Investigations into Holdfast development in α-proteobacteria

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Motivations

My intentions for this course were to gain skills in the fundamentals of microbiology and understand the historic development of the field. My background composed mostly of bioinformatic modeling as well as basic molecular and biochemical techniques. My basal understanding of microbiology was at the level of one model organism, *Caulobacter crecentus*. Thus, I wanted to understand mechanisms of growth in other microbial organisms within their natural habitats to better connect my myopic knowledge of my organism and broaden my perspectives by tackling questions at an evolutionary and ecological level.

Introduction

Comparative genomics is a powerful tool to elucidate genetic functions. With the increasing number of genomes sequenced each year, comparative genomics approaches are in higher demand than ever. This approach, however, is dependent on well-characterized gene annotations. Thus, model genetic systems across several organisms need to be developed in order to improve our understanding of comparative genomic approaches.

Polar holdfast synthesis, dimorphic cell cycle division, and stalk development are three characteristics that are highly conserved within the α-proteobacteria clade. As seen in figure 1, taken from a review in 2012, the development of these three traits can be found in wide range of α-proteobacteria, however, losses of these traits have also been observed. Furthermore, these three traits have been study extensively in my model organism and other model α-proteobacteria for over half a century.

My goal for this course was to investigate holdfast development in three different α-proteobacteria: *Caulobacter crecentus*, Hyphomicrobia, and *Rhodopseudomonas palustris*. Holdfast is an outer-membrane structure that forms at a single pole of a cell in some α-proteobacteria species; it is comprised of adhesive polysaccharides that allow them to attach to surfaces or each other (Hughes 2012). My hopes were to understand genetic determinates of holdfast in *Rhodopseudomonas palustris* TIE-1, which has been previously uncharacterized, and compare these identified components with those of Hyphomicrobia and *Caulobacter*. Additionally, I attempted to isolate a new strain of Hyphomicrobia in order to see how much variability can arise from a tamed laboratory strain of Hyphomicrobia and a novel environmental sample. In a functional perspective, I wanted to see if holdfast development and structure within each of these α-proteobacteria have diverged functionally to the point that they could not attach to other α-proteobacteria.

Methods

Strains and Media Conditions.

Wild type *Rhodopseudomonus palustris TIE-1* strains were obtained from the lab of Dianne Newman (DKN-379). Aerobic chemoheterotroph medium (ACM) comprised of 3g Peptone, 3g Yeast Extract, 50 mM MOPS buffer per liter of nanopure water. Aerobic conditions consisted from 30°C shaking incubator, windowsill, and 860 nm light chamber. Anaerobic photoautotrophic medium comprised of 10 mL of 100x Fresh Water Base Medium (FWB:

40g $MgCl_2$•$6H_2O$, 10g $CaCl_2$•$2H_2O$, 100g NaCl, 50g KCl per liter of nanopure water), 5.6 mL of 1M NH_4Cl, 36 mL of 100mM KH_2PO_4. After medium was autoclaved and degassed with CO_2, the photoautotrophic medium was buffered with 22 mL of 1M $NaHCO_3$ (autoclaved and degassed). 1mL of filter sterilized 100x trace elements solution (3.0g Na_2EDTA•$2H_2O$, 1.1g $FeSO_4$•$7H_2O$, 0.19g $CoCl_2$•$6H_2O$, 0.042g $ZnCl_2$, 0.024g $NiCl_2$•$6H_2O$, 0.018g Na_2MoO_4•$2H_2O$, 0.3g H_3BO_3, 0.002g $CuCl_2$•$2H_2O$, 0.05g $MnCl_2$•$4H_2O$ added to 1 liter of nanopure H_2O), 1 mL of filter sterilized 1000x Vitamin B_{12} solution (5mg in 50 ml nanopure H_2O), 5 mL of filter sterilized 200x Vitamin solution (0.004g 4-Aminobenzoic acid, 0.001g D (+) biotin, 0.01g Nicotinic acid, 0.005g Ca (+) pantothenate, 0.01g Pyridoxamine dihydrochloride, 0.01g Thiamine dichloride, 0.05g Riboflavin in 500 mL of nanopure water). Anaerobic growth consisted of inoculating sterilized and degassed bottles of anaerobic medium from saturated cultures grown at aerobically. Carbon sources for both mediums were added from autoclaved stocks of sodium acetate and succinic acid. Carbon source additions were always added to a concentration of 10 mM. Aerobic agar plates were made with 3g Peptone, 3g Yeast Extract, 15g Agar, and 50 mM MOPDS buffer per liter of water (10mM carbon sources were added as needed).

E. coli transposon conjugators were obtained from the lab of Dianne Newman (DKN-4, DKN-157, DKN-165). DKN-4 and DKN-157 are β2155 strains and DKN-165 is a BW29427 strain. Each strain has a deletion of their *dapA* gene and thus requires di-aminopimelic acid (DAP) to grow; thus, this acts as an antagonistic selective marker. DKN-4 carries a suicide plasmid (pBSL180); this plasmid contains a KmR Tn10 derivative mobile element and transfers kanamycin resistance into the recipient bacteria upon successful conjugation and transposition. DKN-157 carries a suicide plasmid (pSC189); this plasmid contains a mini-mariner transposable mobile element and transfers kanamycin resistance into the recipient bacteria upon successful conjugation and transposition. DKN-165 carries a suicide plasmid (pRL27); this plasmid contains a Tn5 transposable mobile element and transfers kanamycin resistance into the recipient bacteria upon successful conjugation and transposition. Donor strains were grown in LB medium with 300 μM DAP and 50 μg/mL of Kan. Cultures for conjugation were grown overnight at 30˚C shaking.

