My Newfound Love of Microscopy:
Rosette formation in *Rhodobacter capsulatus*

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Introduction

Surface attachment is a dominant and essential feature of bacterial communities in the natural environment. Biofilm, an assemblage of microbial cells enclosed in a self-produced polymeric matrix, is a ubiquitous and dominant mode of existence in nature, with estimates suggesting more than 90% of bacteria existing within biofilms [1,2]. The immobilized, aggregated mode of lifestyle provides bacteria with many advantages for survival and growth; it often facilitates metabolic cooperation, exchange of genetic information, and provides protection against environmental stresses [3,4]. For this reason, bacteria associated with surface-attached communities have been shown to colonize various man-made and medical devices. Considering the importance of this process from an ecological as well as human health perspectives, there has been a growing appreciation to study various determinants of bacterial surface attachment process [3].

A rosette-shaped multicellular growth pattern in which cells are attached to one another by means of polar adhesin (hereinafter referred to as “rosette”) is a frequently observed phenotype across the class of Alphaproteobacteria (Fig. 1) [5]. In addition, rosette formation has been shown to be closely associated with biofilm development [6,7,8]. For example, rosette-forming strains of a marine bacteria *Roseobacter* have been shown to be 13 to 30 times more efficient in attaching to glass and forming biofilm at the air-liquid interface, compared to the strains that do not form rosettes [6]. *Caulobacter crescentus* is another Alphaproteobacterium whose dynamics and control of biofilm formation is well studied. In the case of *C. crescentus*, surface attachment is initiated via means of a polysaccharide-rich, polar organelle known as the holdfast [4]. The formation of holdfast is both a genetically controlled and environmentally triggered process that occurs during the transition from sessile, stalked to flagellated, motile cell types [4]. Cell-to-cell contact upon holdfast development can result in rosette formations in *C. crescentus*.

This study investigated the determining factors and mechanism underlying the formation of rosettes in *Rhodobacter capsulatus*, a non-sulfur phototrophic Alphaproteobacterium closely related to *Roseobacter* and *Caulobacter* (Fig. 1). Different microscopic techniques were applied in combination of various experimental controls. In combination with microscopy-based approach, the chemical nature of the adhesin in *R. capsulatus* rosettes was examined; favorable conditions for rosette formations were identified; and the mechanism for rosette formation was hypothesized through time-lapse imaging.
Figure 1. The phylogenetic tree of Alphatroteobacterial clade. The species known to produce polar adhesins are marked with red stars (★). Modified from Philippot et al. (2010).

Materials & Method

Isolation, Purification and Identification

The studied organism was initially isolated from a pebble collected at the mouth of the Trunk River, Woods Hole, MA. The slimy material on the surface of the pebble was spread on a 5YE agar plate (see Appendix I for composition). After about two weeks of incubation in a 30°C incubator, a reddish purple colony appeared. The pigmented colony was re-streaked on a fresh plate, and the process was repeated over three times to obtain a pure culture.

Once pure colonies were obtained, cells were collected from a colony for 16S rRNA analysis. Cells were first lysed by boiling in 20 ul ALP reagent (alkaline PEG200) for ca. 15 minutes. 2 ul of the boil supernatant was combined with a master solution for 16S rRNA gene amplification. Bacterial forward and reverse primers, 8F and 1391R respectively, were used for amplification.
Prepared sample tubes were placed in a thermocycler for PCR reactions. The master solution composition and detailed thermocycler settings can be found in Appendix II. The PCR product was sent out for sequencing, and the BLAST online search tool was used to determine the sequence identity for 16s rRNA analysis result. The top BLAST search match for the organism was *Rhodobacter capsulatus* SB 1003 strain, with query coverage of 97% and sequence identity of 99%.

