Abstract

An unidentified *Tenacibaculum* species was isolated from seawater and analyzed through a variety of methods including microscopy, spectrophotometry and genetics. Growth on seawater agar formed brilliant green iridescence and the cells exhibited gliding motility, as seen under a microscope. Transposon mutagenesis and chemical mutagenesis were performed in an attempt to obtain non-iridescent and non-gliding mutants. Chemical mutagenesis using 40 mM methyl methanesulfonate resulted in nine potential mutants. One of these mutants appeared more orange, and contained less green iridescence, than the wild type.

The genome of *Tenacibaculum sp.* was sequenced by Lisa Cohen using the Oxford Nanopore MinION resulting in 3.3 Mbp with 54x coverage. The genome was assembled by Dr. Harriet Alexander using Canu into two contigs. Annotation of the genome via RAST reported 3,332,108 bases, a GC content of 31.9% and 7275 coding sequences. Annotation using Prokka delivered 7163 coding sequences, 40 tRNA and 1 tmRNA.

Future work aims to compare the available *Tenacibaculum sp.* genomes to the genome sequenced here using Anvi'o. Additionally, the genome of the mutant will be sequenced and compare to the genome of the wild-type sequenced via MinION and MiSeq.

1. Background

Iridescence is largely unstudied in bacteria despite its widespread occurrence in various genera (Kientz et al. 2012c). The phenomenon seems to be relatively undefined and poorly understood, at least in the microbial world. Kientz et al. (2013; 2012a; 2016; 2012c) have attempted to characterize and understand the iridescence exhibited by an isolated marine organism, *Cellulophaga lytica*, in comparison to other species known to have “glittering” or “shimmering” qualities. The authors have noted that the unique coloration is often dependent on the angle of illumination and suggest that a structural mechanism is responsible for the iridescence (Kientz et al. 2012c). In addition, the authors suggest that intercellular communication mechanisms may also play a role in the multicellular organization that gives rise to the effect (Kientz et al. 2012c). In an additional study, Kientz et al. (2012b) looked at a variety of abiotic factors, including salt concentration and temperature, to further understand the conditions under which *C. lytica* exhibited iridescence. High temperature (35-40 °C) inhibited iridescence, as did salted (NaCl)
media. Agar concentration also affected iridescence, as well as the agarolytic and gliding motility properties of the organism. Lastly, cell density appeared to affect the color of iridescence, which included shades of yellow, green, blue, red and violet (Kientz et al. 2012b).

An interesting characteristic noted by Kientz et al. (2012a) is that, of the organisms studied from the Bacteriodetes phylum, all iridescent organisms also exhibited gliding motility. The authors hypothesize that iridescence is uniquely tied to gliding motility and cell-to-cell communication is likely necessary for both phenomena (Kientz et al. 2012a).

Not surprisingly, the organism isolated in this study, Tenacibaculum sp. is a member of the Bacteriodetes phylum and exhibits both iridescence and gliding motility. Members of the Tenacibaculum genus are typically isolated from marine environments, and many are associated with pathogenesis in fish (Habib et al. 2014). Colonies of these species are typically characterized as “greenish” and “glistening” but no further mention or analysis of this iridescence is noted (Parte et al. 2011). This study attempted to further analyze the iridescence of the isolated organism using multiple approaches including microscopy and genetics.

2. Methods

2.1 Sample Collection and Isolation

Seawater samples were obtained at the river/ocean interface near Trunk River and housed in within 50-mL Falcon tubes. A serial dilution onto Seawater-complete (SWC) agar media (see below) was performed using 1 mL seawater. In short, 100 μL seawater was dispensed directly onto one SWC agar plate, spread with an L-shaped glass spreader, and allowed to dry (1x plate). Another 1 mL aliquot of seawater was centrifuged for 5 min at 10000 rpm and 900 μL of the supernatant was removed (pellet plate). The pellet was resuspended in the remaining 100 μL supernatant, and this was dispersed and spread onto an SWC agar plate. Lastly, a 1 mL aliquot of seawater was added to 9 mL sterile SWC medium, resulting in a 10⁻¹ dilution. Of this dilution, 100 μL was plated onto an SWC agar plate. Serial dilutions were carried out to 10⁻³ and plated as above. All plates were incubated at 30 °C for one day.