Hyphomicrobium enrichment culture isolated from several sources around Woods Hole, MA. Hyphomicrobium was enriched in minimal oxygen medium in Pfennig bottles. Enrichment medium consisted of 10 mL of 100x FWB, 2mM MOPS buffer, 1 mL of 1000x HCl-Dissolved Trace Elements Solution (20 mM HCl, 7.5 mM $FeSO_4$•$7H_2O$, 0.48 mM H_3BO_3, 0.5 mM $MnCl_2$•$4H_2O$, 6.8 mM $CoCl_2$•$6H_2O$, 1.0 mM $NiCl_2$•$6H_2O$, 12 μM $CuCl_2$•$2H_2O$, 0.5 mM $ZnSO_4$•$7H_2O$, 0.15 mM Na_2MoO_4•$2H_2O$, 25 μM $NaVO_3$, 9 μM $NaWO_4$•$2H_2O$, 23 μM Na_2SeO_3•$5H_2O$), 0.2 mL 1 M Na_2SO_4, 1.0 mL 100 mM Potassium phosphate (pH 7.2), 5.0 mL 1 M NH_4Cl, 5.0 g KNO_3).

Caulobacter crecentus CB15 wild type and xylose inducible GFP strains were obtained from the lab of Sean Crosson (FC-19 and ACC 158 respectively). *Caulobacter crecentus* was grown in 2 g Peptone, 1 g Yeast extract, 1 mL of 1M $MgCl_2$, and 500 μL of 1M $CaCl_2$. To induce production of green fluorescent protein (GFP) in FC, .15% (w/v) of xylose was added to the medium. Cultures were grown in a shaking incubator at 30˚C.

Library construction
Plasmids with transposable elements were mated with TIE-1. Each donor strain was inoculated and incubated overnight. Various saturated aerobic TIE-1 cultures grown in complex medium were pooled together in order to generate enough recipient volumes to perform conjugations. Ratios of donor to recipient varied to find the optimal conjugation efficiency to construct the transposon mutant library; details are elaborated further in Results. Donor and recipient cultures were pelleted and washed with medium once before mixing. Once ratio of donor to recipient mixture was achieved, cells were spun at 8000g for 3 min at room temperature. Conjugation pellets were resuspended in 100μL of YP-rich medium. Suspended conjugations were spotted anaerobically on complex medium plates with additions of succinate, acetate, and DAP as specified earlier. Spotted conjugations on these plates were incubated overnight in the 30˚C incubator. Overnight conjugations were resuspended in complex medium and plated on rich medium plates with additional carbon sources, kanamycin, and no DAP. Suspension of the over night conjugation was always done to twice the volume of total conjugation volume plated on the overnight plates. Colonies were usually observed after 5-7 days and were picked into 96-wells with 100 μL of rich medium with acetate, succinate, and kanamycin.

Spot Dilutions
Spot dilutions were used in several aspects over the course of these experiments. To evaluate transposition efficiency, 10-fold dilutions were conducted with 1.5 mL tubes transferring 100μL of culture into 900μL of media. From each dilution, 10μL of each dilution in the series was plated.

Crystal violet stain assay
96-well plates with cultures of R. palustris were subjected to crystal violet staining of cells attached to the sides of the 96-well plates. First, cultures in the 96-well plates were dispensed. The wells are then stained with crystal violet solution by addition of a volume that is 25μL more than the culture volume dispensed. 0.1% weight by volume of crystal violet solution in water was used to stain wells. Stain was dispensed and the 96-well plate was rinsed in two separate water baths of RO-H_2O. Plates were left upside down to dry. Once dried, the wells were then filled with destain (45% H_2O, 45% Methanol, 10% Acetic Acid). Volume of destain was 25μL more than volume of crystal violet solution.

Assay Development
Determination of the linear relationship between crystal violet staining of cells in 96-well plates compared to od600 of TIE-1 cultures. Serial dilutions of saturated inoculum were conducted in 96-well plates. The first column of the well was filled with blank medium with no inoculated cultures. Column 2 was filled with 100 μL of saturated TIE-1 cultures. Columns 3-12 were filled with 80μL of medium. 5-log dilutions were conducted by transferring 20 μL of the previous well into the subsequent well. Dilutions started with column 2 into 3 and so forth. Plates are then examined after 4-6 days and subjected to crystal violet stain. OD600 of TIE-1 was measured in the PacBio plate reader. OD560 of the crystal violet destain was measured in the PacBio plate reader.

Results and Discussion

Genetic screen to elucidate Holdfast development in Rhodopseudomonas palustris

At a phenotypic level, *Rhodopseudomonas palustris* holdfast development is similar to a wide diversity of α-proteobacteria (Whittenbury 1967). It has an asymmetrical cell cycle in which a single swarm cell collapses its flagellum, forms a polar-localized, adhesive polysaccharide, and begins forming a daughter cell at the opposing pole. Although it's asymmetrical cell-cycle morphology and adhesive qualities are similar to other α-proteobacterium, R. palustris lacks a stalk often associated with other asymmetrically dividing alphas (Hughes 2012).

