

Characterization of the iridescence and motility mutants in *Tenacibaculum sp. strain ECSMB88*.

Nadia Herrera

California Institute of Technology

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Introduction

Since the establishment of the *Tenacibaculum* genus with the discovery of two distinct species in 2001, the number of species added to the genus has greatly increased to 23 individual species (1). The distinct *Tenacibaculum* species have been reported as isolates from many parts of the world—from China to Peru-- as parts of marine organisms, rocky shores, and recently, deep sea sediments (2-7). *Tenacibaculum* was initially identified as originating from marine sponges, but the genus was quickly populated by species which were being isolated from fish pathogens (3,4,6,7). It was not until more recently that a group started identifying a “glitter-like” iridescence in the *Tenacibaculum* genus, as originated by studies on *Cellulophaga lytica*, which later developed means for enrichment for iridescent *Tenacibaculum* isolates (2,8-10).

Iridescence, however, had not gone unnoticed and had been described in the literature in reports, as early as 1950 (11). A challenge in presenting iridescence instances in a bacterial isolate was accompanied by the lack of color photography which could be used in those reports. This resulted in varied means by which authors reported and described the characteristic of iridescence, and as such, it has remained a relatively understudied facet of microbiological physiology. Interestingly, aside from iridescence, bacteria which have this phenotype also utilize gliding motility. Gliding motility has been much less studied than flagellar motility, and as such remains another understudied facet of microbial physiology (12). In recent literature, both gliding and iridescence have been studied in *Cellulophaga*, *Tenacibaculum*, and *Aquimarina* (8).

An iridescent *Tenacibaculum* isolate, corresponding to *Tenacibaculum sp. strain ECSMB88*, has been isolated by a student in the 2017 cohort of Microbial Diversity, Melissa Kardish from the Marine Biological Laboratory’s Stony Beach in Woods Hole, Massachusetts. Intriguingly enough, *ECSMB88* was previously isolated from the East China Sea Biofilm by Yang, J.L., and Guo, X.P. in the Fisheries and Life Science institution in China, as stated in their deposition, KU982641, in the GenBank database. This iridescent *Tenacibaculum* isolate readily grows gliding colonies which show iridescence upon the first 12 hours of incubation at 30°C. As such, this particular isolate presents a model organism in which to study genes behind gliding and iridescence using observations on whole colonies. This report presents 7 mutants to which defects in gliding motility and iridescence were introduced by use of a chemical mutagenesis

screen using Methyl Methanesulfonate to modify the DNA in *Tenacibaculum sp. Strain ECSMB88*.

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Materials and methods

Isolation of *Tenacibaculum sp. Strain ECSMB88*

Samples for isolation were collected by Melissa Kardish from Stony Beach in Woods Hole, Massachusetts at 41.529 °E, 70.674 °N, at 8 A.M. during the low tide. A seagrass root was collected from the east end of the beach, next to the jetty. After collection, the seagrass root was stored in seawater until it was transported to the lab. Once in the lab, the sample was subjected to thorough mixing using the vortex. The resulting supernatant was serially diluted and plated onto Seawater complete medium plates and incubated at 30 °C. The sequence of the sample was found by doing 16s PCR with primers 8F and 1391 R, as described before (13). The PCR product was submitted for sequencing and the result was aligned using NCBI BLASTn. A phylogeny tree comparing the relation of the top 10 hits of 16s RNA sequencing result was made using a MUSCLE alignment followed by use of MEGA7 with default parameters to generate a tree based on maximum likelihood based on previous work (14,15).

MMS Mutagenesis

Methyl methanesulfonate (MMS) was acquired from Sigma-Aldrich, and used at a final concentration of 40 uM in all reaction. 10 uL of a liquid overnight culture of *Tenacibaculum sp. Strain ECSMB88* was used to inoculate a 5 mL culture in Seawater complete medium and was grown for around 7 hours or until it reached to an $OD_{600nm} = 0.5-0.8$, so as to obtain the organism in exponential phase. Once exponential phase was reached, a 1mL aliquot of cells was removed from the culture and subjected to a final concentration of 40 uM MMS (4.44 uL of 99% MMS into 1 mL of cells) for a total of 5 minutes at 30 °C. The completed reaction was centrifuged using a tabletop centrifuge operating at 12,000RPM for 2 minutes. Supernatant was discarded and the remaining colony was re-suspended with seawater complete medium. The re-suspension was serially diluted up to 10^{-8} . A total of 80 plates were used for spreading the cells, to distribute the number of cells attained and screen every dilution for the emergence of individual colonies. To distribute the cells spread, 100 uL of inoculum was spread in each of 20 plates per dilution: 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} . An additional seawater complete medium plate was inoculated with wildtype *Tenacibaculum sp. Strain ECSMB88*, so as to analyze wildtype colonies. After a 2-day incubation period at room temperature (~ 20 °C) colonies were analyzed and picked for further analysis.

Mutant selection and plating analysis

Initially, colonies were picked on the basis of three visible phenotypes, loss or iridescence, loss of motility, or gain of motility. In the latter case, the colony would appear as more diffuse than a wildtype standard, whereas loss of motility would be manifested in a colony which lost the irregular edges and formed a less motile and compact arrangement. Loss of iridescence would be analyzed by visually inspecting colonies while tilting the plate to allow

light to shine through the colonies under inspection. From the initial screen, 34 colonies were picked, and streaked for isolation to verify the phenotype. Once verified, 6 mutant colonies were used for subsequent analysis on seawater complete medium supplemented with 1% paper mate ink. Each colony was plated by pipetting 2-5 uL of either re-suspended colony in seawater complete medium, or liquid culture. The resilience of the mutation was further observed by plating the cells on ½ diluted seawater complete medium plates with 1%, 1.5% (same as regular plates), 2%, and 2.5% agar concentrations.