SWC media contains, per liter: 1 L seawater (SW) base, 5 g tryptone, 1 g yeast extract, 3 mL glycerol, and 15 g agar (for solid media). Seawater base consists of the following, per 20 L: 400 g NaCl, 60 g MgCl₂•6H₂O, 3 g CaCl₂•2H₂O and 10 g KCl.

Tenacibaculum sp. was isolated from the 1x SWC agar plate described above. Fresh cultures were maintained by streaking on fresh SWC agar plates.

2.2 Growth and Motility Characterization

Pure cultures were maintained on SWC agar over 35 days at both 30 °C and room temperature (~22 °C). Growth and motility were assessed in both solid and
liquid SWC cultures, as well as on LB agar medium, 5YE agar medium and modified SW LTY 5.5 agar medium.

Modified SW LTY 5.5 agar medium is composed of: 1 L SW base (see above), 0.1 g tryptone, 0.1 g yeast extract, and 15 g agar, per liter. 5YE agar medium consists of: 5 g yeast extract and 15 g agar per liter of water.

For better visibility under a microscope, agar pads were created on microscope slides. These agar pads were made out of SWC agar medium and were the height of a microscope slide. The agar pads were allowed to solidify and *Tenacibaculum* sp. was inoculated across the pad in a single streak and left at room temperature overnight.

A growth curve was created using OD600 absorbance measurements from a Promega Glomax Discover GM3000.

Microscopy was performed using a Zeiss Imager.A2 with an Axiocam 503 mono and an Axio Zoom.V16 with an Axiocam 503 color, both utilizing Zen software.

### 2.3 Culture Preservation

Pure cultures of *Tenacibaculum* sp. were preserved in two cryovials using either glycerol or dimethyl sulfoxide (DMSO). The cryovial with glycerol contained 750 μL pure culture (in SWC medium) and 750 μL 50% glycerol. The cryovial with DMSO contained 900 μL pure culture in SWC medium and 100 μL DMSO. Both cryovials were stored at -80 °C.

### 2.4 Colony PCR and 16S Sequencing

Colony PCR and 16S sequencing was performed from a pure culture of *Tenacibaculum* sp. Briefly, a colony was touched with a sterile 10-μL pipette tip and suspended in 25 μL ALP reagent (alkaline PEG200). The ALP cell suspension was boiled for 5 min in a MJ Research PTC-200 Thermal Cycler. Following boiling, the ALP cell suspension was vortexed briefly and pelleted in a microcentrifuge.

A “master mix” was prepared for PCR amplification. The mix consisted of: 12.5 μL GoTaq HotStart Green, 2 μL forward primer (8F), 2 μL reverse primer (1391R) and 6.5 μL nuclease-free H2O, per reaction. The template DNA was added to the master mix in the amount of 1 μL and pipetted up and down three times. The resulting solution was then placed in the thermal cycler for the PCR to occur. The PCR protocol is given in Table 1.

A gel was prepared by combining 0.5 g agarose and 50 mL deionized water (creating a 1% agarose gel). The gel solution was microwaved to dissolve the agarose, cooled, and poured into an appropriate gel casing (including a gel comb). After the gel solidified, the gel comb was removed and 1x TAE buffer was poured over the top of the gel to submerge the gel entirely. After the completion of the PCR, the gel was loaded. In the first well, the DNA ladder was combined with a loading dye. This was mixed separately on a piece of parafilm, combining 1 μL DNA ladder and 9 μL loading dye. The 10 μL solution was then pipetted into the first well in the gel. Next, 5 μL DNA+master mix solution was added to the adjacent well. No loading dye was required because the GoTaq HotStart Green already contains dye. The gel
was then plugged into a voltage source and ran for 15 minutes at 100 V. After 15 minutes, the voltage was turned off and the gel was visualized on a UV light box. PCR product purification was performed by Kate Hargreaves.

