Genetic and Environmental Modulation of Vibrio fischeri Motility and Biofilm Formation via CRISPRi and Phenotypic Assays

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Jason Cho, Tailai Cong, Alex Liu

Abstract

Vibrio fischeri is a model bacterium widely utilized for studies on biofilm formation and motility—the factors that play key roles in its symbiotic host-microbe interactions with Hawaiian bobtail squid. In this study, we have tested the effects of different environmental conditions–IPTG, calcium chloride, para-aminobenzoic acid (PABA), cephalexin, and temperature–on the surface behaviors of various V. fischeri strains. A series of biofilm and motility assays were run using CRISPRi knockdown strains, insertion mutants, and various supplements to evaluate phenotypic changes. Motility assays demonstrated minimal phenotypic differences among most strains, except for a modest reduction in CrvY. Biofilm assays revealed that only BinK mutants formed cohesive biofilms in the presence of calcium, whereas all other strains exhibited either no cohesion or only early-stage of biofilm formation. No conjugants were obtained through triparental mating. Overall, our findings underscore the complexity of biofilm and motility regulation in V. fischeri, the importance of environmental conditions, while emphasizing the need for further optimization to clarify gene-environment interactions that govern symbiotic behavior.

Introduction

Vibrio fischeri is a marine bacterium best known for its bioluminescence and its role in the symbiotic relationship with the Hawaiian bobtail squid (Euprymna scolopes) (Septer & Visick, 2024). Its ability to emit blue-green light fascinates scientists for centuries, and research into its light production mechanisms has led to major discoveries in bacterial genetics, symbiosis, and host-microbe interactions (Septer & Visick, 2024). Although Vibrio fischeri is a non-pathogenic bacterium, measurements commonly used with pathogens such as motility assays and biofilm formation can still be utilized (Septer & Visick, 2024). It is a powerful model organism for studying microbial ecology and symbiosis.

Biofilms are bacterial communities attached to surfaces, which may form beneficial partnerships with other organisms. For instance, Vibrio fischeri colonizes the Hawaiian Bobtail Squid by forming a transient biofilm on the squid's light organs, which helps them to survive and reproduce in the long term (Fung et al., 2024). The biofilm formation process is controlled by different components, such as symbiosis polysaccharide (SYP), cellulose, Bmp proteins, and surface structures (like flagella) (Fung et al., 2024). To regulate biofilm formation, several external factors can be explored.

According to Dial et al. (2021), yeast extract can inhibit biofilm development, while combining para-aminobenzoic acid (PABA) and calcium ($CaCl_2$) may induce improved biofilm production in wild-type Vibrio fischeri (ES114), since c-di-GMP may be over-produced, and enhances biofilm matrix formation while suppressing motility (an aspect worth noting due to an often negative correlation with biofilm).

During preliminary observations, we noticed a special "bumpy" phenotype in the strains cultured by RIBS students, especially VxrB (see Figure 1), which "VxrB G4" is used in our experiments. Besides, several other possible mutant strains (named as "VxrB 4A", "VxrB 4B", and "VxrB Fake") grown by different groups caught our attention as well. This specific phenotype is consistent with previously described "wrinkled and clumped biofilm structures", as mentioned in Dial et al. (2021). As a result, we have tested out how genetic mutations and environmental factors influence the biofilm and motility expression.