The model strain for the genetic screen was TIE-1 isolated in Woods Hole from School Street Marsh over a decade ago. Isolated for it's unique ferrous Iron [Fe(II)]-oxidizing bacterium, TIE-1 was subjected to genetic manipulations by transposon mutagenesis to elucidate the genetic components that help R. palustris utilize ferrous Iron (Jiao 2005, Jiao & Newman 2007). Thus, this system seemed amenable to tackle the genetic determinates of holdfast development within R. palustris.

Although TIE-1 has been subjected to transposon mutagenesis previously with a mariner transposon, I investigated other transposon plasmids and donor strains. Motivation for this exploration would ultimately save time in order to generate a sufficient library of transposon TIE-1 mutants. The results of this exploration are summarized in figure 2.

In figure 2A, pRL27 yielded more colonies on the kanamycin selective medium plates than pBSL180 and pSC189. As described in the methods section, pRL27 contains a hyper active mutant of T_N5 on its plasmid and transfers its kanamycin resistance upon successful integration into the genome (Larson 2002). From the literature, pSC189 contains a C9 transposition system that has not been modified (Chiang 2002). Additionally pBSL180 has been adapted to reduce the specificity of transposition compared to its wildtype counterpart (Alexeyev 1995). Based on several library construction methods, pRL27 seems to the more successful transposition plasmid in my hands. Additionally pRL27 is in the *E. coli* strain BW29427 and the other two plasmids are in a β2155. Controls of wildtype TIE-1 on kanamycin selection plates are presented in figure 2B.

To increase the efficiency of transposition of pRL27 transposon mutagenesis, conjugation ratio of donor (BW29427) to recipient (TIE-1) was explored. I also used the better donor from the β2155 strains, pSC189, to examine if its transposition efficiency could also increase with increased recipient ratios. pRL27 and pSC189 conjugations were mixed with in volumes of 1:1, 1:2, 1:5, and 1:10 of donor:TIE-1. The results of each conjugation are presented by spot dilutions (in magnitudes of 10) on figure 3A. From this figure we can observe that the number of kanamycin resistant colonies increases with transposon efficiency. Colonies were observed with the pRL27 conjugation down to the 10^{-2} spot dilutions on kanamycin, while pSC189 did not form any visible colonies that day.

Furthermore, to see if this trend would hold when scaling up the total volume of the conjugation, I performed the same experiment in larger quantities but with a smaller range of ratios: 1:2, 1:3, 1:4, and 1:5. For this scaled up approach, colonies were counted over 5 replicates of each donor strain as well as a sixth plate that was plated with a 10^{-1} amount of the overnight conjugation. The same trend holds as seen in figures 3B and 3C. Note that counts were done on the same day. While pSC189 did not seem to produce too many mutants, micro-colonies were observed on the plates.

Previous genetic investigations into holdfast with other alphaproteobacteria have utilized a crystal violet screening technique developed in the biofilm field (O'Toole 2000, Toh 2008). Thus, I adapted the assay to identify phenotypes of TIE-1 mutants that showed extraordinary differences in holdfast development. As seen in figure 4A, crystal violet staining of the attached cells has a linear relationship with the OD600 of the original culture. Additions of extra carbon sources did not seem to affect the formation of rosettes or holdfast; however, the addition of extra carbon sources allowed for TIE-1 to grow faster (data not shown). The assay was performed in anaerobic photoautotrophic conditions with succinate, acetate, and both as carbon sources; however, lack of a constant light source did not allow for fast growth of the 96-well plates even after 9 days in the anaerobic chamber at 27˚C, figure 4B.

Of the 15 mutants screened, none showed deficiencies in holdfast development. Evidence for such can be seen in microscopic images of each of the mutants where rosette formations were observed figure 5A. Additionally, the crystal violet assay showed evidence of holdfast attachment to the 96-well surface figure 5B. The crystal violet stain presented in 5B was performed on the initial inoculum plate, since the passaged plate intended for crystal violet stain was dropped on floor. This plate also showed visual evidence of holdfast rosettes as visualized in 5C.

Hyphomicrobia enrichment and isolation attempts

Hyphomicrobia isolation attempts were conducted with Bingran Cheng. Most of my work contributed to this project was to help make the minimal 337 medium, methanol plates, and passaging enriched cultures of hyphomicrobium. Original enrichment bottles were given to us by Apollo Stacy and were labeled as: "Soil", "Tarpac", "Garbage Beach", and "Swamp."

Hyphomicrobia enrichment attempts to tackle two metabolic restrictions that are specific to hyphomicrobia. Hyphomicrobia can be found in fresh water, soils, and on plants (Phillippot 2010). They are able to utilize one carbon compounds such as methanol as their sole carbon source. Additionally, they can reduce nitrate into nitrogen gas (denitrification) under anaerobic conditions (Sperl 1971). Thus our enrichment medium aimed to utilize these characteristics in order to obtain isolates of hyphomicrobia.

