glycerol stock in cryovial (see Basic Protocol 3)
LBS plate (see recipe)

Applicator stick, sterile
28°C incubator

1. Use a sterile applicator stick to scrape a small amount of *V. fischeri* cells from the frozen stock in the cryovial. Transfer bacteria and make a heavy streak on a small area of the surface of an LBS plate.
2. With a second sterile applicator stick, make a second streak by gently passing it through the heavy streak once. Repeat twice more with additional sterile sticks to generate four quadrants of decreasing bacterial concentration with the goal of isolating single colonies.
3. Invert plate and incubate upside down in a 28°C incubator overnight.

   Other temperatures, such as 24°C or room temperature, will also permit growth.

4. Store plate at 4°C for up to 1 week.

GROWTH OF *V. fischeri* IN COMPLEX LIQUID MEDIUM

BASIC PROTOCOL 2

V. fischeri is routinely grown in complex media like LBS or SWT (a seawater-based medium). Depending on the assay, the choice of medium may result in significantly different phenotypic outcomes (Marsden, Grudzinski, Ondrey, DeLoney-Marino, & Visick, 2017).

Materials

V. fischeri on LBS agar plate (see Basic Protocol 1) or from frozen stock (see Basic Protocol 3)
LBS or SWT medium (see recipe)

Applicator stick, sterile
Glass culture tube with cap, sterile (routinely 18 × 150–mm)
28°C shaking incubator

1a. *From agar plates:* Pick a single colony of *V. fischeri* from an LBS streak plate using a sterile applicator stick.

1b. *From frozen stocks:* Scrape some *V. fischeri* cells from the frozen culture stock with an applicator stick.

2. Inoculate a liquid culture by suspending cells from the stick in 5 ml medium in a sterile 18 × 150–mm glass culture tube. Remove stick and cap test tube.

   V. fischeri grows better with good aeration. Therefore, smaller tubes can be used with a proportionally smaller volume.

3. Grow culture overnight at 28°C with aeration by shaking at 220 rpm.

GROWTH OF *V. fischeri* IN MINIMAL MEDIUM

ALTERNATE PROTOCOL 1

Nutritional requirements or auxotrophies of wild-type and mutant strains of *V. fischeri* can be assessed by growth in a defined minimal medium. Similarly, minimal medium allows one to control the exact composition of metals, nitrogen sources, and carbon sources provided to the culture.

Materials

V. fischeri on LBS agar plate (see Basic Protocol 1) or from frozen stock (see Basic Protocol 3)
Minimal medium (TMM or HEPES minimal medium [HMM]; see recipe)

Applicator stick, sterile
Glass culture tube with cap, sterile (routinely 18 × 150–mm)
28°C shaking incubator

1a. *From agar plates:* Pick a single colony of *V. fischeri* from an LBS streak plate using a sterile applicator stick.

1b. *From frozen stocks:* Scrape some *V. fischeri* cells from the frozen culture stock with an applicator stick.

2. Inoculate a liquid culture by suspending cells from the stick in 5 ml TMM (or HMM) in a sterile 18 × 150–mm glass culture tube. Remove stick and cap test tube.

3. Grow culture overnight at 28°C with aeration by shaking at 220 rpm.

The cells may not recover well from the frozen stock. Addition of casamino acids to 0.1% can help cells to recover.

BASIC PROTOCOL 3

STORAGE OF *V. fischeri* IN FROZEN STOCKS

For long-term storage of *V. fischeri*, the bacteria can be stored in a 20% glycerol stock at −80°C and subsequently recovered using Basic Protocol 1.

Materials

V. fischeri on LBS agar plate (see Basic Protocol 1)
LBS medium (see recipe)
Glycerol
LBS plate (see recipe)

Applicator stick, sterile
18 × 150–mm glass culture tube with cap, sterile
28°C shaking incubator
Plastic or glass cryovial, sterile

1. Using a sterile applicator stick, pick a single *V. fischeri* colony from an LBS agar plate.

2. Inoculate an 18 × 150–mm glass culture tube containing 5 ml LBS by suspending cells from the applicator stick in the medium. Remove applicator stick, and cap test tube.

3. Aerate inoculated tube at 28°C at 220 rpm overnight.

To maintain the health of the culture, incubate for only 14 to 18 hr.

4. The following day, harvest 1 ml overnight culture, and add to a sterile cryovial containing 335 µl of 80% (v/v) glycerol. Pipette up and down or invert tube a few times to ensure complete mixing of glycerol and culture.

5. Store cryovial containing the strain stock at −80°C.

6. Check viability of the frozen stock the next day by streaking onto an LBS agar plate and incubating the streak at 28°C overnight.

This is particularly important if LBS medium containing glycerol was used for growth of the culture.

BIOFILM ASSAY ON SOLID AGAR

Biofilm formation by *V. fischeri* is a critical step in establishing symbiosis with its host, and a correlation has been drawn between host colonization and biofilm formation under laboratory conditions (Shibata, Yip, Quirke, Ondrey, & Visick, 2012; Yip, Geszvain, DeLoney-Marino, & Visick, 2006). Wild-type *V. fischeri* strain ES114 does not form robust biofilms under these common laboratory conditions. However, certain genetically altered ES114 derivatives as well as some other squid isolates are biofilm competent and exhibit colony morphologies such as colony cohesion, adherence to the plate, and/or wrinkles or corrugation on solid agar plates (Fig. 2). For further reading on this technique, see Ray, Morris, & Visick (2012).

Materials

V. fischeri on LBS agar plate (see Basic Protocol 1)
LBS medium (see recipe)
LBS agar (or LBS agar containing $CaCl_2$) plate (see recipe)

Applicator stick, sterile
18 × 150–mm glass culture tube with cap, sterile
24°C and 28°C incubator with shaking capabilities
Spectrophotometer
Dissecting (stereoscopic) microscope
Toothpick