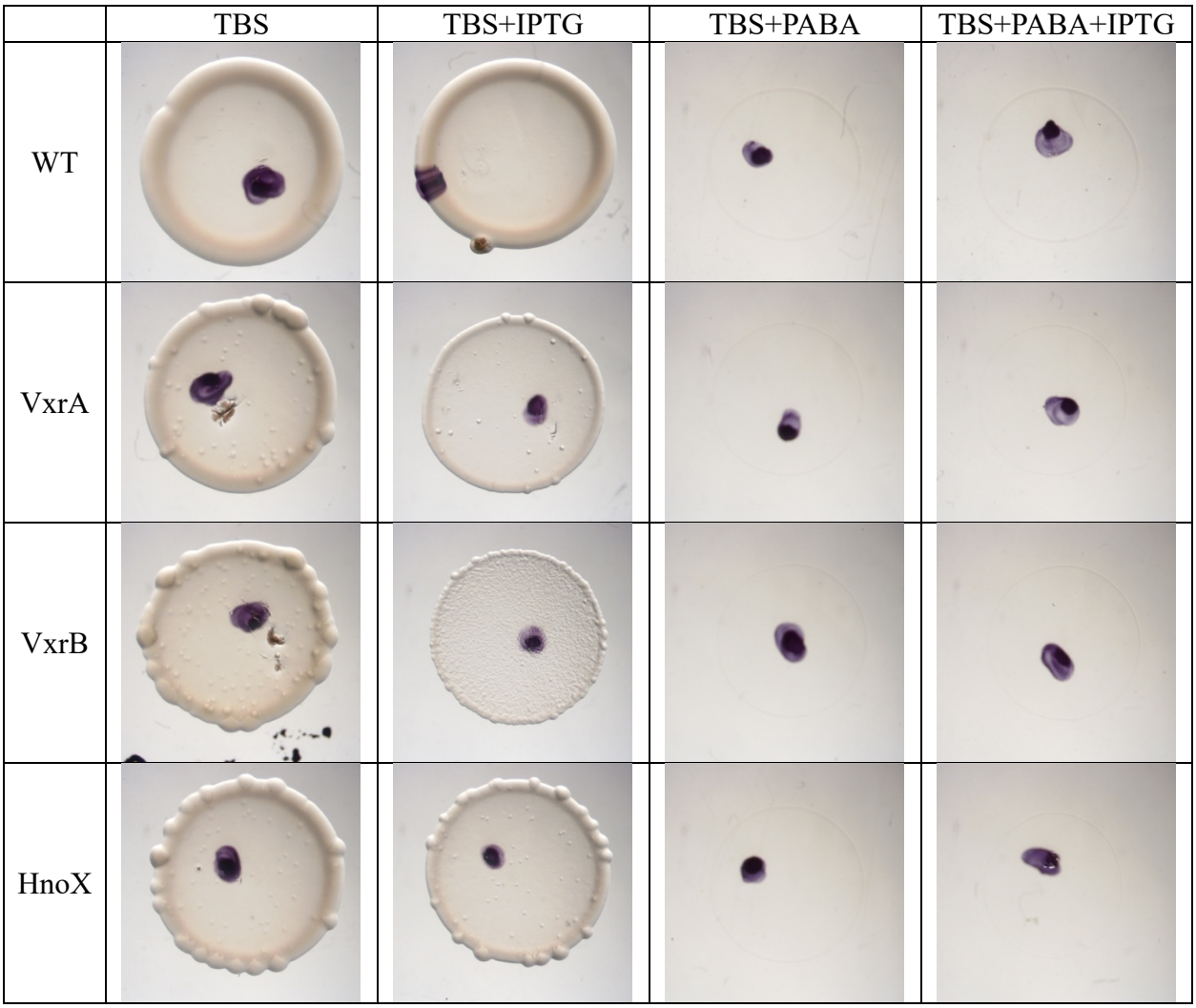


Figure 1. Strains cultured by RIBS students in the first two weeks. TBS and TBS+IPTG are from group 7, TBS+PABA and TBS+PABA+IPTG are from group 9. A unique bumpy shape can be observed in the VxrB strain on TBS + IPTG plates.

In our experimental design, we mainly examined the role of the VxrB G4 and derivatives, including CRISPRi modified mutants, insertion mutants, and double-hit mutants generated through triparental mating in surface behaviors such as motility and biofilm. We will evaluate these VxrB-related strains with other known mutants such as AmiB, VxrA, CrvY, BcsA, and BinK.

Environmental conditions are also changed in different sessions, such as the presence or absence of $CaCl_2$, with or without PABA, media containing Isopropyl β-D-1-thiogalactopyranoside (IPTG) or not, and the temperature of bacterial culture. These factors may affect c-di-GMP levels, which in turn influence biofilm matrix production and motility.

On the other hand, we would also like to examine the impact of antibiotics called cephalexin on Vibrio fischeri's cell curvature, which might be related to biofilm matrix production at the signaling pathway level. As previously known in Vibrio cholerae, the VxrA receptor indirectly suppresses cell curvature (leading to straight cells), as well as biofilm production (Peschek et al., 2020). We will validate if the same pathway and phenotype is also present in Vibrio fischeri.

Methods

Materials

Vibrio fischeri Strains:

- ES 114 Wild Type
- Wild Type + pJMP empty vector
- RscS Overexpression
- AmiB mutant
- VxrA
- VxrB (Group 4; our gene)
- VxrB 4A
- VxrB 4B
- VxrB Fake
- VxrB Insertion mutant

(VxrB 4A, VxrB 4B, and VxrB Fake are variants of VxrB mutants)

Supplements:

- $CaCl_2$
- Para-aminobenzoic acid (PABA)
- IPTG
- Cephalexin

Procedures Overview

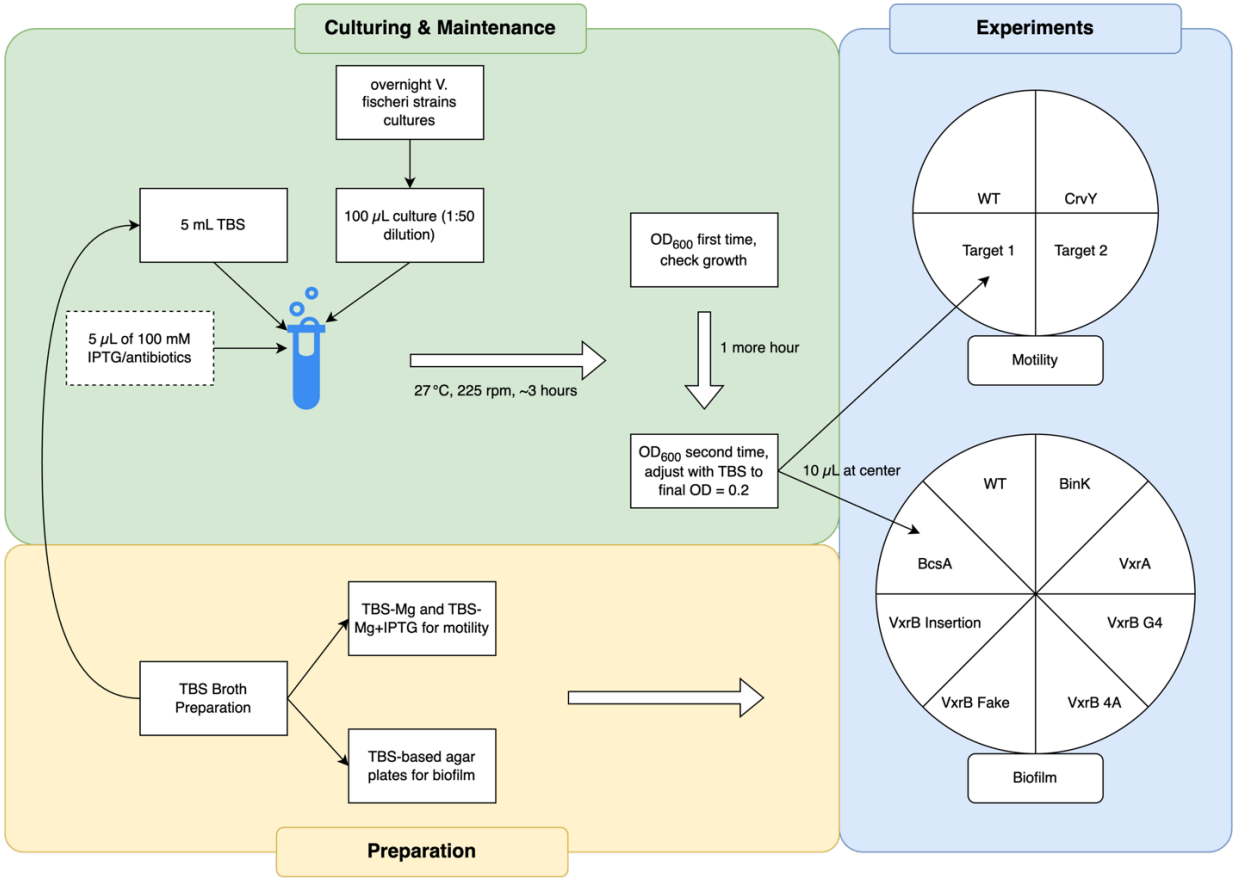


Figure 2. Experimental workflow for Vibrio fischeri phenotypic assays. The study began with the preparation of TBS broth and TBS-based agar plates. During the culturing and pretreatment phase, we inoculated overnight cultures into fresh TBS, with optional supplementation of IPTG or antibiotics, followed by incubation with shaking and then optical density adjustment. The resulting cultures were spotted on plates for biofilm & motility assay.

Detailed Procedures

TBS Broth Preparation

To prepare a standard tryptone broth salt (TBS) solution, we began by placing a weighing boat on a digital scale and taring it. We weighed 5.00 g of tryptone (1% final concentration) and 10.00 g of sodium chloride (2% final concentration) and added them to a sterile beaker. Using a graduated cylinder, we measured 500 mL (500 g) of distilled water and added it to the same beaker to achieve the final volume.

The beaker was autoclaved under a standard sterilization cycle lasting 74 minutes. Following autoclaving, the solutions were incubated at room temperature, allowing them to cool before further use.

Preparation of TBS-Mg and TBS-Mg + IPTG Plates for Motility Assay

Reagents: Bacto-Tryptone, Difco, 1% final concentration; NaCl, 2% final concentration; Bacto-agar, Difco, 0.25% final concentration; and Magnesium sulfate, Heptahydrate $MgSO4 \cdot 7H2O$, 35mM.

The reagents above were dissolved in distilled water in a flask containing at least twice the final intended volume. The solution was autoclaved on a 30-minute liquid cycle, then cooled in a water bath to 55–60 °C.

For TBS-Mg + IPTG plates, after cooling, IPTG was added to one batch of molten agar in a flask to a final concentration of 100 μM (0.25 mL of 100 mM IPTG was added).

The resulting molten agar was dispensed into sterile 100 mm plastic Petri dishes, labeled to distinguish between IPTG-containing and non-IPTG batches, with 25 mL per plate. Avoid making bubbles. Plates were dried and stored upright at room temperature. Plates were handled carefully to avoid cracking or drying, as soft agar is fragile.

Preparation of TBS-Based Agar Plates for Biofilm Assays

- TBS Agar Plates ± IPTG

Reagents: Bacto-Tryptone, Difco, 1% final concentration; NaCl, 2% final concentration; Bacto-agar, Difco, 1% final concentration; and Tris (1 M), 50mM.

To prepare standard TBS agar plates with and without IPTG, the reagents were combined in a flask for a final volume of 250 mL. The mixture was prepared in a flask with at least 2× the final volume capacity, autoclaved, and cooled in a water bath to 55 °C. Non-IPTG plates were prepared by dispensing 25 mL of molten medium into 5 sterile 100 mm plastic Petri dishes. To make IPTG-supplemented media, 0.125 mL of 100 mM IPTG stock was added to the rest of the media (125 mL left), achieving a final concentration of 100 μM. Swirl the flask and pour the rest of the media by dispensing 25 mL of molten medium into 5 different sterile 100 mm plastic Petri dishes. Avoid making bubbles. Store plates at room temperature and use within two days to maintain proper moisture levels.

- TBS + Calcium Chloride Agar Plates ± IPTG

Reagents: Bacto-Tryptone, Difco, 1% final concentration; NaCl, 2% final concentration; Bacto-agar, Difco, 1% final concentration; Tris (1 M), 50mM; and $CaCl_2$, 10mM.

TBS-calcium agar plates were prepared using the same base as TBS agar, with the addition of calcium chloride: Calcium chloride (1 M): 2.5 mL per 250 mL final volume (10 mM final concentration). After autoclaving and cooling to 55 °C, add the appropriate amount of calcium chloride to the molten media. After pouring 5 plates, for IPTG-supplemented versions, 0.125 mL of 100 mM IPTG was added to the rest of the solution as described above. Plates were poured and stored using the same procedure as for standard TBS ± IPTG agar.