Stereomicroscopy

Stereomicroscopy images of individual colonies on plates were taken using a Zeiss SteREO Discovery V.8 microscope supplemented with an external light box for angled illumination of the iridescent colonies.

Spectroscopy

As a light source, we used an OSRAM M11 Halogen light bulb 8V/20W. The light energy was directed in a ZEISS mobile fiber and focused by a microscope condenser lens with an aperture of 1.25 under an angle of 38° onto the horizontally positioned colony on a plate. Iridescent light was collected into a glass fiber mounted at a distance of 1cm vertically above the object and connected to a Spectral Evolution SR-1900 Spectroradiometer SN 15782C0. Spectra were recorded and analyzed by the DARWIN software of Spectral Evolution (1 Canal Street, Unit B1, Lawrence, MA 01840).

Light microscopy

Liquid mount slides were prepared shortly before imaging. Light microscopy images of individual cells from liquid culture were taken on a Zeiss Axio Imager 2 microscope, using 100x magnification and a phase contrast plate for ease of visualization.

Scanning electron microscopy

Whole colonies which had been growing for 12 hours were dehydrated for scanning electron microscopy (SEM) studies utilizing an osmium tetroxide and glutaraldehyde fixation method, followed by dehydration with ethanol and hexamethyldisilazane (HMDS). Dehydrated colonies were then mounted onto a specimen stub using double sided carbon tape. A 10nm coat of platinum was applied using a Leica sputter coater. For a detailed protocol, please see the end of this report. Imaging of the samples was done on a SUPRA™ 40VP Field Emission SEM, equipped with GEMINI column technology supplemented with the Thermo Noran System SIX EDS.

Results

Phylogeny tree based on 16S rRNA sequencing

The phylogeny tree (Figure 1) indicated that our isolated has a close relation with *Tenacibaculum sp strain ECSMB88*, and *Tenacibaculum mesophilium strain HMG1*. Due to the similarity of these results, we have opted for using the name of the first hit in the entirety of this report. As such, the isolated strain is referred to as *Tenacibaculum sp strain ECSMB88*.

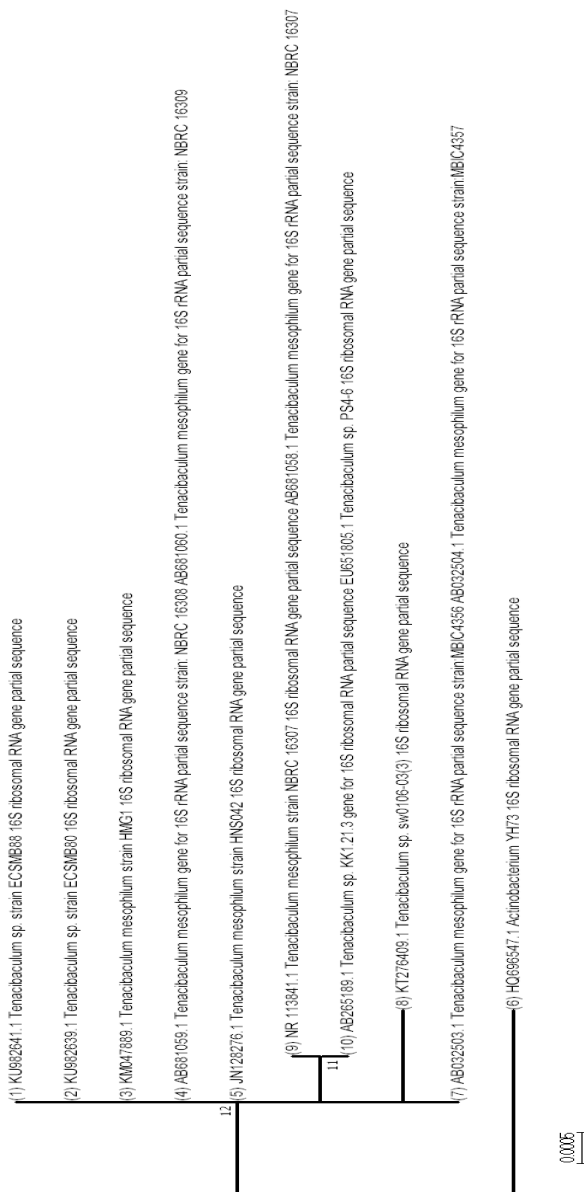


Figure 1- Phylogeny tree calculated from the top ten hits of 16s rRNA sequencing of our isolate. The phylogeny tree displays a comparison of 10 hits from the NCBI BLASTn results of 16s rRNA sequencing data obtained from our isolate. The scale bar indicates the branch distance scale.

First round of mutants selected

The first series of mutants were selected individually from an 80 plate array. Colonies that spread out relative to a wild-type comparison were considered to be gain of gliding mutants and, and re-struck on seawater complete medium, as shown in blue boxes in figure 2. On the other hand, colonies which appeared as loss of gliding mutants had very round colonies lacking an irregular edge, these colonies were picked and are shown in orange boxes in figure 2. Finally, colonies which appeared to lose iridescence or change iridescence from the wildtype green/yellow hue were picked for further analysis, and are shown as yellow boxes in figure 2.