*Table 1. PCR Program*

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>2</td>
<td>95 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>3</td>
<td>55 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>4</td>
<td>72 °C</td>
<td>1.5 min</td>
</tr>
<tr>
<td>5</td>
<td>Cycle steps 2-4 20 times</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>7</td>
<td>12 °C</td>
<td>Hold</td>
</tr>
</tbody>
</table>

2.5 DNA Extraction and Genome Sequencing

DNA was extracted according to the Qiagen Genomic-tip 500/G protocol. Initially, 500 mL SWC medium was inoculated with *Tenacibaculum sp.* and incubated overnight in a shaker at 30 °C.

DNA was amplified and visualized by Dr. Scott Dawson. Library preparation and sequencing was performed by Lisa Cohen using the MinION Mk1B. Genome assembly was performed by Dr. Harriet Alexander using Canu.

2.6 Genome Annotation and Analysis

Genome annotation was performed using both RAST and Prokka (Seemann 2014). Genome comparison between *Tenacibaculum sp.* and other species is currently being conducted using Gepard (Krumsieck et al. 2007) for dot matrix analyses and Anvi’o (Eren et al. 2015) for pangenome analyses.

2.5 Spectrophotometry Analysis

Spectrophotometry of *Tenacibaculum sp.* was performed using the Spectral Evolution SM-1900 Series Spectrometer and the Spectral Evolution DARWin SP Application Software (Version 1.3).

Spectra of colonies were taken using the lowest light setting on the light source. The fiber optic cable for the spectrometer was situated just above the desired colony where the light source was also focused. Blank spectra were taken using a clean agar surface.

2.6 MALDI-ToF Mass Spectrometry

MALDI-ToF mass spectrometry data were taken by Kurt Hanselmann.
2.7 Transposon Mutagenesis

2.7.1 Antibiotic Resistance

Mutagenesis was attempted using *Escherichia coli* as a donor and various available transposons. First, the antibiotic sensitivity of *Tenacibaculum sp.* was tested using SWC agar plates with 25, 50, 100, or 200 μg/mL kanamycin, 25, 50, 100 or 200 μg/mL gentamycin, 25, 50, 100, 200 μg/mL neomycin and 5, 10, 50, 100 μg/mL tetracycline. *Tenacibaculum sp.* was streaked onto half of each plate (plates were shared with another student), and plates were incubated at 30 °C.

2.7.2 Growth on Variable Media

*Escherichia coli* β2155 carrying the pSC189 (mini-mariner) plasmid was streaked onto LB+kan+DAP (50 μg/mL kanamycin and 200 μL DAP; diaminopimelic acid). *Escherichia coli* β2155 carrying the pFAC plasmid was streaked onto LB+gent+DAP (50 μg/mL gentamycin and 200 μL DAP) plates. *Pseudomonas aureofaciens* and *Tenacibaculum sp.* were streaked onto an LB agar plate. Both *E. coli* β2155 and *P. aureofaciens* were streaked from -80 °C freezer stocks.

*E. coli* β2155 containing the pTnTet plasmid and *E. coli* β2155 containing the pSC189 plasmid were streaked onto LB+tet+DAP (10 μg/mL tetracycline and 200 μg/mL DAP) plates. *E. coli* β2155 containing the pTnTet plasmid was streaked from a -80 °C freezer stock.

*Tenancibaculum sp.* and *P. aureofaciens* were streaked on ½-concentrated SWC agar plates (in this medium, only 500 mL SW base were used, with 500 mL deionized water, per liter). *E. coli* β2155 containing the pTnTet plasmid was streaked on ½x-SWC+tet+DAP (50 μg/mL tetracycline and 300 μM DAP).