"Soil" enriched cultures were produced from a pea size sample of soil from a fire hydrant near MBL/Stony Beach. "Tarmac" enriched culture was inoculated with soil adjacent to the driveway of a resident of Woods Hole close to the "Soil" sampling sight. "Garbage Beach" enriched cultures came from soil near palm trees on garbage beach. "Swamp" enriched cultures came from soil near the edges of School St. Marsh. Bottles had been transferred into fresh methanol enrichment medium once before we inherited the enrichments.

Bottles were transferred in total 4 times since we inherited the bottles. Multiple attempts at plating enriched cultures aerobically and anaerobically were attempted to isolate a pure colony of hyphomicrobia. Several colonies visualized over several days did not show characteristic hyphomicrobia morphologies under the light microscope. These background organisms that outgrew the hyphomicrobia were other methylotrophs.

The enrichment bottles, however, showed a significant amount of hyphomicrbia compared to other methlytrophs. Bingran Cheng has more quantitative data on this. This can also be seen under the microscope as seen in figure 6.

Heterogeneous Rosette formation

Rosette formation in alphaproteobacteria is a communal structure often observed in species that contain a holdfast. Rosettes are commonly formed when adhesive polysaccharides at the poles of individual bacterium attach to one another's holdfasts, as seen in figures 7A-C.

Based on individual observations of holdfast formation, I mixed *Caulobacter crecentus* CB15 mutant that contains a xylose inducible green fluorescent protein with *Rhodopseudomonas palustris* TIE-1 and the enriched culture of Hyphomicrobia. Protein fluorescence was detected under the Zeiss HE GFP + DsRed shift Filter set. I mixed different ratios (by volume) of each culture and let them grow on my bench at various amounts of time; however, upon initial mixing and visualization on slides of the 1:1 ratio, I was able to identify heterogeneous rosettes, figure 7D-I. More specifically, figure 7D-F shows *Caulobacter* and Hyhomicrobia and figure 7G-I shows *Caulobacter* attached to *Rhodopseudomonas*.

These heterogeneous rosettes as seen in figure 7D-I only show *Caulobacter* attached to *Rhodopseudomonas* and *Hyphomicrobia* rosettes. Observation of the other direction, *Rhodopseudomonas* and *Hyphomicrbia* attached to Caulobacter rosettes were hard to identify since both strains did not contain any kind of detectable marker under the microscope.

Discussion

Holdfast development has many charasmatic characteristics that make it a potentally good genetic system to experimentally and computationally use comparative genomics to study evolution and ecology. Throughout the various studies conducted within this course, I believe that studying the loss, gain, and adaptation of holdfast, asymmetrical cellular division, and stalk formation could be developed to study how clusters of genes evolve and could elucidate some first principles of studying biological evolution at a broader scope genome rather than a single gene level.

Although each of the projects outlined above were small in scope in terms of the questions being asked, they do contribute to an understanding of a system that I think is very attractive to study. From my investigations into holdfast from a genetic, species, and community level, I've gained some insights on how I could begin formulating bioinformatic models and developing experimental systems to study the loss, gain, and conservation of large portions of genes within a genome.

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My main collaborator in this project was with Bingren Cheng. Hannah Naught also provided some extra hands to the project. Thank you to the Microbial Diversity Class, Facutly, and Teaching Assistance of 2014. Appreciation for funds go to NSF, Crosson Lab, and The William Randolph Hearst Educational Endowment.