- TBS + Calcium Chloride + PABA Agar Plates ± IPTG

Reagents: Bacto-Tryptone, Difco, 1% final concentration; NaCl, 2% final concentration; Bacto-agar, Difco, 1% final concentration; Tris (1 M), 50mM; and $CaCl_2$, 10mM.

For TBS-calcium + PABA plates, we used the same base as standard TBS-calcium agar, but with the additional reagent 0.3325g PABA (for 9.7mM final concentration) to a 250 mL batch. After dissolving the reagents and autoclaving, calcium chloride and IPTG were added to the cooled media as described in the TBS-calcium agar part. Plates were poured and stored using the same procedure.

Reculturation of Vibrio fischeri

Each strain was assigned to a sterile 15 mL culture tube, which was labeled accordingly. For all the Vibrio strains, 3 mL of tryptone broth salt (TBS) was dispensed into each tube using a serological pipette.

Once the media were added, individual colonies were picked from previously streaked plates using sterile inoculating loops and transferred into their respective tubes. Cultures were mixed by flicking to ensure proper suspension.

After inoculation, E. coli tubes were incubated at 37 °C, while Vibrio fischeri tubes were incubated at ~27 °C, both under shaking conditions overnight. This reculturing step ensured that all strains would be available in sufficient quantity and in log-phase growth for use in upcoming experimental procedures.

Motility Assay Media Preparation & Vibrio Motility Assay

Pre-cultured V. fischeri strains were used to inoculate 5 mL of fresh tryptone broth salt (TBS) in sterile 15 mL tubes. For each tube containing 5 mL TBS, 100 µL of the corresponding overnight culture was added to achieve a 1:50 dilution.

For the preparation of cultures that are pretreated with IPTG and/or cephalexin (pretreatment), in addition to 100 µL of culture, add 5µL of 100mM IPTG (1:50 dilution) and/or cephalexin to the final concentration of 10 µg/mL. Cultures were incubated at 27 °C with gentle shaking (~225 rpm) for approximately 3 hours. Following incubation, optical density at 600 nm (OD_{600}) was measured for each culture using a spectrophotometer.

The volume of TBS to be added to each culture was calculated to normalize the OD using the formula:

$$\text{Volume of TBS to add} = \left(\frac{\text{OD}_{600} \times \text{measured volume}}{0.2}\right) - 4\text{mL}$$

The adjusted TBS volume was added to bring each culture to a final standardized optical density of 0.2, which is suitable for the motility assay. Cultures were gently mixed.

For spotting, TBS-Mg motility plates with and without IPTG were used. Each plate was divided into four sectors using a permanent marker, and a dot was drawn at the center of each sector to indicate the inoculation spot. Each sector has one strain labeled.

Then, we pipetted 10 µL of each standardized culture onto the center of their assigned sectors. After inoculation, the spots were allowed to soak for around 10 minutes at room temperature. Plates were then incubated right side up at 27 °C for approximately 4 hours. Motility was assessed by measuring the diameter of the bacterial spread from the central inoculation point at both 3 and 4 hours post-inoculation.

Biofilm Assay Media Preparation & Vibrio Biofilm Assay

Eight V. fischeri strains were included: WT with empty vector, ΔBinK, VxrA, VxrB (G4), VxrB (4A), VxrB–Fake, VxrB insertion mutant, and ΔBcsA.

Cultures were grown overnight and then diluted 1:50 by adding 100 µL of each overnight culture to 5 mL of TBS broth in sterile 15 mL tubes. To prepare cultures that are pretreated with IPTG, in addition to 100 µL of culture, add 5µL of 100mM IPTG (1:50 dilution). Cultures were incubated at 27 °C with shaking at ~225 rpm for 3 hours.

After incubation, the optical density at 600 nm (OD_{600}) was measured for each strain using a spectrophotometer. To normalize the OD for downstream applications, the volume of TBS to be added was calculated using the same formula used for motility assay preparation. The calculated TBS volume was added to each culture to bring the final OD to 0.2 in 4 mL. Samples were gently inverted several times.

For inoculation, Biofilm plates containing various conditions were used: TBS only, TBS + IPTG, TBS + $CaCl_2$, TBS + $CaCl_2$ + IPTG, TBS + $CaCl_2$ + PABA, TBS + $CaCl_2$ + PABA + IPTG. Plates were inverted and divided into 8 sectors. Each sector was labeled with the strain name, and a small dot was placed in the center of each sector to mark the inoculation point. Plates were assigned to two incubation temperatures: 24 °C and 28 °C. 10 µL of each normalized culture was spotted onto the designated sector of each biofilm plate. The spots were allowed to soak into the agar for approximately 30 minutes before the plates were inverted and incubated at the appropriate temperature for 48 hours. Biofilms were assessed by measuring their size and characteristics (cohesiveness, adhesiveness, etc.).

Results

Motility

Motility data was collected by measuring the diameter in pixels. The same scanner and imaging settings are applied to all scans. ± IPTG plates are scanned side by side.