**Media Composition**

Four different types of liquid media were used for different experimental purposes: FW-PNSB, SW-PNSB, 25%Ac-PNSB, and 5YE-PNSB media. All four types were modified versions of the FW-AcMet media used for the enrichment of purple non-sulfur bacteria in 2016 Microbial Diversity course (Appendix I). FW-PNSB media contained all components of the FW-AcMet media, except for DCMU and methanol. DCMU (photosystem II inhibitor) was omitted, for there was no longer a need to prevent the growth of anoxygenic phototrophs in the pure culture. Methanol was omitted after confirming the consumption of acetate in the media with HPLC and cell growth under the microscope; this was an attempt to reduce the risk of enriching unwanted methanol-utilizing microorganisms. SW-PNSB media contained the same ingredients as the FW-PNSB media, with the FW base replaced by SW base. Instead of adding 970 ml of deionized water and 10 ml of 100X FW base per liter of media, 980 ml of 1X SW base was used. The major difference between the FW-PNSB and SW-PNSB media was salinity, containing 17.1 mM and 342.2 mM NaCl, respectively. 25%Ac-PNSB media contained the same ingredients as the FW-PNSB media, with reduced amount of acetate. The total amount of acetate was reduced to 25% of the amount used in the original FW-AcMet media recipe (i.e. 1.25 g C$_2$H$_3$NaO$_2$ per liter of media). For 5YE-PNSB media, acetate was replaced by yeast extract (5 g yeast extract per liter of media). Detailed compositions of the media can be found in Appendix I.

**eSEM Imaging**

Environmental scanning electron microscope (eSEM) housed in the Loeb laboratory was used for high-resolution imaging of the cells. Sample preparation involved the following procedure. Cells in 100 ul of liquid culture were fixed with 900 ul of 3% formaldehyde solution for ca. 1.5 hours. Fixed cells were washed with deionized water by centrifuging the tube with the cells suspended in fixative at 1,5000 g for 2 minutes, removing the supernatant, and resuspending the cells in 900 ul of deionized water. The washing step was repeated twice. Washed cell suspension was then slowly injected through a 0.2-micron filter attached to a syringe. The filter was made sure to stay moist throughout the serial drying steps with 30%, 50%, 70%, and 100% ethanol. After ca. 10 minutes of incubation in 100% ethanol, the filter was transferred to a critical point dryer housed in the Lillie building for the final drying step. The dried sample was then mounted on an aluminum stub and was sputter-coated with platinum with 5 nm thickness. Hitachi
TM3030 eSEM was used for imaging and the EDX software SwiftED was used for elemental composition analyses.

Lectin Staining

A fluorescently labeled lectin Wheat Germ Agglutinin (WGA-Alexa595) was used for the identification of adhesive structure. WGA binds N-acetylglucosamine, a major component of the holdfast in Caulobacter species. Unfortunately, the tube containing the stain obtained from the previous course lacked information about its concentration, and the optimal stain-to-sample ratio had to be determined arbitrarily by trial and error. The background fluorescence was too high for signal identification, so the initial concentration in the tube was diluted 10,000 times until a reasonable signal-to-noise ratio was obtained. After the incubation at room temperature in the dark for ca. 15 minutes, samples were prepared on a microscope slide and imaged with the EGFP filter cube using the fluorescence microscope.

Physical Disturbance Experiment

In order to investigate the effect of physical disturbance on the rosette formation and/or surface attachment process, cultures were grown in the same type of media with the presence and absence of agitation. Two different types of media (FW-PNSB and 25%Ac-PNSB; Appendix I) were used. Each of the test tubes containing 10 ml of FW-PNSB and SW-PNSB media was inoculated with 0.5 ml of active culture, and the tubes were kept still inside a 30°C incubator. Duplicates of both tubes were made and kept in agitation inside a 30°C shaking incubator. The growth and aggregation patterns were observed using phase contrast light microscopy.