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Figures

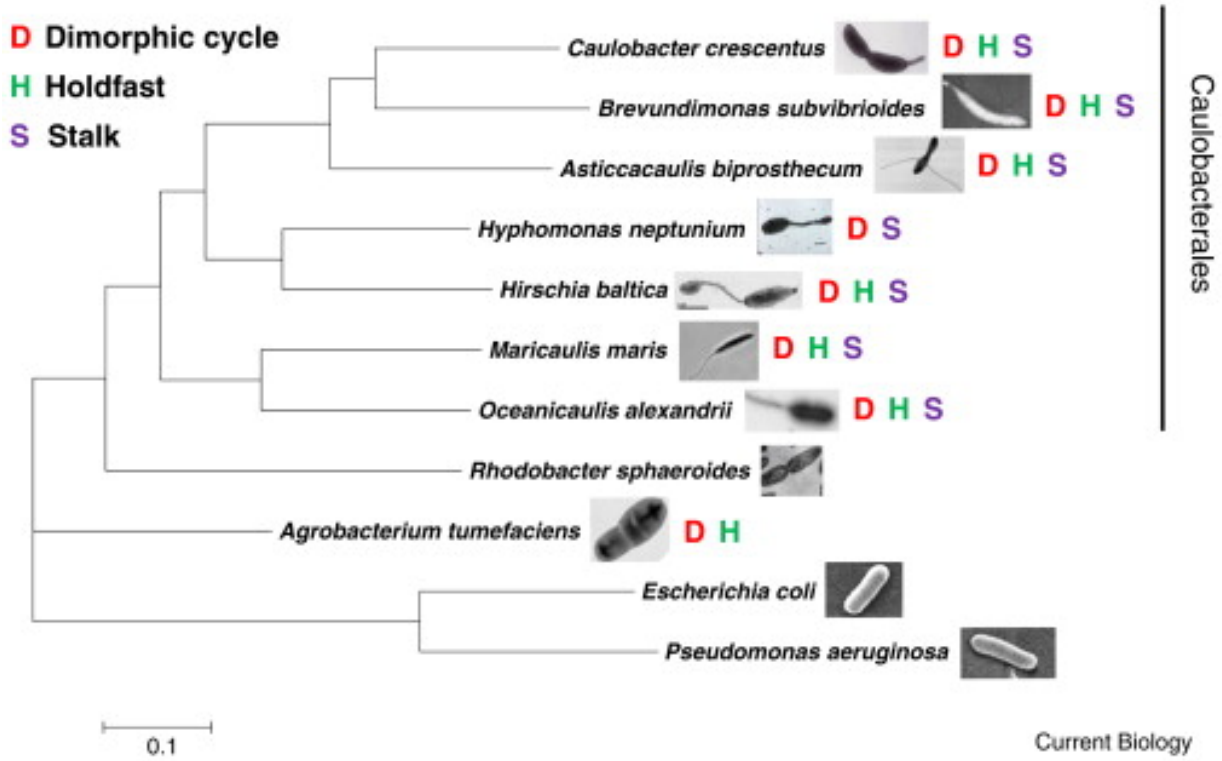

Figure 1. Motivation to use holdfast as a model system for comparative genomics. Image taken from Hughes 2012.

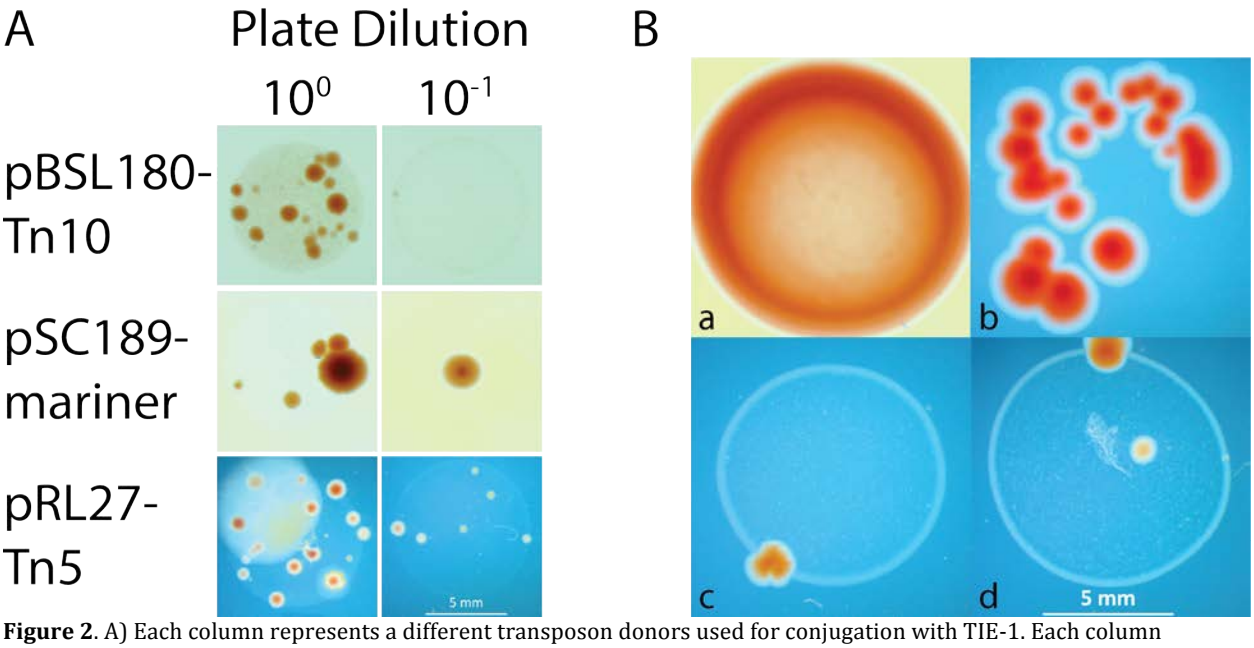

Figure 2. A) Each column represents a different transposon donors used for conjugation with TIE-1. Each column represents the dilution conjugation plated. Spots are 10µL of each dilution. B) (a)10 µL spot of 10^0 dilution of TIE-1 wildtype after several days on rich medium plate with no kanamycin (b) 10 µL spot of 10^{-10} dilution of TIE-1 wildtype after several days on rich medium plate with no kanamycin (c) 10 µL spot of 10^0 dilution of TIE-1 wildtype after several days on rich medium plate with 200 µg/mL kanamycin. (d) 10 µL spot of 10^0 dilution of TIE-1 wildtype after several days on rich medium plate with 400 µg/mL kanamycin.