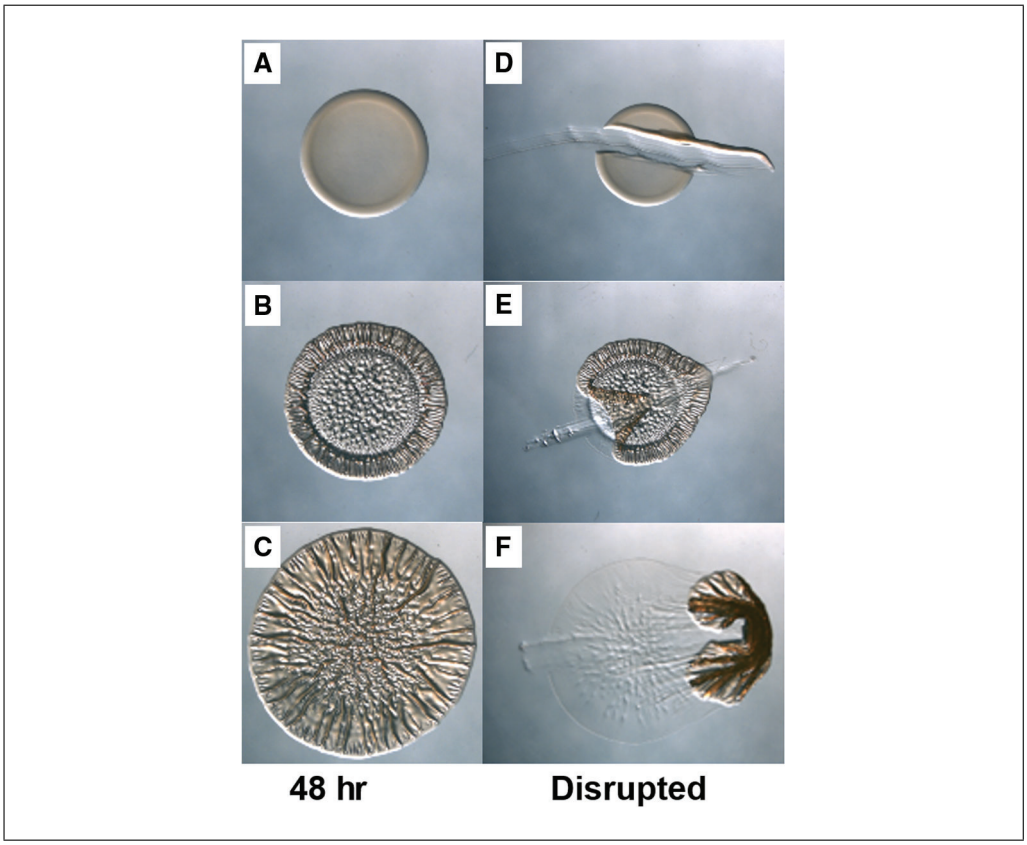

Figure 2 Examples of different biofilm phenotypes on solid medium. Images were taken after growth for 48 hr and before (left) and after (right) toothpick disruption. (**A**) Smooth colony. (**B** and **C**) Wrinkled colonies. (**D**) No biofilm, as evidenced by lack of adherence and coherence. (**E**) Colony adherent to plate. (**F**) Coherent colony. Images A, B, and C are magnified relative to images D, E, and F.

1. Using a sterile applicator stick, pick a single *V. fischeri* colony from an LBS agar plate.
2. Inoculate an 18 × 150–mm glass culture tube containing 5 ml LBS by suspending cells from the applicator stick in the medium. Remove applicator stick, and cap test tube.
3. Aerate inoculated tube at 28°C at 220 rpm overnight.

   Try not to exceed 16 hr to maintain the health of the culture.
4. Subculture overnight culture by pipetting 50 μl from the overnight culture into an 18 × 150–mm glass culture tube containing 5 ml LBS.

   This results in a 1:100 dilution of the overnight culture.
5. Aerate cultures until they reach log phase. Determine OD_{600} using a spectrophotometer, and normalize cultures to an OD_{600} of 0.2 using LBS.

   For example, if a culture reaches an OD_{600} of 0.3, add 67 μl culture to 33 μl LBS. If another culture reaches an OD_{600} of 0.4, add 50 μl culture to 50 μl LBS. When this is done, mix the cell suspensions by vortexing.

   V. fischeri grows rapidly and should only take about 1 to 2 hr to achieve the desired OD_{600}. However, time to achieve log phase should be independently determined for each strain via growth curve analysis.
6. Divide an LBS plate into four to eight sections. Evenly spot 10 μl normalized culture onto the LBS agar plate.

   For consistent results within an experiment that requires more than one plate and between experiments, the agar plates should be made with a standard volume, such as 25 ml.

   LBS medium can be supplemented with a nutrient or compound of interest (such as 10 mM $CaCl_2$, which is known to promote V. fischeri biofilm formation) prior to autoclaving or prior to dispensing the autoclaved and cooled agar into petri dishes, or it can be substituted with a distinct medium of choice.

   Samples can be spotted in duplicate or other multiples on the same plate, both to evaluate reproducibility and to permit multiple "endpoint" analyses as described in step 8.
7. Leave plate upright until the spot soaks in, and then invert plates and incubate at 24°C. Observe spots at various times after spotting using a dissecting microscope.

   A biofilm-competent strain may produce spots with visible wrinkles that continue to develop over time.

   Some strains will form biofilms at higher temperatures, such as 28°C, so this condition can be modified as needed.

   The timing of biofilm formation varies depending on the choice of strain, genetic manipulation, and medium or growth conditions. If the timing is unknown, a reasonable time course would be to evaluate colony morphology every 24 hr for 3 to 5 days.
8. After the last time point, disrupt colony with a toothpick by gently dragging it across the agar surface and through the colony from one side to the other.

   This "toothpick assay" can sometimes reveal colony cohesiveness (cells in the colony stick together) or adhesiveness (cells in the colony stick to the plate) that cannot be observed by eye or by using the dissecting microscope. Alternatively, in some cases architecture is observed without a corresponding "stickiness" (cohesiveness or adhesiveness). The "toothpick assay" thus permits an assessment of colony stickiness, permitting a determination of whether or not the colony has formed a biofilm (is cohesive or adhesive).

   The "toothpick assay" can be used at multiple time points if replicate samples were spotted.

BIOFILM ASSAY IN SHAKING LIQUID CULTURE

In addition to forming biofilms on solid agar, *V. fischeri* can produce biofilms in a shaking liquid culture in a test tube (Tischler, Lie, Thompson, & Visick, 2018). While the wild-type *V. fischeri* strain ES114 does not form robust biofilms, genetically altered, biofilm-competent strains form white, opaque rings at the air–liquid interface of a shaking culture and/or clumps of cells at the bottom of the test tube, which may be cohesive (Fig. 3). $CaCl_2$ (10 mM final concentration) is commonly used as an inducing signal for certain ES114 mutants (e.g., Δ*binK*; Tischler et al., 2018).