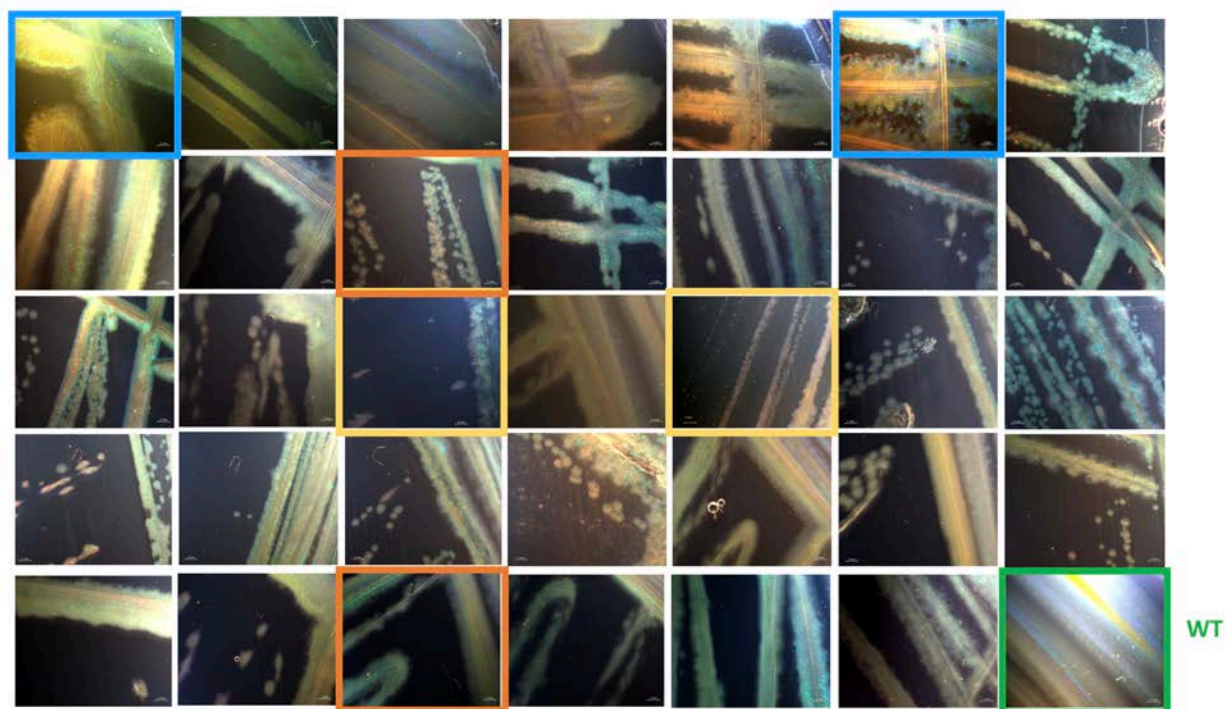


Figure 2- Stereomicroscopy images of mutants selected for analysis by re-streaking of a single mutant colony. Instances of gain of gliding, loss of gliding, and loss of iridescence are highlighted in blue, orange, and gold boxes, respectively.

Analysis of selected mutants from first round

To understand the physiological changes behind the different physical observations at the colony level, the mutants of interest were plated on seawater complete medium supplemented with ink. The black ink showed the striking difference in iridescence as the colony grows onto the plate, with green iridescence typically residing in the center of the colony and red iridescence residing on the edges of the colony (Figure 3). In our mutations of interest, this iridescence was only changed in mutants 4 and 5, which had been highlighted as loss of iridescence mutants in the screen illustrated in figure 2. Once a basal phenotype was observed, the colonies were plated on low-nutrient medium, $\frac{1}{2}$ seawater complete with the same amount of agar as is used in typical seawater complete medium plates, 1.5%. These plates

showed that with lower nutrients, the edge of the gliding colonies was spread widely while maintaining a corrugated shape, as seen in wildtype, and mutants 1, 2, 4, and 5 (Figure 3).

On the other hand, mutants which had been identifying as having a loss of gliding motility were spread out, but had evenly circular edges as seen in mutants 3 and 6 (Figure 3). In addition, it was clear that colonies 3 and 5 had strikingly different organization than the rest, highlighting a circular colony surrounded by a thinner defined ring and tapered with a thinner layer of iridescent cells. This phenotype was much reduced when analyzing the same colonies in $\frac{1}{2}$ seawater complete and 1% and 2.5% agar (figures 5-6). A striking observation was that in $\frac{1}{2}$ seawater complete medium with 1% agar, most of the colonies spread thoroughly, and even displayed loss of iridescence in mutants 2 and 5 (figure 5). Intriguingly, mutant 3 did not spread thoroughly, but instead remained in a small and circular colony shape (figure 5). Lastly, upon increasing the agar concentration. In $\frac{1}{2}$ seawater complete medium to 2.5%, iridescence was very distinct from the other conditions, and a green hue was observed on the outside of wildtype, and mutants 1-3, and 5-6 (Figure 6). On the other hand, the mutant which had been selected for a loss of iridescence, mutant 4, was pigmented completely differently and displayed very little iridescence amidst a red hue (figure 5). Collectively, the effect of agar concentration and nutrient availability greatly impacts how the mutations manifest themselves. This indicates that it is critical to “make nice with your bug” and plate it on multiple media in the pursuit of investigating the effects of a mutation.