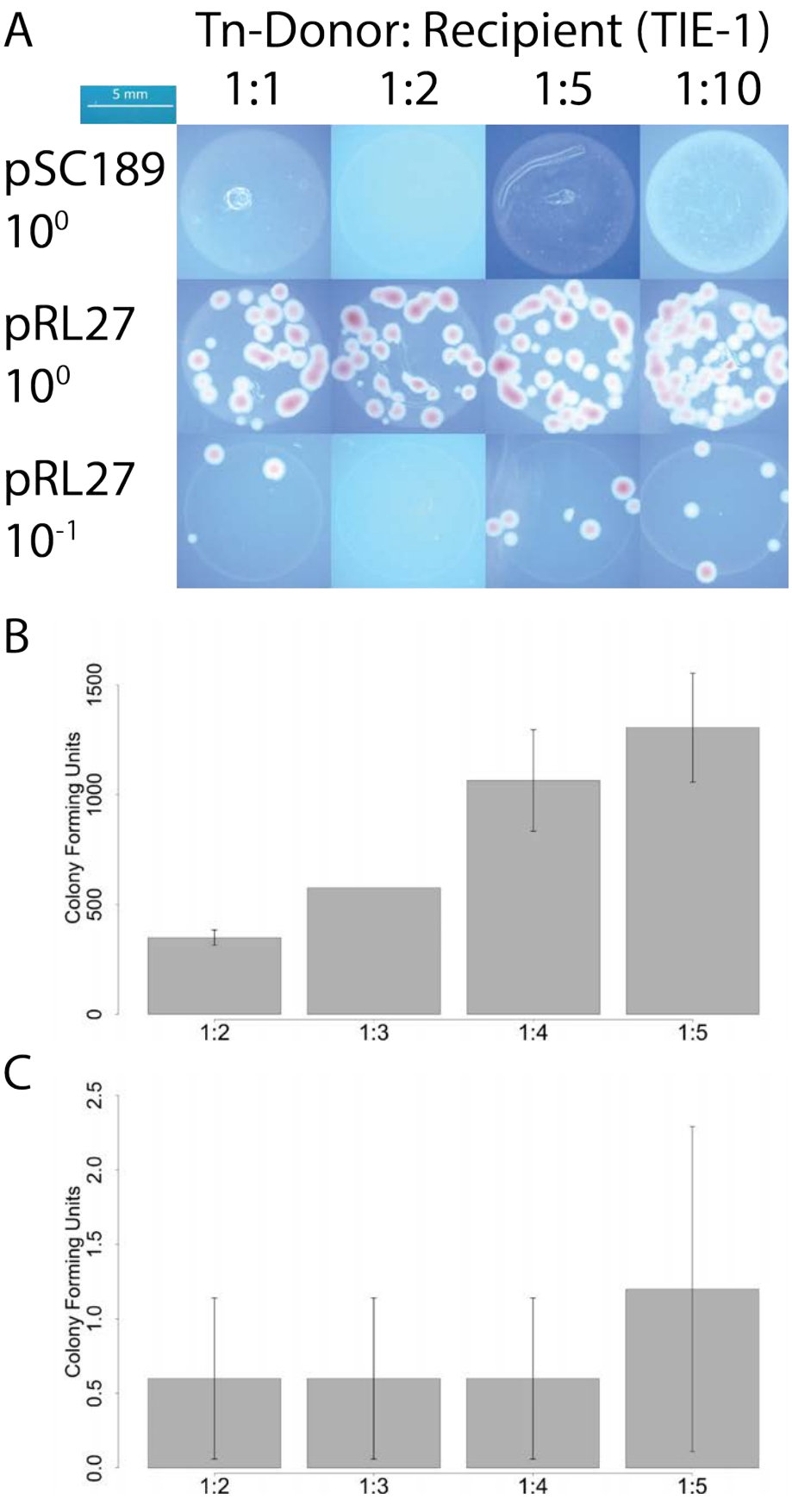

Figure 3. A) Increased recipient volumes increases transposon efficiency. Rows represent the donor plasmid and the dilution of conjugation plated. Columns represents different ratios of Donor to Recipient. All 10 µL spots were plated on rich medium containing 400 µg/mL of kanamycin. Increasing recipient ratio yields more kanamycin resistant colonies. This is a measure of transposon efficiency.
B) Increased recipient volume increases transposon efficiency in scaled up conjugation.of pRL27. X-axis represents Donor:Recipient ratio of conjugation. Y-axis represents Colony forming units counted across 5 plates of replicates (except for 1:3 dilution) in which four of the five plates showed contamination. The donor from these plates, contained 200 µL of resuspended conjugation at the 10^0 dilution (undiluted)
C) Increased recipient volume increases transposon efficiency in scaled up conjugation.of pSC189. X-axis represents Donor:Recipient ratio of conjugation. Y-axis represents Colony forming units counted across 5 plates of replicates. The donor from these plates, contained 200 µL of resuspended conjugation at the 10^0 dilution (undiluted)