Materials

V. fischeri on LBS agar plate (see Basic Protocol 1)
LBS medium (see recipe)
1 M $CaCl_2$

Applicator stick, sterile
18 × 150–mm and 13 × 100–mm glass culture tube with cap, sterile
24°C and 28°C shaking incubator
Spectrophotometer

1. Using a sterile applicator stick, pick a single *V. fischeri* colony from an LBS agar plate.
2. Inoculate an 18 × 150–mm glass culture tube containing 5 ml LBS by suspending cells from the applicator stick in the medium. Remove applicator stick, and cap test tube.
3. Aerate inoculated tube at 28°C at 220 rpm overnight.
4. The following day, prepare a volume of LBS supplemented with 10 mM $CaCl_2$ sufficient for the needs of the experiment. For example, add 1 ml of 1 M $CaCl_2$ to 100 ml LBS.

   Making this medium fresh helps to prevent precipitation of calcium that can be observed during extended storage of the medium.

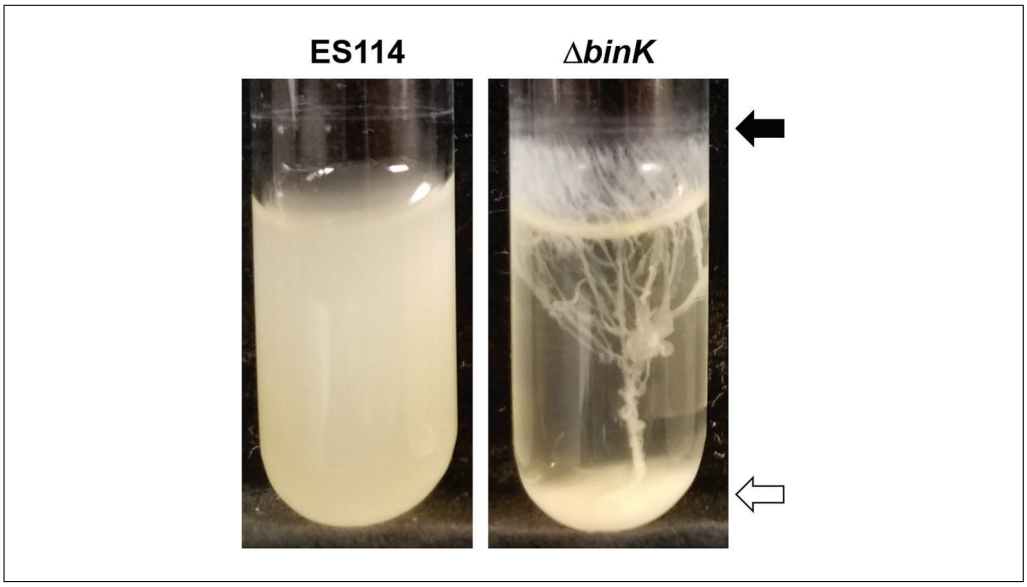

Figure 3 Biofilm formation during growth with shaking in liquid medium. Following growth with shaking in LBS medium supplemented with 10 mM $CaCl_2$, wild-type strain ES114 (left) presents a very faint ring and no clump, while the Δ*binK* mutant (right) exhibits clumps (white arrow) and rings (black arrow) connected by "trees." These cultures were imaged after 24 hr of shaking at 24°C.

5. Pipette 2 ml LBS supplemented with 10 mM $CaCl_2$ into a 13 × 100–mm glass culture tube with cap.
6. Determine OD_{600} of the overnight culture using a spectrophotometer, and pipette an appropriate volume of overnight culture to achieve a final OD_{600} of 0.05 in the 2 ml LBS supplemented with 10 mM $CaCl_2$.

   For example, if an overnight culture reaches an OD_{600} of 5, add 20 µl of that culture into a tube containing 2 ml LBS. If another culture reaches an OD_{600} of 6, add 16.7 µl of that culture into another tube containing 2 ml LBS.

7. Aerate tube with shaking at 220 rpm at 24°C for 24 hr.

   Timing can be important for observing this phenotype. Earlier time points may reveal deficiencies for certain mutants that may be missed at later time points.

8. Visually assess and/or photo-document the biofilms formed.

   The optical density of the planktonic cells in the culture medium can be measured with a spectrophotometer, if desired, for a quantitative measure of biofilm formation; the lower the optical density of the planktonic cells, the greater the biofilm formation.

   If measuring optical density, a no biofilm control should be included for comparison.

   If a biofilm has formed, do not disturb the biofilm when taking optical density readings of the culture medium.

ALTERNATE PROTOCOL 3

BIOFILM ASSAY IN STATIC LIQUID CULTURE

A third method to assess biofilms formed by *V. fischeri* is by observing biofilms formed during static liquid culture (i.e., pellicles; Fig. 4). Wild-type *V. fischeri* strain ES114 does not form a robust pellicle, but genetically altered biofilm-competent ES114 or strains that are naturally biofilm competent will form a film at the air–liquid interface of a bacterial culture that may develop wrinkles. $CaCl_2$ is commonly required to induce robust pellicles for certain ES114 mutants (e.g., Δ*binK*; Tischler et al., 2018), though it may not always be required.

Materials

V. fischeri on LBS agar plate (see Basic Protocol 1)
LBS medium (see recipe)
1 M $CaCl_2$

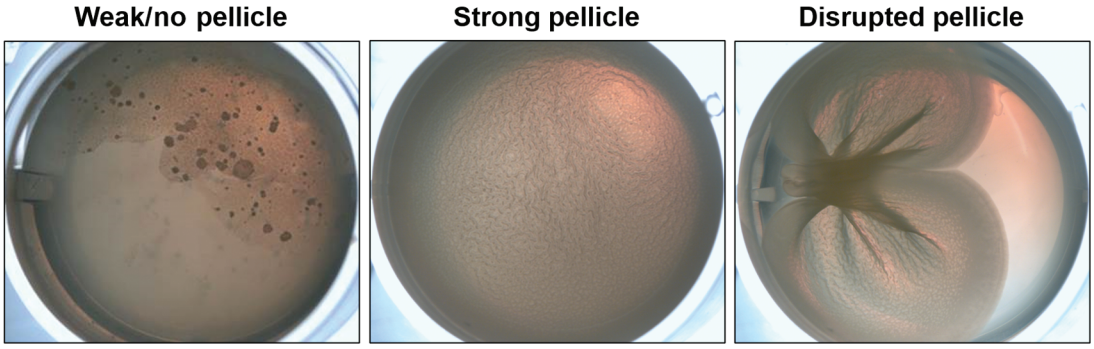

Figure 4 Biofilm formation during growth in static culture. Pellicles of *Vibrio fischeri* in static cultures grown at 24°C. Left: image of a poor pellicle-producing strain with a brittle pellicle and dark microcolonies following disruption with a toothpick. Middle: image of a strain with a pellicle with visible architecture (wrinkles). Right: image of the pellicle in the middle panel following disruption by a toothpick to assist visualization. This "toothpick test" permits an assessment of pellicle robustness/strength that is not possible to determine by visual examination alone.