Chemical Disturbance Experiment

The chemical nature of the adhesive was further characterized by observing the effects of four different chemicals on the degree of cell surface attachment. The tested chemicals include 20% sodium dodecyl sulfate (SDS), 0.05 M D-(+)-glucosamine-HCl, 5 M NaCl, and EDTA solution. Liquid culture was transferred to a MatTek dish such that just enough liquid covers the entire cover slip at the bottom (ca. 500 ul for a 60-mm-diameter dish). The dish was kept still on the inverted microscope stage for ca. 10 minutes to allow the cells to settle. After surface-attached cells were identified at the bottom of the dish, 500 ul deionized water was added directly to the sample in the dish, with a minimum amount of disturbance to the set up. The bottom cells were simultaneously imaged in a live stream video before, during, and after the addition of water. The purpose of documenting the addition of water was to make sure that the bottom cells in focus were in fact attached to the surface and are not subjected to disturbance caused purely by the physical flow of added liquid. To minimize unwanted physical disturbances, it’s been found helpful to insert the pipette tip in the middle of the sample, neither touching the coverslip at the bottom nor creating bubbles on the surface, and gently injecting the liquid. After repeating the
water addition twice, the same procedure was repeated with the addition of different types of chemicals.

**Time-lapse Microscopy**

FW-PNSB liquid media was made into a 2% agar plate and was cut into ca. 10 mm x 10 mm square blocks. Each block was inoculated with 1µl of liquid culture. After the inoculum was sufficiently absorbed into the agar block, the pad was transferred and placed upside-down on the glass bottom of the MatTek dish. After placing a total of ca. 100 µl of deionized water droplets around the edge of the dish to maintain humidity, the dish was sealed with parafilm. Detailed instruction for the sample dish preparation can be found in Polka & Silver (2014) [9]. Once the region of interest was identified, time lapse acquisition was started and the cells were grown for 12 hours or longer at room temperature.

**Results & Discussion**

**Characterization of the Adhesive**

*Figure 2.* eSEM images of *R. capsulatus* cells collected from the planktonic water column of a 2-day-old culture. (A): Thread-like structure connecting two cells at ca. 15 um distance; (B): putative adhesive material binding a cluster of cells together. Note the mucilaginous texture and the thread-like extension similar to the one observed in (A). Both images were taken at different spots within the same sample stub; similar thread-like structure was observed in other samples as well (data not shown).

In order to get high-resolution images of the cell and rosette surface structure, samples were prepared for and examined under the eSEM. Structural and textural details that were not visible under the light microscope were observed using the eSEM. Putative adhesive material with a mucilaginous texture was observed at the center of a cell cluster (Fig. 2B). Cells appear to be connected to one another via means of this adhesive material, as fibrous extension of the
adhesive material connects the poles of the cells associated with the cluster. The thread-like structure is most apparently visualized when the connected cells are at a distance (Fig. 2A). A plausible interpretation is that these cells were more closely attached to one another by means of the adhesive and were pulled apart, going through the physical disruptions involved in the sample preparation procedure. The artifact, however, beautifully reveals the nature of the adhesive.

Additionally, EDX analysis was done to further characterize the adhesive structure. The field of view including both the adhesive and surrounding cells was scanned for the detection of major biomass elements including carbon (C), nitrogen (N), oxygen (O), phosphorous (P) and sulfur (S). P peak coincided with the platinum (Pt) peak and, therefore, unambiguous identification of P distribution was not possible. (Pt signals were strong and ubiquitous, as Pt was used to sputter coat all samples.) Besides P, the remaining elements showed up as relatively clear signals.

![Figure 3. eSEM-EDX results displaying the elemental composition and distribution of carbon, oxygen, and nitrogen. (A): distribution of carbon in red, false color; (B): distribution of oxygen in blue, false color; (C): superimposed distribution of carbon in red and oxygen in blue, false color; (D): superimposed distribution of carbon in red and nitrogen in green, false color. Note the difference in the relative distributions of C and O in the cell biomass (C-a) and in the adhesive material (C-b).](image-url)
It is important to note that the relative distributions of C and O in the vicinity of the adhesive and the cell biomass are different. When the signals of C and O are superimposed (Fig. 3C), it becomes clear that the major component of cell biomass is C, whereas the adhesive has a more even distribution of C and O. The average biomass composition of phototrophic sulfur bacteria is $C_{380}H_{580}O_{153}N_{67}P_{3}S_{2.5}M$, where M stands for minor elements [9]. On the other hand, extracellular polymeric substances (EPSs) have distinct elemental compositions. For example, N-acetylglucosamine is a major component of the holdfast structure in *C. crescentus* and has an elemental composition of $C_8H_{15}NO_6$. In other words, representative C to O ratio of bulk biomass and EPSs can be taken as 2.48 and 1.33, respectively. Considering the relatively low C to O ratio in the adhesive structure from eSEM-EDX analysis, it is reasonable to conclude that the adhesive is composed of carbohydrate structures that are typically found in EPSs.