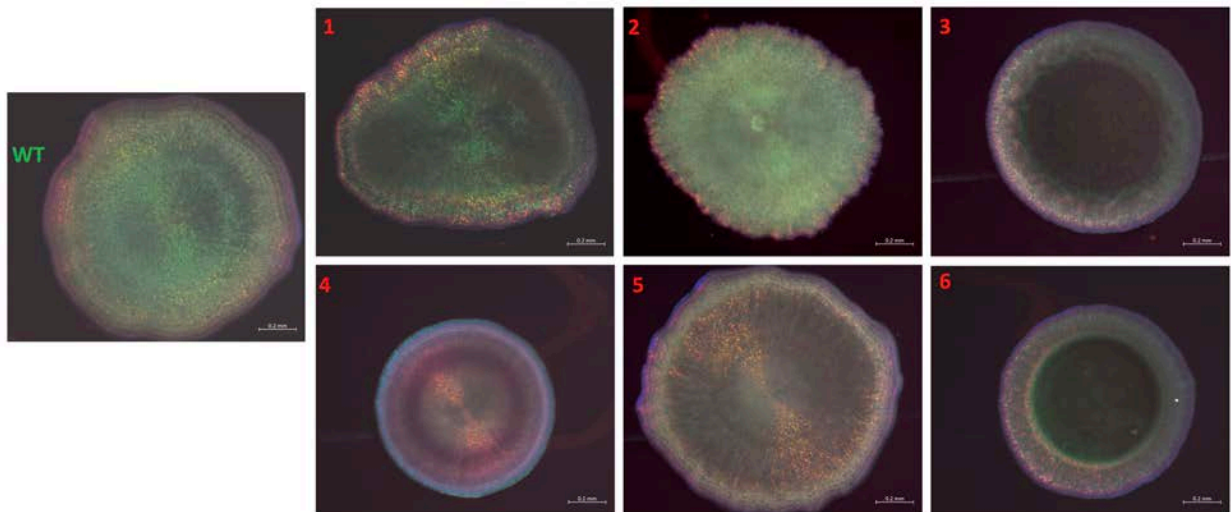


Figure 3- Colony comparison in seawater complete medium, using 1.5% agar. Wildtype is displayed on the left-hand panel, whereas the selected mutants are on the right-hand panel. Iridescent properties are highlighted by the use of black ink on the plates, demonstrating the variability of colors in different regions of the colony.

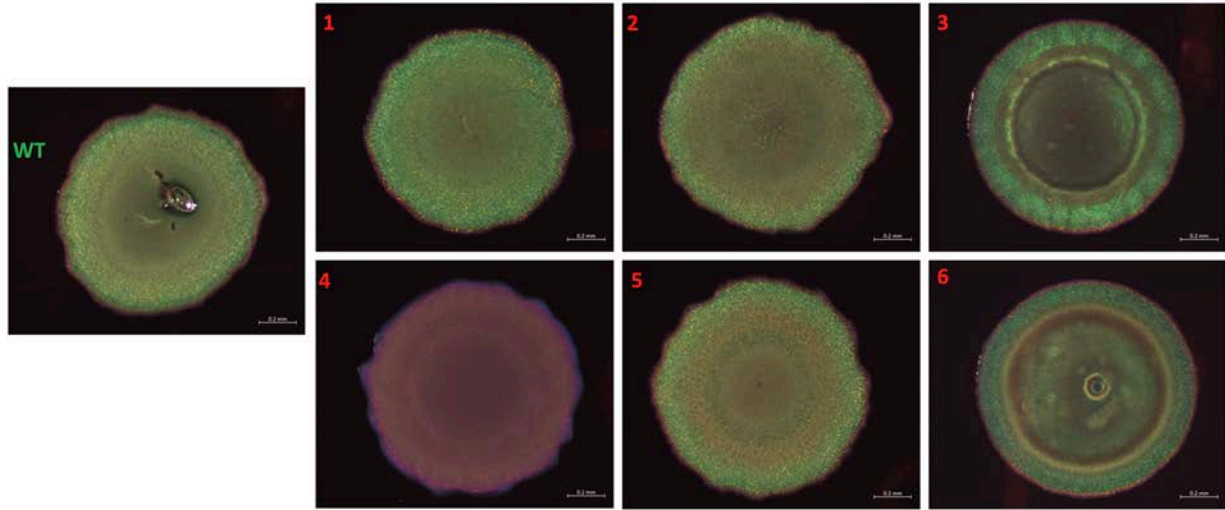


Figure 4- Colony comparison in seawater complete, using $\frac{1}{2}$ concentration of medium nutrients while maintaining the rigidity of the agar from regular seawater complete plates. These plates highlight additional gliding in all of the mutants analyzed, with defined changes in colony morphology in mutants 3 and 6.