Applicator stick, sterile
18 × 150–mm glass culture tube with cap, sterile
24°C and 28°C incubator with shaking capabilities
24-well microwell plate
Spectrophotometer
Resealable plastic bag
Dissecting (stereoscopic) microscope
Toothpick

1. Using a sterile applicator stick, pick a single *V. fischeri* colony from an LBS agar plate.
2. Inoculate an 18 × 150–mm glass culture tube containing 5 ml LBS by suspending cells from the applicator stick in the medium. Remove applicator stick, and cap test tube.
3. Aerate inoculated tube at 28°C by shaking at 220 rpm overnight.
4. If using LBS supplemented with 10 mM $CaCl_2$, add a volume of sterile 1 M $CaCl_2$ to LBS to achieve a final concentration of 10 mM $CaCl_2$.

   Making this medium fresh helps to prevent precipitation of calcium that can be observed during extended storage of the medium.

5. Pipette 2 ml LBS or LBS supplemented with 10 mM $CaCl_2$ into a well of the 24-well plate.

   To avoid differences in aeration between the outer wells and inner wells, only the inner eight wells should be used.

6. Determine OD_{600} of the overnight culture using a spectrophotometer, and pipette an appropriate volume of overnight culture into the well to achieve a final OD_{600} of 0.2.
7. Place plate in a resealable plastic bag. Incubate 24-well plate at 24°C, and observe pellicles under a dissecting microscope at intervals, such as at 24 and 48 hr.

   The plate can be incubated for shorter or longer times, although longer periods may result in evaporation of the medium.

   Care should be taken not to disturb the pellicles when observing pellicle growth at the 24-hour time point.

8. After the last time point, drag a toothpick or plastic pipette tip across the pellicle from one side to the other to disrupt the pellicle.

   Disruption of the pellicle helps to visualize the biofilm atop the turbidity of the culture and can impart information about the stickiness, cohesion, and robustness of the biofilm.

MOTILITY ASSAY

BASIC PROTOCOL 5

Migration of *V. fischeri* must occur for the bacteria to travel into the light organ of the squid (Graf et al., 1994; Nyholm et al., 2000). To study the migration of *V. fischeri* in the laboratory, low-percentage agar plates are used to observe flagella-based motility of the bacteria on a macroscopic scale (Fig. 5).

Materials

V. fischeri on LBS agar plate (see Basic Protocol 1)
LBS medium (see recipe)
Tryptone broth salt (TBS) medium (see recipe)
Low-percentage (0.25%) TBS containing magnesium (TBS-Mg) or tryptone broth seawater (TBSW) agar plate (see recipe)

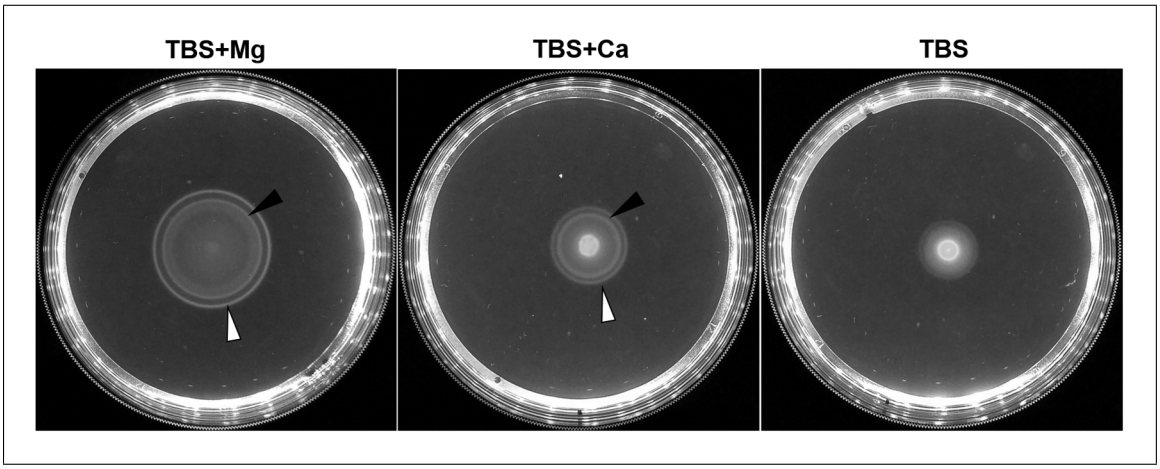

Figure 5 Motility in semisolid agar medium. Migration of *Vibrio fischeri* across TBS soft agar plates supplemented with 35 mM $MgSO_4$ (left) or 10 mM $CaCl_2$ (middle) or without supplementation (right). The inner ring (black arrow) and outer ring (white arrow) can be readily seen for magnesium and calcium supplementation and represent cells migrating to serine and thymidine, respectively. The rings take longer to form when cells are inoculated onto plates containing TBS without supplementation (right).

Applicator stick, sterile
18 × 150–mm glass culture tube with cap, sterile
24°C and 28°C incubator with shaking capabilities
Spectrophotometer
Ruler

1. Using a sterile applicator stick, pick a single *V. fischeri* colony from an LBS agar plate.
2. Inoculate an 18 × 150–mm glass culture tube containing 5 ml LBS by suspending cells from the applicator stick in the medium. Remove applicator stick, and cap test tube.
3. Aerate inoculated tube at 28°C by shaking at 220 rpm overnight.
4. Subculture 50 μl overnight culture in an 18 × 150–mm glass culture tube containing 5 ml TBS (for a 1:100 dilution of the overnight culture).
5. Aerate culture by shaking at 220 rpm at 28°C until cells have reached log phase.
6. Determine OD_{600} of the overnight culture using a spectrophotometer, and normalize culture to an OD_{600} of 0.2 using TBS as diluent.
7. Spot 10 μl normalized culture onto either a low-percentage TBS-Mg or TBSW plate.

   The plate can be divided into three or four parts to spot three or four strains on a single plate.

   A control strain should be included on each plate to take into account plate-to-plate variation.