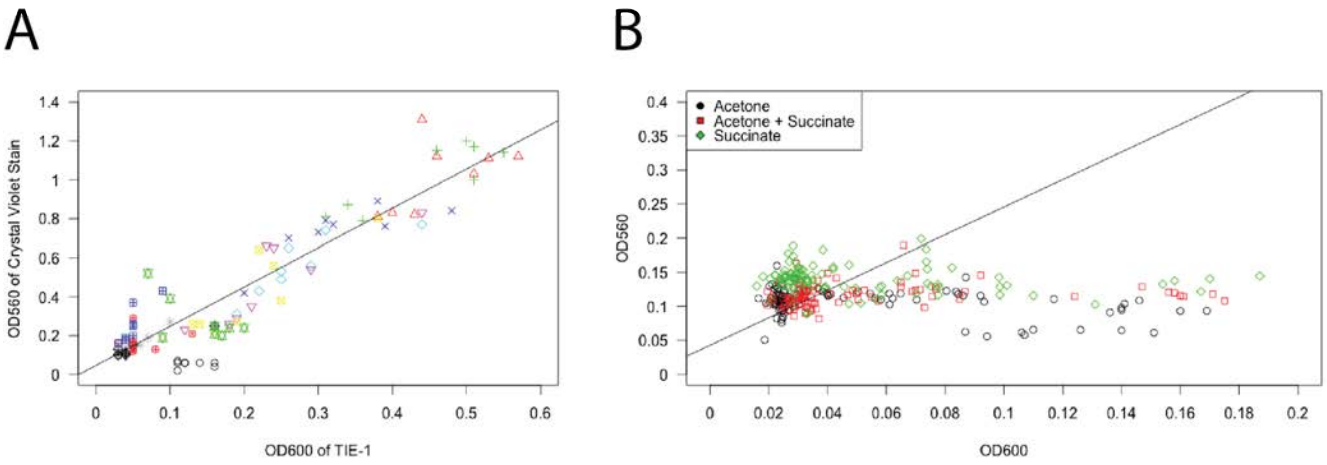

Figure 4. A) Cell density of TIE-1 (OD600) has a linear relationship with crystal violet stain (OD560) in 96-well plate. X-axis is the OD600 of TIE-1 taken by a plate reader in a 96 well plate in rich media. Y-axis is the OD560 of the crystal violet destain of TIE-1 attached to the surface. X-intercept of line is 0.043 and slope is 2.026 B) Cell density of TIE-1 (OD600) has a linear relationship with crystal violet stain (OD560) in 96-well plate. X-axis is the OD600 of TIE-1 taken by a plate reader in a 96 well plate in minimal media. Line is the same line as in A. Not much growth or holdfast attachment.

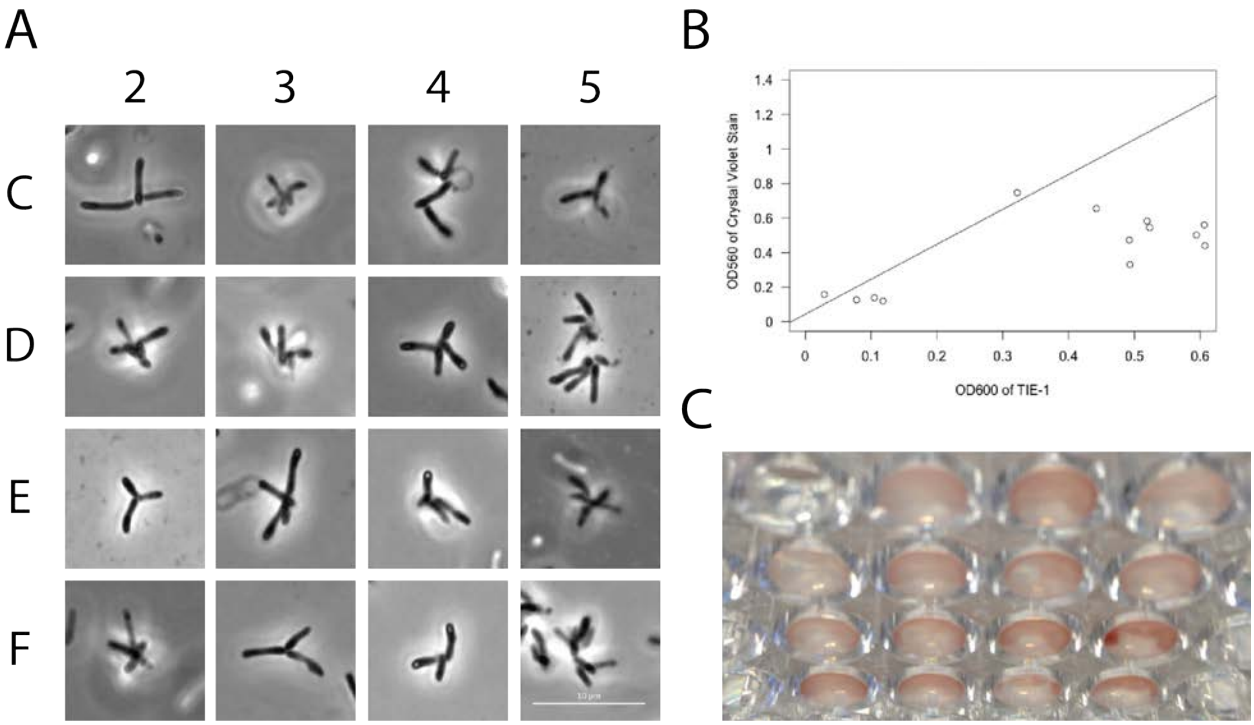

Figure 5. A) Rosettes in transposon mutants of TIE-1. Rows and columns correspond to the alpha-numeric wells in a 96-well plate. F5 is a wildtype.rosette. B) Off results of crystal violet stain of transposon mutants. X-axis is the OD600 of TIE-1 taken by a plate reader in a 96 well plate in rich media. Y-axis is the OD560 of the crystal violet destain of TIE-1 attached to the surface. Line is the described in 4A. C) 96-well plate shows biofilm/holdfast attachment to bottom and sides. 96 well plate was innoculated with mutants that have incurred kanamycin resistance after conjugation with pRL27 donor strain. They grew in 400 µg/mL kanamycin in rich medium with acetate, and succinate. Plates were parafilmed and kept in the 850 nm light box.