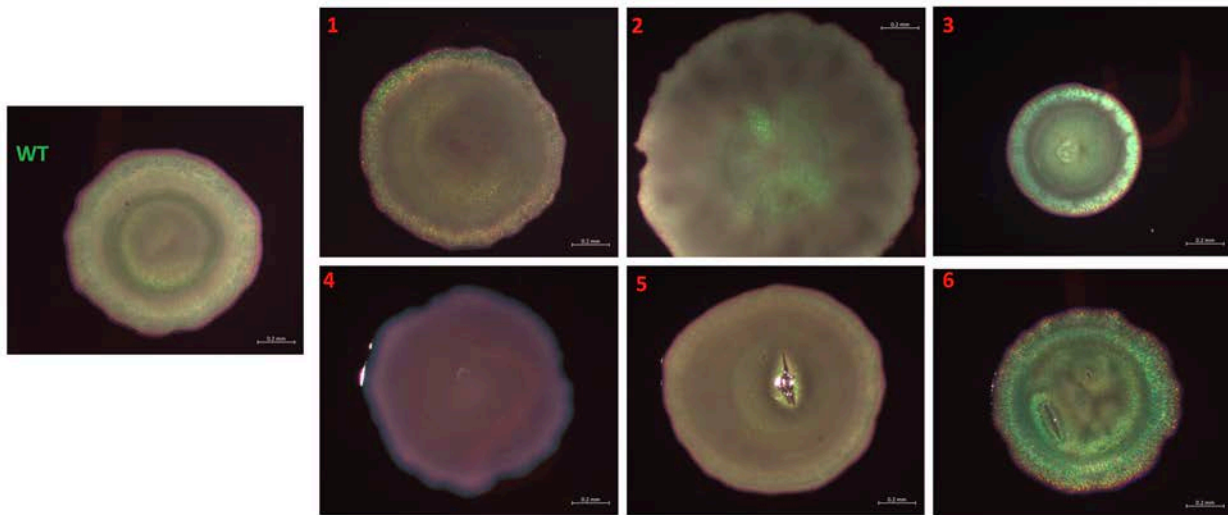


Figure 5- Colony comparison in seawater complete, using $\frac{1}{2}$ concentration of nutrients in medium and 1% agar. In this medium, colonies which were characterized as gain of gliding were much spread, whereas colony 3 was further established as a loss of motility colony. The iridescent hue in the colonies becomes diminished in wildtype, and mutants 1-2, 4-5.

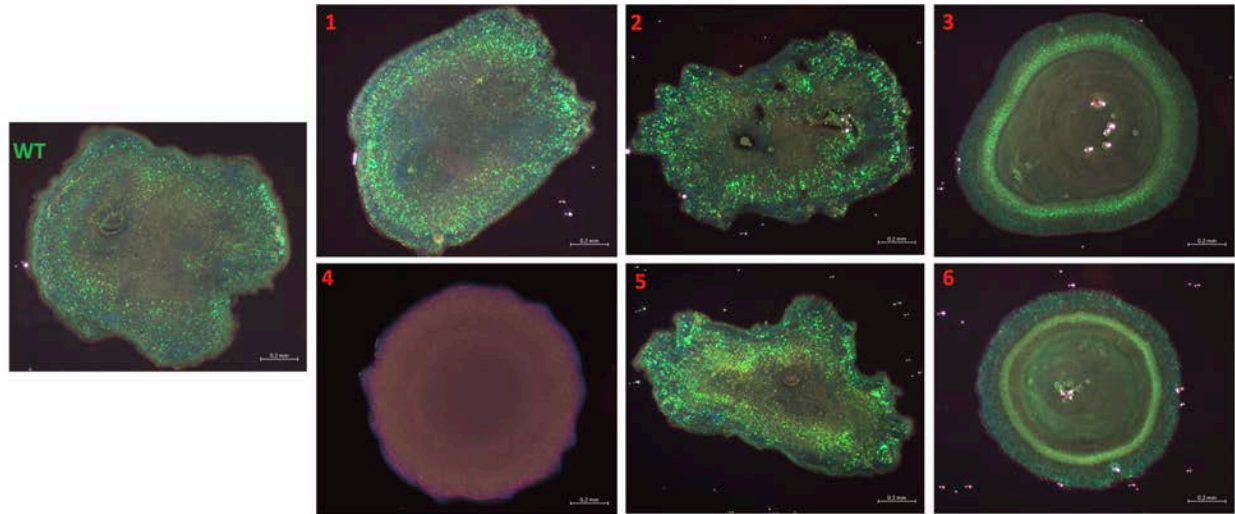


Figure 6- Colony comparison in seawater complete, using $\frac{1}{2}$ concentration of nutrients in medium and 2.5% agar. In this medium, a harder surface prevents most colonies from gliding far. In addition, iridescence is manifested as a green pigment along the edges of most colonies, whereas the loss of iridescence is prominent in mutant 4.

Analysis of cell morphology in individual colonies

Light microscopy on cells obtained from liquid culture of varying mutants showed that they all contained a mixed population of rods with varying lengths, as highlighted in Figure 7. In this analysis, it was evident that a mixed population of rods was homogeneous amidst all cells, with wildtype, and mutants 2, 4 and 5 displaying both an instance of a long rod and a formation of cellular organization on a glass slide. Interestingly, mutants 1-3, and 6 only shared one of those characteristics with wildtype. Since cellular organization appears to be very varied amidst the mutants, it was imperative to analyze their organization at the whole colony level, so as to understand cellular organization at the colony level.

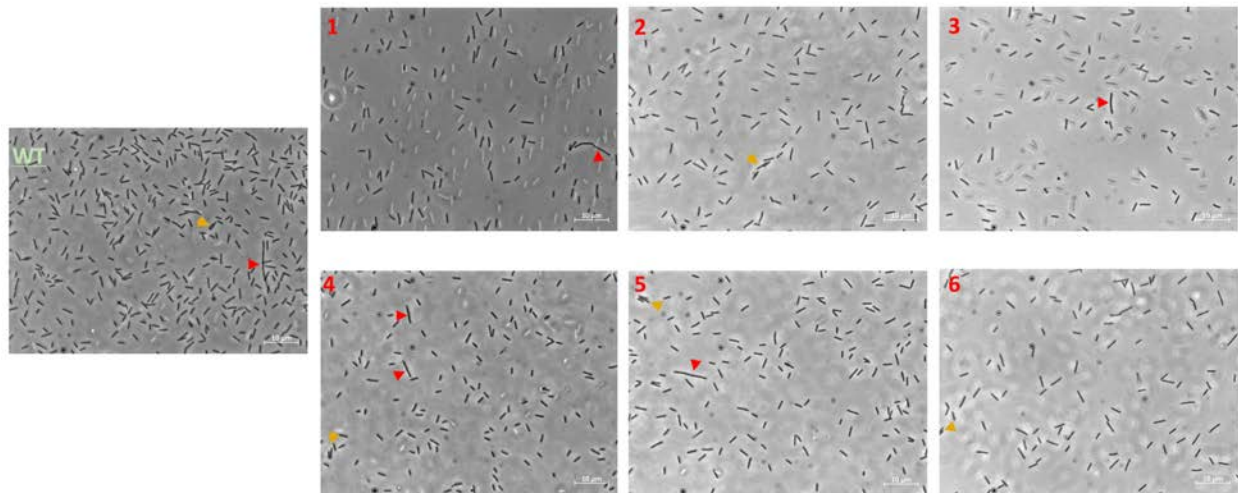


Figure 7- Light microscopy images of the mutants selected for analysis. Elongated cell shapes are highlighted using red arrows, whereas events that appear to be early cellular organization are highlighted using yellow arrows. In general, most mutants share either one or both of these characteristics with wildtype.