![Figure 4](image)

*Figure 4. Lectin staining assay for the identification of adhesin. The fluorescently-labeled WGA-Alexa595 stain specifically binds N-acetylglucosamine. (A) and (B) are phase contrast images of the same culture, and (B) and (D) are images of (A) and (B), respectively, taken using the EGFP filter. Strong signals from cells in the aggregates were detected. Note that not all cells show signals and that some cells have much stronger signals.*

Fluorescently-labeled lectin (WGA-Alexa595) staining result further supported the hypothesis that cells in rosettes and larger aggregates are bound together by means of extracellular...
carbohydrate substances. WGA is a lectin that specifically binds N-acetylglucosamine. Fluorescence signals were mostly concentrated at the center of cell aggregates (Fig. 4). Although the exact identification of the adhesive awaits more rigorous chemical analysis, lines of evidence from eSEM-EDX analysis and stain assay suggest that the adhesive structure in *R. captulatus* cell aggregates is similar in composition to N-acetylglucosamine. However, unlike in the case of *C. crescentus* where signals are concentrated only on the holdfast at the tip of the stalked cells [4], signal distribution was rather unspecific. Not all cells in an aggregate carry signals; some cells are entirely lit up with signals; whereas some cells are only lit up at the pole (Fig. 4). Several possibilities may have contributed to the phenomena. It is possible that the background signal was so high that some cells were lit up entirely with false signals. In fact, the high background noise a significant problem in the initial troubleshooting step. On the contrary, it may simply be that the adhesive produced by *R. capsulatus* is different from the holdfast produced by *C. Crescentus*. If the adhesive structure creates an extracellular polymeric matrix around the cells, the signal need not necessarily be concentrated at a specific locus on the cell surface. Better staining protocol in the future can provide more insights regarding the localization of adhesive on the cell surface of *R. capsulatus*.

**Chemical Disturbance Experiment**

![Figure 5](https://www.youtube.com/watch?v=w1LNQfqlkMM (A, entire field of view); https://www.youtube.com/watch?v=A0TDj35RCbY (B, entire field of view); https://www.youtube.com/watch?v=adnbpeMguPM (A, zoom-in); https://www.youtube.com/watch?v=uwW3VmcDxCe (B, zoom-in).)

*Figure 5*. Effects of the addition of deionized water and 20% SDS on cell surface attachment. (A): time-lapse images taken as 500 ul deionized water was added to ca. 2 ml culture; (B): time-lapse images taken as 500 ul 20% SDS was added to ca. 2 ml culture + 1 ml deionized water. All images were taken with an inverted microscope Axio Observer.Z1 with a Plan-Apochromat 63X/1.40 Oil DIC M27 objective. Each interval between consecutive images is approximately 3.6 sec.
The changes in cell surface affinity upon the addition of different chemicals were monitored to examine the chemical properties of the adhesive. When deionized water was added to the sample, individual cells and rosettes in the planktonic water column moved with the flow of water whereas the surface-attached cells at the bottom remained immobile (Fig. 5A). On the other hand, the attached cells were significantly disturbed and some were even removed from the surface upon the addition of 20% SDS (Fig. 5B). It should be noted that chemicals were always added after water had been added, such that the starting volume of liquid was different from the initial volume. However, possible changes in buoyancy effect and/or surface tension were likely negligible, since the addition of other chemicals did not result in decreased cell surface affinity.