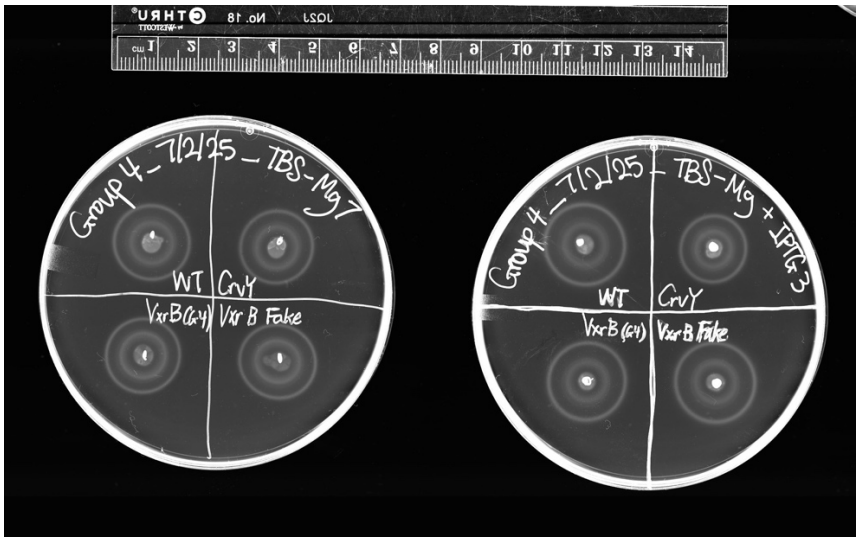


Figure 3. Example Motility Image Scan

The following motility data is at the 4-hour point. A clearer pattern is shown compared to the 3-hour point. We also calculated the average for each strain to obtain a more reliable result: WT and CrvY is measured on every single plate; VxrB G4 is measured on two out of three plates with the same condition; Other strains are measured only once. Additionally, we collected partial data during the experiment on the previous day, at the 4-hour point: WT, CrvY, and VxrB G4 for twice, VxrA and VxrB G4 for once.

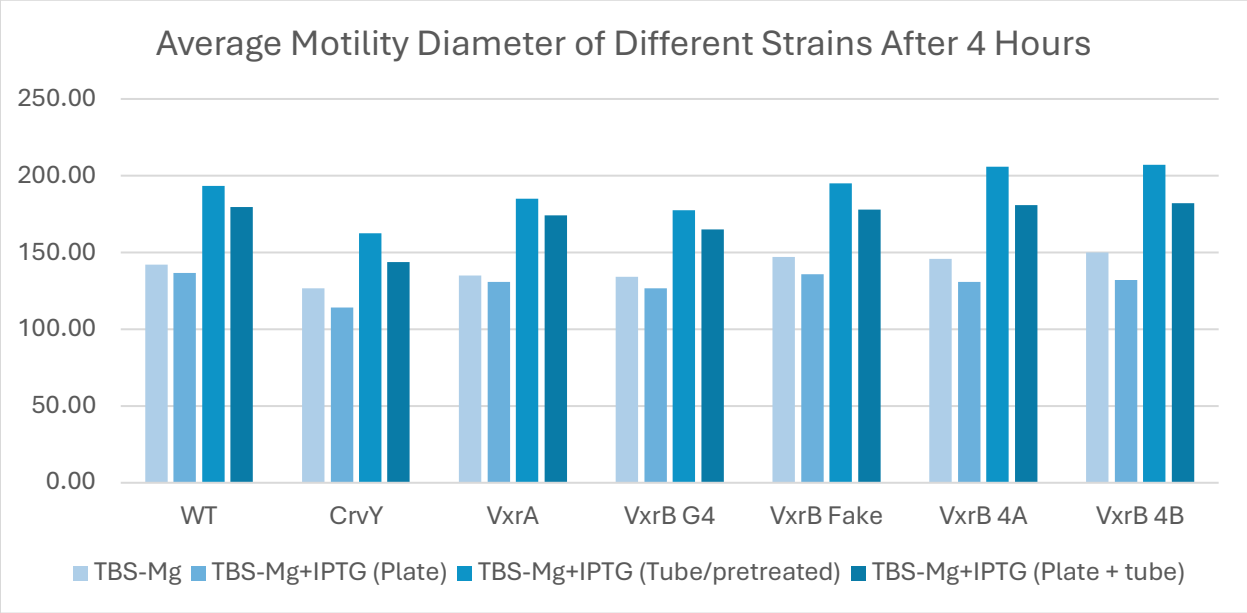


Figure 4. Average Motility Diameter of Different Strains After 4 Hours. No notable difference other than CrvY.

A similar pattern is displayed among every strain: TBS-Mg+IPTG (Tube/pretreated) has the largest diameter, followed by TBS-Mg+IPTG (Plate + tube), TBS-Mg, with TBS-Mg+IPTG (Plate) with the smallest diameter. While comparing among different strains, CrvY have a slightly smaller motility diameter compared with other strains as expected, however, WT and experimental groups does not have a significant difference.

To visualize the results better, we compared strains with the respective control groups (WT and CrvY) on the same plate. The difference in percentage to the control groups is calculated. For control groups under the same conditions, due to multiple spotting on different plates, we also calculated their average and standard deviation, displayed using error bars.