SEM analysis of colonies and mutants

SEM analysis of our colonies allowed for the investigation of subcellular organization in the entirety of a colony. In preparation for SEM, it was noted that the iridescent properties of the colonies appeared to survive dehydration, as shown in figure 8. Once analyzed with SEM, immediate colony organization was not evident in any portion of the colony for any of the mutations observed. A homogeneous mixture of cell size in colony formation was observed in wildtype and mutants 1,2, 4-6 (Figure 9). This result likely explains the glitter-like iridescence observed in colonies on agar, as the iridescence is likely the result of a small group of colonies forming an ordered array amidst an array of cells in a given colony. Interestingly, our loss of iridescence mutant displayed an ordered array of cells which seemed to have a periodic structure within individual clusters and within cells themselves (Figure 9). This result is indicative of a structural difference correlated with a change of pigment in a colony. On the other hand, the cells in mutant 3 appear to release a material which is likely exopolysaccharide used in biofilm formation. Characterization of iridescent properties in these mutants, is therefore of interest for further differentiation.

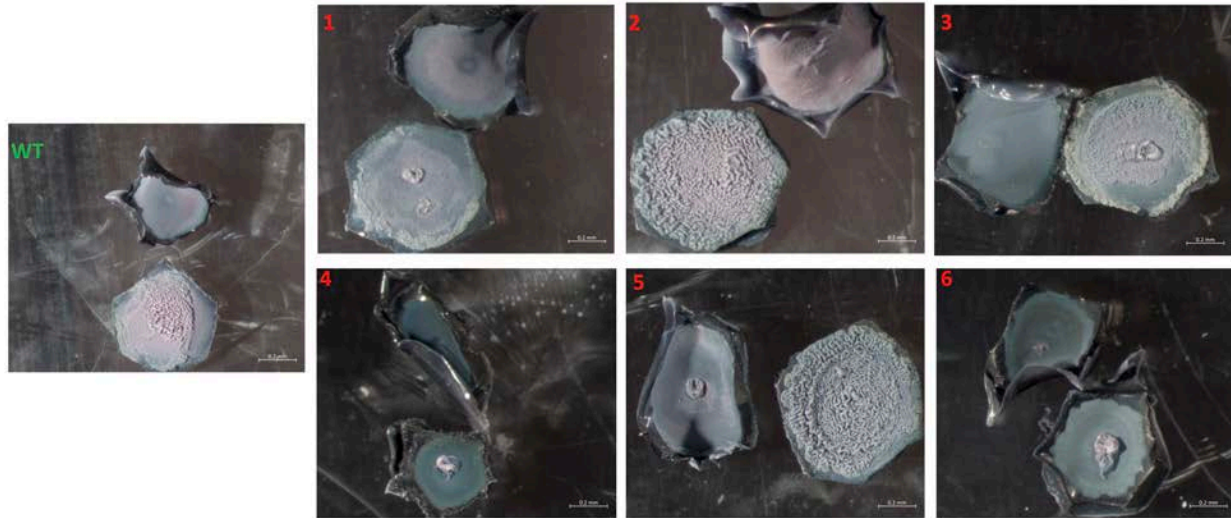


Figure 8- SEM colony dehydration after fixation. Hues of pink and green on the colony surface appear to manifest a retention of colony iridescence through the procedure of dehydration. Colony size was reduced to about 1/10 of the original size and colonies became corrugated from dehydration after preparation.

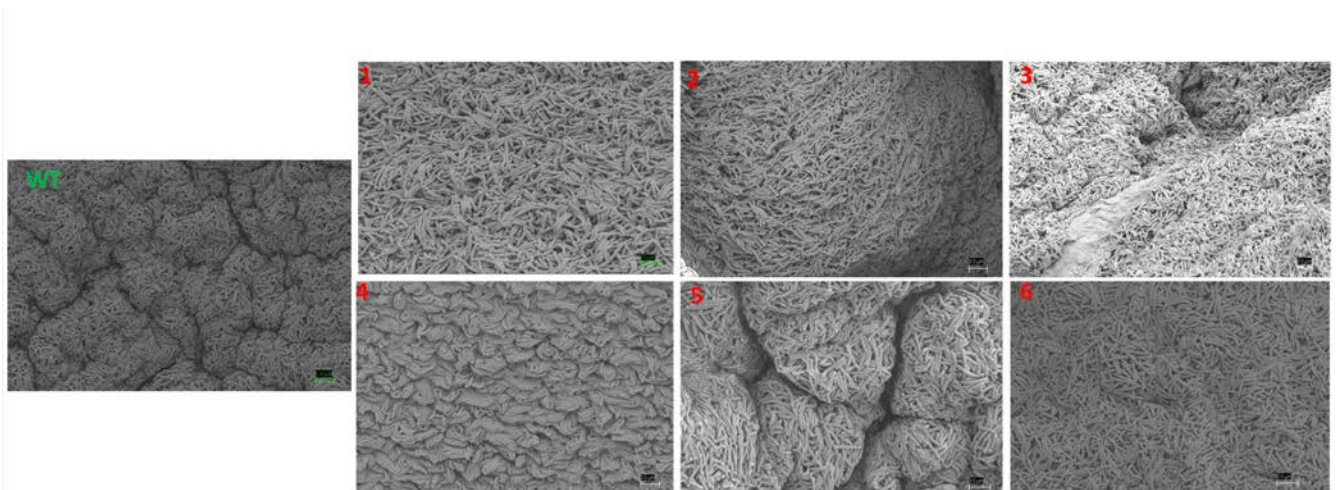


Figure 9- SEM of colonies.