8. Incubate plate right side up at 24°C or 28°C.

   Be careful and gentle with the low-percentage plates as the agar gel can break easily. They should NOT be inverted during incubation.

9. At a fixed time point or hourly, measure the zone of migration using a ruler and/or image plates with a camera.

   ES114 migrates rapidly through TBS-Mg (or TBSW) motility agar, visibly moving within 1 or 2 hr and reaching the edges of the petri plate within 8 or 9 hr. Two major

chemotaxis rings are formed that are comprised of cells sensing thymidine (outer ring) or serine (inner ring; DeLoney-Marino, Wolfe, & Visick, 2003).

LUMINESCENCE ASSAY

BASIC PROTOCOL 6

In the symbiosis between *V. fischeri* and its squid host, the squid uses the bacterially produced light to avoid detection by predators (Jones & Nishiguchi, 2004). As a result, bacteria that cannot produce light appear to be "sanctioned" by the host, as they fail to form long-term associations with the host (Bose, Rosenberg, & Stabb, 2008; Koch, Miyashiro, McFall-Ngai, & Ruby, 2014; Visick, Foster, Doino, McFall-Ngai, & Ruby, 2000). In culture, wild-type *V. fischeri* ES114 does not produce visibly detectable levels of luminescence, but within the squid the bacteria induce the production of light that is about 1000-fold higher than in laboratory culture (Boettcher & Ruby, 1990; Bose et al., 2007). This phenomenon suggests a lack of understanding of signals and conditions present in the squid (or lacking in standard laboratory culture conditions). This experimental design can be used to assess the bioluminescence produced by various strains.

Materials

V. fischeri on LBS agar plate (see Basic Protocol 1)
Seawater tryptone at high osmolarity (SWTO) medium (see recipe)

Applicator stick, sterile
18 × 150–mm glass culture tube with cap, sterile
24°C and 28°C shaking incubator
Spectrophotometer
250-ml baffled flask, sterile
1.5-ml cuvette (10-mm path length)
20-ml glass scintillation vial or appropriately sized container for luminometer
Luminometer

1. Using a sterile applicator stick, pick a single *V. fischeri* colony from an LBS agar plate.
2. Inoculate an 18 × 150-mm glass culture tube containing 5 ml SWTO by suspending cells from the applicator stick in the medium. Remove applicator stick, and cap test tube.
3. Aerate inoculated tube at 28°C by shaking at 220 rpm overnight.
4. Determine OD_{600} of the overnight culture using a spectrophotometer. Pipette a volume of the overnight culture into 30 ml SWTO in a 250-ml baffled flask to achieve a final starting OD_{600} of 0.005.
5. Aerate flask at 24°C by shaking at 220 rpm.
6. At desired time points, take an OD_{600} reading and a luminescence reading: Transfer 1 ml culture to a 1.5-ml cuvette and measure OD_{600}. To take a luminescence reading, transfer 1 ml culture to a scintillation vial or an appropriate container suitable for the luminometer. Shake container for 5 s to aerate sample, and then measure luminescence using a luminometer.

   The luminescence reaction requires oxygen. If the sample is not adequately aerated, the luminescence readings will be inaccurate.

   Hourly time points can provide initial details about the dynamics of growth and luminescence. In future experiments, time points can be taken more or less frequently.

REAGENTS AND SOLUTIONS

Artificial seawater (ASW), 2×

Add 4 L distilled water into an 8-L carboy. Dissolve 197.6 g $MgSO_4 \cdot 7H_2O$ in 1 L distilled water, and add to carboy. Dissolve 23.2 g $CaCl_2 \cdot 2H_2O$ into 1 L distilled water, and add to carboy. Dissolve 280.8 g NaCl and 12 g KCl directly into carboy. Bring final volume to 8 L. Store at room temperature for up to 3 months.

The final concentrations of each salt in this stock are 100 mM $MgSO_4$, 19.7 mM $CaCl_2$, 600 mM NaCl, and 20.1 mM KCl.

LBS agar plates

Combine 10 g tryptone, 5 g yeast extract, 20 g NaCl, 15 g agar, 975 ml distilled water, and 25 ml of 2 M Tris, pH 7.5, to a 2-L flask. Autoclave mixture. Allow to cool until the flask can be comfortably held. As desired, add an antibiotic to the cooled medium and mix well. Pour ~30 ml into sterile plastic 100-mm petri dishes, and allow agar to solidify. Let plates sit for 1 to 2 days at room temperature until sufficiently dry. Store plates at 4°C for up to 3 months.

For solid agar–based biofilm experiments, pipette 25 ml medium into sterile petri dishes. For calcium-induced biofilms, add 10 ml of sterile 1 M $CaCl_2$ to the autoclaved medium and mix well. Store plates at room temperature for up to 2 days to retain proper moisture.

Pipetting exactly 25 ml agar medium into each petri dish helps to ensure reproducibility of biofilm assays from plate to plate and from experiment to experiment.

LBS medium

Combine 10 g tryptone, 5 g yeast extract, 20 g NaCl, 975 ml distilled water, and 25 ml of 2 M Tris, pH 7.5, in a 2-L flask or beaker. Stir mixture until solids are completely dissolved. Distribute medium into clean bottles at the desired volume. Autoclave bottles and store at room temperature for up to 3 months.

Some researchers include glycerol in this recipe to a final concentration of 0.3% (v/v). If added, V. fischeri will metabolize the glycerol and secrete acid into the medium. With prolonged growth under these conditions, V. fischeri will begin to die, and thus glycerol addition should be carefully considered and strain growth carefully monitored, as the Tris buffer may not sufficiently prevent acidification produced by V. fischeri.

When performing a shaking biofilm assay, 10 ml of sterile 1 M $CaCl_2$ can be added after autoclaving, for a final concentration of 10 mM.

Minimal medium (HMM or TMM)

75.3 ml distilled water
10 ml of 1 M HEPES, pH 7.5, or 1 M Tris, pH 7.5
6 ml of 5 M NaCl
5 ml of 1 M $MgSO_4 \cdot 7H_2O$
100 µl of 33 mM K_2HPO_4
100 µl of 10 mM ferrous ammonium sulfate
500 µl of 20% (w/v) NH_4Cl
1 ml of 20% (w/v) D-glucose
1 ml of 1 M KCl
1 ml of 1 M $CaCl_2 \cdot 2H_2O$
Store at 4°C for up to 2 days

Filter sterilize 1 M HEPES (or 1 M Tris) before use. Prepare water and other component stocks in individual bottles and autoclave each.