2.7.3 Conjugation

Cells of *Tenancibaculum sp.* were inoculated into 5 mL SWC liquid medium and kept in a 30 °C shaker overnight. *E. coli* β2155 containing the pTnTet plasmid was inoculated into 5 mL LB liquid medium containing 50 μL DAP and 25 μL tetracycline and incubated at 37 °C overnight. *P. aureofaciens* was inoculated into 5 mL LB liquid medium and kept in a 30 °C shaker overnight.

A total of seven conjugations were conducted. All conjugations were performed on ½x SWC+DAP plates, except for the control 00 plate, which was LB+DAP (see Table 2).

Cells of *Tenancibaculum sp.*, *E. coli* β2155 containing the pTnTet plasmid, and *P. aureofaciens* were washed according the following procedure. Aliquots of 1 mL were removed from each of the overnight cultures and placed into a 1.5 mL eppendorf tube. The 1-mL aliquots were pelleted in a centrifuge. The supernatant was poured off, and the cells were resuspended in ½x SWC (500 μL SWC, 500 μL deionized water) and pelleted again. The supernatant was disposed of and the cells were resuspended in ½x SWC medium.

Following washing, donor and recipient cells were pipetted into 1.5 mL eppendorf tubes according to the conjugations listed in Table 2. Each conjugation was spotted with 20 μL donor+recipient total. The donor+recipient mixtures were pipetted up and down three times to mix, and the 20 μL was spotted into the middle
of a \( \frac{1}{2} \times \) SWC+DAP plate for each conjugation. The plates were dried at room temperature and then incubated at 30 °C for ~24 hours.

After 24 hours, cells were recovered from each conjugation plate by scraping with a sterile loop. The entire “colony” was scraped into 1 mL SWC in a 1.5 mL eppendorf tube. Next, serial dilutions were carried out to \( \times 10^8 \) for each conjugation. This was performed in a 96-well plate. Using a multi-channel pipette, five serial dilutions \((1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8})\) were “dripped” onto two separate plates for each conjugation (10 μL from each dilution is spotted at the top of the plate, the plate is held vertically to allow the drops to travel the length of the plate and then the plates are left to dry on the benchtop). The 1-\( 10^{-4} \) dilutions were plated onto an SWC+tet plate and the \( 10^{-4} \)-\( 10^{-8} \) dilutions were plated onto an SWC plate. The plates were incubated at 30 °C for 24-48 hours, after which the plates were assessed for colony formation. This procedure was conducted three times.

### Table 2. Conjugations

<table>
<thead>
<tr>
<th>Donor Amount (μL)</th>
<th>Recipient</th>
<th>Recipient Amount (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 00 (LB+DAP)</td>
<td>E. coli β2155 w/ pTnTet plasmid</td>
<td>10</td>
</tr>
<tr>
<td>Control 0</td>
<td>E. coli β2155 w/ pTnTet plasmid</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>E. coli β2155 w/ pTnTet plasmid</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>E. coli β2155 w/ pTnTet plasmid</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>E. coli β2155 w/ pTnTet plasmid</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>E. coli β2155 w/ pTnTet plasmid</td>
<td>10*</td>
</tr>
<tr>
<td>5</td>
<td>E. coli β2155 w/ pTnTet plasmid</td>
<td>15</td>
</tr>
</tbody>
</table>

*Cells of E. coli and Tenacibaculum sp. were pelleted together in the washing procedure.

### 2.8 Chemical Mutagenesis

Chemical mutagenesis was conducted using methyl methanesulfonate (MMS) as a mutagen. Initially, a flask containing 50 mL SWC was inoculated with Tenacibaculum sp. and incubated in a 30 °C shaker overnight. Next, the optical density of the Tenacibaculum sp. culture was measured at OD600 using a ThermoScientific Spectronic 200. The culture was diluted to OD ~ 0.3 using sterile SWC and 5 mL was pipetted into five separate 15-mL Falcon tubes. The tubes were transferred to the chemical hood where the mutagen was added. Each tube received a different amount of MMS: 0 (control), 10 mM, 20 mM, 40 mM, 80 mM. The caps to
the Falcon tubes were loosened one quarter-turn and taped in place. The tubes were incubated in a shaker at 30 °C for 38 min.