Each chemical was tested for the following different properties of the adhesive; SDS for hydrophobicity of the adhesive, D-glucosamine-HCl for the affinity to sugar molecules, EDTA for possible roles of metals, and NaCl for the effect of salinity. Given the apparent disruptive effect of SDS on cell surface attachment, it can be inferred that the surface adhesive is hydrophobic in nature. This hypothesis is further supported by general observations of cells at the water-air interface. Whenever there were bubbles trapped between the microscope slide and the coverslip, a strong tendency to attach to the water-air interface at their poles was observed (Appendix III).

**Long-Term Environmental Effects on Cell Growth and Rosette Formation**

In addition to observing the instant effects of chemical disturbances, the changes in growth patterns in different media composition were examined. Long-term exposure of the organism to chemically distinct types of media should reflect the organism’s response and adaptation to different environmental conditions.

Cell dimension analysis using the fiji/imageJ software revealed significant differences in the length and width of the cells among the three types of media used. Cells in the FW-PNSB media were largest in the overall dimension (1.67 um x 0.82 um; n = 1,672). Cells grown in the 5YE-PNSB media were similar in length yet thinner (1.59 um x 0.75 um; n = 1,998) than those grown in FW-PNSB. Lastly, cells grown in 25%Ac-PNSB media exhibited a dramatic decrease in cell length; the cells were shorter but the thickness did not change significantly compared to those grown in FW-PNSB. Rosettes and larger aggregates of cells were present in all three conditions, but the shorter cells in 25%Ac-PNSB media formed much tightly packed aggregates compared to the other two conditions. Overall, the correlation between nutrient availability, cell dimension and arrangement
Physical Disturbance Experiment

Since this organism was isolated from a well-aerated, moderately turbulent environment (see Materials & Method), physical disturbances must have an ecological relevance to the development of rosettes and surface-attached communities in the organism’s native habitat. Moderate degree of physical disturbance always had positive effects on growth under, whereas it had differential effects on cell aggregation depending on the nutrient availability. When cells are grown in FW-PNSB media (0.06 M sodium acetate as a carbon source), the cells grown with agitation in a shaker had a notable decrease in large aggregate formation compared to a replicate grown under a static condition (Fig. 6A and 6B). Considering the fact that agitated tube had faster growth and higher cell density and yet less aggregation than the static tube, physical disturbance definitely seems to have an inhibitory effect on cell-to-cell attachment under this particular experimental condition.

Figure 6. Physical disturbance experiment. Four representative 10X objective views of culture grown in: (A) FW-PNSB media under static conditions; (B) FW-PNSB media under turbulent conditions; (C) 25%Ac-PNSB media under static conditions; (D) 25%Ac-PNSB media under turbulent conditions.
In contrast, no significant difference in cell aggregation was observed between the static and agitated conditions under nutrient-limited conditions (in 25%Ac-PNSB media; 0.015 M sodium acetate). In both cases, cells exhibited a strong tendency to aggregate (Fig. 6C and 6D). It should be noted that the cell density of both cultures grown in 25%Ac-PNSB media was significantly less than the ones grown in FW-PNSB media. Therefore, the extent of large-scale aggregation observed in 25%Ac-PNSB media (Fig. 6D) is rather significant. Under higher magnification (100X objective), cells were rarely observed as individuals but almost always occurred in rosettes or larger aggregates (data not shown).

These observations may suggest an important ecological implication about the lifestyle of *R. capsulatus* in the environment. The amount of acetate provided as a carbon source in FW-PNSB media (0.06 M) is highly unlikely to encounter in natural environments; estimates suggest that the acetate concentration in fresh water can be as low as tens to hundreds of uM [10]. In most cases, nutrient availability is much lower in the environment compared to laboratory conditions with pure culture and rich media composition. Reflecting on the environmental conditions from which this organism was originally isolated, it is plausible to imagine that cells would grow in aggregates of various sizes and readily develop surface-attached communities against the forces of physical turbulence and challenges of nutrient limitation.