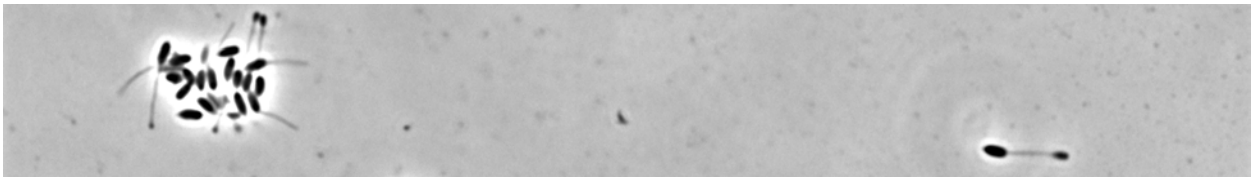

Figure 6. Hyphomicrobia in enrichments. The clump of cells in the left side of the image is a rosette of hyphomicrobia. The clump of cells on the right side of the image is a single cell of hyphomicrobia.

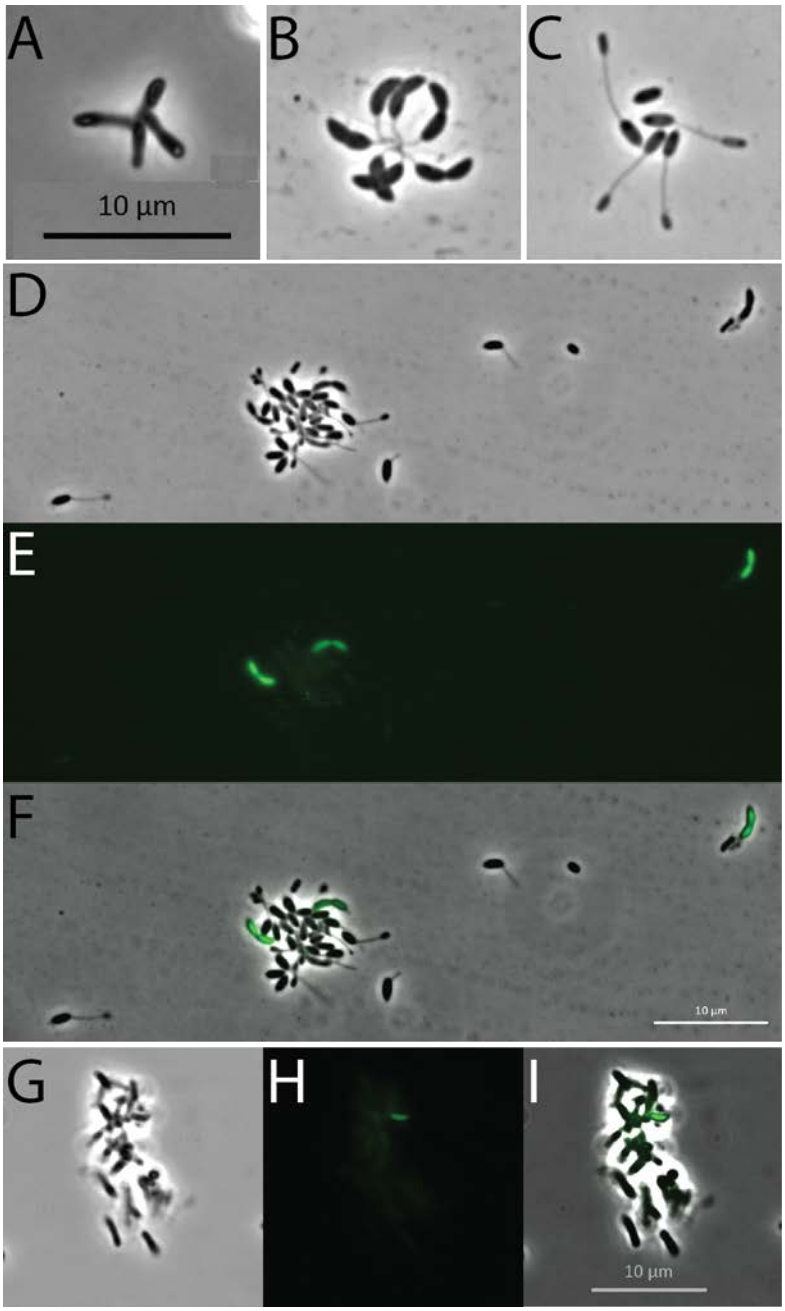

Figure 7. A) *Rhodopseudomonas palustris* TIE-1 rosette.
B) *Caulobacter crecentus* CB15 rosette
C) Hyphomicrobia rosette
D-E) *Caulobacter* and Hyphomicrobia rosettes. *Caulobacter* cultures with inducible GFP production were mixed in a 1:1 ratio with Hyphomicrobia enrichment. D shows a rosette and some single cells with transmitted light. E shows the same slide with GFP excitation and emission. F shows the overlay of the heterogeneous rosette formation.
G-I) *Rhodopseudomonas* mixed with *Caulobacter* at a 1:1 ratio. G shows an image of a *Rhodopseudomonas* rosette with transmitted light. H shows the same slide with GFP excitation and emission. I shows the overlay of the heterogeneous rosette formation.