After incubation, the Falcon tubes were returned to the chemical hood. An aliquot of 1 mL was transferred from each tube to a 1.5 mL eppendorf tube. The cells were washed three times. In short, the cells were pelleted at 11,000 RCF for 1 min, and the supernatant was poured off. SWC was added to each tube and the cells were pelleted again. After the washing procedure, a dilution series was carried out to 10^-9. The dilutions were again drip-plated (see 2.6.3 Conjugation, above) onto two separate SWC plates: 1 to 10^-4 on one plate, and 10^-5 to 10^-9 on a second plate. The dilution plates were incubated at 30 °C overnight.

The chemical mutagenesis procedure was repeated, using solely 40 mM MMS. The protocol was similar to the one above, except that during the washing portion, cells were pelleted at 5000 RPM for 3 min. Additionally, only three dilutions were plated out: 10^-3, 10^-4, and 10^-5 with ten replicates for each dilution set.

2.8.1 Mutant Screen and Analysis

Dilution series plates were visually analyzed for mutant colonies according to either morphology or color. Nine potential mutants were selected for isolation, streaked onto separate SWC plates and stored at 30 °C overnight. Of the nine isolates, one was selected for further analysis. This isolate was streaked for isolation and analyzed via microscopy, spectrophotometry and MALDI-ToF mass spectrometry. A growth curve was created using OD600 absorbance measurements from a Promega Glomax Discover GM3000.

3. Results and Discussion

3.1 Morphology and Growth

Cells are long and rod-shaped (Fig. 1). Length is variable between 2 and 10 μm, averaging around 3-4 μm. Colonies are irregular and spreading. *Tenacibaculum sp.* wild-type colonies display a brilliant green iridescence (Fig. 2), but are initially pale yellow, turning a deeper yellow over time. *Tenacibaculum sp.* mutants also show green iridescence but to a much lesser extent than wild-type colonies and appear more orange (Fig. 2).

Cells of *Tenacibaculum sp.* also exhibit gliding motility.

Growth curves for both the *Tenacibaculum sp.* wild-type and *Tenacibaculum sp.* mutant were created (Fig. 3). The doubling time for *Tenacibaculum sp.* wild-type is 3.9 hours. The doubling time for *Tenacibaculum sp.* mutant is 4.5 hours. The measured absorbances (OD600) become significantly different after 3.7 hours (p < 0.05, t-test).

3.2 16S Sequencing

Performing a BLAST search on the 16S fasta file yielded “*Tenacibaculum sp.* sw0106-02 16S ribosomal RNA gene, partial sequence” as the top hit with 99% identity and 99% query cover.
3.3 Genome Sequencing

Using the assembled contigs, a BLAST search yielded “Tenacibaculum dicentrarchi strain AY7486TD, complete genome” as the top hit with 87% identity and 26% query cover. There are other hits with greater identity but lower query coverage.

Figure 1. Tenacibaculum sp. wild-type (left) and mutant (right) from liquid culture.

Figure 2. Views of Tenacibaculum sp. wild-type and Tenacibaculum sp. mutant colonies at different angles. Top row: incubated overnight at room temperature. Bottom row: incubated overnight at 30 °C. Left column: Tenacibaculum sp. mutant. Right column: Tenacibaculum sp. wild-type.

3.4 Genome Annotation and Analysis

Genome annotation by RAST displays a genome size of 3,332,108 bases, a GC content of 31.9% and 7275 coding sequences. Annotation using Prokka (Seemann 2014) delivered 7163 coding sequences, 40 tRNA and 1 tmRNA.