Substitute glucose for any carbon source desired. If carbon sources are not being studied, 1 ml of 10% casamino acids stock can be added to enhance growth in this medium.

The individual reagents can be made up separately, so that the concentrations of each component may be adjusted depending on the experimental design. This strategy also prevents precipitation of calcium and phosphate. Alternatively, NaCl, $MgSO_4$, KCl, and $CaCl_2$ can be replaced with 35 ml of 2× ASW (see recipe) per 100 ml, with the amount of water adjusted accordingly.

Ferrous ammonium sulfate will settle to the bottom of a bottle of stock solution after autoclaving and must be resuspended prior to addition.

SWT medium

Combine 5 g tryptone, 350 ml of 2× ASW (see recipe), and 3 g yeast extract in a 2-L bottle, and bring volume to 1000 ml with distilled water. Sterilize by autoclaving. For best effect, prepare solution the day before use.

Some researchers include glycerol in this recipe to a final concentration of 0.3% (v/v). If added, V. fischeri will metabolize the glycerol and secrete acid into the medium. With prolonged growth under these conditions, V. fischeri will begin to die, and thus glycerol addition should be carefully considered and strain growth carefully monitored.

Note that ASW is used at <1× in this recipe.

SWTO medium

Combine 5 g tryptone, 350 ml of 2× ASW (see recipe), 3 g yeast extract, and 20 g NaCl in a 2-L bottle, and bring volume to 1000 ml with distilled water. Sterilize by autoclaving. For best effect, prepare solution the day before use.

The addition of NaCl brings the solution near to the osmolarity of seawater.

Some researchers use Instant Ocean instead of 2× ASW.

As with SWT, some researchers include glycerol in this recipe to a final concentration of 0.3% (v/v). V. fischeri will metabolize the glycerol and secrete acid into the medium, which causes cell death with prolonged exposure. However, glycerol addition may also increase luminescence production by V. fischeri.

Note that ASW is used at <1× in this recipe.

TBS medium

Combine 10 g tryptone and 20 g NaCl in a 2-L bottle, and bring volume to 1000 ml with distilled water. Stir mixture until solids are completely dissolved. Distribute medium into clean bottles at the desired volume. Autoclave bottles and store at room temperature for up to 3 months.

TBS-Mg plates

Combine 10 g tryptone, 20 g NaCl, 2.5 g agar, and 8.6 g $MgSO_4 \cdot 7H_2O$ in a 2-L flask, and bring volume to 1000 ml with distilled water. Autoclave and allow to cool until the flask can be comfortably held. Pipette 25 ml medium into sterile plastic 100-mm petri dishes. Store plates at room temperature for up to 2 days to retain proper moisture.

These plates are very fragile and should never be inverted.

Pipetting exactly 25 ml agar medium into each petri dish helps to ensure reproducibility from plate to plate and from experiment to experiment.

V. fischeri requires magnesium for optimal motility, and its omission from the medium results in a severely decreased rate of migration through the soft agar (O'Shea et al., 2005).

TBSW plates

Combine 10 g tryptone, 350 ml of 2× ASW (see recipe), and 2.5 g agar in a 2-L flask, and bring volume to 1000 ml with distilled water. Autoclave and allow to cool until the flask can be comfortably held. Pipette 25 ml medium into sterile plastic 100-mm petri dishes. Store plates at room temperature for up to 2 days to retain proper moisture.

These plates are very fragile and should never be inverted.

Pipetting exactly 25 ml agar medium into each petri dish helps to ensure reproducibility from plate to plate and from experiment to experiment.

Note that ASW is used at <1× in this recipe.

COMMENTARY

Background Information

Biofilm assays

Efficient colonization by *V. fischeri* depends on the ability of this microbe to form a biofilm on the surface of the squid's symbiotic light organ and to subsequently disperse from the biofilm to migrate inside where colonization occurs (Nyholm et al., 2000; Yip et al., 2006). The ability to form a biofilm in laboratory culture strongly correlates with colonization competence, with mutants that fail to form biofilms in vitro exhibiting colonization defects and strains with enhanced biofilm formation exhibiting increased colonization proficiency (e.g., Shibata et al., 2012; Yip et al., 2006). Studies with genetically altered strains of ES114 with increased biofilm capability in vitro and the assays described here have permitted a mechanistic understanding of the requirement for symbiotic biofilm formation and colonization as well as the identification of the *syp* (symbiosis polysaccharide) genes as required for biofilm formation and symbiotic colonization.

There are numerous assays of biofilm formation. Strains of ES114 with increased biofilm capability (e.g., overexpressing *rscS*) form wrinkled colonies on solid agar and strong pellicles in static liquid culture (Yip et al., 2006). Null mutants defective for the *syp* genes form smooth colonies and weak pellicles, and they colonize squid poorly. Thus, these two assays are highly predictive of symbiotic competence. In contrast, the crystal violet assay (O'Toole, 2011) is not a good predictor of symbiotic competence (Shibata et al., 2012) and is not included here. Finally, certain strains can form biofilms upon exposure to calcium under shaking liquid culture conditions (Tischler et al., 2018). Recent work has shown that this assay also can provide insights into the requirements for symbiotic colonization (Thompson, Tischler, Tarnowski, Mandel, & Visick, 2019).

Motility assay

To date, motility is the one phenotype that appears to be absolutely critical for colonization by *V. fischeri*: nonmotile strains fail to colonize (Graf et al., 1994). Other strains with altered motility or with chemotactic defects also have shown altered colonization dynamics (e.g., Mandel et al., 2012; Millikan & Ruby, 2002). While other assays exist, we present here the simplest assay for motility: tryptone-based soft agar plates. This simple assay can be performed by the most junior researcher and will yield results within a short time frame (<6 hours). By measuring the diameter of the outer migrating band of cells at specific intervals (with a low-tech instrument; i.e., a ruler), subtle defects in the motility of a given strain relative to another can be uncovered. Furthermore, this assay can reveal differences in behavior, such as differences in the response or chemotaxis to nutrients in the medium. This assay permitted both the discovery that magnesium promotes migration by ES114 and the identification of two chemoattractants, serine and thymidine, sensed by *V. fischeri* (DeLoney-Marino et al., 2003; O'Shea et al., 2005). This assay can be adapted by using different media such as a minimal medium to test specific nutrients as chemoattractants.