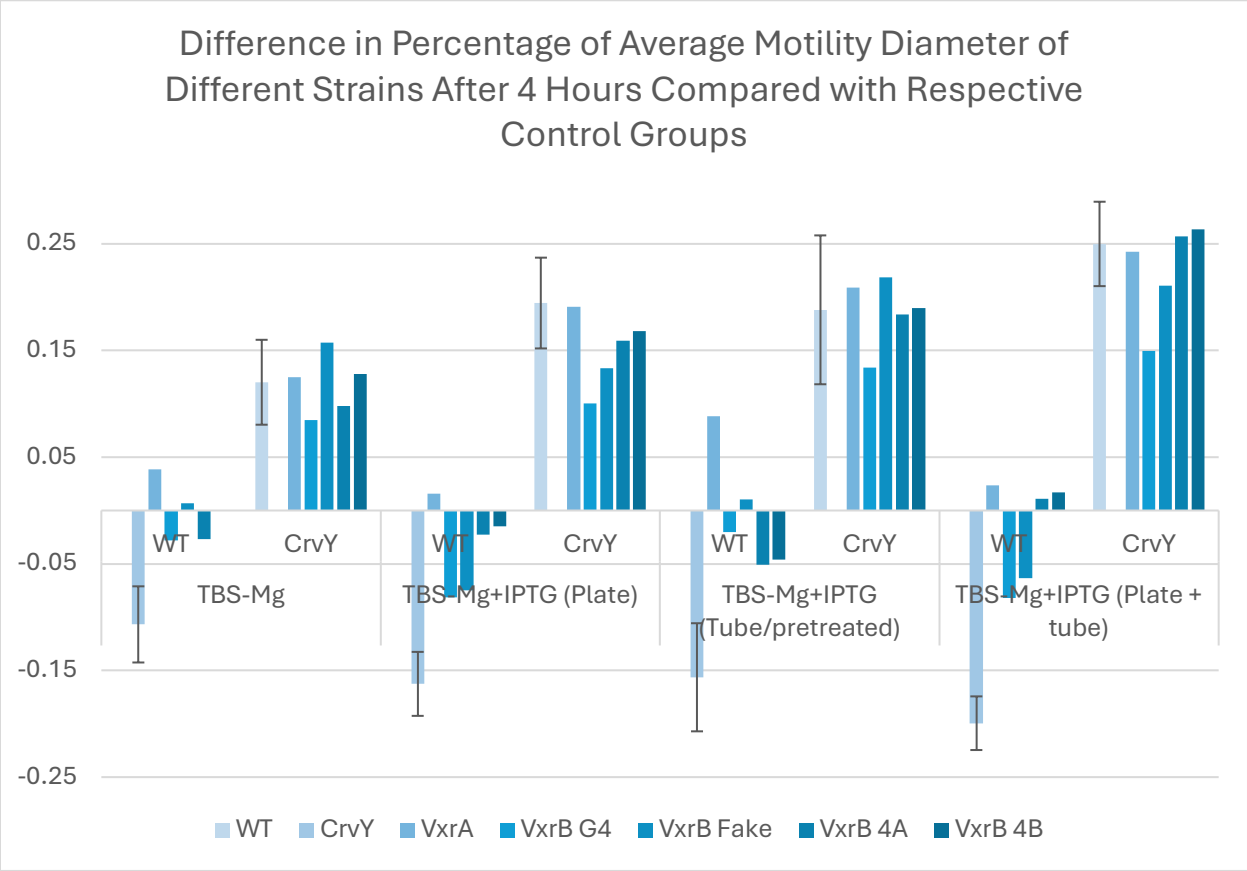


Figure 5. Difference in Percentage of Average Motility Diameter of Different Strains After 4 Hours Compared with Respective Control Groups.

Figure Explanation: For example, under the TBS-Mg conditions, the bin clusters on the left named WT, all strains' diameter is compared with WT using percentages: $\left(\frac{\text{Actual}}{\text{WT}} - 1\right) \times 100\%$. As a result, the left-most WT strain compared with WT gets 0% of difference.

For confirmation, all CrvY strains have a lower percentage compared to WT, while variations of both strains do not have a statistical significance, suggesting that the experiment proceeds correctly. However, the changes in experimental groups are still not very notable.