**Time-lapse Imaging and Model for Rosette Formation Mechanism**

A 2D view of the colony progression was monitored using the inverted microscope and an agar block (see Materials & Method). This approach enabled the visualization of the life cycle of individual cells. The time-lapse video is available online at: https://www.youtube.com/watch?v=i22mgNLOIFM.

An important observation to be made is that two distinct (motile and sessile) cell types co-exist during a period of active growth. Anchored cells start division via binary fission. The daughter cell is motile and is immediately released from the mother cell, rather in a rapid launching motion, just as the division is completed. The daughter cell eventually becomes permanently immotile, either by attaching onto a surface or joining other cells at its pole, creating a rosette-shaped cluster. It is not until the daughter cell loses its motility and anchors at a surface or makes a cell-to-cell contact that it initiates the division process and becomes a mother cell. Such life cycle is very similar to that of *C. crescentus*, which also has a flagellated, motile and a sessile, stalked cell type with occasional holdfast development [4]. However, *R. capsulatus* is different in that it divides via binary fission rather than asymmetric division as is the case in *C. crescentus*. In addition, the time-lapse video showed that an initial cell-to-cell contact or cell-to-surface contact renders a cell immotile and that consecutive additions of immotile cells to the cluster can result in a rosette-shaped structure.
The temporal association between cell cycle and motility have never been fully appreciated using the snapshot approach with a typical wet mount slide sample, because the resolution of exposure time inevitably fails to capture the progression through cell cycles. Therefore, time-lapse imaging provides useful complementary information and helps improve our understanding of the rosette formation mechanism.

**Conclusion & Future Study**

In this study, the mechanism behind the rosette formation in *R. capsulatus* was investigated through a series of microscopic observations along with growth and perturbation experiments. The presence of adhesive material at the center of a medium-sized rosette was confirmed with high-magnification eSEM images. The elemental composition with a relatively low C to O ratio in the adhesive, compared to the cell biomass, suggests that the adhesive is likely composed of carbohydrate structures. Chemical disturbance experiments revealed that, out of the four chemicals tested (SDS, surfactant; glucosamine, sugar; EDTA, chelating agent; and NaCl, salt), only SDS has a significant impact on the cell surface attachment affinity. This suggests that the adhesive is hydrophobic in nature. Physical disturbance experiments revealed that physical disturbance has a certain degree of preventive effect on rosette formation, but has less effect under low-nutrient conditions. A tendency to develop a more compact cluster was also observed under nutrient-limited conditions. Therefore, it was speculated that rosette formation and surface-attached communities may be prevalent lifestyle of *R. capsulatus* in its natural habitat.

Through time-lapse image and video analyses, two distinct cell types were identified. A sessile mother cell produces a motile daughter cell via binary fission. Finally, temporal progression towards the formation of rosette-like structure was observed from the time lapse video. These findings altogether suggest that *R. capsulatus* may exist in a range of sizes and morphologies at a cellular level and form rosettes and larger aggregates at a population level by means of cell-to-cell or cell-to-surface contact.

As for future investigations, it would be helpful to examine the following aspects: 1) the exact identification of the chemical composition and local distribution of the adhesive material; 2) possible regulatory mechanism behind the adhesive production and surface attachment process. Studies have demonstrated that the holdfast elaboration in *C. crescentus* is a function of both upstream regulatory control and post-translational environmental triggers [4]. The result of this study suggests that chemical and environmental factors have significant effects on the development of rosettes and larger cell aggregates; it also suggests that the population growth is closely associated with the bimodal life cycle of the organism, as is the case in *C. crescentus*. Therefore, it is plausible that a tight control over the development of adhesive and promotion of cell aggregation exists at a genetic level. The pursuit of such research question will broaden our understanding about the diversity of multicellular arrangement behavior within the Alphaproteobacteria.
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References

Appendix I. Media Composition

5YE agar plate
Per liter of deionized H$_2$O
- 5 g yeast extract
- 15 g agar

FW-AcMet media
Per 2.9 liter of deionized H$_2$O
- 30 ml 100X FW Base *
- 15 g sodium acetate
- 15 ml 1 M NH$_4$Cl solution
- 0.6 ml 1 M sodium sulfate
- 30 ml 100 mM potassium phosphate, pH 7.2
- 4 ml 1 M MOPS Buffer, pH 7.2 *
- 3 ml trace elements *