Luminescence assay

Luminescence is the key "product" of symbiosis between *V. fischeri* and its squid host. Mutants of *V. fischeri* that are defective for light production can initially colonize but fail to sustain long-term colonization (Koch et al., 2014; Visick et al., 2000). Examination of *lux* gene expression in the light organ has revealed distinct patterns in the different crypts,

a result that provides insight into the environments found in the three crypts (Dunn, Millikan, Adin, Bose, & Stabb, 2006). Finally, symbiotic ES114 produces about 1000 times the amount of light relative to that which it produces in laboratory culture (Boettcher & Ruby, 1990). This phenomenon permitted the discovery of ArcA as one factor that inhibits bioluminescence in culture (Bose et al., 2007). In addition to control by ArcA, light production depends on stimulation by multiple autoinducers that are produced when the cells reach high cell density (Verma & Miyashiro, 2013). Thus, understanding regulation of light production provides insight into the behaviors of *V. fischeri*—and the underlying mechanism—during colonization of the light organ. The protocol included here uses a complex medium that contains high levels of NaCl and other salts present in seawater, as this combination of salts was shown to result in increased light production by ES114 (Bose et al., 2007).

Critical Parameters and Troubleshooting

Biofilm assay on solid agar

To obtain reproducible results within and across experiments, the agar plates should be made with a standard volume, such as 25 ml. Additionally, the plates should be fresh but dry. Differences in moisture of the agar plates can affect the ability of *V. fischeri* to form biofilms. Thus, the duration between pouring and use should be noted for each experiment. Finally, it is possible that different batches of tryptone or yeast extract from different lots or companies may result in differences in the exact timing in the development of the wrinkled colony phenotypes. In our biofilm work, we use Gibco™ Bacto™ Yeast Extract and Gibco™ Bacto™ Tryptone. The consistent use of the same reagents with positive and negative controls should permit reproducible data collection.

If two or more nutrients (e.g., $CaCl_2$ and $MgSO_4$) are to be compared for their effects on biofilm formation, one single flask of medium should be used. Following autoclaving, the medium can be split into sterile flasks, followed by delivery and mixing of specific additives. Alternatively, additives may be added to each plate individually from liquid stocks. These approaches avoid inconsistencies that can arise from preparing independent flasks of medium that may have slight differences in composition due to measurement errors or liquid loss from autoclaving. If the volume of additives is different or a no addition condition is compared, a corresponding volume of sterile water should be added to each flask or plate to ensure equivalent final volumes. These additives can be added to sterile flasks or petri dishes while the medium is autoclaving and cooling. It is critical to ensure the additives are fully integrated into the medium in the plate either by swirling or pipetting. Otherwise, uneven pockets of the nutrient may be found across the plate.

Samples should be spotted as equidistantly as possible. Proximity of the spots to one another and to the edge of the plate may affect their phenotypes. Do not puncture the agar or introduce air bubbles when spotting.

Biofilm assay in shaking liquid culture

The size of tube can affect results of this assay. A biofilm-competent *V. fischeri* strain grown in a 13 × 100–mm glass tube with 2 ml medium often results in a strong biofilm with a clear culture. However, the same strain in an 18 × 150–mm glass tube with 5 ml medium will often show reduced biofilm phenotypes, with the culture retaining some turbidity. A positive control with a known behavior should be used whenever altering conditions.

Biofilm assay in static liquid culture

Cultures grown in the outer wells of a 24-well plate may not produce the same pellicle phenotypes as the same strains grown in the inner wells, and thus the outer wells should not be used for bacterial culture. The differences in aeration and/or evaporation rates between these inner and outer wells may account for the observed differences in pellicle formation. Placing the plate in a sealed plastic bag or plastic box helps to mitigate these differences. In two to four outer wells, uninoculated medium controls should be added that flank the samples. These negative controls help to ensure that there was no contamination during pipetting into the wells.

Take care when moving the plate once pellicles have formed. Jostling of the plate may cause the pellicles to detach from the sides of the well.

When disrupting pellicles with a toothpick, the pellicle may stick to the toothpick. To overcome this issue, pull the adherent part of the pellicle to the edge of the well in the direction of disruption. Then, gently wipe the pellicle off of the toothpick on the edge. Repeat until the pellicle has dislodged from the toothpick.

Motility assay

To obtain consistent results, exactly 25 ml agar-containing solution should be dispensed into each petri plate. These plates must be made fresh and used within 1 to 2 days after pouring. Differences in moisture can have significant effects on reproducibility between experiments. Once solidified, care must be taken with motility plates as the low-percentage agar is very easily broken. Do not puncture the agar when spotting. Ideally, if multiple plates are used in a single experiment, each plate should have a control spot, such as a wild-type strain, as a standard to permit comparisons across plates. The experiment thus typically ends when a wild-type control spreads to or into the growth of a less-motile strain. If it is necessary to verify that a strain is fully nonmotile, then that strain may also be placed on its own plate to permit extended incubation.

V. fischeri requires magnesium for optimal motility. Tryptone powder from different companies or lots from the same company may have differing amounts of magnesium, which may influence migration. Growth of the cells in LBS instead of TBS prior to spotting may affect the migration rates and/or patterns of migration.

Luminescence assay

It is essential that the samples are aerated for 5 s prior to reading luminescence (by vigorous pipetting or shaking the vial). Aeration is required for an accurate measurement of light produced.

Understanding Results

Biofilm assay on solid agar

Biofilm-competent colonies may be adherent to the plate, cohesive, and/or produce architecture or wrinkles (Fig. 2). Strains that cannot form biofilms will be smooth and lack adherence, cohesion, or architecture (Fig. 2A,D). The degree of wrinkling can vary from a minor phenotype (Fig. 2B) to a strong phenotype (Fig. 2C) for different strains at the same time point. Disrupting a spotted colony with a toothpick is a critical tool for assessing biofilm formation, as some smooth-appearing colonies are cohesive (and thus categorized as biofilm forming) and some colonies exhibit a nonsmooth architecture but remain noncohesive (and thus categorized as nonbiofilm forming). Furthermore, a time course provides valuable information about the dynamics of biofilm formation.

Biofilm assay in shaking liquid culture

A strain of *V. fischeri* that cannot form a biofilm in this assay will remain as a turbid culture. If a strain can form a biofilm (Fig. 3), the culture will be mostly clear with a ring at the air–liquid interface, a clump at the bottom of the test tube, or both. When both are present, a stringy "tree" can connect from the ring to the clump. For biofilm-competent derivatives of ES114, the ring at the top of the tube depends primarily on the polysaccharide cellulose, while the clump at the bottom of the tube depends primarily on the Syp polysaccharide (Tischler et al., 2018).