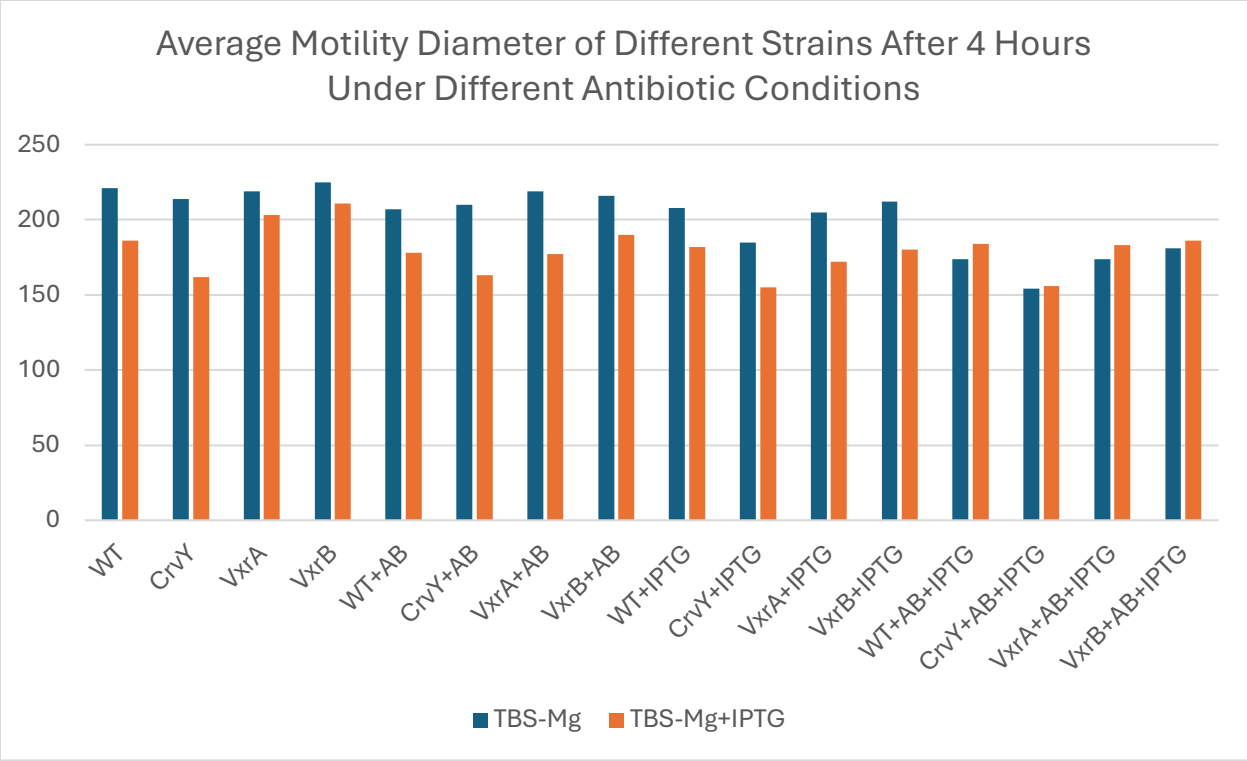


Figure 6. Average Motility Diameter of Different Strains After 4 Hours Under Different Antibiotic Conditions.

From the data above, antibiotics (Cephalexin) slightly reduce the motility results, however, the changes are not remarkable. IPTG alone has similar effects, while both IPTG and antibiotics does not yield a greater difference.

Biofilm

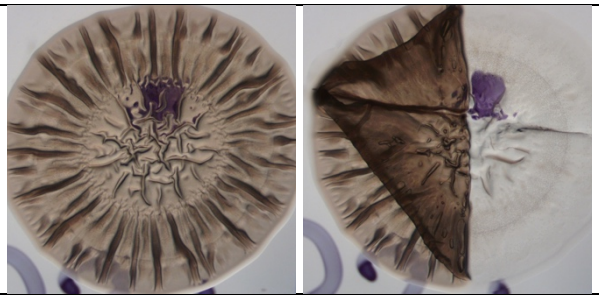
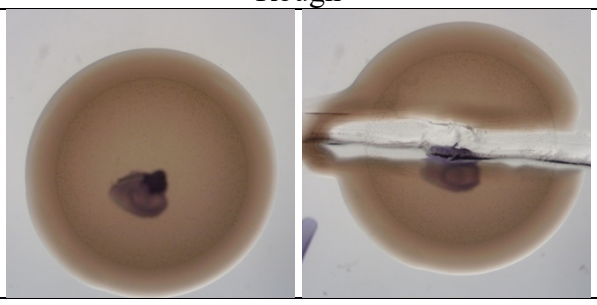
BinK	
Biofilm	Rough
	
$TBS+CaCl_2$ $TBS+CaCl_2$+IPTG $TBS+CaCl_2$+PABA TBS+ $CaCl_2$+PABA+IPTG	TBS Only TBS+IPTG

Figure 7. BinK Phenotype Under Different Environmental Conditions.

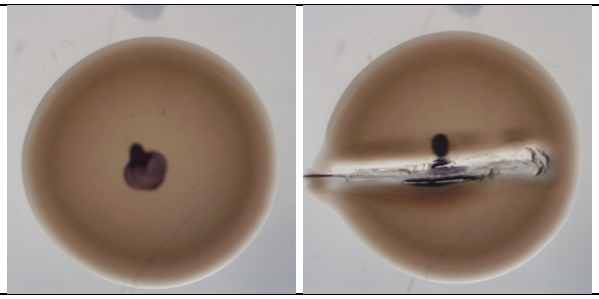
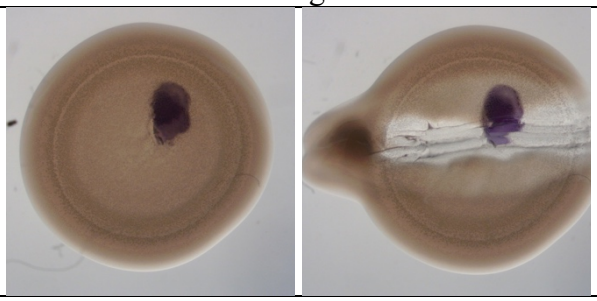
WT/VxrA/VxrB G4/VxrB 4A/VxrB Fake/BcsA	
Smooth	Rough
	
TBS Only TBS+IPTG TBS+$CaCl_2$+PABA TBS+$CaCl_2$+PABA+IPTG	TBS+$CaCl_2$ TBS+$CaCl_2$+IPTG

Figure 8. Phenotype of Strains Other Than BinK Under Different Environmental Conditions.

We conducted biofilm assay on the following strains: WT, BinK, VxrA, VxrB G4, VxrB 4A, VxrB Fake, VxrB Insertion, and BcsA. For BinK, a control group to expect biofilm to form, all strains with $CaCl_2$ formed wrinkled and cohesive biofilm; while others only formed a rough phenotype with particles inside the colony. For the other strains, only conditions with $CaCl_2$ and without PABA formed the rough phenotype; other conditions do not contain any particles.