Autoclave
After cooling, add:
- 3 ml multi-vitamin solution *
- 10 ml 1 M sodium bicarbonate
- 50 mg DCMU
- 2 ml fresh methanol

* these stock solution compositions can be found in the 2016 Microbial Diversity lab manual

FW-PNSB media
Per 2.9 liter of deionized H$_2$O
- 30 ml 100X FW Base *
- 15 g sodium acetate
- 15 ml 1 M NH$_4$Cl solution
- 0.6 ml 1 M sodium sulfate
- 30 ml 100 mM potassium phosphate, pH 7.2
- 4 ml 1 M MOPS Buffer, pH 7.2 *
- 3 ml trace elements *

Autoclave
After cooling, add:
- 3 ml multi-vitamin solution *
- 10 ml 1 M sodium bicarbonate
No DCMU or methanol
* these stock solution compositions can be found in the 2016 Microbial Diversity lab manual

**SW-PNSB media**

**Per 2.9 liter of 1X SW Base** *

15 g sodium acetate
15 ml 1 M NH4Cl solution
0.6 ml 1 M sodium sulfate
30 ml 100 mM potassium phosphate, pH 7.2
4 ml 1 M MOPS Buffer, pH 7.2 *
3 ml trace elements *

**Autoclave**

**After cooling, add:**

3 ml multi-vitamin solution *
10 ml 1 M sodium bicarbonate

* these stock solution compositions can be found in the 2016 Microbial Diversity lab manual

**25%Ac-PNSB media**

**Per 2.9 liter of deionized H2O**

30 ml 100X FW Base *

5 g sodium acetate
15 ml 1 M NH4Cl solution
0.6 ml 1 M sodium sulfate
30 ml 100 mM potassium phosphate, pH 7.2
4 ml 1 M MOPS Buffer, pH 7.2 *
3 ml trace elements *

**Autoclave**

**After cooling, add:**

3 ml multi-vitamin solution *
10 ml 1 M sodium bicarbonate

* these stock solution compositions can be found in the 2016 Microbial Diversity lab manual

**5YE-PNSB media**

**Per 2.9 liter of deionized H2O**

30 ml 100X FW Base *

15 g yeast extract
15 ml 1 M NH4Cl solution
0.6 ml 1 M sodium sulfate
30 ml 100 mM potassium phosphate, pH 7.2
4 ml 1 M MOPS Buffer, pH 7.2 *
3 ml trace elements *
Autoclave
After cooling, add:
   3 ml multi-vitamin solution *
   10 ml 1 M sodium bicarbonate
* these stock solution compositions can be found in the 2016 Microbial Diversity lab manual

Appendix II. PCR Amplification

Master solution ingredients
Per reaction
   12.5 ul Promega GoTaq G2 Hot Start Green Master Mix
   1.0 ul 16S_8F (15 pmol) bacterial forward primer
   1.0 ul 16S_1391R (15 pmol) bacterial reverse primer
   20.0 ul nuclease-free H2O

PCR thermocycler program

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Appendix III.

Hydrophobic poles of the cells attaching to the water-air interface of the bubble created under the coverslip. Phase contrast light microscope image.
Appendix IV. Long-term effects of salinity on cell growth and rosette formation.

(A) Culture grown in FW-PNSB media (17 mM NaCl);
(B) Culture grown in SW-PNSB media (342 mM NaCl).
Appendix V. Effects of Nutrition Availability.

(Top) Cells grown in 25% Acetate_PNSB media
Avg. cell dimension (n = 1343)
length = 1.44 um
width = 0.84 um

(Middle) Cells grown in FW_PNSB media
Avg. cell dimension (n = 1672)
length = 1.67 um
width = 0.82 um

(Bottom) Cells grown in 5YE_Acetate_PNSB media
Avg. cell dimension (n = 1998)
length = 1.59 um
width = 0.75 um