Biofilm assay in static liquid culture

The expectation is that a biofilm-forming strain will form a pellicle that extends across the air–liquid interface of the well. Like in the solid agar biofilm assay, these pellicles may be smooth or have wrinkles. After disruption with a toothpick, a pellicle may tear or remain coherent. In some cases, the pellicle may stick to the toothpick. The stickiness and cohesion of the pellicle is primarily imparted by the polysaccharide Syp (Shibata et al., 2012).

Motility assay

Upon spotting normalized cultures onto motility agar plates, the bacteria will swim outward from that spot. Hourly measurement of the diameter of the spot quantifiably measures migration by the bacteria. The distance that the bacteria migrate from the initial spot depends on when the cells synthesize flagella as well as the frequency of the bacteria swimming in a single direction. Monitoring the distance moved over time can help to inform whether the cells start swimming earlier or whether they swim faster.

Luminescence assay

Strains may either produce luminescence or they may not. For the strains that do luminesce, luminescence should increase with optical density. At some point, the luminescence may plateau and/or may decrease.

Time Considerations

Growth of V. fischeri from frozen stocks

The time to grow *V. fischeri* from frozen stocks is very short at permissive conditions: after streaking and placing the plate at 28°C, a healthy *V. fischeri* strain will produce large colonies after an average overnight of 16 hr, though colonies may arise in even less time. If the cells are incubated at 24°C or room temperature, it will take more than 16 hr for the

development of large colonies. On antibiotic-containing media, resistant colonies will take longer to achieve the same size as their counterparts on LBS alone.

Growth of V. fischeri in rich, undefined liquid medium

V. fischeri cultures started from frozen stock or taken from a single colony on a plate will take very little time (~8 hr) to achieve high optical densities. Routinely, cultures incubated at 24°C or 28°C should be grown overnight for no longer than 16 to 18 hr to preserve the health of the culture.

Growth of V. fischeri in minimal medium

As with many other bacteria, *V. fischeri* grows more slowly in minimal medium than it does in rich medium. The transition from a frozen stock or a rich, plate-bound lifestyle may be stressful for the bacteria, and growth may be poor or variable. However, overnight growth at 24°C or 28°C in this medium for 14 to 18 hr often will yield dense cultures, with an OD_{600} of about 1.0 to 1.2, that are readily subcultured the next day. A shorter overnight growth period of around 14 hr results in "young" cells that have not yet reached late stationary phase and that will regrow more quickly and reliably in the subculture, an approach that can substantially reduce the lag phase of growth. When *V. fischeri* is growing well in minimal medium, the culture will reach an OD_{600} of about 0.5 in about 5 to 6 hr following a 1:50 dilution from an overnight culture; higher inoculum will decrease the time to reach that optical density.

Storage of V. fischeri in frozen stocks

Frozen stocks are typically generated with fully turbid cultures (i.e., OD_{600} >3) of *V. fischeri*, which can take as little as 8 hr or as many as 16 to 18 hr to obtain. Thus, the time required to take a colony from a plate and prepare a frozen stock depends on how long it takes to obtain a turbid culture. Glycerol cryovials should be prepared in advance by pipetting 80% glycerol into plastic or glass cryovials and sterilizing by autoclaving. Alternatively, 80% glycerol and cryovials can be sterilized separately, and then 80% glycerol can be transferred into the sterile cryovials.

Biofilm assay on solid agar

The time required to perform a biofilm assay is between 2 and 3 days of setup with 3 to 5 days of analysis. For a quicker exploratory experiment, an overnight culture can be started directly from frozen cells on the first day. On the second day, the overnight culture is subcultured 1:100 in fresh medium for about 1 to 2 hr. Depending on the number of strains being spotted, the optical density readings, normalization, and spotting could take minutes or up to an hour. To achieve accuracy in assessments of the timing of biofilm formation, experiments should be designed to have sufficiently few strains or conditions such that it takes <1 hour to spot the cultures. To speed up this process, tubes and plates can be labeled before or during growth of the subculture. For a more precise experiment, the first day will instead require streaking the cells from frozen stocks onto LBS plates, and then the work can proceed as described for the subsequent 2 days.

After spotting onto the plates, analysis can be performed at a single time point each day for up to 5 days. The amount of time each day for analysis and imaging will be proportional to the number of spots. In some cases, differences will be observed in a time frame of <24 hr, and thus subsequent assessment could be hourly from (e.g., 16 to 24 hr).

Biofilm assay in shaking liquid culture

The shaking liquid culture biofilm assay will take 3 to 4 days. Like the solid agar biofilm assay, this timing depends on whether frozen cells are directly inoculated to make an overnight culture (1 day) or instead are streaked onto a plate for single colonies and the following day inoculated into a liquid medium (2 days). On the following day, the overnight culture will be normalized and subcultured. After a specified (consistent) period of incubation time (e.g., 16 or 24 hr), the tubes can be assessed for biofilm formation.

Biofilm assay in static liquid culture

Starting with single colonies on a plate, a static liquid culture assay will take 2 days with 1 to 3 days for analysis. The day after growth of the overnight culture, optical densities of each strain are determined, and the overnight culture is subcultured into a 24-well plate containing fresh medium. The pellicles may develop after only 24 hr, or they may take up to 3 days. The length of time for analysis is dependent on the number of wells used.

Motility assay

The motility assay is more time intensive than the biofilm assays if performing a time course. If starting with single colonies on a plate, the assay will take only 2 days. On the first day, a single colony will be used to start an

overnight culture in TBS. The low-percentage agar plates should also be made on this day. On the second day after subculturing from the overnight culture, the culture will be grown for 1 to 3 hr to achieve an OD_{600} of 0.2. Depending on the number of strains or conditions, normalization of the cultures and spotting may only take minutes and should take less than an hour. Once the spotted plates are incubating, the experiment may continue for as many hours as desired. Every time point taken requires measurement of the diameter of each spot, which takes an amount of time proportional to the number of spots. The duration of migration will depend on how closely strains were inoculated onto a single plate, as the migrating cells will run into each other, preventing further accurate measurement.

Luminescence assay

Like the motility assay, the assay will only take 2 days when starting from single colonies on a plate. The first day requires starting an overnight culture. The subsequent day, optical densities are taken to permit standardization of the subculture. The subcultured cells are grown throughout the day, with time points taken each hour or potentially more frequently. The duration of each time point will depend on the number of samples, as each sample requires time for harvesting, an optical density reading, and a luminescence reading.

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