		WT	BinK	VxrA	VxrB G4	VxrB 4A	VxrB Fake	VxrB Insertion	BcsA
24°C	TBS Only	S	R	S	S	S	S	S	S
	TBS+$CaCl_2$	R	B	R	R	R	R	S	R
	TBS+IPTG	S	R	S	S	S	S	S	S
	TBS+$CaCl_2$+IPTG	R	B	R	R	R	R	S	R
	TBS+$CaCl_2$+PABA	S	B	S	S	S	S	S	S
	TBS+ $CaCl_2$+PABA+IPTG	S	B	S	S	S	S	S	S
28°C	TBS Only	S	R	S	S	S	S	S	S
	TBS+$CaCl_2$	R	B	R	R	R	R	S?	R
	TBS+IPTG	S	R	S	S	S	S	S	S
	TBS+$CaCl_2$+IPTG	R	B	R	R	R	R	S	R
	TBS+$CaCl_2$+PABA	S	B	S	S	S	S	S	S
	TBS+ $CaCl_2$+PABA+IPTG	S	B	S	S	S	S	S	S

Legend: S=Smooth, R=Rough, B=Biofilm

Table 1. Phenotypes of All Strains Under Different Environmental Conditions.

By combining all data in the table, we can make the following assumptions. The rough particles may be a precursor to Biofilm. To approach the pre-biofilm stage, $CaCl_2$ is necessary, while PABA might inhibit its formation. VxrB Insertion, as an "outlier", displays a smooth phenotype under every condition, which suggests that the insertion induced mutated expression of SYP/cellulose. Only the BinK strain showed cohesive biofilm properties after 48 hours at 24°C and 28°C, while other samples lacked biofilm/cohesion.

Analysis

When examining our biofilm results, every sample, other than the BinK mutant, did not present a cohesive property after incubation at 24°C and 28°C for 48 hours. There are numerous reasons for what might cause this–a dead or inactive cell culture being used for the assay, a poorly

prepared Stock solution to grow the cell culture, contamination in various stages, miscalculated dilutions, etc.

Among them, after a close examination of our experimental procedures and the intermediate results from similar experiments, we ended up concluding that the most likely reason our samples did not demonstrate a cohesive biofilm property was insufficient incubation time.

In a relevant study, Dial et al. (2021) were able to successfully grow Vibrio fischeri biofilm that demonstrated a cohesive property under the same conditions as our experiments, but in an extended incubation time. Dial et al. (2021) examined their biofilm 3 separate times: 72 hours and 96 hours after incubation, and post-disruption. We examined our biofilm only twice, once 48 hours after incubation and the other right after disruption. When comparing our sample images to those from Dial et al. (2021), we observed that, although examined 24 hours earlier, our samples displayed similar phenotypic traits, seen as the mold-like dots on the biofilm.

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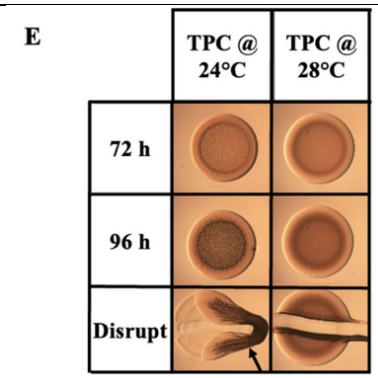
<td> 
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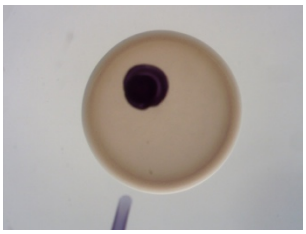
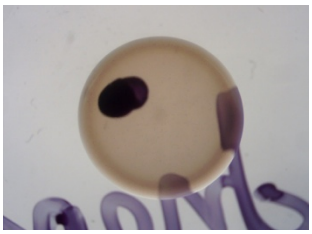
<td>  </td>
<td>  </td>
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<td>Image credit: Dial et al. (2021)</td>
<td>24°C 48hrs</td>
<td>28°C 48hrs</td>
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Figure 9. Biofilm Results Comparison

It can be reasonably assumed that with longer incubation time, our Vibrio fischeri sample would yield similar, if not the same, result on biofilm cohesive property. As for the mutants' effect on motility, though inconclusive on our sample images, they do not affect motility in any statistically significant way.

Conclusion

Our findings suggest that the VxrA and VxrB regulatory elements do not significantly impact cell shape or motility in Vibrio fischeri under the tested conditions. However, the appearance of a bumpy colony phenotype may indicate early-stage changes related to motility or biofilm initiation.

While V. cholerae exhibits dramatically increased biofilm production with $CaCl_2$+PABA, V. fischeri displayed a more modest biofilm response, potentially due to species-specific media requirements or environmental factors.

On the other hand, our triparental mating experiments failed to yield colonies, possibly due to reduced strain viability, suboptimal media conditions, or differences in PABA concentration. Although antibiotic stress is known to activate Vxr systems in V. cholerae, its role in V. fischeri remains unclear and may differ from other species.

Overall, the modest biofilm phenotype and failed indicates the need of further optimization of experimental conditions, such as media composition, incubation time (72–96h), and chemical pretreatments, to better understand biofilm regulation and gene transfer efficiency in V. fischeri.

Acknowledgements

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