SEM on individual colonies showed a mesh-like structure of cells of varying sizes on the colony, regardless of location of imaging on the colony. Mutant 3 showed a particularly dense bed of extracellular material accumulation which is likely exopolysaccharides.

Spectroscopic characterization of mutants

Spectroscopy studies on individual colonies

Spectroscopy measurements were taken on individual colonies grown on seawater complete plates in black agar. These plates provided a base for reflection measurements off of the iridescence of the colony. We observed that wildtype has strongest reflections at 540 nm, which is right in the green zone of the light spectrum and in accordance with the color that we see by eye. This peak is shifted in most mutants, but the most striking change is in mutant 4,

where the peak moves to the 582 nm range, which is closer to the yellow spectrum and differentiates this colony as having different spectroscopic reflections. Our results suggest that this procedure of measuring iridescence is a way to provide quantification of differences observed in iridescence, and could be a way to truly discern between different iridescence types.

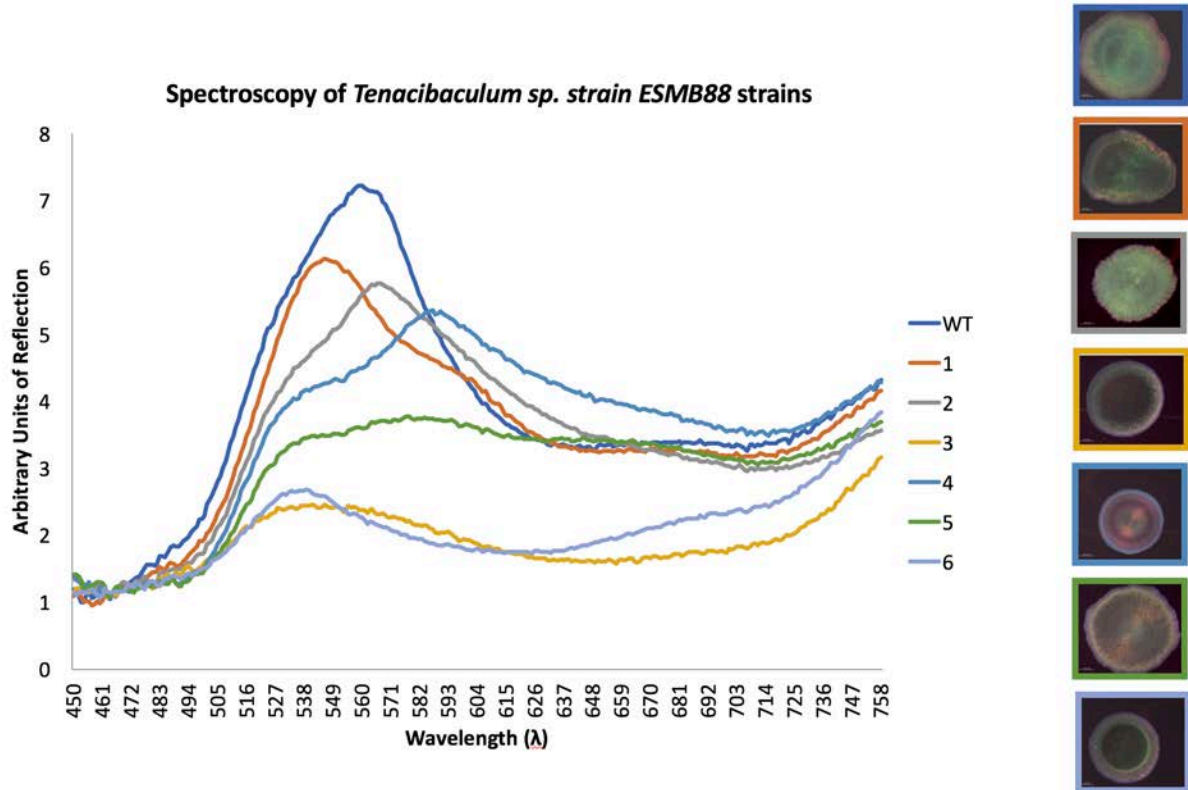


Figure 10- Spectroscopy on individual colonies grown on seawater complete agar plates supplemented with ink.

Discussion

We have isolated colonies that have defects in gliding ability and iridescent color. Studying the genetic basis behind this will shed light on two facets of bacterial physiology which are not well understood to date. Though it is initially observed that colonies form little order, they appear to retain iridescence after desiccation. In addition, their iridescent properties seem to remain unchanged after dehydration. In order to further analyze colony organization at the cellular level, it would be necessary to image a growing colony using an inverted microscope setup, or studying a whole colony using transmission electron microscopy, which could shed light on cellular organization at the ultrastructural level.