Dot matrix analysis was used to compare the genome of Tenacibaculum sp. to other Tenacibaculum genomes available online. Analyses were conducted using Gepard (Krumseik et al. 2007). The organisms with the greatest similarity to Tenacibaculum sp. are Tenacibaculum soleae strain UCD-KL19 (Fig. 4) and Tenacibaculum mesophilum HMG1 (Fig. 5).
Pangenomic analysis using Anvi’o (Eren et al. 2015) is still being conducted.

Figure 3. Growth curve for *Tenacibaculum sp.* wild-type (green) and *Tenacibaculum sp.* mutant (orange). The error bars represent one standard deviation.

Figure 4. Dot matrix analysis between *Tenacibaculum sp.* (horizontal) and *Tenacibaculum soleae* strain UCD-KL19 (vertical).
Figure 5. Dot matrix analysis between *Tenacibaculum* sp. (horizontal) and *Tenacibaculum mesophilum* HMG1 (vertical).

3.4 Spectrophotometry

Spectra of *Tenacibaculum* sp. wild-type colonies and *Tenacibaculum* sp. mutant colonies (Fig. 6) were taken for comparison. Absorbance spectra were measured at various locations on a SWC agar plate, in order to fully capture different colony characteristics. A portion of the agar plate that remained uninoculated was used as a blank. Older cultures exhibit greater variation in absorbance intensity at different locations across the plates as compared to younger cultures (Fig. 6).

3.5 MALDI-ToF Mass Spectrometry

Data are still being collected.

3.6 Transposon Mutagenesis

3.6.1 Antibiotic Resistance

*Tenacibaculum* sp. was resistant to both gentamycin and kanamycin up to the highest concentration tested, 200 μg/mL. The organism was also resistant to 25, 50, 100, and 200 μg/mL neomycin. *Tenacibaculum* sp. showed growth at the initial inoculation point on all plates of tetracycline (up to 100 μg/mL), however growth
was to a much lesser extent on the 50 μg/mL and 100 μg/mL plates, compared to the 5 μg/mL and 10 μg/mL plates. Thus, 50 μg/mL tetracycline was chosen for the ensuing conjugations.

Figure 6. Absorbance spectra of *Tenacibaculum* sp. wild-type (left) and *Tenacibaculum* sp. mutant (right). The blank (red line) refers to an uninoculated agar surface. The other lines correspond to different locations containing *Tenacibaculum* sp. **Top:** five-day-old culture. **Middle:** three-day-old culture. **Bottom:** two-day-old culture.

3.6.2 Growth on Variable Media

The growth of *Tenacibaculum* sp. was tested on various media. After two days at 30 °C, growth was apparent on LB agar plates, although to a much lesser extent than growth on SWC agar plates after 24 hours at either 30 °C or room temperature. The organism failed to grow on 5YE agar plates at 30 °C.
3.6.3 Conjugations

The conjugation procedure was attempted three times. The first procedure failed due to the lack of DAP on the conjugation plates. The next two conjugations also failed to produce any exconjugates.

3.7 Chemical Mutagenesis

The effect of four different concentrations of methyl methanesulfonate (MMS) on Tenacibaculum sp. was tested to determine the appropriate concentration to be used for chemical mutagenesis. Of these initial concentrations (10 mM, 20 mM, 40 mM, 80 mM), 40 mM was chosen as the concentration to be used for future mutagenesis experiments based on the number of possible mutant colonies recovered.

Nine potential mutants were identified via microscopy. After streaking for isolation, one mutant (Tenacibaculum sp. mutant) was chosen for additional observation. Growth of the mutant on SWC agar plates was generally more orange than the Tenacibaculum sp. wild-type strain at both 30 °C and room temperature. Further comparison between Tenacibaculum sp. wild-type and Tenacibaculum sp. mutant using various techniques such as microscopy, spectrophotometry, MALD-ToF mass spectrometry.

References


Habib C et al. (2014) Multilocus sequence analysis of the marine bacterial genus Tenacibaculum suggests parallel evolution of fish pathogenicity and endemic colonization of aquaculture systems. Applied and environmental microbiology 80:5503-5514