This organism has a lot of interesting features which are traceable, and many characteristics and properties left to explore. For instance, throughout this time, it is difficult to use centrifugation at 3400 RPM to pellet a 50mL mass of cells, even after extended spinning. It is likely that these cells are being kept in suspension by their production of exopolysaccharide in the growth medium once they reach lag phase, as this is not observed in cells grown to exponential phase. In fact, studies have been done where a foam bead is seen gliding along a cell in liquid medium, so as to suggest that their gliding motility is active while growing on liquid medium, despite the lack of a surface to glide on (16).

In order to better-understand these phenotypic mutations, it essential to sequence the genome of the colonies of interest, so as to study the genes which are being mutated to create the effects seen. Out of the mutants presented in this study, there are three distinct colonies that should be studied to start to understand patterns of change, and those are wildtype, a loss of motility mutant 3, and a loss of iridescent mutant 5 (Figure 11). Once we are able to map the mutations to the genome, complementation studies will allow us to establish the genes behind the mutations observed. Fortunately, in the last year a genetic system has been established for a similar type of bacteria which can be used for complementation studies (17). Ultimately, these constructs can prove to be the beginning of an investigation towards two bacterial phenotypes which have yet to be thoroughly understood.

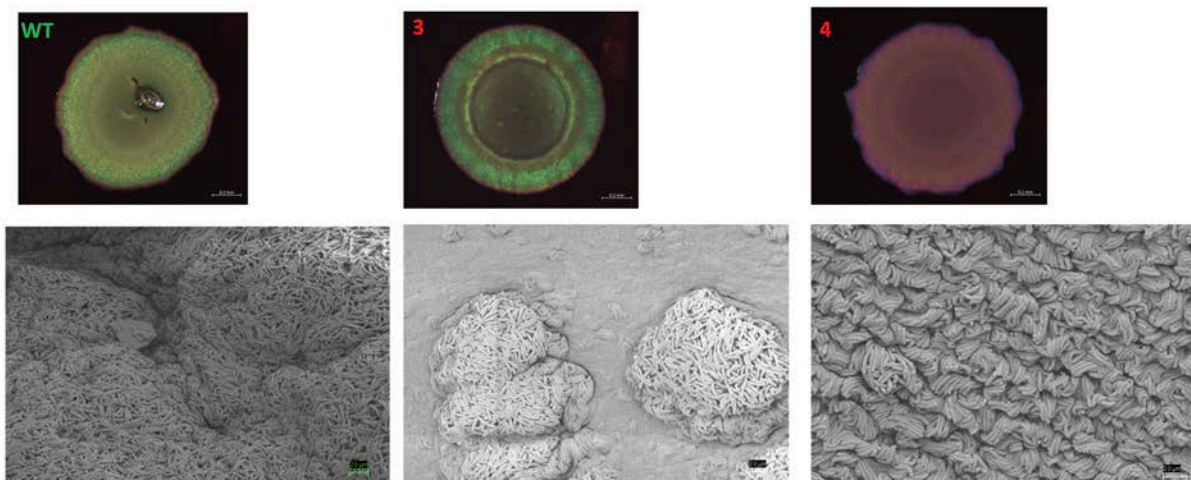


Figure 11- Whole genome sequence candidates, colony and SEM images. Amidst all of the mutations in this study, these three candidates would allow for initial understanding of the machinery behind motility.

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Supplemental information

Protocol for SEM dehydration and fixation of a bacterial colony

- 1- Using a razor blade, cut out the colony from an agar plate.
- 2- Scoop the colony onto the lid of a 1cm diameter petri dish
- 3- Add 500 uL of fixative agent, Osmium tetroxide in water at 1% concentration to the bottom of the petri dish and spread throughout plate evenly.
- 4- Carefully place the colony over the liquid in the bottom of the plate, and add 300 uL of additional 1% osmium tetroxide so as to form a meniscus around the colony. Do not re-suspend colony.
- 5- Allow fixation to proceed for 1 hour at room temperature, cover your dish.
- 6- Pipette out the fixative, and replace with 800 uL of 3.5% glutaraldehyde in 0.1M cacodylate buffer.
- 7- Allow fixation to proceed for 2 hours at room temperature, cover your dish.
- 8- Pipette out the fixative and replace with 800 uL of 0.1 M Cacodylate buffer.
- 9- Allow washing to proceed for 15 minutes, and repeat step 8 for an additional 15 minutes.
- 10- At this point, your colony should be darkened. Pipette out washing solution and add 800 uL of 1% Osmium tetroxide in 0.1M cacodylate buffer.
- 11- All fixation to proceed for an hour at room temperature, cover your dish.
- 12- Remove supernatant and add 800 uL of distilled water.
- 13- Allow washing to proceed for 30 minutes, and repeat step 12 for an additional 30 minutes.
- 14- To dry your sample, pipette gently, use an electric pipette that will allow for dispensing at low speed. Pipette out the water and replace with 4mL of 50% ethanol.
- 15- Allow drying to proceed for 15 minutes.
- 16- Pipette out ethanol, and replace with 4 mL of 70% ethanol.
- 17- Allow drying to proceed for 15 minutes.
- 18- Repeat steps 16-17 with 80%, 90%, 95%, and 100% ethanol (twice for 100%).
- 19- After final step, remove ethanol and replace with 4mL of a 1:2 solution of Hexamethyldisilazane (HMDS): Ethanol.
- 20- Allow drying to continue for 20 minutes.
- 21- Pipette out first solution of HMDS:Ethanol and replace with 4mL of a 2:1 HMDS:Ethanol solution.

- 22- All drying to occur for 20 minutes.
- 23- Repeat steps 21 and 22 with 100% HMDS.
- 24- Once you reach your final step of 100% HMDS, pipette out liquid carefully, and allow colony to dry slowly by leaving the cover on the petri dish overnight. Once dry the colonies will be brittle and much reduced in size.